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The effect of maternal diet on offspring vascular smooth muscle polyunsaturated fatty acid synthesis and vasoconstriction

by

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ABSTRACT

FACULTY OF MEDICINE

HUMAN DEVELOPMENT AND HEALTH

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THE EFFECT OF MATERNAL DIET ON OFFSPRING VASCULAR SMOOTH MUSCLE POLYUNSATURATED FATTY ACID SYNTHESIS AND VASOCONSTRICTION

By Nicola Alice Irvine

Cardiovascular disease (CVD) is a major global health problem. Maternal nutrition during pregnancy, including fat intake, has been shown to affect the CVD risk in the offspring. However, the relative effects of quantity and quality of maternal dietary fat on the vascular function of the offspring have not been investigated. Furthermore, the mechanism by which maternal dietary fat induces persistent changes in offspring vascular function is largely unknown.

The overall aim of this study was to test the hypothesis that differences in quality and quantity of maternal fat intake alter vascular function in the offspring via persistent changes in vascular cell membrane composition. Pregnant and lactating rats were fed diets enriched with different amounts of either saturated, monounsaturated or polyunsaturated fatty acids. The findings showed that the type of fat influenced blood pressure, whilst a high fat diet induced increased *ex vivo* phenylephrine (Pe) –mediated sensitivity and vasoconstriction in the aortae of the offspring. Offspring of dams fed a high fat diet exhibited lower proportions of arachidonic acid and docosahexaenoic acid in their aortae than those of dams fed a low fat diet. These changes were not reflected in the plasma composition, which suggested altered regulation of polyunsaturated fatty acid (PUFA) biosynthesis in the aortae. Maternal high fat diet increased expression of FADS1 and decreased level of FADS2 mRNA in offspring aorta, suggesting dysregulation of the pathway and a possible mechanism linking maternal diet to altered regulation of vascular tone.

The activity of the PUFA biosynthesis pathway in non-developmentally primed arteries was investigated using specific delta-6 or delta-5 desaturase inhibitors. This showed for the first time that PUFA biosynthesis inhibition reduced Pe-mediated vasoconstriction in rat aortae and human femoral arteries *ex vivo* by at least 50%. This effect was localised to the vascular smooth muscle layer. Calcium release from the sarcoplasmic reticulum is a key event in vasoconstriction. Inhibition of delta-5 or delta-6 desaturase in mouse vascular smooth muscle cells (VSMC) induced a dose-related decrease in calcium release. This suggests that in addition to any role in maintaining membrane composition, PUFA biosynthesis *de novo* is required for calcium release and hence vasoconstriction in response to Pe stimulation in VSMC.

Analysis of the mRNA expression of the four key genes involved in PUFA biosynthesis detected transcripts for FADS1, FADS2 ELOVL5, but not ELOVL2, in mouse VSMC, untreated adult mouse aorta, human primary VSMC and untreated adult rat aorta. All four genes were expressed in liver. Silencing of ELOVL2 transcription was not due to hypermethylation of its promoter in VSMC or aorta. Direct measurement of linoleic acid conversion showed that the same metabolites were synthesised *de novo* in VSMC as in liver. Thus another elongase enzyme may be responsible in VSMC for catalysing the step catalysed by elongase 2 in liver, which suggests the possibility of VSMC-specific regulation of PUFA biosynthesis.

Overall, this study identified a novel pathway in the regulation of vascular tone that has implications for understanding the effect of maternal dietary fat intake on vascular function in the offspring.

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I, Nicola Alice Irvine declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

The role of vascular smooth muscle polyunsaturated fatty acid synthesis in vasoconstriction

I confirm that:

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3. Where I have consulted the published work of others, this is always clearly attributed;
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   Kelsall CJ, Hoile SP, Irvine NA, Masoodi M, Torrens C, et al. (2012) Vascular Dysfunction Induced in Offspring by Maternal Dietary Fat Involves Altered Arterial Polyunsaturated Fatty Acid Biosynthesis. PLoS ONE 7(4): e34492.

   Hoile SP, Irvine NA, Kelsall CJ, Sibbons C, Feunteun A et al. (2013) Maternal fat intake in rats alters 20:4n-6 and 22:6n-3 status and the epigenetic regulation of Fads2 in offspring liver. Journal of Nutritional Biochemistry Jul;24(7):1213-irvine20.

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Irvine N.A., Sibbons C.M., Grenfell L.R., Lilycrop K.A., Hanson M.A. et al. (2014) Polyunsaturated fatty acid synthesis de novo is required for calcium release in vascular smooth muscle. 11th Congress of the International Society for the Study of Fatty acids and Lipids. 2014 June, Stockholm, Sweden.

Signed: ……

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List of Abbreviations:

AA- Arachidonic acid

ACh- Acetylcholine

ALA- Alpha linolenic acid

aPKCζ- Atypical protein kinase Cζ

cDNA- Complimentary DNA

CE- Cholesterol esters

cGMP- Cyclic guanosine monophosphate

CHD- Coronary heart disease

COX-1/COX-2- Cyclooxygenase-1/2

cPKC- Conventional protein kinase C

cPLA2- Cytosolic phospholipase A2

CRC- Concentration- response curve

CVD- Cardiovascular disease

DAG- Diacyl glycerol

DGLA- Di homo gamma linoleic acid

DHA- Docohexaenoic acid

DNA- Deoyribonucleic acid

DOHaD- Developmental origins of health and disease

ECM- Extracellular matrix

EDHF- Endothelial hyperpolarising factor

ELISA- Enzyme-linked immunosorbent assay

ELOVL- Elongation of very long fatty acids

EPA- Eicosapentanoic acid

ET1- Endothelin

FADS1/FADS2- Fatty acid desaturase 1/2

FAMEs- Fatty acid methyl esters

FO- Fish oil

GC- Gas chromatography

GPCR- G coupled protein receptor

GTP- Guanosine triphosphate

HASMCs- Human aortic smooth muscle cells

HDL- High density lipoprotein

Hepa1-6- Mouse hepatoma cells

HETEs- Hydroxyeicosatetraenoic acids

HSO- Hydrogenated soyabean oil

IP3- Inositol triphosphate

LA-Linoleic acid

LDL- Low density lipoprotein

L-NAME- L-Nitro arginine methyl-ester

MLC- Myosin light chain

MLCK- Myosin light chain kinase

MLCP- Myosin light chain phosphatase

MOVAS- Mouse aorta smooth muscle cells

mRNA- Messenger RNA

MUFA- monounsaturated fatty acid

NEFA- Non-esterified fatty acid

NO- Nitric oxide

NOS- Nitric oxide synthase

nPKC- Novel protein kinase C

Pe- Phenylepherine

PGE_2- Prostaglandin E2

PGF_{2a}- Prostaglandin F2a

PKC- Protein kinase C

PLC- Phospholipase C

PUFA- Polyunsaturated fatty acid

RFU- Relative fluorescence unit

RNA- Ribonucleic acid

ROS- Reactive oxygen species

RT-PCR- Real time polymerase chain reaction

SAO- Safflower oil

SFA- Saturated fatty acid

SNP- Single nucleotide polymorphism

SPE- Solid phase extraction

TAG- Triacylglycerol

TP- Thromboxane/prostaglandin endoperoxide receptor

TSS- Transcription start site

TXA_2- ThromboxaneA_2

VSMCs- Vascular smooth muscle cells

Chapter 1-

Introduction

1. Introduction

Cardiovascular disease (CVD) is a major global health problem, accounting for 30% of all deaths. It is not confined to developed countries, but is a leading cause of death in developing countries too, with mid and low income countries contributing 80% of CVD deaths (1). Maternal nutrition during pregnancy has been shown to affect the risk of CVD (2). Excessive fat intake is a common dietary imbalance worldwide and has resulted in an increase in obesity. In 2008, 300 million women were obese (3), this has led to an increase in obesity in women of a childbearing age (4). It has been shown that maternal fat intake can cause persistent changes in vascular function in the offspring (5). Evidence has shown that a high saturated fat diet during pregnancy leads to vascular dysfunction in the offspring and a predisposition to CVD (5). Deficiencies in other types of maternal fat, including n-3 polyunsaturated fatty acids have been associated with increased blood pressure and thus increased risk of CVD in the offspring (6). Typically studies investigate a specific fat type or a high fat maternal diet and the effect it has upon the offspring's cardiovascular function. However, the effect of both quantity and quality of maternal dietary fat on the vascular function of the offspring has not been investigated. In addition to this, the mechanism whereby maternal dietary fat leads to persistent changes in offspring vascular function is unknown. Previous studies have investigated offspring endothelial dysfunction following enriched or deficient maternal diets (7;8). However, very few studies have investigated changes within vascular smooth muscle cells and how permanent changes within these cells could lead to an increased risk of cardiovascular disease within the offspring. It has also been shown that a maternal high saturated fat diet leads to changes in the fatty acid composition of the offspring aorta, specifically arachidonic acid and docosahexaenoic acid (8). The composition changes were proposed to be responsible for the changes in vascular function, however, the mechanism was not explored. Therefore, this thesis is going to investigate the effect of different types and quantities of maternal diet on offspring vascular function and determine whether changes in the vascular smooth muscle cell polyunsaturated fatty acid synthesis are responsible for changes in vascular function.

1.1 Arterial structure and function:

1.1.1 The vascular system:

The vascular system is a complex network of arteries, capillaries and veins that play an important role in the movement of blood through the cardiovascular system. The Cardiovascular system is made up of the heart and blood vessels. The role of the vascular system is to carry blood from the heart and around the body with the crucial role of

maintaining homeostasis. Homeostasis is maintained by removing cellular and metabolic waste products and allowing absorption of essential nutrients and maintaining cellular functions (9).

1.1.2 Blood vessel structure:

The arteries, capillaries, veins, venules and arterioles that make up the vascular system have a specific cellular composition and structure to facilitate their physiological roles within the body. However, most blood vessels share a common basic structure. Generally, the vessels are composed of three distinct layers, tunica intima, tunica media and tunica adventitia (fig 1.1). The tunica intima is the thinnest layer; consisting of a single layer of endothelial cells mounted on a basal lamina that are directly in contact with the blood. Below the endothelial cells there is fibro-elastic connective tissue and a layer of elastic lamina which provide flexibility and stability (9). Around the endothelial cells lie the pericytes which are multipotent stem cells. Pericytes are important for maintaining the endothelium. The endothelial cells do not have their own blood supply, so are reliant upon the pericytes for providing nutrients and a balanced cellular environment. Pericytes are also capable of dividing into many types of cells including smooth muscle cells and connective tissue. The distribution and structure of pericytes differs in different vessel types (10). The tunica media is made up mainly of smooth muscle cells and elastin which can be more highly organised in large arteries as they have to move greater volumes of blood and withstand the pressure generated by the heart (9). Surrounding the vascular smooth muscle cells (VSMC's) is the extracellular matrix (ECM) which consists of elastin and collagen which are important in maintaining tissue structure and function (11). The final layer is the tunica adventitia and this is composed of fibro-elastic connective tissue. Arterial walls are thicker and contain more VSMCs than the cell walls of veins. This is because the arteries are responsible for providing rapid distribution of blood to all tissue and organs throughout the body. Muscular arteries contain a large layer of smooth muscle to ensure complete distribution of blood to all organs. However, elastic arteries which are the arteries present near the heart, such as the aorta, are composed of a high density of elastic tissue and relatively lower amounts of smooth muscle. This is to accommodate large changes in blood volume expelled from the heart (9).

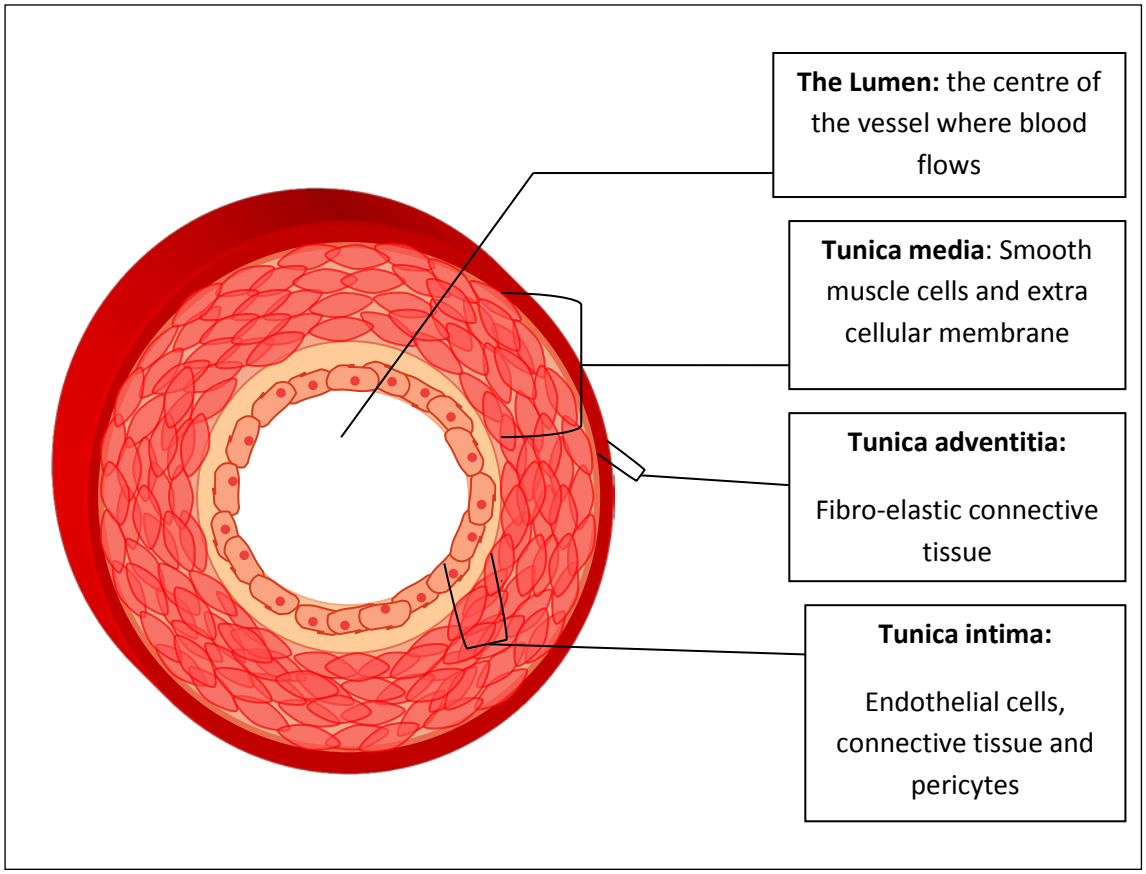


Figure 1.1: A diagram of a blood vessel, highlighting the endothelium and smooth muscle.

1.1.3 Vascular smooth muscle cells:

The VSMCs are important for both structure and function of the blood vessels. They perform two functions, a synthetic function and a contractile function. The primary function of VSMCs is contraction and they are responsible for maintaining blood vessel tone and blood pressure, allowing them to distribute blood around the body. They do this by changing the diameter of the vessel by contraction or dilation which results in a change in blood pressure (12). VSMCs have a phenotype to match their function; in contractile VSMCs a majority of their volume is made up of contractile machinery such as actin filaments, myosin heavy chain and sarcoplasmic reticulum, with synthetic organelles sparse (13). They also express a range of signalling molecules, ion channels and contractile proteins which are necessary for vasoconstriction (14).

VSMC's with a synthetic function are composed mainly of synthetic organelles such as golgi apparatus and rough endoplasmic reticulum and contain relatively fewer contractile filaments (13). The synthesis role of the VSMCs is required to produce ECM such as collagen and elastin which surrounds the VSMC and also to allow proliferation and differentiation. The rate of ECM synthesis is greatest during development and also during injury, with high levels of

synthesis and differentiation to aid repair of the vascular wall (12). In contrast, VSMCs in adults have a low synthetic activity and express contractile proteins, ion channels and signalling molecules required to perform the cell's contractile function (14). However, the VSMCs are not terminally differentiated and retain plasticity, the contraction state and synthetic state are two extremes of a spectrum and there can be a range of intermediate phenotypes at different stages of development and even as in adult organisms (14). The VSMC phenotype can undergo reversible changes in phenotype which depend on environmental cues including inflammatory mediators, growth factors and cell-cell interactions. This is not always beneficial as switching phenotypes can play a role in a number of diseases including atherosclerosis (14).

1.1.4 The endothelium:

The endothelium is a monolayer of endothelial cells that form the inner wall of the vascular system, between the blood vessel wall and the blood stream (fig 1.1). It senses both physical stimuli e.g. pressure, stress and chemical stimuli (hormones) within the vascular system and maintains the homeostasis of the system by releasing modulators or by changing the shape of the vessel (15). The first evidence of the importance of the endothelium in regulating vascular tone was shown by Furchgott et al. who demonstrated that acetylcholine (ACh) induced relaxation of rabbit thoracic aorta and other blood vessels requires the presence of an intact endothelium (16). They proposed that this was due to ACh acting on muscarinic receptors on endothelial cells and thus stimulated the release of substances that cause the vascular smooth muscle to relax (fig 1.2).

The endothelium releases vasoactive factors that control inflammation, modulate hemostasis and regulate vasomotor function as well as contributing to mitogenesis, vascular angiogenesis, vascular permeability and fluid balance. The vasoactive substances include the dilatory nitric oxide (NO), prostacyclin (PGI_2), endothelium derived hyperpolarising factors (EDHF) (17), and the vasoconstrictors; endothelin (ET_1), angiotensin II, thromboxane A_2 (TXA_2) and reactive oxygen species. The balance of these factors modulates vascular tone and thus regulates blood flow and pressure. Additionally, NO, PGI_2, TxA_2, alongside other endothelial factors such as von Willebrand factor, plasminogen-activator inhibitor-1 and fibrinogen, control haemostasis and contribute to normal endothelial function (18).

A healthy endothelium is important in maintaining cardiovascular homeostasis. Dysfunction in the endothelium is a recognised marker of CVD and an important factor in its progression. Endothelial dysfunction is believed to be the first step in atherosclerosis (19).

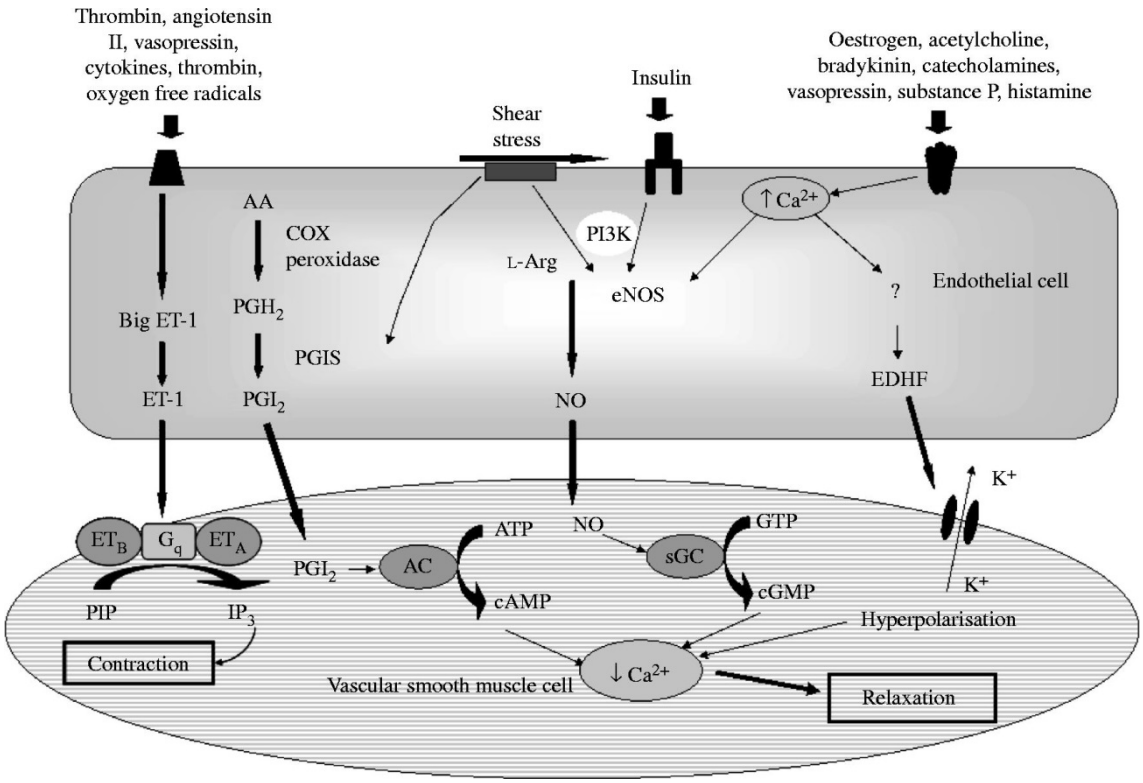


Figure 1.2: Vasoactive factors released by the endothelium and their interaction with the VSMCs to produce vasorelaxation or vasoconstriction. Image from Hall et al. (2009) (20). AA: arachidonic acid, PGH2: prostaglandin H2, PGI2: prostacyclin, ET-1: endothelin, COX: cyclooxygenase, PGI: prostacyclin synthase, L-Arg: L-arginine, eNOS: endothelial nitric oxide synthase, PI3K- phosphoinositide-3 kinase, NO: nitric oxide, EDHF: endothelium-derived hyperpolarising factor, ETb: endothelin receptor A, endothelin receptor B, Gq: Gq protein, PIP:phosphatidylinositol 4,5-biphophate, IP3: inositol 1,4,5 triphosphate, AC: adenylyl cyclase, ATP: adenylyl triphosphate, cAMP: cyclic adenylyl monophosphate, sGC: soluble guanylate cyclase, GTP: guanylate triphosphate, cGMP: cyclic guanylate monophosphate.

1.1.5 Vasoconstriction:

Vasoconstriction is the narrowing of blood vessels resulting from smooth muscle contraction in the wall of the blood vessel. Vasoconstriction is controlled by a range of mechanical, nervous and humoral factors. These factors include noradrenaline from sympathetic nerves, circulating angiotensin II, endothelin-1 (ET_1) and eicosanoids such as thromboxane (TxA_2) and prostaglandin E_2 (PGE_2). There is evidence to show that the vascular smooth muscle is also capable of producing pro-constrictor eicosanoids, such as PGE_2 and Prostaglandin I_2 (PGI_2). However, they produce lower amounts than the endothelium and this is thought to be due to a lower concentration of prostaglandin H (PGH) synthase, which is required to produce PGH_2, this is the precursor for prostaglandins, prostacyclin and thromboxanes (21).

The common pathway of vasoconstriction is through the Gq G protein-coupled receptor (GPCR) and results in the stimulation of phospholipase C (PLC) which catalyses the subsequent formation of inositol triphosphate (IP_3) and diacyl glycerol (DAG) from the membrane lipid phosphatidylinositol 4,5-bisphosphate. Once formed, IP_3 interacts with IP_3 receptors on the sarcoplasmic reticulum which results in the release of Ca^{2+} from the sarcoplasmic reticulum into the cytosol. DAG, in the presence of Ca^{2+} then activates protein kinase C (PKC) which phosphorylates a number of specific target proteins (22). Ca^{2+} also binds to calmodulin which leads to activation of the myosin light chain (MLC) kinase. MLC kinase phosphorylates the MLC which binds to actin and cross-bridge cycling occurs. This causes shortening and contraction of the smooth muscle cell. The contractile activity is primarily determined by the phosphorylation state of the MLC. Myosin light chain phosphatase (MLCP) causes the dephosphorylation of MLC and thus terminates the contraction. A decrease in intracellular Ca^{2+} concentration is also required for termination of the contraction (fig 1.3) (23). phenylepherine (Pe) is a synthetic alpha-1 adrenergic receptor agonist which is a Gq coupled and causes vascular smooth muscle contraction through this pathway (24).

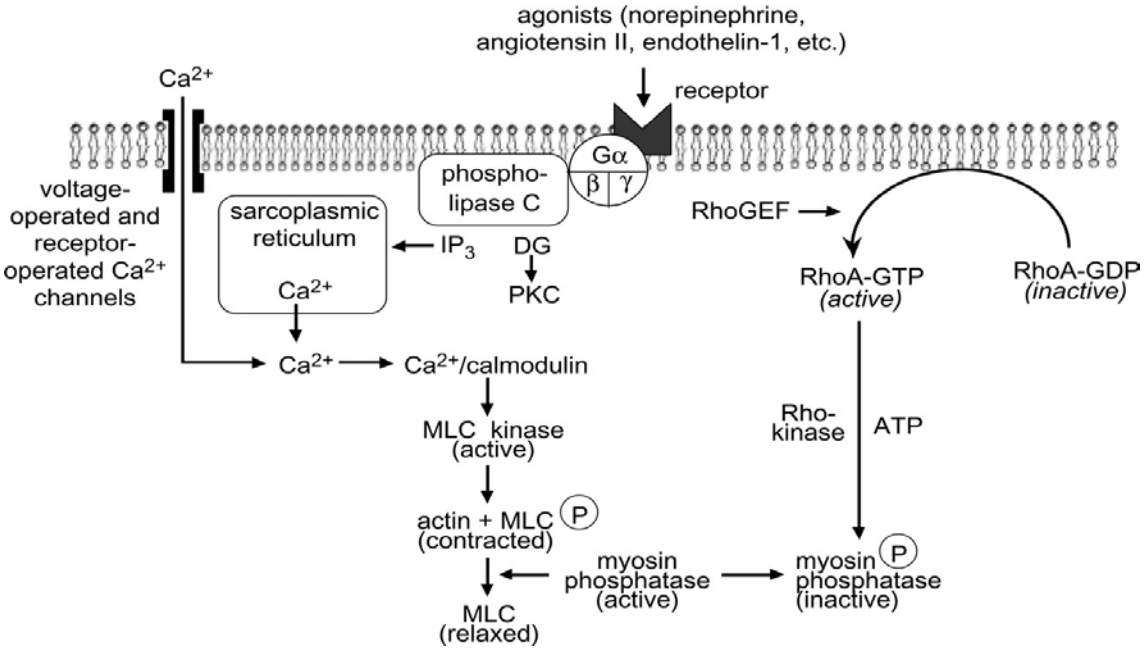


Figure 1.3: Regulation of smooth muscle contraction. Inositol triphosphate: IP3, diacyl glycerol: DG, protein kinase C: PKC, myosin light chain: MLC, guanosine triphosphate: GTP, guanosine diphosphate: GDP, ATP: adenosine triphosphate, Ras homolog gene family member A: RhoA, Rho gunanine nucleotide exchange factor: RhoGef. Image from Webb et al. 2003

Contractile force is maintained by Ca^{2+} sensitisation, this is controlled by the RhoA/Rho kinase pathway. RhoA is a G-protein regulated by guanosine triphosphate (GTP) binding. When RhoA

is activated, the RhoA-GTP complex activates Rho Kinase. Rho kinase can then phosphorylate the myosin-binding subunit of MLCP, which inhibits and prevents dephosphorylation of myosin light-chain (MLC), thus sustaining the contraction (25). Studies have shown that alterations in the activity of the Rho kinase pathway lead to increased vasoconstriction and increased peripheral vascular resistance in hypertension (26;27).

1.1.6 Eicosanoids:

Eicosanoids are signalling molecules and include prostaglandins, thromboxanes, hydroxyeicosatetraenoic acids (HETEs) and leukotrienes (28). Eicosanoids are produced through an oxidative pathway from polyunsaturated fatty acids (PUFAs) produced in the n-3 and n-6 pathway. The fatty acids from which eicosanoids are produced include arachidonic acid (AA), eicosapentaenoic acid (EPA), dihomo-γ-linolenic acid (DGLA) and docosahexaenoic acid (DHA). These fatty acids are released from phospholipids in the cell membrane (synthesis of eicosanoids discussed in more detail in section 1.6.3) (28). The eicosanoids are short lived and very potent molecules which have very diverse biological actions throughout the body. These actions include physiological functions such as regulation of smooth muscle tone, platelet aggregation and vascular permeability. They are also very important for immune and inflammation function within the body (29).

The products of AA eicosanoid synthesis are considered pro-inflammatory, and have atherogenic and pro-thrombotic effects. However, the eicosanoid products from EPA are anti-inflammatory and products which oppose the products of AA, see table 1.1 (30). PGF_{2a}, PGE_2 and TXA_2 are products of AA and have been shown to have pro-arrhythmic actions including increased beating rate, chaotic activity and contractions of myocytes. But Thromboxane B_3 (TXB_3), PGI_3, prostaglandin F_{3a} (PGF_{3a})and prostaglandin E_3 (PGE_3) which are eicosanoid products of EPA have anti-arrhythmic actions (31)

The eicosanoid TXA_2 which is released from the endothelium plays an important part within the vasculature; it diffuses toward the vascular smooth muscle cell and activates the thromboxane/prostaglandin endoperoxide receptor (TP) which is a Gq coupled and acts through stimulation of PLC and production of IP_3 to cause a contraction. It is also thought that activation engages the calcium independent RhoA signalling to maintain the contraction (32). Prostaglandin H_2 (PGH_2), Prostglandin $F_{2\text{-alpha}}$ ($PGF_{2\alpha}$), PGE_2, prostaglandin D_2 (PGD_2) and PGI_2 have also been shown to stimulate the TP receptor and cause vascular smooth muscle contraction (33).

Lipoxins are also produced from AA by a series of reactions mediated by lipoxygenases. Lipoxins are formed during cell-cell interactions, such as platelet-leukocytes interactions and display anti-inflammatory effects and are signalling molecules for the resolution phase of acute inflammatory responses (34). Lipoxins activate mononuclear cell recruitment without stimulating the release of pro-inflammatory chemokines or activation of pro-inflammatory gene pathways. They also stimulate polymorphonuclear leukocytes and activate anti-microbial defence mechansisms and clear mucosal surfaces. These actions of the lipoxins stimulate overall resolution of inflammation (35).

Resolvins and protectins are also two families of lipid mediators which resolve inflammation. Resolvins are produced from EPA and DHA, the E-series are produced from EPA and D-series from DHA. Protectins are also produced from DHA and demonstrate anti-inflammatory and neuro-protective actions (36). Resolvins and protectins both act locally and are potent agonists of endogenous anti-inflammation and are pro-resolving chemical mediators of interest in human disease (34). Resolvins actions include reducing neutrophil traffic, regulating cytokine and reactive oxygen species and lowering the magnitude of the response. Protectins were given their name due to the protective action they have in neural systems, stroke and Alzheimer's disease (36).

Table 1.1: Table showing effects of n-6 and n-3 derived eicosanoids. (30). RvE**:** Resolvin E series, RvD: Resolvin D series, PGE: prostaglandin E, LXA: lipoxin A.

N-6 Fatty acid- AA		**N-3 fatty acid- EPA**		**N-3 Fatty acid- DHA**	
Eicosanoid	**Physiological affect**	**Eicosanoid**	**Physiological affect**	**Eicosanoid**	**Physiological affect**
Thromboxane B_2	Vasoconstriction	**Thromboxane B_3**	Vasorelaxation	**Resolvins** **eg. RvE and RvD**	Anti-inflammatory, regulate leukocyte trafficking and immunoregulatory.
Prostaglandins e.g. PGE_2	Pro-arrhythmic, vasoconstriction	**Prostaglandins e.g. PGE_3**	Vasorelaxation, Anti arrhythmic and decrease platelet aggregation	**Protectins** **PD1**	Anti-inflammatory and neuroprotective
Thromboxane A_2	Increase platelet aggregation and vasoconstriction	**Thromboxane A_3**	Decrease Platelet aggregation		
Leukotriene B_4	Inflammation, chemoattractant	**Leukotriene B_5**	Anti-inflammatory		
Lipoxins **eg. LXA_4 and LXB_4**	Anti-inflmmatory pro-resolving mediators- stop				

1.2 Cardiovascular disease (CVD):

CVD covers a range of diseases of the heart and blood vessels. It is usually a result of vascular dysfunction and a result of atherosclerosis, thrombosis or high blood pressure. CVD diseases include coronary heart disease (CHD), peripheral vascular disease, congestive heart failure, atrial fibrillation and stroke. CHD affects the blood vessels supplying the heart and peripheral vascular disease affects the blood vessels supplying the arms and legs. CVD is associated with the major risk factors of; obesity, poor diet, hypertension, hypercholesterolemia, smoking, ageing and diabetes mellitus (37). CVD accounts for 30% of all deaths in the UK (1), with CHD alone as the most common cause of premature death in the UK (38).

Atherosclerosis is one of the main causes of CVD; it is the underlying disease process in the blood vessels that results in CHD and stroke. It is a chronic, inflammatory disease of the

arterial wall which is initiated by infiltration of lipid particles, endothelial activation, infiltration of macrophages and foam cell formation. The foam cell formation is known as a 'fatty streak' and this is followed by smooth muscle migration and proliferation and deposition of ECM (39). This process leads to lesion formation and ultimately an atherosclerotic plaque and the narrowing of arteries. It eventually leads to a rupture of the atherosclerotic plaque, resulting in a myocardial infarction or stroke (40). The pathology of atherosclerosis begins with endothelial damage which could be caused by shear stress or high blood pressure (41), obesity, smoking and hypercholesterolemia. The endothelial damage caused by these factors promote atherogenesis and result in dysregulation of vascular tone, increased inflammation and thrombosis, this is discussed in more detail in section 1.2.2 (42).

It has been shown that atherosclerotic lesions appear from a young age, they appear in 1 in 6 teenagers and no symptoms are seen from these lesions. These lesions are an accumulation of fatty streaks which contain lipid engorged macrophages and lymphocytes in the intima of the arteries. The fatty streaks may progress and further lipids may accumulate leading to the formation of an atherosclerotic plaque, alternatively the fatty streak may regress (43). Children generally have low cholesterol, but studies have shown that maternal hypercholesterolemia during pregnancy is responsible for alterations in the fetal aorta and children developing fatty streaks that increase their risk of atherosclerosis (44). A study on 204 patients aged 2-39 years of age showed that the prevalence of fatty streaks in the coronary artery increased with age from 50% at ages 2-15 to 85% at 21-39 years olds. The prevalence of raised fibrous-plaque lesions also increased with age from 8% at 2-15 years old to 69% in 26-39 years old. Increased body mass index, blood pressure and levels of serum cholesterol and low density lipoproteins (LDL) were all associated with the increased prevalence and extent of atherosclerosis (45). This is supported by another study which had a larger cohort of 2876 people, aged 15-34 years. This study also showed that the prevalence of fatty streaks also increased with age, raised fatty streaks were present in aorta of 20% of people aged 15-19 years old and 40% of subjects aged 30-34. The percent of intimal surface involved with raised fatty streaks increased with age and was associated with hypertension, obesity and impaired glucose tolerance (46). These studies both show that atherosclerosis prevention should begin in childhood as it is demonstrated that obesity, increased triglycerides and high LDL are associated with accelerated and more extensive atherosclerotic changes in the aorta and coronary artery during childhood (45;46). Therefore, a healthy lifestyle during childhood may prevent or minimise atherosclerotic vascular changes.

1.2.1 Endothelial Dysfunction:

Endothelial dysfunction is a shift from normal function towards reduced vasodilation, a pro-inflammatory state and pro-thrombotic properties (18). It is often characterised by a reduced endothelium dependent vasorelaxation in response to an endothelium dependent vasodilator such as ACh. However, as the endothelium is responsible for producing a number of vasoactive factors, including eicosanoids, a number of other changes can be observed. These changes can include an increase in production of pro-inflammatory molecules and adhesion molecules (47). The imbalance between endothelium derived contracting and relaxing factors can cause a shift towards vasoconstriction, platelet aggregation, vascular inflammation and mitogenesis (48). This can involve a reduction in the bio-availability of nitric oxide (NO) which is a key endothelium-derived relaxing factor. A reduction in NO may be due to a reduction in NO synthesis or increased breakdown (49). NO is a product of the reaction between L-arginine and nitric oxide synthase (NOS). NO activates cyclic guanosine monophosphate (cGMP) causing smooth muscle relaxation by regulating cytosolic Ca^{2+} and thus causing vasorelaxation (see Fig 1.4). NO also has the ability to reduce vascular permeability, platelet aggregation, tissue oxidation, tissue inflammation and activation of thrombogenic factors amongst other roles (15).

1.2.2 CVD and endothelial dysfunction:

A number of studies have documented impaired endothelium-dependent vaso-relaxation in CVD, these include, hypertension, lipid disorders and heart failure, as measured with strain gauge plethysmography (50). The level of endothelial dysfunction also indicates the likelihood of a CVD patient suffering from a cardiac event, which included myocardial infarction, heart failure and surgical or percutaneous coronary revascularisation. The more severe the endothelial dysfunction, the greater the chance of a cardiac event (51). Endothelial dysfunction is an important step in the development of atherosclerosis. The first study to show evidence of this demonstrated that in transplant patients, impaired endothelium-mediated vasodilation precedes the development of coronary atherosclerosis and any angiographic evidence of atherosclerosis (52). These findings were extended in a later study which demonstrated that severe endothelial dysfunction in the absence of obstructive coronary artery disease were associated with an increase in cardiac events. From this study it was hypothesised that endothelial dysfunction represents the stage of rapid atherosclerosis progression, which may be due to the loss of various protective physiological roles of endothelial cells, including a loss of NO bioavailability. In addition to vasodilation, NO also results in inhibited platelet aggregation and adherence, smooth muscle proliferation and endothelial cell-leukocyte

interaction, all of which are anti-atherogenic. Therefore, a reduction in NO bioavailability leads to atherosclerosis progression and inflammation in the vessel wall, which could result in plaque rupture (51). As well as reduced NO, increased reactive oxygen species (ROS) and the release of adhesion molecules, either caused by the dysfunctional endothelium or as a consequence of endothelial dysfunction, also aid in the initiation and progression of plaque formation in atherosclerosis and also trigger cardiovascular events (18). Patients with endothelial dysfunction have a higher incidence of hospitalisation for heart failure and death due to cardiac problems, so endothelial dysfunction is linked to an increase in clinical events and accelerated progression of heart failure (53).

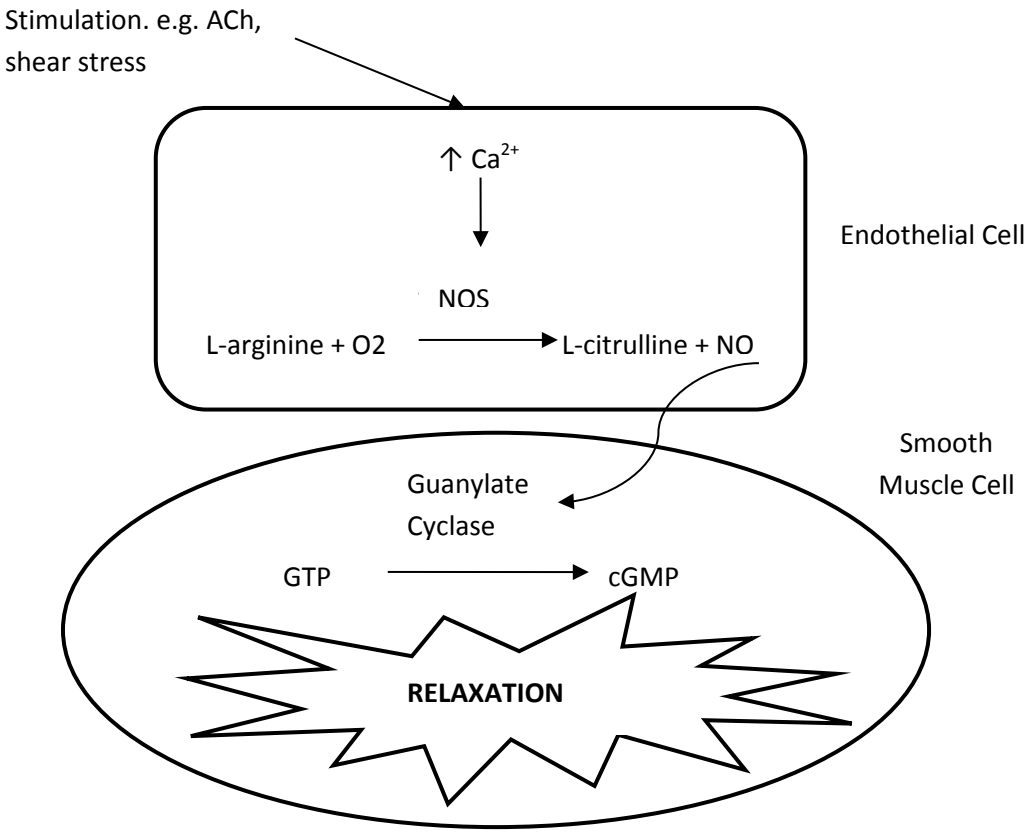


Figure 1.4: The cGMP pathway by which Nitric oxide (NO) induces relaxation of the smooth muscle cells in the cardiovascular system. Abbreviations: Guanosine triphosphate: GTP, cyclic guanosine monophosphate: cGMP, nitric oxide synthase: NOS.

1.2.3 CVD and the role of vascular smooth muscle:

The vascular smooth muscle also plays an important role in the pathogenesis of CVD. Hypertension is a well-recognised risk factor for CVD. One explanation for the cause of

hypertension is due to abnormalities in the VSMCs which lead to increased vascular resistance. Increased vascular resistance is the principle cause of hypertension (54). These abnormalities in the VSMCs alter the contractile state of the cell, this causes abnormal vascular tone and disorders in blood pressure regulation, such as hypertension (26). There is evidence that changes to the contractile state are due to up-regulation in Rho-kinase which causes an increase in calcium sensitisation in vascular smooth muscle cells. This increase in calcium sensitivity leads to increased vasoconstriction and thus vascular resistance, which results in hypertension in humans (27).

VSMC also play a role in the development of atherosclerosis. As discussed in section 1.1.3, the VSMCs in adults are 'contractile' VSMCs which are needed to carry out the vessels contractile function and regulate blood vessel diameter. However, in atherosclerosis, endothelial injury which can be caused by hypertension or diabetes leads to the release of growth factors and cytokines. These factors promote the change in state of the contractile VSMCs into an active synthetic state (14). This type of VSMC can migrate, proliferate and have a greater ability to synthesise ECM, collagen, elastin, growth factors and cytokines. These synthetic VSMCs have a reduced ability to maintain normal contractile function and instead control vascular construction (55). The VSMCs migrate from the media to the intima and proliferate and produce ECM. ECM accumulates over the course of the lesion progression, ECM has also been shown to retain lipoproteins and thus increase the lipid content of the lesions and accelerate progression (56). This leads to the production of an atherosclerotic plaque. VSMCs interact with inflammatory cells such as macrophages and macrophages filled with lipid droplets called foam cells (57). Increased infiltration of inflammatory cells such as macrophages and T-cells produce apoptosis promoting mediators which lead to the apoptosis of VSMCs (58). As well as this, the lesions contain oxidised LDL which induces apoptosis of vascular cells (59). Together, this causes the plaques to weaken and rupture which leads to myocardial infarction and stroke.

Arterial stiffness is described as the reduced capability of an artery to expand and contract in response to pressure change within the cardiovascular system (60). Arterial stiffness is the result of a number of changes involving structural and cellular elements of the vessel wall which are influenced by shear stress, increased luminal diameter and lipids, as well as hormones and glucose regulation. The changes that occur include increases in VSMC and collagen and decreases in elastin in the media, increases in collagen and inflammatory mediators in the intima and increases in collagen and fibroblasts in the adventitia. Endothelial dysfunction and increased endothelial permeability can also lead to arterial stiffness (61).

Arterial stiffness is involved in the development of atherosclerosis and also hypertension, large artery stiffness can be used to predict cardiovascular events (62). Vascular stiffness has long been considered to involve the endothelium (63) and the ECM (64). However, a novel study in rats has demonstrated that aortic stiffness in hypertension is due to stiffness or reduced contractility of individual VSMCs. It has been postulated that the increased aortic stiffness is due to changes in actin cross-bridge cycling within the VSMCs (65). In an earlier study looking at arterial stiffness in aging of non-human primates, older primates had higher stiffness of the aorta compared to younger monkeys. Expression of α-smooth muscle actin was increased in those monkeys with greater aortic stiffness, suggesting that it is the changes in α-smooth muscle actin that are responsible for VSMC stiffness (66). This mechanism may also be responsible for vascular stiffness in other disease states such as hypertension.

1.2.4 Rodent models for studying the vascular system:

The benefits of using rodents as models is that they have a short gestation and lifespan so allow studies to be carried out relatively quickly, they a relatively inexpensive, easy to maintain and breed and they also share many anatomical and physiological similarities with humans (67).The ideal animal model for cardiovascular disease research should have human-like anatomy, hemodynamics and physiology and develop human disease characteristics and complications in a timely or accelerated fashion. No species can fit perfectly into this specification, therefore experimental models can be used to dissect and isolate various factors associated with the regulation of the cardiovascular system, genetic factors and cellular responses to injury (68). There are many advantages for using rodents as animal models for cardiovascular disease including, their size, larger blood volume than that of mice and they are low cost compared to larger animal models. In addition to this, there is a large body of information available describing cardiovascular physiology and pathology as well as disease linked genome sites (69). Rodent models also are susceptible to environmental factors such as diet (70) (71) and stress (72) which modify the cardiovascular system and cause disease-like pathology similar to that seen in humans.

No model mimics exactly all of the symptoms of human disease, but most models mimic some aspect of the human disease. As diseases of the cardiovascular system have many genetic and environmental causes, a number of rodent models may need to be studied to fully understand the pathology (73). Common pathophysiological changes in the human cardiovascular system including hypertension (74), cardiac hypertrophy (75) and heart failure (76), have all been successfully reproduced in rat models. Although it is possible to mimic these human diseases in rodents, some differences between the diseases within the species do

exist. Human diseases such as hypertension often have a slow onset and affects middle aged-old people, however rats often show acute symptoms and at an earlier stage of development compared to humans, hypertension is often seen in young adult rats (77). It is not possible to mimic all cardiovascular disease in rodents. Rodents including rats and mice are resistant to the development of atherosclerosis, however some inbred lines of rats, for example the spontaneously hypertensive rat model, are susceptible to the development of atheroma and result in a similar end points to those of humans (69). Mice generally have high levels of HDL and low levels of LDL and therefore do not develop atherosclerosis. However, murine models such as apo-lipoprotein E knockout model do develop atherosclerotic lesions (78). Through changes to diet it is possible to induce a metabolic phenotype in rodents that is similar to that observed in human subjects, even though it does not progress to atherosclerosis (79). Therefore, it is important and useful to use rodent models to look at specific aspects of disease pathology. Endothelial dysfunction is thought to underlie CVD in humans (80) and has been shown to occur in both rat (81) and mouse models (82), it provides a pathological change which can be measured as a marker of cardiovascular disease. Many studies use animal models to investigate the vascular system and cardiovascular disease. However, the cell culture has replaced some animal studies as this allows the study of a single cell type in the absence of homeostatic mechanisms over a range of experimental conditions.

There are also developmental differences between humans and rodent models. Including the number of young born, rats and mice have large litter sizes, compared to humans which usually give birth to single offspring. This is a disadvantage as this affects the space within the uterus of the rodent, resulting in each pup developing a in a slightly different environment due to differences in nutrition exposure and can also effect the exposure to hormones (79). The effect of litter number is hard to control for in the pre-natal period, but in the post-natal period litter number is reduced to a set number at birth to reduce post-suckling variability. There is also a difference in developmental timing. Rodent models are altricial species, thus are relatively immature at birth compared to humans. This means that rats and mice are born with a poorly developed central nervous system and autocrine system and development of organs continues into postnatal life. Therefore, the vulnerability of the developing systems differs between species; interventions directed at the same stage of gestation are not comparable. However, the altricial nature of rodents can be advantageous as development of certain organs continues into postnatal life, so it is more accessible for experimental manipulation than in species in which the critical window lies earlier in gestation (79). In human hearts at late gestation the cardiomyocytes are terminally differentiated, this is characterised by myofibril assembly, sarcomeric formation and enhanced cellular contractility.

Following this, growth of the myocardium happens by hypertrophy and deposition of ECM. In rats heart cardiomyocytes are still proliferating at birth and complete the maturational switch during the first two weeks of postnatal life (83). Therefore, developmental timing needs to be taken into consideration in studies comparing humans to rodents, especially in maternal feeding studies, as carried out within this thesis. Therefore, the diet is fed throughout pregnancy and lactation to ensure exposure is comparable to humans.

Although many differences exist between humans and rodents, they still provide useful information regarding the vascular system and disease progression. Despite the fact that they do not perfectly mimic human disease, they allow specific aspects and pathways of disease to be investigated and do exhibit a number of aspects of human disease following a range of interventions.

1.3 The Pathways that lead to CVD:

1.3.1 CVD risk factors- Lifestyle:

In many cases the "classical" CVD risk factors play a big part in the development of CVD. The "classical" risk factors include age, high blood pressure, smoking, obesogenic diet, hypercholesterolemia and obesity (37). A person's lifestyle encompasses a number of these risk factors, such as, smoking, poor diet and obesity. It also includes other factors which can lead to CVD such as excessive alcohol consumption and physical inactivity (84). Industrialisation, urbanisation, economic development and market development have accelerated over the last decade and this is having a significant impact on the health and nutritional status of people living in developing countries (85). Whilst standard of living, access to services and food availability have improved, there have been a number of negative impacts, such as increased availability to tobacco, unhealthy diet and decreases in physical activity (84). One key problem is an increase in consumption of energy-dense diets which are high in fat; this is coupled with a decrease in energy expenditure. A decrease in energy expenditure is associated with an increase in motorised transport, less labour-intensive jobs and leisure time which is more sedentary. All of these factors lead to an increase in diet related chronic disease, including CVD (85). It is commonly seen that people often have a combination of three or four risk factors, for example, smoking, drinking and low vegetable and/or fruit intake (84).

Physical inactivity is a major risk factor for CVD, it was estimated that sedentary living is responsible for a 30% of deaths due to CVD in the United States (86). A study by Stampfer et al. demonstrated that middle aged women who did not smoke, were not overweight, maintained a healthy diet, exercised moderately or vigorously for 30 minutes a day and had a moderate consumption of alcohol showed an 80% lower rate of coronary incidence compared women with a poorer quality lifestyle (87). This was a thorough study which used a very large cohort of 84,129 women and a long follow up period of 14 years, which increases the reliability of these data. One limitation however, is that it relies on self-reporting questionnaires which are a less reliable method of data collection, as it can lead to misclassification. But due to the study design this would likely lead to under-estimates of the effect and so the data outcome of the study remains the same. Further evidence to support the role of physical activity and CVD risk is shown by a meta-analysis carried out by Li and Siegrist on prospective cohort studies, investigating physical activity and CVD incidence between 1980 and 2010. They found that a high level of leisure time physical activity reduced the overall risk of CHD and stroke in men and women by between 20-30% compared to low level leisure physical activity, whilst moderate level leisure physical activity decreases the risk by 10-20%. This demonstrates a dose dependent relationship between leisure physical activity and risk of CVD. They also found that moderate level of occupational physical activity may reduce the risk of CVD by 10-20% (88).

Cigarette smoking is another major risk factor for CVD and it is responsible for 140,000 premature deaths from CVD every year. The risk of CVD produced by smoking is measured by relative risk which estimates the ratio of CVD in smokers compared to people who have never smoked (89). A study on 1592 men and women aged 55-74 were selected and risk factors in the development of peripheral arterial disease and coronary artery disease were measured. They found that both conditions were more prevalent in moderate and heavy smokers compared to non-smokers. The relative risk for coronary artery disease was 1.59 for moderate smokers and 1.66 in heavy smokers, whilst the relative risk for peripheral artery disease was 1.87 in moderate smokers and 3.94 in heavy smokers (90). A number of studies have observed that stopping smoking can reduce the risk of CVD; a systematic review looked at 20 studies and assessed the size of the risk reduction achieved by stopping smoking in patients with coronary heart disease. They concluded that there was a 36% reduction in relative risk for patients with coronary heart disease who stopped smoking compared to those who continued to smoke (91).

1.3.2 Diet and CVD:

A person's diet is also a major factor in the risk of developing CVD. As discussed in the previous section, the availability and dietary patterns have changed a lot in recent decades with industrialisation, market development and globalisation; examples of these changes include the growth of the fast food and soft drink industry across the globe. Generally, diets are now becoming much more energy-dense and containing a greater amount of sugar, there is also a shift away from diets high in fibre towards processed food. In developed countries there is also an increase in snacking, take-aways and portion size (92). Between the years of 1977 to 2001 in an American population, the energy intake from sweetened beverages increased by 135%, with a total increase of 278 calories and this was associated with increased portion sizes and an increase in the number of sweetened beverages consumed per day (93). In addition to this, another study investigating dietary patterns between 1977 and 2006 in children from the US, demonstrated that total energy intake has increased by 184 kcal/d in 2-18 year olds. There is also an increase in the portion size of key foods which are commonly eaten at fast food outlets, such as; pizza, hamburgers, cheeseburgers and Mexican fast food, which accounts for up to an extra 150 kcal/d. Consumption of these foods represents 36% of the daily energy intake of this population (94). Fast food is considered energy dense, typically with an energy density more than twice the recommendation of healthy diets and they also contain high amounts of industrially produced trans fat (95). A study on Singaporean people demonstrated that the intake of a western diet which is calorie dense, served in large portions, contains processed meat and is high in cholesterol and refined carbohydrates was associated with an increased risk of coronary heart disease when consumed two or more times per week (96). However, this study is based on mortality from CHD and so one limitation is that they did not investigate lipid or blood profiles of the participants. Another study looked at fast food intake in an Iranian population and also demonstrated that an increased intake of fast food was associated with CVD risk factors, including increased serum triacylglycerides and atherogenic index of plasma (97).

The intake of fruit and vegetables is also a key factor in CVD, the UK government recommends eating 5 portions of fruit and vegetables a day. This is because there is evidence that a high consumption of fruit and vegetables has the ability to reduce the risk of CVD (98). The World Health Organisation (WHO) decided there was enough supporting evidence for fruit and vegetable intake lowering the risk of CHD. Therefore, they stated the recommended daily intake of fruit and vegetables to be 400-500g, which equates to 5-6 portions a day (85). There have been a number of studies investigating the intake of fruit and vegetables and the risk of

CVD. One study used a large cohort to study the relationship between intake of fruits and vegetable and the risk of mortality from ischemic heart disease (IHD). The study covered a cohort of 519,978 men and women across Europe between 1992 and 2000. The study showed that each portion increment of fruit and vegetable led to a 4% lower risk of dying from IHD, those participants that consumed at least 8 portions a day had a 22% lower risk of IHD compared to those participants eating three portions or less a day (99). Another large-scale study investigating 39,876 females over five years also showed that a higher intake of fruit and vegetable were associated with a lower risk of CVD and this was also more evident in preventing myocardial infarctions (100). Studies into food intake are not always the most reliable and are hard to accurately measure as they rely on questionnaires filled in by the cohort. Intake may be overestimated or intake may change during the duration of the study. However, it is hard to measure in any other way. Therefore, these studies are not entirely accurate as they both rely on questionnaire answers. They have tried to take this into account by correcting for this, which may mean the association between fruit and vegetable intake and CVD may be different from that reported and may actually be higher. It is not fully understood what is the protective factor within the fruit and vegetables, but it is believed that it may be down to the combination of fibre, micronutrients and antioxidants.

The content of salt in food is also considered a risk for CVD; this is especially high in a lot of processed food. A systematic review carried out into the association between CVD risk and sodium intake in a number of populations. This demonstrated that sodium intake is linked to CVD. By reducing sodium intake a reduction in the risk of CVD was also seen in the general population and this reduction in risk is greater in susceptible groups of the population, such as those with elevated blood pressure, older patients and those with an increased body mass. The reason salt intake can affect the risk of CVD is due to sodium causing water retention which causes and increase in blood pressure. Controlling blood pressure reduces the risk of CVD (101).

Fat intake is considered to be a classical CVD risk factor. The aim over the past decade has been to reduce fat intake, to reduce the risk of CVD and other health issues. However, this has been comprehensively reviewed and the type of fat is indicated as having a more important role in determining risk of CVD than the total amount of fat in the diet (102). Saturated fat and trans fats have been shown in a number of studies to be associated with an increased risk of CVD, whilst many studies have shown that PUFA and monounsaturated fat can in fact be protective in the risk of CVD (103). Replacing trans fats and saturated fats with monounsaturated fats and PUFAs can be more effective at altering the lipid profile and

preventing CVD than just reducing overall fat intake which has little effect (104). The effect of fatty acids on CVD will be discussed in more detail in section 1.4.1-1.4.3.

1.4 Fatty acids

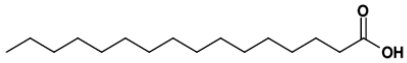
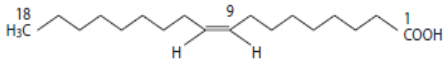
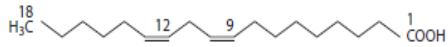
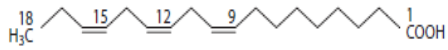
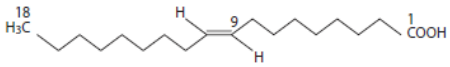
Fatty acids are hydrocarbon chains with a methyl group at one end and a carboxylic acid group at the other (105). Fatty acids are grouped depending on their structure and thus their behaviour; if they contain one double bond they are termed monounsaturated (MUFA). If they contain more than one double bond then they are termed polyunsaturated fatty acids (PUFAs). Those containing no double bonds are termed saturated (SFA). In unsaturated structures such as MUFAs and PUFAS, if the hydrogen atoms are on the same side of the double bond they are termed cis-fatty acids and if the hydrogen atoms are on opposite sides of the double bond they are termed trans fatty acids (see table 1.2) (106). Fatty acids from dietary fats play important roles within the body; they are incorporated into blood lipids and fat deposits (105). In addition, fatty acids are components of biological membranes and are also synthesised into lipid mediators, including eicosanoids which are signalling molecules that regulate a number of physiological responses and pathological processes (28).

Physical and chemical characteristics of the fatty acids are determined by their structure. Saturated fatty acids are stable and have a low reactivity with other molecules such as water and oxygen. The chain length also affects the characteristic of the fatty acid, the melting point of the fatty acid increases with the chain length of fatty acid. The presence of double bonds in the unsaturated fatty acids results in a more reactive fatty acid than the saturated fatty acids. This reactivity increases as the number of double bonds increases (106). Therefore, saturated fatty acids are considered relatively inert compared to unsaturated fatty acid, for this reason unsaturated fatty acids are oxidised more rapidly than saturated fatty acids, long-chain fatty acids are also oxidised at a slower rate than shorter chain fatty acids (107).

Some fatty acids are considered ‘essential fatty acids’, these are structural components of all tissue and are needed for membrane synthesis. The essential fatty acids are the n-6 PUFA; linoleic acid (LA) and the n-3 PUFA; alpha-linolenic acid (ALA) (108). Neither can be made from other substrates in the body so must be supplied from dietary sources (see table 1.2). Humans need LA and ALA to produce highly unsaturated fatty acids through metabolism made up of a series of desaturation and elongation steps which occur in the liver (see fig 1.7)(109). The products of this process of reactions are a number of derivatives which

have potent biological activities and are essential for optimal development of a range of tissues (110). Specialised membranes such as heart myocytes and synaptic terminals contain high amounts of arachidonic acid (AA) (n-6). As discussed previously AA can also be metabolised to produce a number of eicosanoids. Docosahexaenoic acid (DHA) (n-3) is found in phospholipids, structural lipids for membranes, receptors, sperm and cardiomyocytes. A change in fatty acid composition of the membrane can lead to changes in fluidity, membrane thickness, lipid-phase properties, membrane microenvironment and protein interactions (106).

Table 1.2: Summary of the different types and properties of each type of fatty acid. (Information in table from: (106))

Type of fat	Example structure	Properties
Saturated (SFA)	Palmitic acid: 16:0 	• Less readily oxidised • Classified into subclasses depending on the length of the chain. • Found in milk fats, vegetable oil and animal fats. • Example: palmitic acid
Mono-unsaturated **(MUFA)**	Oleic acid: 18:1n-9 	• Obtained from animal, vegetable and marine oil. • Common examples are palmitoleic acid, vacceneic acid and oleic acid.
Polyunsaturated **(PUFA)**	Linoleic acid (LA): 18:2n-6  α-Linolenic acid (ALA): 18:3n-3 	• 12 different families depending on double bond position. • Most important in human health and nutrition are the n-6 and n-3 families. • Linoleic (LA) is the parent of the long chain n-6 PUFAs. • n-6 from vegetable oils. Eg. Safflower, sunflower and soy-bean oil. • α-Linolenic acid (ALA) is the parent of the long chain n-3 PUFAs. • LA and ALA Cannot be interconverted, this can only be done in plants. • n-3 found in soybean oil, flaxseed oil, fish oils, marine fish.
Trans	*trans*-oleic 9t-18:1 	• Small amounts occur in nature. • Also arise from technological treatment, such as hydrogenation of oils. • Human diets contain naturally occurring trans-fats and those from hydrogenation.

1.4.1 Saturated fats and cardiovascular function:

Butter and lard are examples of foods containing high proportions of saturated fat; high intakes of saturated fat have been linked to an increased risk of CVD (111). Replacement of saturated fat with PUFA has been shown to lead to a reduction in low density lipoprotein (LDL)

and the LDL/High Density Lipoprotein (HDL) ratio (112) resulting in a reduction in the risk of CHD (113). In another study investigating the effect of saturated fat on CVD in humans, it was demonstrated that a single meal of containing a high concentration of saturated fat was enough to have an impact on cardiovascular function. This study showed that following a high saturated fat meal there was a decrease in the anti-inflammatory effects of HDL and a non-significant trend towards impaired endothelial function (91). However, this study was only carried out on a small cohort of 14 people who did not suffer from any cardiovascular risk factors or disease. Therefore it is hard to extend these findings to 'at-risk' groups of people and the small cohort is also a disadvantage of the study. However, if this was carried out on a bigger cohort, more significant data may be achieved with regard to endothelial function (114).

Endothelial dysfunction has also been shown to be a result of a diet high in saturated fat and thus may lead to CVD. Reduced flow mediated dilatation is one indication of endothelial dysfunction and this has been shown to deteriorate with a high saturated fat diet fed for 3 weeks (92). However, this study had a limitation which brings question to its validity; around 3-5% of trans fat was present in the saturated fat diet which may have added to the endothelial dysfunction. So this raises doubt over how much of an affect the saturated fat had on the flow mediated dilation. However, these data is supported by another study which showed that a high saturated fat diet caused a 27% decrease in flow mediated dilation in humans. This study is better as it did not contain any known trans fat, so there is no confusion of whether the trans fat is contributing to the decrease in flow mediated dilation and it can be concluded that the effect is due to saturated fat (116).

It has also been suggested that saturated fat can induce an inflammatory reaction; similar to trans fats. Saturated fat can induce nuclear factor κB (NFκB) activation, expression of cyclooxygenase-2 (COX-2) and other inflammatory markers through activation of toll like receptors (117). A study by Seo et al. has shown that saturated fat increases the uptake of cholesterol in the arterial wall of mice which accelerates the initial development of atherosclerosis. This is another example of saturated fat negatively affecting vascular function (118).

Recently it has been suggested that current nutritional guidelines on the risk of saturated fat on CVD may need re-assessing. The outcome of a large meta-analysis of 32 observational studies, covering 530,525 participants did not support cardiovascular guidelines which advise a reduction in the consumption of saturated fatty acids. The meta analysis showed no association between consumption of total saturated fatty acids and coronary risk in human studies measuring dietary saturated fatty acid intake by self-report questionnaires. In

addition to this, there was no association between saturated fatty acid biomarkers in the blood and coronary disease, an inverse association was observed between circulating margaric acid and coronary disease. Margaric acid is associated with milk and dairy fat consumption. This suggests that fatty acids which reflect milk or dairy consumption may have a less deleterious effect in the risk of coronary heart disease. However, there are some limitations of this study which raises questions of its reliability and may provide a reason for the lack of association between saturated fatty acid intake and coronary risk. Many of these studies in the meta-analysis relied on self-report questionnaires, which are open to biases especially regarding snacks which are high in saturated fat. This may be responsible for lack of association in self-reported studies of saturated fat and CVD. The study of biomarkers may provide more accurate assessment of consumption compared to questionnaires. However, there is also uncertainty with studies looking at circulating saturated fat, as this represents both fatty acids from consumption and those synthesised de novo, both of these different sources can have different associations with coronary heart disease (119). Therefore, there are a number of limitations within this study and so there is no clear evidence and it is not a conclusive study. Nutritional guidelines will not be changed without more firm evidence as many studies including those shown above have demonstrated the effects of saturated fat on endothelial function and cholesterol, all of which are risk factors of CVD.

1.4.2 Trans fats and cardiovascular function:

There is increasing evidence that consumption of trans fatty acids increases the risk of CVD (120). A meta-analysis showed that a 2% increase in energy intake of trans fats was associated with a 23% increase in CVD in humans (121). Trans fats have a number of effects on the body which are responsible for their role in CVD. A meta-analysis of four prospective cohort studies, which in total included 140,000 participants, demonstrated that with each 2% increase in energy intake of TFA there was a 23% increase in the incidence of myocardial infarction and CHD death in humans. The risk of CHD was even greater when the retrospective case-control studies were taken into account for the meta-analysis (122).

High intake of trans fats has been linked to endothelial dysfunction. In a study by Lopez-Garcia et al. it was demonstrated that human subjects who had a high intake of trans fats exhibited increased plasma concentrations of E-selectin, soluble intercellular and vascular cell adhesion molecules (sICAM-1 and sVCAM-1). These are all biomarkers of endothelial dysfunction. The proposed biological mechanism for this is that the trans fats are incorporated into the endothelial cell membranes. This incorporation leads to altered vascular tone, hyper-adhesiveness and production of inflammatory markers. Another suggested reason is that

intake of trans fats may lead to a reduction in HDL (123). This study by Lopez-Garcia et al. was a very large cohort study. Although there were limitations with working with such a large cohort, such as the reliability of the questionnaires, measures were taken to overcome these, which add to the dependability of the study. To improve reliability of the questionnaire, repeated questionnaires were carried out which reduced within-person random error, the intake of trans fat was also accurately worked out by considering type and brand of foods listed. To more accurately account for trans fat intake biomarker measurements were taken and these remained stable during the study. Therefore this study is a thorough one and strongly supports the adverse effect trans fats have on endothelial function and the risk for CVD. In support of the role of trans fat causing reductions to HDL, Mensink et al. showed that trans fatty acids had the greatest effect out of all the fatty acids of raising the total:HDL cholesterol ratio in humans. This is a predictor of CHD (124). This is consistent with observations in another study that showed that trans fatty acid consumption caused a raise in LDL cholesterol, reduced HDL and increases the ratio of total cholesterol to HDL cholesterol (125). Another study demonstrated that trans fatty acids impair NO production in human endothelial cells, which supports the understanding that trans fatty acids cause endothelial dysfunction. This study also showed that the endothelial cells also had increased activation of NF-κB when treated with trans fats. This is a sign of vascular inflammation (126). It has also been demonstrated that trans fats promote inflammation, such as activation of the tumour necrosis factor α (TNFα) system and also increased levels of interleukin-6 and C-reactive protein (CRP) (123). In patients with heart disease, membrane levels of trans fats were associated with increased levels of inflammatory biomarkers (127). The changes in endothelial function and also increases in systemic inflammation together and separately increase cardiovascular risk and the likelihood of developing CVD. Information from a meta-analysis shows that calorie for calorie; trans fatty acids have a stronger association with CHD risk than SFA, MUFA and PUFA. The meta-analysis was carried out on four prospective cohorts for TFA and two prospective cohorts for SFA, MUFA and PUFA. Together the studies demonstrated that the risk of CHD is far greater when carbohydrate was isocalorically replaced with trans fatty acids than when it was replaced with SFA, MUFA or PUFA (128).

As discussed previously, there are two main sources of trans fat; naturally occurring trans fats which occur in meat and dairy products and those industrially produced by the partial hydrogenation of vegetable fat (106). It has been suggested that natural and industrially produced trans fats may have different effects on the risk of CVD. A study on 3686 participants over 18-years, demonstrated that the was no association with ruminant trans fatty acid and CHD. In women there was an inverse association between ruminant trans fatty acid and risk of

CHD, however this association was not seen in male participants. From this study it was concluded that ruminant trans fats are not associated with CHD and may be protective against it (129). In addition to this study, a meta-analysis was carried out on all the prospective cohort studies which assessed ruminant or industrial trans fat and CHD in adults. This study concluded that total trans fat was associated with a 20% increased risk of CHD. Specifically the meta-analysis showed that industrial trans fat may increase the risk of CHD and ruminant-trans fat did not affect the risk of CHD and may be protective (130). However, there are a number of problems with this outcome; the meta-analysis is based on very few studies, as few cohort studies have investigated the association between ruminant-trans fatty acids and industrial-fatty acids. In addition to this, ruminant trans fatty acid is consumed in much lower quantities than industrial trans fatty acids, it accounts for 3-6% of total fatty acids in dairy products and 3-9% of total fatty acids in beef and lamb (131), whilst partially hydrogenated fat may contain up to 60% of trans fatty acids (132). Therefore, it would be difficult to consume high enough amounts of ruminant trans fatty acids. Also, the dairy products which contain the trans fat may be cardio protective themselves. Therefore, there is not enough reliable or consistent data to provide conclusive evidence for the differential effects of ruminant trans fat and industrial trans fat on CVD, however, the effect of total trans fat and increased risk of CVD remains conclusive.

1.4.3 N-3 and n-6 polyunsaturated fats and cardiovascular function:

Polyunsaturated fatty acids (PUFAs) are considered to be cardioprotective, a higher intake of PUFA is associated with a decreased risk of CVD (120). n-3 PUFA studies have been thoroughly reviewed and they are believed to have beneficial effects on CVD, this is thought to be through different biological pathways (133). However, the effect of n-6 fatty acid on CVD is more controversial (134). LA is the most abundant PUFA in most diets; it is 5-10 fold more abundant than ALA. ALA is 10 fold more abundant than EPA and DHA (135). There are variable effects from consuming high levels of LA, some studies find that it is cardioprotective whilst others claim that that it could be pro-inflammatory. Therefore, an n-6 PUFA intake of above 5% and around 10% of total energy is advised (136).It has been shown in a study of 4584 human participants that the greater the intake of LA, the lower the risk of cardiovascular disease. This study also looked at LA and ALA together and found the combined intake was associated with a greater decrease in cardiovascular risk (137). A study using pooled data of 11 cohort studies found that substituting saturated fats with PUFAs, which mainly consisted of n-6 PUFAs, resulted in a decrease in coronary heart disease in humans (138).

The cardioprotective effect of N-6 PUFAs is thought to be due to its cholesterol lowering effects. N-6 PUFAs have been shown to have hypocholesterolaemic properties, by lowering LDL cholesterol and increasing the HDL concentration. A large meta-analysis of 60 controlled trials in humans, has shown that replacing carbohydrates with n-6 PUFAs caused the biggest change to total cholesterol:HDL-cholesterol ratio and LDL cholesterol concentrations compared to other fatty acids and is therefore associated with a decreased risk of CVD (139). This is supported by another study which showed that replacing saturated fat with n-6 PUFAs lowered total cholesterol by 19% and LDL cholesterol by 22% (140).

N-6 PUFAs are the main precursors for eicosanoids which are involved in inflammatory responses and pro-aggregatory actions. Therefore, high n-6 PUFA intake may increase the risk of CVD (136). It is also suggested that a higher intake of n-6 PUFAs may lead to competition between n-6 and n-3 PUFAs. Both pathways require the same desaturase and elongation enzymes for production of longer chain fatty acids such as AA and DHA. This could result in a reduced production of anti-inflammatory molecules from n-3 PUFAs (141). The prostaglandins PGE_2, PGI_2 and TXA_2 are all derived from AA and are more pro-inflammatory than the EPA derived prostaglandins PGE_3, PGI_3, and TXA_3 (142). PGE_2 induces cyclic oxygenase2 (COX-2) and increases its own synthesis and the production of pro-inflammatory cytokines (143). TXB_2 is a potent vasoconstrictor and platelet activator whereas TXB_3 inhibit TXB_2-mediated platelet aggregation and promotes vasodilation. Leukotriene B_4 (LTB_4) which is produced from n-6 PUFAs, increases vascular permeability, is a chemotactic agent for leukocytes and accelerates reactive oxygen species production and also leads to the production of inflammatory cytokines like interleukin-1 and interleukin-6 (142). However, not all of the products of AA are pro-inflammatory, PGE_2 has been shown to have both pro and anti-inflammatory effects, it has been shown to inhibit 5-lipoxygenase and thereby decrease the production of pro-inflammatory leukotriene 4 series species (144). PGE_2 also induces 15-lipoxygenase which promotes the formation of lipoxins. Lipoxins are formed from AA, they display anti-inflammatory effects and are signalling molecules for the resolution phase of acute inflammatory responses (34). Lipoxins activate mononuclear cell recruitment without stimulating the release of pro-inflammatory chemokines or activation of pro-inflammatory gene pathways (35). Therefore, lipoxins are beneficial products from n-6 PUFAs and resolve inflammation, which is contrary to the belief that n-6 PUFAs are pro-inflammatory. There is little evidence for high intakes of n-6 fatty acids being associated with elevated levels of inflammatory markers. A study investigating the effect of a combination of n-3 and n-6 PUFAs on inflammatory markers in humans showed that n-6 PUFAs did not inhibit the production of anti-inflammatory markers. However, those with high levels of n-6 and very low levels of n-3

had the highest level of inflammatory markers. When high amounts of both n-6 and n-3 were taken together, these participants had the lowest levels of inflammatory markers. Therefore, this study does not support the hypothesis that n-6 PUFAs antagonise the effect of n-3 PUFAs (145). This study does however support the findings of Djouss et al. which were discussed previously. They showed a combination of n-3 and n-6 PUFAs was associated with a decreased risk of CVD (137). In contrast to these findings, another study showed that intake of n-6 and n-3 PUFAs had no effect on inflammatory cell numbers. However, it does show that n-3 PUFAs can reduce markers of endothelial activation in humans (146). Therefore, the role of n-6 PUFAs in CVD risk remains unclear as not all studies are consistent. It has been demonstrated that they reduce cholesterol and studies have shown that the hypotheses regarding n-6 PUFAs increasing inflammatory markers are not correct and some products of n-6 PUFAs can in fact have anti-inflammatory effects, such as in the production of lipoxins.

As briefly discussed previously, consumption of n-3 PUFAs are also considered to be protective against CVD (147). High proportions of n-3 fatty acids in serum lipids are associated with a lower risk of death in patients with coronary artery disease (148). There are many factors which may be accountable for the protective effect of n-3 PUFAs, these include, lowering triacylglycerol (TAG) concentrations, chemo-attractants, growth factors, adhesion molecule production and they are anti-inflammatory (110). A human study investigating fish oil intake in patients with hypertriglyceridemia showed that intake of fish oil decreased triglyceride concentrations and resulted in an increase in HDL. However, this study was carried out on a relatively small cohort of only 21 patients and so a bigger cohort would be better to confirm this observation (149). However, a meta-analysis of 21 trials, which is more comprehensive than the smaller trial, investigated n-3 PUFA intake and triglyceride concentration. This supports the beneficial effect of n-3 PUFA intake on triglyceride serum content (150). The suggested mechanisms for n-3 PUFAs lowering triglyceride concentrations may be due to a combination of decreased hepatic secretion, coupled with enhanced clearance of triglycerides from the plasma (151).

N-3 PUFAs are also found to have favourable effects on blood pressure. A large and comprehensive meta-analysis examining thirty-six human trials demonstrated that fish oil reduced blood pressure and the greatest effect was seen in older and hypertensive individuals. The mean duration trial duration was 11.7 years and used a dose of 3.7 g/d of n-3 fatty acids (152). Another study on human females with metabolic syndrome showed that intake of fish oil displayed a reduced blood pressure; this was coupled with an increase in NO. The increase in NO is presumed to be responsible for the reduced blood pressure as it has a vasodilatory

effect (153). However, a limitation of this study was that it was based on a small cohort. But studies on a larger cohort also show decreases in blood pressure with n-3 PUFA intake. Changes in blood pressure were even seen at moderate levels of n-3 PUFA intake, which is far more achievable in normal life (154). A typical recommendation of n-3 PUFAs are 0.2-0.65 g/day for general good health and 1 g/day for prevention of myocardial infarction (155). Many intervention studies which show the beneficial effects of n-3 PUFA supplementation on blood pressure use doses of 4-5g/day, which is very hard to achieve, even when a daily fish meal is consumed (156). The study by Ramel et al investigated salmon consumption three times a week, which provides a daily amount of 1-1.5 g/day of n-3 PUFA. Regular intake (salmon three times a week) of oily fish and thus n-3 is easier to sustain as it requires a less drastic change in dietary habits than consumption of oily fish every day and it still provides a decrease in diastolic blood pressure (157). N-3 PUFAs have also been shown to cause a reduction in arterial compliance in humans. This is another mechanism in which blood pressure and CVD risk can be reduced (158). In addition to this n-3 PUFAs have been shown to improve endothelium-dependent vasorelaxation, via a NO-dependent pathway (159). This is supported by a study which shows that intake of n-3 fish oil has beneficial effects on endothelial function, even in healthy subjects. This study was a thorough study which had a relatively large cohort and the level of n-3 in the diet was that of an achievable amount (160).

N-3 fatty acids are thought to exert effects at many of the steps associated with CVD, a number of mechanisms have been suggested to contribute to these protective effects of n-3 fatty acids. One suggested mechanism is by increasing availability of n-3 fatty acids in the body which results in higher concentrations of n-3 fatty acids in cellular phospholipids compared to AA and so a greater production of anti-inflammatory and vasodilatory lipid mediators. As some products of AA metabolism are pro-inflammatory, pro-arrhythmic and promote vasoconstriction, including TXA_2 which promotes platelet aggregation and vasoconstriction and prostacyclin PGI_2, which is pro-arrhythmic. So, an increase in n-3 long chain PUFAs decreases TXA_2 and PGI_2. EPA acts as a substrate for COX-2 and produces TXA_3 and PGI_3. TXA_3 has a less potent pro-thrombotic activity and PGI_3 is anti-aggregatory. Therefore a less thrombotic environment is produced, so n-3 PUFAs are considered anti-thrombotic. Other suggested mechanisms include n-3 PUFAs having anti-arrhythmic properties and also anti-inflammatory effects (110). Previous studies have shown that a fish oil supplementation in humans leads to decreased production of PGE_2 which is a pro-constriction eicosanoid (161), this was also seen in another study whereby a fish oil diet inhibited plasma concentrations of both PGE_2 metabolites and TXA_2 (162). In addition to these studies, a randomised controlled study on men with increased risk of CVD, were fed fish oil or daily fish and the effect on platelet

aggregation and platelet thromboxane was observed. It showed that n-3 fatty acids reduced collagen induced platelet aggregation and platelet activating factor regardless of how the n-3 fatty acids were consumed. The study also showed that dietary n-3 fatty acids also reduced platelet TXB_2 release from collagen aggregated platelets (163). This group also demonstrated that subjects having consumed an n-3 diet had a reduction in both systolic and diastolic blood pressure compared to control subjects (164) and improvements in triglycerides and lipoprotein profiles (165). In addition, resolvins and protectins also play a role in the beneficial and anti-inflammatory effects of n-3 PUFAs on CVD. Resolvins and protectins are produced from DHA, both act locally and are potent agonists of endogenous anti-inflammation and are pro-resolving chemical mediators of interest in human disease (34). As atherosclerosis is an inflammatory disease of the vascular wall, it has also been shown that the biosynthesis of the pro-resolving lipid mediators; resolvin D1 and protectin D1, exert potent agonist actions on macrophages and vascular endothelial cells that control the inflammatory response in atherosclerosis in humans. Therefore, these mediator have a beneficial effect on controlling atherosclerosis progression, failure of these pro-resolving mechanisms may underlie the inflammation observed in atherosclerosis (166). Another study has shown that resolvins and protectins confer a switch of VSMCs to a protective phenotype and demonstrated that resolution failure may be directly related to atherosclerosis (167). This shows that n-3 PUFA's have a number of beneficial effects on the CVD risk.

Most studies that investigate the effects of n-3 PUFA and the potential protective effect for CVD look at long chain PUFA, particularly EPA and DHA (168). However, fewer studies have investigated how ALA relates to CVD outcomes. As previously mentioned, ALA is an essential fatty acid that is not synthesised by humans and is obtained from plant sources such as soya beans, canola oil and walnuts (169). A study that involved feeding a diet high in ALA to humans had cardioprotective effects, increases in serum levels of ALA, EPA and DPA were observed as well as decreases in lipid and lipoprotein levels and decreases in VCAM-1 and pro-inflammatory c-reactive protein. The study concluded that ALA decreases the risk of CVD by inhibiting vascular inflammation and endothelial activation in addition to its lipid lowering effects (170). However, this study only observed these changes in a small cohort or 20 men and 3 women. In an 18 year follow up study of 76,763 women, it was shown that higher intakes of ALA may reduce the risk of sudden cardiac death, but was not associated with any other types of fatal coronary heart disease or myocardial infarction. Women with the highest intake of ALA had a 38% to 40% lower risk of sudden cardiac death. As the effects of ALA were so specific in this cohort, the effect may be due to ALA having antiarrhythmic properties (171). The antiarrhythmic properties of ALA have previously been demonstrated in

a study on dogs, whereby ALA prevented fatal ventricular arrhythmias in a dog model of sudden cardiac death (172).

In contrast to these findings, a cohort study of 3277 women and men showed no association between the intake of ALA and the risk of ischaemic heart disease. The reason no effect was seen may be due to the competitive actions of LA for delta-6 desaturase in the PUFA biosynthesis pathway (173). In another study on a cohort of men expressing a high-risk atherogenic lipoprotein phenotype, the effect of an ALA diet compared to a fish oil diet on markers of CVD was explored. Fish oil produced changes in plasma lipids and LDL, but these changes were not reproduced by ALA and so did not have the same beneficial effect on CVD, although it did not have a negative effect either (174).

Therefore, the benefits of ALA on CVD outcomes are inconsistent. A meta-analysis to assess the effect of ALA on CVD risk which included 27 studies and 251,049 people demonstrated that ALA is associated with a moderately lower risk of CVD (169). However more large clinical trials are needed to provide more information to evaluate the effects of ALA on CVD.

1.5 The developmental origins hypothesis and CVD:

CVD is usually considered an adult disease, however, it is now understood that the pathology of the disease begins in childhood (175). More recently the importance of early developmental environment has been demonstrated in the risk of developing CVD. Offspring of low birth weight (a surrogate marker for fetal nutrition and fetal growth) are found to be at greater risk of developing CVD, suggesting that poor uterine environment predisposes people to CVD (176). An increase in birth weight by 1 kg is associated with a reduction in CVD risk by 10-20% (177). This concept of under-nutrition during development leading to disease, specifically CVD in later life is termed as the 'Developmental origins hypothesis '.

The first evidence that CVD may originate in utero and during infancy came from a study carried out on a cohort of men from Hertfordshire. The study showed that death rates from coronary heart disease fell between those with lowest and highest weights at 1 year (178). This finding was supported by a later study which confirmed these findings. This study also showed that low birth weight, which reflects fetal under-nutrition, is linked to cardiovascular disease later in life in both men and women (fig 1.5) (2). This observation led to the hypothesis that environmental factors, particularly nutrition, act in early life to prime the

fetus to adverse health outcomes in adult life (178). One example of this in humans is The Dutch Hunger winter cohort which shows that offspring exposed to famine during early gestation had an increased risk of CHD (179). Links have been found between early nutritional environment and CVD risk factors, diabetes and the metabolic syndrome in adulthood (180).

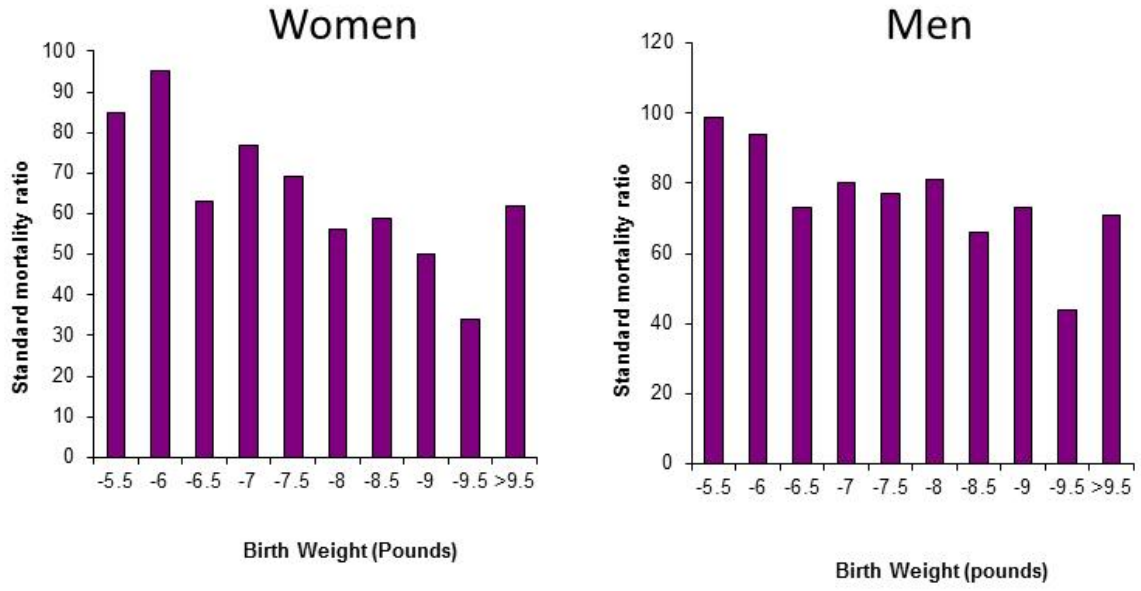


Figure 1.5: Standardised mortality ratios for coronary heart disease in men and women compared to birth weight. (2)

The 'thrifty phenotype' hypothesis was put forward as an explanation for the observation that low birth weight was associated with increased risk of disease in adult life. It was proposed that when there is intrauterine deprivation the fetus will grow more slowly, optimising organ growth and leading to permanent changes which increase the chances of short term survival (181). This was later expanded upon to account for certain adaptations which do not have an immediate survival advantage. This predictive adaptive response (PAR) hypothesis suggests the fetus can make physiological changes based on the predicted postnatal environment in response to maternal cues. While such changes have little short term benefit they confer a survival advantage to offspring in the postnatal environment, allowing the offspring to optimally thrive (182). However, the developmental changes which are proposed by both the thrifty phenotype hypothesis and the PAR theory may be disadvantageous if the postnatal environment is 'mismatched' to the pre-natal environment, predisposing the offspring to adult disease (fig 1.6).

This risk of disease depends on the degree to which the environments are mismatched. This risk of disease is due to the inability of the offspring to respond to the

challenges posed by the environment (183). The adverse intrauterine environment leads to fetal adaptations that include; insulin resistance, altered exercise and feeding behaviours, reduced skeletal muscle mass, central fat deposition, reduced vascularity in some tissues and alterations in the hypothalamic-pituitary adrenal axis. This is to prepare the fetus for a deprived environment in postnatal life in order to aid survival. It does this to conserve resources via reduced growth and diverts substrates from metabolism to fat stores to provide energy (184). When offspring are born to a richer environment than predicted, which is seen with modern diets that are high in calories and fat, a great deal of 'mismatch' occurs. The offspring are not are not adapted to cope with this environment and are more susceptible to disease. This can lead to obesity, diabetes, hypertension, metabolic syndrome and cardiovascular disease (185).

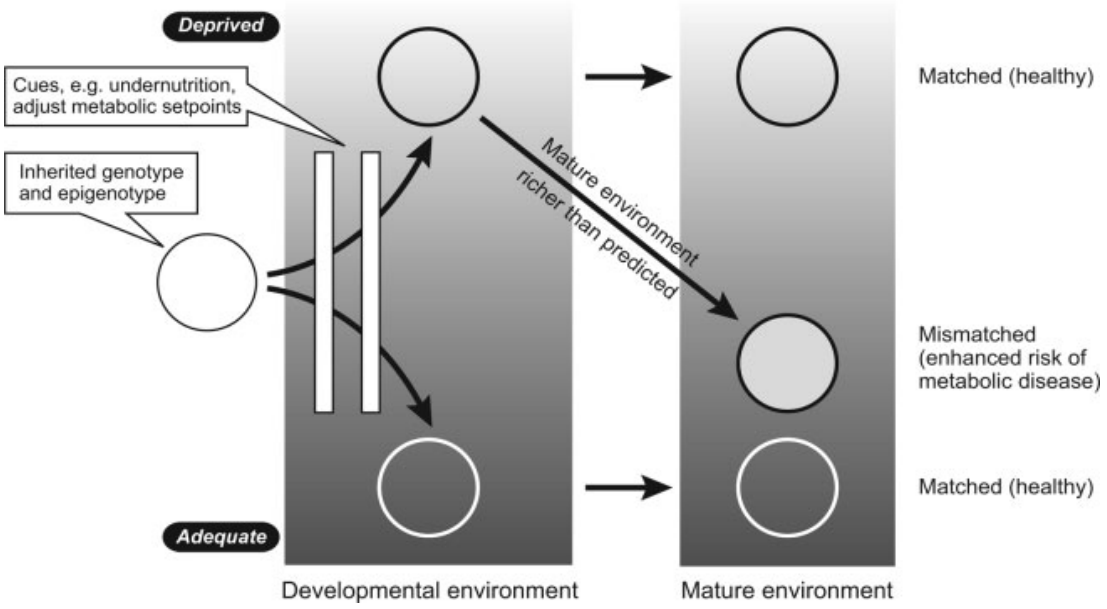


Figure 1.6: A model to show the mismatch concept. (183).

1.5.1 Animal models demonstrating DOHaD and CVD

The human epidemiological studies such as the Hertfordshire cohort study and The Dutch hunger winter cohort have provided much of the evidence to support the developmental origins hypothesis. But these epidemiological studies often rely on historical data and on studies in which maternal nutrition has not been measured. Animal models are therefore both invaluable and necessary for investigating the underlying mechanisms of how offspring are more susceptible to disease. Animal models allow manipulation of diet, allow studies of the full life span and following generations and allow more invasive measurements to be taken. Therefore, animal models are important for demonstrating the biology behind the human observations and supporting the developmental origins hypothesis.

Various animal models have been developed to investigate the developmental origins hypothesis and the changes to physiological function that are associated with an increased risk of CVD. Most models have investigated the developmental origins hypothesis by manipulating maternal dietary intake. Studies have investigated maternal undernutrition and/or postnatal high-fat on CVD. It has been shown that global undernutrition; rats fed a restricted diet of 30% diet less than the control, led to offspring significantly smaller at birth than control offspring. These offspring, which after birth were fed an ad libitum diet, also exhibited a significantly elevated blood pressure during adulthood, a risk factor for CVD (186). This finding is consistent with the observations in humans from The Dutch winter cohort, as previously discussed. It is a very useful study as it provides direct experimental support for the theories which were previously derived from human epidemiological studies and criticized as they were from retrospective studies. It also provided a useful model for studying CVD and maternal nutrition.

Other studies have looked at restriction in specific nutrients such as protein restriction and CVD risk. Rats fed a protein restricted diet during pregnancy have been shown to produce offspring with a significantly elevated blood pressure compared to control offspring. The protein restricted offspring also demonstrated endothelial dysfunction and showed abnormalities in the vascular smooth muscle function. Therefore, protein restriction during pregnancy has been shown to program the development of vascular dysfunction in offspring which may lead to the development of CVD. However, in this study it was only investigated in male offspring so conclusions cannot be drawn for females (187). However, another study using the same rat experimental model demonstrated that protein restriction in pregnancy programmes vasodilator dysfunction in resistance arteries of female offspring when they become pregnant. This is consistent with the outcome in the male offspring. However, this does not look at females who aren't pregnant. This study is comprehensive as it does look at both resistance and conduit arteries. The changes are seen in resistance arteries which have greater determination over blood pressure (188).

A study by Torrens et al. showed that rat offspring exposed to under-nutrition in utero with a high fat post weaning diet showed endothelial dysfunction. Endothelial dysfunction is associated with atherosclerosis and hypertension, so a key marker for CVD. This is the first study to investigate the effect of mismatched nutrition on postnatal endothelial function (7).

Although many studies have looked at under-nutrition during pregnancy, the most common dietary problem in Western populations is an excessive intake of dietary fat. Therefore, studies have been carried out to investigate whether a maternal diet high fat during pregnancy predisposes the offspring to CVD. A diet rich in lard was fed to rats during

pregnancy and lactation. Blood pressure measurements were taken using radiotelemetary during adulthood. Radiotelemetary is a thorough technical approach as it has the advantage of allowing recordings to be taken in an unrestrained manner without raising the blood pressure of the animal due to stress and it allows continuous data to be collected day and night. Therefore the blood pressure data is reliable and showed that blood pressure in female offspring of lard fed dams was significantly raised. This study also demonstrated endothelial dysfunction in both male and female offspring of lard fed dams was observed. This demonstrates signs of CVD in offspring of dams fed nutritionally imbalanced diet. It was proposed in this study that the mechanism for hypertension may be due to changes in the hypothalamic-pituitary-axis (HPA) in offspring. Another suggestion is that as this study is using a high amount of lard for treatment, which is rich in saturated fat. This may lead to a deficiency of cardioprotective n-3 PUFAs such as DHA during development. Or it may be due to the imbalance between other nutrients such as carbohydrates and protein (5).

A later study was carried out which used a cross fostered rat model of offspring from fat-fed dams to normally fed dams consuming standard chow and offspring of standard chow dams were cross fostered to fat-fed dams. There was also a control group and a high fat fed during both suckling and pregnancy. The study showed that abnormal endothelial function and blood pressure in offspring exposed to high fat during pregnancy and also when exposure was confined to the suckling period. Showing the suckling period is also important in the induction of CVD in offspring. This study also supported the 'predictive adaptive response' model; endothelial function was most severely affected in offspring only exposed to fat-rich diet during suckling. Mesenteric arteries showed almost complete failure to relax to ACh in those exposed only during suckling. This suggests those exposed to a fat rich diet during pregnancy confers a level of protection against a fat-rich diet in postnatal life (189). This is a particularly good study as the blood pressure measurements were carried out using radiotelemetary which as discussed, is the most reliable technique for blood pressure measurements. The size of the groups was also large which provides reliable data. The endothelial studies were performed on mesenteric arteries which are peripheral resistance vessels and thus give a better indication in the effect this would have on systemic blood pressure compared to conduit arteries. This study also has similarities and support other developmental models which all show signs of metabolic syndrome. Therefore this is a good study which shows 'predictive adaptive response' and provides strong support for the developmental origins hypothesis in adults.

A number of animal studies have been used to support and investigate the developmental origins hypothesis and the human studies in this field. Many of these studies

clearly show the increased risk of offspring developing CVD following exposure to an under-nourished in-utero environment. Now, with the increase in obesity and high fat diets being a common problem, there is a great deal of evidence to support the hypothesis that an imbalanced maternal diet can also increase the risk of the offspring developing CVD.

1.5.2 Types of fatty acid consumption and developmental origins:

Excessive fat intake is a common dietary imbalance worldwide; this has resulted in an increase in obesity. The number of obese cases has more than doubled since 1980. In 2008, 1.5 billion adults were overweight. This includes over 200 million men and nearly 300 million women who were obese, one of the key causes of this is high saturated fat intake (3). This results in an increase in obesity in pregnancy. Evidence has suggested that a high saturated fat diet during pregnancy may predispose the offspring to CVD. Feeding female rats a diet rich in saturated fat before, during and after pregnancy produced normally fed offspring with gender related cardiovascular dysfunction. The changes observed were an increase in blood pressure and endothelial dysfunction (5). Even though total dietary fat increased, an induced deficiency of the cardio-protective DHA during the perinatal period was reported. This was associated with the development of raised blood pressure. A similar rat model also showed the effect of saturated fat on offspring cardiovascular function. This study involved feeding a lard rich diet to dams during pregnancy and suckling. The offspring of these lard fed dam's demonstrated increased aortic stiffness and reduced smooth muscle cell number and endothelial cell volume. It is expected that aortic stiffness in the offspring would be related to increased medial thickness and smooth muscle hypertrophy, this results in increased blood pressure and altered vascular function such as reduced vaso-relaxation (190).

Maternal trans fatty acid intake and the affect it has on offspring cardiovascular function has been investigated less than other fatty acids. It has been suggested that intake of trans fatty acids inhibit the desaturation of LA to AA and ALA to DHA. This would have an impact on offspring CVD risk, as these fatty acids are essential for membrane composition and eicosanoid production. A study by Larque et al. showed reduced concentrations of DHA in rat maternal and fetal liver and plasma and a decrease in maternal delta-6 desaturase activity in rats fed high amounts of trans fats. The proportion of LA in the diet was kept constant. Delta-6 desaturase is a key enzyme in the conversion of LA to AA and ALA to DHA (191). However, other studies have shown that high concentrations of trans fatty acids relative to LA are needed in order to inhibit LA desaturation (192). This does not typically translate into humans as the level of LA intake in human diets is much higher than the intake of trans fatty acids (193).

The importance of n-3 PUFA during the perinatal environment on blood pressure control has been investigated. Rats that were deficient in n-3 PUFA demonstrated a higher blood pressure than those who were not n-3 PUFA deficient. However, the mechanism whereby this happens was unknown (6). This study was supported by another study which demonstrated hypertension in rat offspring of n-3 PUFA deficient dams. This study also showed that the hypertension could be exacerbated by persistent n-3 PUFA deficiency after weaning, but was improved by fish oil supplementation (194). However, a human study looking at n-3 PUFA supplementation in the third trimester of pregnancy showed no correlation between supplements and offspring blood pressure or heart rate at 19 years of age. This study was not the most reliable to draw conclusions from, as it used supplements at a late stage of pregnancy and an age of 19 is still relatively young in terms of developmental origins studies, an affect may not be seen until later in life. This study also had a number of participants drop out so may have affected the results (195). From animal studies it is shown that supply of n-3 PUFA in early life is important and may reduce the risk of hypertension later in life. Therefore, the composition of the fat may be the determining factor, excessive SFA or low PUFA intake may be responsible for the impacts observed in the offspring (196). However, the mechanism for these persistent changes in the offspring is not clear and need to be elucidated.

1.5.3 Epigenetics and developmental origins of health and disease:

Epigenetics refers to chemical alterations to DNA or histone proteins which lead to changes in the structure of the chromatin and alter the readability of the DNA, without altering the DNA sequence. These alterations are stable and heritable and remain through cell cycle divisions, but they can also be reversed (197). Epigenetic modifications include; DNA methylation, histone acetylation, miRNA and polycomb group proteins. Changes to epigenetic regulation of genes may lead to changes in phenotype (198).

1.5.4 DNA Methylation:

DNA methylation is a heritable covalent modification which is highly stable and alters DNA without changing the sequence (197). DNA methylation is typically associated with gene silencing, this involves the addition of methyl groups to the 5’-position of cytosines of the CpG dinucleotide, and this alters the appearance of the major groove of DNA. It is this major groove of DNA where DNA binding proteins bind. Therefore changes in the methylation of DNA, changes the interaction of these proteins with the DNA. These changes cause alterations in chromatin structure and can either increase or decrease the rate of transcription (199).

Hypermethylation is generally associated with transcriptional repression, hypomethylation is associated with increased transcriptional activity (198). DNA methylation is mediated by DNA methyltransferases, these include; DNMT1, DNMT3A, DNMT3B and DNMT3L. DNMT3a and DNMT3b are de novo methyltransferases which target unmethylated CpGs to initiate methylation (199). DNMT1 maintains methylation marks throughout life, during mitotic cell division and DNMT1 catalyses the transmission of the methylation patterns (200). DNA methylation patterns are mainly established in utero. Therefore, fetal and embryonic environment may alter DNA methylation and cause stable changes in gene expression that may be sustained throughout the lifetime of the offspring (201). Throughout the genome there are areas which have elevated CpG content but these are often predominantly unmethylated, these sequences are approximately 1kb in length and overlap the promoter regions of 60-70% of all human genes, these are called CpG islands. CpG islands co-localise with promoters of all constitutively expressed genes and 40% of those with tissue specific restricted expression. This allows transcription factor binding for the expression of most housekeeping genes and regulated genes. The CpG island also provide a means to distinguish the transcription start site of a gene (202).

1.5.5 Histone modifications:

Chromatin consists of DNA wrapped around an octamer of core histone proteins, two units of the following histones; histone 2A (H2A), H2B, H3 and H4. Histones are small, positively charged proteins that have an affinity for DNA, which is negatively charged. Post translational modifications of the histone tail, which may be acetylation, methylation, ubiquitylation and phosphorylation cause a change in the structure and function of the chromatin as it changes the interactions between the histones and DNA (197).

Histone acetylation and phosphorylation are associated with transcriptional activation, histone methylation and ubiquitylation may be repressive or activating, this depends on which residue (197). Lysine residues of the N-terminal of histone tails can be actylated by histone acetyltransferase enzymes (HATs) or deacylated by histone deacetylases (HDACs). The positively charged lysine residues bind tightly to DNA to form a closed chromatin structure which prevents transcription factor binding. Acetylation removes the positive charge and thus reduces the affinity for DNA and therefore produces an open chromatin structure, allowing easier access of transcription factors (203). Histone Methylation occurs on different lysine residues and can result in the addition of up to 3 methyl groups. The effect of histone methylation on gene function depends on the specific lysine modified. Histone methylation at

H3K4me, H3K36me or H3K&9me result in transcriptional activation. Methylation at H3K9me, H3K20me or H4K27me is associated with gene silencing (204).

1.5.6 Polycomb group proteins:

Polycomb group proteins are present in multicellular organisms and are required for normal development and target hundreds of developmentally important genes. The polycomb group proteins are responsible for cellular differentiation during development by transcriptional repression (197). Polycomb proteins form two major repressive complexes (PRC); PRC1 and PRC2. These complexes include a number of proteins which play a role in the polycomb interaction with chromatin. The polycomb binding involves PRC2 binding to chromatin and its catalytic subunit; EZH2 trimethylates H3K27. H3K27 is then recognised by a chrombox-domain (CBX) which is a component of PRC1. The PRC1 components; RING1 and E3 ligase monoubiquitylates the H2AK119sub, this leads to chromatin compaction and inhibits RNA polymerase II dependent transcriptional elongation. This results in transcriptional repression (205).

1.5.7 miRNA:

miRNA is non coding RNA, around 22 nucleotides long that play a role in controlling gene expression. miRNAs are transcribed by RNA polymerase II into primary miRNAs and form a hairpin structure. Upon transcription, the primary miRNA is processed in the nucleus by RNase III Drosha and the microprocessor complex DGCR8 which cleaves the hairpin and produces precursor miRNA. Exportin-5 exports the precursor miRNA into the cytoplasm where they are processed by an RNase III Dicer into functional miRNa duplex (206).

The miRNA then binds to a protein called Argonaute2 to form the RNA-induced silencing complex (RISC). One strand of the duplex miRNA is retained by RISC to target mRNA and this leads to down regulation of mRNA, this may be due to imperfect complimentary of miRNA and mRNA or by degradation (197).

Small interfering RNAs (siRNAs) are considered to be closely related to miRNAs and are involved in DNA methylation and histone modifications (206). siRNAs act through RNA interference pathways (RNAi) to silence gene expression. Silencing of gene expression can happen at a transcriptional level or post-transcriptional level. Both pathways are activated by the detection of double stranded RNA (dsRNA) by RNAi machinery. Dicer ribonucleases process the dsRNA into siRNA duplexes that bind to argonaute. One strand of the double stranded siRNA is eliminated and the other strand binds to the target RNA and causes it to be cleaved

and degrade. This process is similar in both post-trancriptional gene silencing and transcriptional silencing. However, in post-transcriptional silencing, this process happens in the cytoplasm and has no epigenetic affect as it does not act directly on the transcription rate of the gene. Transcriptional silencing acts in the nucleus and causes chromatin modifications which persist through generations (207).

1.5.8 Epigenetics and CVD:

Epigenetic changes are required for normal development and health, but global or gene-specific changes can be responsible or a result of some disease states. One example of this is cancer, epigenetics play a major role in this disease (208). There is now substantial evidence that epigenetic changes are involved in CVD, however, this is an area that needs more research (209). DNA methylation has been associated with CVD, including atherosclerosis and vascular inflammation (200). In a study carried out on APOE-null mice, which are genetically prone to atherosclerosis, DNA methylation patterns were investigated to determine whether changes in methylation were observed before the appearance of vascular lesions. The data showed that there were alterations in DNA methylation profiles in the aorta which were detectable at 4 weeks of age, with no detectable lesions. This study also demonstrated that in a human monocyte cell line, atherogenic lipoproteins promote DNA hypermethylation on a global scale. Together the data demonstrates that changes in DNA methylation may play a causative role in atherosclerosis and may be early markers of the disease (210). In a human study, the atheroprotective oestrogen receptor gene ESR1 and ESR2 which are expressed in VSMCs, have been found to be hypermethylated in atherosclerosis. This results in a loss of atheroprotective factors which are expressed by these genes and therefore increase the risk of vascular damage (211).

Some studies have also identified histone modifications which are associated with CVD. Genome wide analysis of histone methylation profiles has shown that tri-methylation of H3K4 or H3K9 in cardiomyocytes is associated with the development of heart failure in both humans and rats (212). Non coding RNA has also been implicated in CVD. ANRIL is a non-coding RNA which is expressed in endothelial, smooth muscle and immune cells and has been linked to CVD. Its role in atherosclerosis has been investigated by knocking down its expression in human aortic VSMCs. Gene expression changes caused by ANRIL knockdown were investigated and it was concluded that genes involved in cell proliferation, apoptosis, ECM remodelling and inflammatory pathways were affected by the silencing of ANRIL. Therefore, ANRIL may affect the progression of CVD; this may be due to remodelling of vascular tissue or during plaque formation and may lead to myocardial infarction (213).

These are just a number examples of how epigenetic changes are related to cardiovascular disease, there are a number of modifications that take place that have an impact upon the cardiovascular system and this is still a growing area of research.

1.5.9 Epigenetics and developmental origins:

It has been shown that maternal nutrition can lead to changes in offspring methylation and may explain disease phenotypes later in life. One example of this is in the Dutch Hunger Winter famine, where individuals were prenatally exposed to famine. These individuals exhibited a hypomethylation of insulin-like growth factor (IGF2) which is a key gene in human growth and development, this observation was made 6 decades after the exposure and was compared to same sex siblings who were not affected by the famine (214). Further studies demonstrated that in the same subjects there was hypermethylation of a number of gene including IL-10, LEP and MEG3 (215). Together these findings demonstrate that environmental conditions during gestation can lead to persistent changes in epigenetic information. A number of studies have been carried out investigating maternal diet and the effect on offspring epigenetic status in animals, these include feeding a protein restricted diet during pregnancy demonstrated hypomethylation of key metabolic transcriptional regulators such as the PPARα and glucocorticoid receptor promoters in the liver (198). In another study, offspring of hypercholesterolemin apoE(-/-) mice were more susceptible to dietary induced atherosclerotic changes than offspring born to wild type mothers. These changes are associated with altered histone methylation in vascular endothelial cells and smooth muscle cells (215). In addition to this, a further rat study has shown that maternal fat intake during pregnancy and lactation leads to altered methylation of Fads2 in the liver of the offspring and thus alterations in PUFA metabolism. This results in long-term changes in membrane PUFA content, including altered proportions of AA and DHA in the liver. These changes in Fads2 transcription may affect the capacity to maintain both plasma and liver AA and DHA. This may result in changes in tissue function and increase the risk of disease (216). Therefore, epigenetic mechanisms are shown to play an important role within the developmental origins of health and disease and CVD.

1.6 N-6 and N-3 fatty acid biosynthesis:

In mammals the production of long chain fatty acids happens through conversion of the essential fatty acids, LA and ALA to longer chain n-6 PUFAs and n-3 PUFAs respectively, which has previously been described in rat liver following the injection of labelled PUFA (109). This happens via a number of desaturation and elongation reactions which take place in the endoplasmic reticulum (fig 1.7). The LA and ALA are both substrates for delta-6 desaturase which inserts a double bond into the fatty acid at the delta-6 position to produce 18:3n-6 (γ-linoleic acid) and 18:4n-3 (stearidonic acid). This is the rate limiting reaction of the pathway. This is followed by the addition of two carbons by Elongase 5 and then further addition of a double bond in the delta-5 position by delta-5 desaturase to produce 20:4n-6 (AA) and 20:5n-3 (eicosapentaenoic acid (EPA)). AA and EPA are precursors for eicosanoid synthesis. A further 2 carbons are then added, again by elongase 5 to produce 22:4n-6 (adrenic acid) and 22:5n-6 (docosapentaenoic acid (DPA)). Then a further elongation step takes place and another delta-6 desaturation occurs to produce 24:5n-6 and 24:6n-3. At this point the 24 carbon PUFA is translocated to the peroxisome where it undertakes one cycle of β-oxidation to produce 22 carbon PUFAs, 22:6n-3 (DHA) and 22:5n-6. The 22 carbon PUFAs are then transported back to the endoplasmic reticulum where they can then be used for membrane lipid biosynthesis. DHA is also a precursor for the synthesis of resolvins and protectins. PUFAs can be used at any point during the pathway for membrane lipid synthesis by enzymes in the endoplasmic reticulum (109).

It has been shown that the same delta-5 and delta-6 desaturase enzymes are present in the pathway for both the n-3 and n-6 pathway (217). ALA is the preferred substrate for delta-6 desaturase. However, it is typical for humans to have an excess intake of LA as it is the most abundant PUFA, found in vegetable oils and products containing these oils. Therefore, this leads to a greater net-conversion of LA compared to ALA (218).

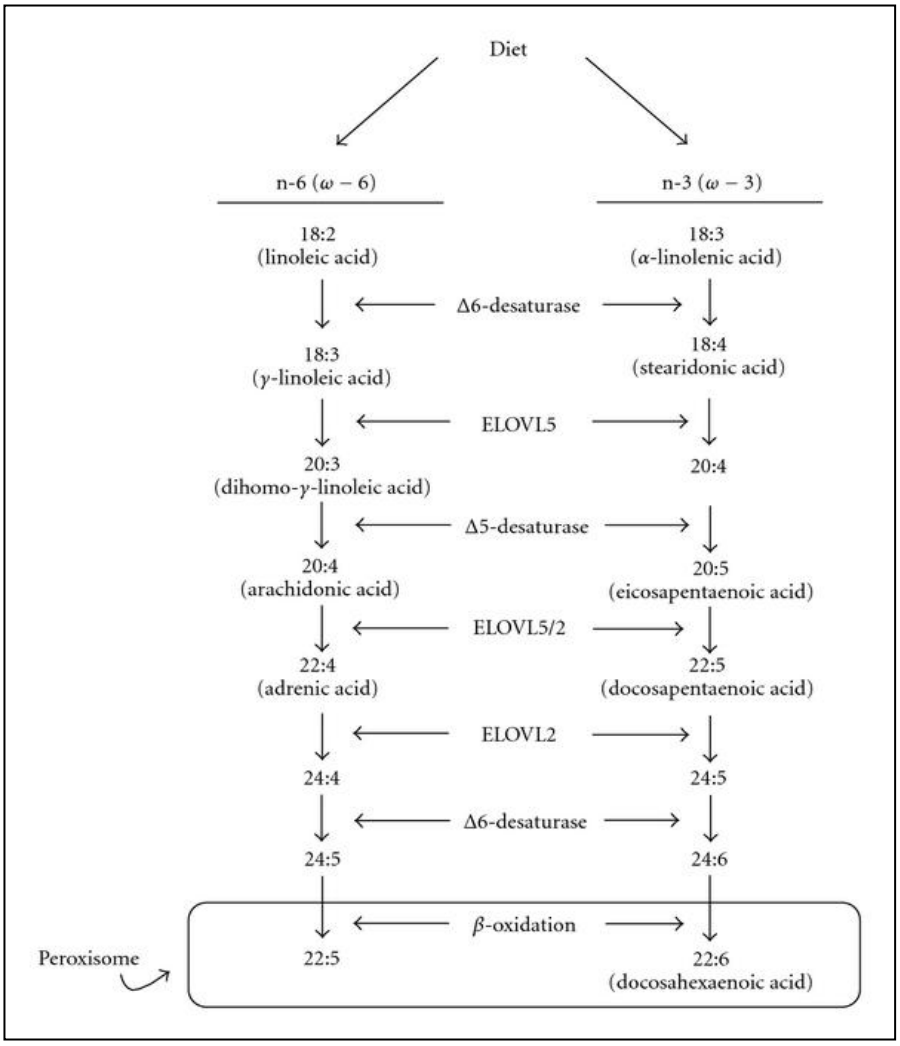


Figure 1.7: Pathway of metabolism of linoleic acid and α-linolenic acid into n-6 and n-3 PUFA fatty acid derivatives. (110)

1.6.1 Delta-5 and Delta-6 desaturase:

Delta-5 and Delta-6 desaturase are the enzymes responsible for the main regulation of the n-6 and n-3 PUFA pathway. Their function is to insert double bonds at specific locations in the carbon chain of the fatty acid; delta-6 desaturase at the delta-6 position and delta-5 desaturase at the delta-5 position of the chain. The delta number describes the position from the carboxyl end of the fatty acid. Delta-5 and delta-6 desaturase are encoded by the genes FADS1 and FADS2 respectively. The FADS1 and FADS2 genes are located together in a gene cluster along with FADS3 which encodes for delta-3 desaturase on the human chromosome 11q12-q13.1, together this cluster is 91.9kb (219). They are both membrane bound enzymes. Both enzymes have the highest activity in the liver but are found in a number of other tissues. Delta-6 desaturase is expressed in the brain, liver, skeletal muscle, lung and heart (220) and

delta-5 desaturase expression has also been noted to be expressed in all of these organs but also in placenta, kidney and pancreas. Delta-5 desaturase is most abundant in the liver brain and heart, however, the delta-5 desaturase expression level is much lower than delta-6 desaturase (221). The desaturase enzymes work by using activated molecular oxygen to remove hydrogen from the fatty acid chain; this creates a carbon-carbon double bond and a molecule of water (222).

The presence of delta-6 desaturase activity and where it specifically acts in the n-6 and n-3 pathways was confirmed using the selective delta-6 desaturase inhibitor 2,2-diphenyl-5-(4-{[(1*E*)-pyridin-3-yl-methylidene]-amino}piperazin-1-yl)pentanenitrile (SC-26196). It was shown that the inhibitor was able to suppress the conversion of LA to AA in the n-6 pathway and eicosapentaenoic acid (EPA) to DHA in the n-3 pathway. In addition to this, it was shown that the conversion of docosapentaenoic acid (DPA) to DHA was also reduced. This confirms that delta-6 desaturase is also responsible for the synthesis of the DHA precursor 22:4n-3 (223).

1.6.2 Elongase

The Elongase enzymes are responsible for elongating the fatty acid chains in the n-3 and n-6 PUFA pathway by two carbons. The Elongase enzymes are encoded for by 'Elongation of very long chain fatty acid' (ELOVL) genes. There have been seven ELOVL genes identified to date, these are ELOVL1-7. The ELOVL's can be separated into two groups, dependent on their substrate preference. ELOVL1, ELOVL3, ELOVL6 and ELOVL7 prefer saturated and monounsaturated fatty acids. Whilst ELOVL2, ELOVL4, ELOVL5 and ELOVL7 prefer polyunsaturated fatty acids as substrates (224). The two enzymes involved in the n-6 and n-3 pathway are ELOVL5 and ELOVL2. Following the discovery of enzymes which elongate monounsaturated and saturated fatty acids, ELOVL5 was first to be discovered. It was shown to cause the elongation of PUFAs C18 and c20 in baker's yeast (225). ELOVL2 was then identified as being involved in the PUFA pathway, when ELOVL2 cDNA was expressed in baker's yeast and in mouse cells. In these cells it produced an active enzyme which appeared to be involved in elongation of C20 and C22 PUFA but not saturated or monounsaturated fatty acids (226).

The Elongase enzymes work by elongating the fatty acid by two carbon units. This happens through four separate enzyme reactions, the first is a condensation reaction which is the rate controlling condensation reaction, and this is controlled by the Elongase enzyme. This reaction involves the condensation between the fatty acid and the donor malonyl-coA. This is

followed by a reduction reaction, then a dehydration reaction and the final step is a reduction reaction to produce the elongated fatty acid (224).

There has been some uncertainty as to where the ELOVL2 and ELOVL5 act in the n-6 and n-3 pathways. Using Elovl2 deficient mice, it has since been shown that in the liver, the main function of Elovl2 is the elongation of C22 into C24 PUFAs. This is a step in the formation of 22:5n-6 (DPA) in the n-6 pathway and 22:6n-3 (DHA) in the n-3 pathway. The data also showed that ELOVL5 and FADS1 and FADS2 are the major enzymes involved in the production of the PUFAs up to C22 in the pathway. (227).

Elovl5 is widely expressed in the body and has been found to be expressed in all tissue types that have been tested. The highest expression of Elovl5 is in the adrenal gland, testes, brain, lung and prostate tissue. The lowest expression of Elovl5 was found in the pancreas (225). Compared to Elovl5, Elovl2 is expressed at a much lower level. Elovl2 has only been found to be expressed in the lungs, liver, brain and kidneys, but has not been detected in any other organ (228). The different levels of expression of the genes required for PUFA synthesis is thought to be related to the tissue specific demands for the fatty acids synthesised by the different enzymes (224).

1.6.3 Eicosanoid synthesis:

As discussed in section 1.1.6 eicosanoids are signalling molecules that are produced through an oxidative pathway from C20 fatty acids produced in the n-3 and n-6 pathway; namely AA, EPA, DHA and DGLA. The eicosanoids have diverse biological actions throughout the body, including physiological functions such as regulation of vascular function and are important for immune and inflammation function within the body (29).

Competition between n-6 and n-3 fatty acids occurs for the enzymes responsible for eicosanoid synthesis (229). AA is considered the main eicosanoid precursor within mammals. As described in a previous section, humans have a greater intake of LA. Therefore more AA will be present in membranes compared to EPA, so AA is considered the major substrate for eicosanoid synthesis (142). Supplements with fish oil have been shown to increase EPA and DHA concentrations in the plasma which was coupled with a decrease in AA in plasma and erythrocyte phospholipids and LA in erythrocyte phospholipids. These changes were also accompanied by a decrease in PGE_2 (161). This demonstrates how the n-3 and n-6 ratio in the diet affects eicosanoid production. An increased n-3 fatty acid intake can lead to reduced synthesis of inflammatory eicosanoids produced from n-6 fatty acids, specifically from AA. This clearly demonstrates the competition for the eicosanoid enzymes.

Eicosanoids are synthesised de-novo from AA released from the phospholipids of the cell membrane (fig 1.8). Eicosanoids are not stored but synthesised when cells are activated by medical trauma, cytokines or growth factors (29). Upon stimulation, AA is released from the membrane by cytosolic phospholipase$_2$ (cPLA$_2$). cPLA2 is found in most cells and tissues and hydrolyses phospholipids containing arachidonate in the cell membrane at the sn-2 position (230). cPLA2 requires calcium to cause translocation from the cytosol to the membrane or phospholipids vesicle in order to liberate AA. This happens through cytokines, growth factors of other agonists which mobilise intracellular calcium (231).

The released AA is then metabolised by PGH synthase to PGH$_2$. The PGH synthase is referred to as cyclooxygenase (COX) and exists as two isoforms; COX-1 which is found in most mammalian cells and is responsible for constitutive synthesis of prostaglandins. And also as COX-2 which is an inducible enzyme and increases at sites of inflammation and in inflammatory cells (29). Once the PGH$_2$ is formed, the eicosanoids produced depends upon the cell type in which the PGH$_2$ is formed. The cell type determines the downstream enzymes which metabolise PGH$_2$. Vascular smooth muscle cells, endothelial cells and platelets express PGE synthase and so metabolise the PGH$_2$ to produce PGE$_2$. Platelets express thromboxane synthase and metabolise PGH$_2$ to produce the vasoconstrictor TXA$_2$. Endothelial cells express PGI synthase and so produce the vasodilator PGI$_2$ (232). Prostaglandins are released mainly by facilitated transport using a prostaglandin transporter. The eicosanoids have very short half-lives, especially thromboxane and prostacyclin, with half lives of seconds. So they must act near their site of synthesis (29).

The leukotrienes are synthesised differently to the prostaglandins, they are made mainly by inflammatory cells like macrophages and mast cells. 5-Lipoxygenase (5-LO) is the key enzyme in this cascade and with 5-lipoxygenase-activating protein (FLAP) it transforms AA into LTA$_4$ (29). The synthesis of lipoxins from AA happens during resolution of inflammation and is catalysed by two stages of lipoxygenase action to produce a number of lipoxins. This can be followed by specific lipoxin hydrolase activity which produces different members of the lipoxin family. Lipoxins stimulate the return of vascular permeability to homeostasis and are the first recognised lipid mediators to resolve inflammation (233).

The synthesis of protectins from DHA is catalysed by two stages of lipoxygenase action followed by epoxidation. For the production of resolvins from DHA, two stages of lipoxygenase action also takes place, but this is followed by epoxidation and hydrolases. The protectins and resolvins are produced during resolution of inflammation through transcellular biosynthetic routes which involves the product of one cell is transformed by neighbouring cells (36).

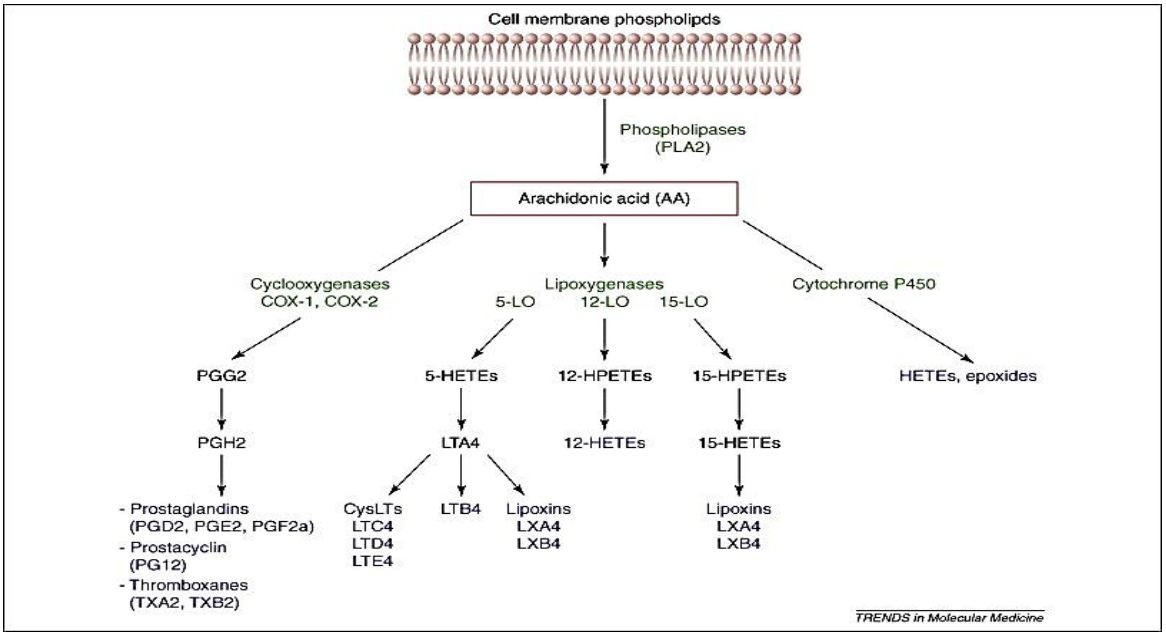


Figure 1.8: Eicosanoid biosynthesis from arachidonic acid (AA) (28)

1.6.4 Desaturase inhibitors:

1.6.4.1 Delta-5 desaturase inhibition:

Sesamin is a lignan which is present in sesame oil and sesame seeds and is a specific inhibitor of delta-5 desaturase, the structure of sesamin is shown in fig 1.9 (234). Early studies demonstrated that incubation of the fungi; Mortierella alpine, with sesame oil caused an increase in DGLA content while the content of AA decreased. This was due to inhibition of the conversion of DGLA to AA and thus inhibition of delta-5 desaturase (235). Sesame oil lignans were isolated and identified and the experiments showed that sesamin is a non-competitive inhibitor, which specifically inhibits delta-5 desaturase at low concentrations in the PUFA biosynthesis pathway. It does not inhibit delta-6 desaturase or delta-9 and delta-12 desaturases (234).

The role of sesamin in PUFA biosynthesis has also been demonstrated in rats. Rats were fed diets containing sesame lignans for 4-weeks. The sesame lignans in the diets caused a reduction in the content of AA and also the n-6/n-3 ratio in the liver. However, this study did not specifically investigate sesamin, but a mixture of sesamin and epi-sesamin (236). Another study also investigated the fatty acid composition of mice liver following the supplementation of sesamin, this study confirmed the previous findings by demonstrating that sesamin supplementation caused an accumulation of DGLA and decrease in AA and inhibition of delta-5 desaturase. This study also showed that, consistent with an increase in DGLA, there was a

reduction in PGE_2 production. DGLA is capable of displacing AA and competing with it in binding cyclooxygenase and thus reducing the formation of pro-inflammatory dienoic eicosanoids. This also results in an increase in the production of 1- series prostaglandins which are less inflammatory (237). A further study also confirmed these findings by showing that mice fed a sesame oil diet had an increase in DGLA content in the liver. Following stimulation with lipopolysaccharide (LPS), which promotes the secretion of pro-inflammatory eicosanoids, a significant reduction in plasma PGE_2 and TXB_2 was demonstrated (238). Therefore, sesamin can causes a reduction in the production of pro-inflammatory 2-series eicosanoids, which are produced from AA and therefore may have therapeutic potential. Sesamin has been shown to enhance n-3 PUFA biosynthesis in mice consuming a diet of alpha-linolenic acid mixed with linoleic acid, whilst decreasing the n-6 PUFA production, these data suggests that n-3 PUFAs are preferentially converted to their delta-5 desaturase products compared to n-6 PUFAs (237). Another rat study also showed enhance n-3 PUFA production in the presence of sesamin and suggested that this may be due to differences in the affinity of delta-5 desaturases in n-3 and n-6 PUFA biosynthesis (239).

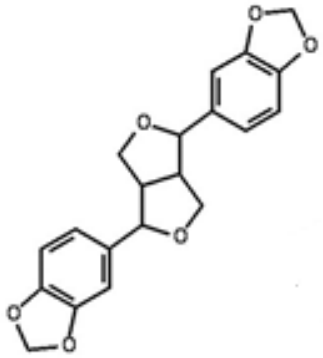


Fig 1.9: The structure of sesamin (240)

1.6.4.2 Delta-6 Desaturase inhibition:

The compound 2,2-dephenyl-5-(4-{[(1E)-pyridin-3-yl-methylidene]amino}piperazin-1-yl)pentanenitrile (SC-26196), as shown in fig 1.10, has been previously shown to inhibit the delta-6 desaturation of isolated rat liver microsomes with an IC_{50} of 200nM. The IC_{50} of SC-26196 for delta-5 and delta-9 desaturase in the liver microsomes was greater than 200 µM, demonstrating the selectivity for delta-6 desaturase (241). The role of SC-26196 in inhibiting delta-6 desaturase was also demonstrated in mice, SC-26196 caused a dose dependent decrease in AA in liver, plasma and peritoneal cells. In this study, it was also observed that SC-26196 was an anti-inflammatory agent, inhibition of delta-6 desaturase caused a decrease in

edema and this corresponded with the decrease in AA (242). This is presumably due to a decrease in eicosanoid production due to a lower availability of AA. Another mouse study confirmed the actions of SC-26196 by demonstrating that dosing mice with SC-26196 resulted in increases in linoleic acid and decreases in AA and DHA in mouse liver and plasma (243).

These findings were extended into a very thorough study on human cells which gave more information on the activity of SC-26196. It was demonstrated that SC-26196 is a potent, selective and long-lasting delta-6 desaturase inhibitor that acts on both 18- and 24- carbon PUFA substrates in human cells. The study demonstrated that SC-26196 does not inhibit the conversion of DGLA to AA, this reaction is mediated by delta-5 desaturase. In human cells, the IC_{50} were found to be in the same range as previous reports and the data also suggested that the inhibitor binds irreversibly to delta-6 desaturase or dissociates slowly after binding. The data also showed that the inhibitor produced very similar IC_{50} values for the inhibition of the conversion of 18- and 24- carbon substrates and so suggests that a single delta-6 desaturase acts on these substrates. It was also demonstrated that SC-26196 inhibited the synthesis of EPA and DHA from n-3 PUFA, the desaturation of α-linolenic acid was reduced to the same extent as linoleic acid and so is evident that the same delta-6 desaturase acts on both n-3 and n-6 PUFAs (223).

As demonstrated in the study by Obukowicz et al. (244) and described for delta-5 desaturase inhibition, the inhibition of delta-6 desaturase would have beneficial effects on inflammation as it would reduce the production of AA and thus the production of pro-inflmmatory eicosanoids. Inhibiting delta-6 desaturase with SC-26196 in mice leads to a reduction in pro-inflammatory AA-derived eicosanoids, such as PGE_2 and also other key inflammatory markers such as interleukin-6 and TNF-α (245). SC-26196 also inhibits the synthesis of EPA in human cells, which can be converted to eicosanoids that have anti-inflammatory and anti-thrombotic properties, very little EPA is normally present in human tissue (223).

As discussed previously, SC-26196 has been shown to be selective and does not inhibit the delta-5 desaturase reaction (223). Sesamin has also been shown to be selective and does not inhibit the activity of delta-6 desaturase (234). Also, both sesamin and SC-26196 have distinctly different structure, therefore the activity of these inhibitors are unlikely to overlap.

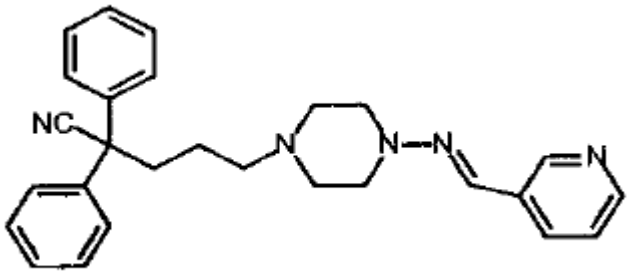


Fig 1.10: The structure of SC-26196 (242)

1.6.5 The functional significance of the PUFA synthesis pathway:

The PUFA synthesis pathway in humans is very important for producing a number of PUFAs which are crucial for normal cell function and for eicosanoid production. Some key components of the pathway include DHA which is found in large quantities in the brain, testes and retina. DHA is known to be important in maintaining the fluidity of cell membranes, which is important for cell signalling and is also important as a modulator of growth factor signalling. AA and EPA are necessary for synthesis of eicosanoids, as discussed previously. The functional significance of this pathway can be best demonstrated by the results of gene knockout experiments of the key genes within the pathway, FADS1, FADS2, ELOVL5 and ELOVL2.

FADS2 is the first step in the PUFA synthesis pathway, this results in the absence of all long chain PUFAs and also eicosanoids. In humans with a deficiency in delta-6 desaturase the symptoms include corneal ulcers, skin abnormalities, growth retardation, gastro-intestinal bleeding and feeding intolerances. Treatment with AA and DHA restored normal growth and eliminated most other symptoms (246). However, this study was based on information from only 1 patient, so observations of the pathology were only based on that specific person and there may be other pathological effects in different people. The study would be better with a bigger cohort as it would confirm that these symptoms are due to that deficiency and add to the reliability of the observation. Therefore it is useful to look at an animal model of this deficiency. This also allows more invasive observations, the known knockout to be confirmed and knowledge that the symptoms are due to that knockout and it also allows for a bigger cohort. It has been shown that the knockout of the FADS2 gene in mice leads to skin lesions which are consistent with that seen in the human deficiency. This is caused by the lack of AA which is needed to maintain normal function of skin, and this is thought to be through the prostaglandin PGD_2. Another symptom was ulceration of the small intestine, which is also consistent with the human case, a lack of prostaglandin synthesis is believed to be responsible

for this, and this could be prevented by AA supplementation. Spermatogenesis was also halted in the knockout mice, so the mice were sterile. AA, DPA and DHA are the main fatty acids in spermatozoa membranes and account for 36% of total fatty acids in mouse sperm and demonstrate the importance of the PUFAs for male fertility (247). This was a good study which provided a useful model for this deficiency which was consistent with the human deficiency. So reinforces the observations seen in humans and allows more observations to be made to enable a greater understanding of FADS2 deficiency.

Another study using this knockout model also showed sterility in male mice, but in addition to this demonstrated female sterility and showed hypogonadism in both male and female mice (248). In this study by Stoffel, the diet contained a level of AA, which is a limitation as it doesn't show the true effect of a total Fads knockout. This may affect the outcome of the study to some extent as the mice were therefore not completely devoid of AA. However, both studies clearly demonstrate the importance of Fads2 within the pathway, especially as ablation of Fads2 affects the whole pathway, not just part of it, so this shows the importance of a number of components within the pathway. These components include the importance of production of AA as a precursor for prostaglandins which are needed to maintain the skin and also the importance for this pathway in fertility.

Knockout of Fads1 in mice led to an increase in DGLA and a systemic decrease in AA, which confirms the success of the knockout. One effect of knocking out FADS1 was a disruption to immune cell homeostasis and increased sensitivity to inflammatory challenges. This is thought to be due to disruption to the eicosanoid PGE_2 and phosphatidylinositol 4,5-bisphosphate (PIP_2) which controls protein activity and is a good source of secondary messengers. This is a result of a decrease in AA. However, the main effect of the loss of this gene was failure to thrive, with most mice dying after 5-6 weeks and none surviving past 12 weeks. This shows the presence of Fads1 is essential for life (249). The reason the studies in Fads2 were able to fully investigate the effect of that knockout is thought to be due to the diet containing a level of AA, allowing the mice to survive. Without the AA in the diet, Fads2 knockout may also have resulted in an inability to survive (248). Therefore, the Fads1 knockout study used AA supplements to give to the Fads1 null mice to see if this increased the survival rate and to determine if an AA deficiency is the reason for poor survival. Supplementation allowed survival to the same ages as wild type mice (249). This clearly demonstrates the importance of the pathway and PUFA production, especially AA which is necessary for survival.

There is also evidence to suggest that defects in the activity of delta-5 and delta-6 desaturase are linked to the development of atherosclerosis. This is again due to decreases in

AA, DHA and EPA and thus a decrease in eicosanoid release. This would result in an imbalance in vasoactive factors in the endothelium and would result in endothelial dysfunction. There would also be a decrease in the eicosanoids required to resolve inflammation and inhibit leukocyte activation (250). This includes the production of lipoxins, protectins and resolvins which are products of AA and DHA and are important in the resolution of inflammation. Previous studies have shown that a reduction in these lipid mediators is linked to the development of atherosclerosis (166). Together, both of these factors would lead to the development of atherosclerosis and other pathological presentations of CVD (250).

Similar to FADS1 knockout, the knockout of Elovl5 in mice resulted in significant decrease in AA and also DHA in the liver. As expected they had an accumulation of 18:3n-6 and 18:4n-6 which are the substrates for Elongase 5 activity. The Elovl5 knockout also leads to activation of the lipogeneic gene, increased lipogenesis and hepatic steatosis. This was caused by decreased AA and DHA concentrations which led to the depression of the sterol regulatory element-binding protein (SREBP-1c), leading to the transcriptional activation of lipid synthesis (251). A novel study looked at knockout of Elovl2 and showed complete arrest of spermatogenesis and an absence of further germ cells in mice rendering the mice infertile. This is due to the absence of 24:4n-6 which is essential for production of 22:5n-6, so therefore demonstrates the importance of this pathway and specifically Elovl2 in spermatogenesis (252). This study was followed by a very important study which using knockout Elovl2 mice, was able to show for the first time the exact position where Elovl2 acted in the pathway and also the effect of Elovl2 ablation and the affect it has on hepatic liver composition and function. As expected from knocking out Elovl2, which is highly expressed in the liver, it leads to decreased levels of DHA and n-6 DPA (22:5n-6) and accumulation of n-3 DPA (22:5n-3) and adrenic acid (22:4n-6) in liver and in serum. There was a 90% fall in DHA serum levels in Elovl2 knockout mice, this also shows that the liver is the main site for DHA in the body and would result in all cells throughout the body being deficient in DHA. In contrast to the Elovl5 knockout, the Elovl2 knockout led to increased SREB-1c and the mice were resistant to hepatic steatosis and demonstrated a diet resistant lean phenotype. This shows that Elovl2 is important in regulating lipid storage. This may be related to the increased levels of EPA and n-3 DPA which were increased in Elovl2 knockout compared to Elovl5 knockout and wild type. EPA and DPA are important in the prevention and improvement of metabolic disorders including hepatic steatosis (227).

In summary, looking at the phenotype and the effect of mice lacking the specific genes in the pathway clearly demonstrate that different phenotypes occur dependent on where the

enzyme acts in the pathway and thus the resulting PUFAs available. Total removal of the pathway or removal of enzymes affecting AA and thus eicosanoid release has the greatest effect.

1.6.6 Tissue specific function of the n-6 and n-3 pathway:

As discussed previously delta-6 desaturase is expressed in the brain, liver, skeletal-muscle, lung, testes and heart (220). Delta-5 desaturase expression has also been noted to be expressed in all of these organs and also in the placenta, kidney and pancreas. However, it is most abundant in the liver brain and heart (221). Elovl5 is ubiquitously expressed, with the highest expression found in the adrenal gland, testes, brain, lung and prostate tissue and the lowest expression found in the pancreas (225). However, Elovl2 is expressed at a much lower level and has a far more tissue specific expression. It has been found to be expressed in the lungs, liver, brain, kidneys (228) and there is evidence for its presence in the testes (252). However, it is absent in other organs. The different levels of expression of the genes required for PUFA synthesis is thought to be related to the tissue specific demands for the fatty acids synthesised by the different enzymes (224).

The brain is one organ which has the highest expression of the genes which encode for the enzymes in the PUFA synthesis pathway. It is shown to express all of the genes in the pathway. The brain is very highly enriched in PUFAs, especially DHA which is crucial for its function. The brain has been shown to synthesise PUFAs, including DHA, which requires the function of all enzymes in the pathway. This has been demonstrated *in-vitro* in rat brain astrocytes, the study showed that astrocytes were able to synthesise DHA from ALA and other intermediates in the pathway (246). This is consistent with other studies showing that endothelial cells and astrocytes cooperate in local synthesis and the release of PUFAs for uptake by the neurons in felines and in cultured cells (253;254). However, the study by Williard et al. also showed that the DHA production in the rat brain reduced, but did still happen, when astrocytes had access to an extracellular supply of DHA, such as in the plasma. The fact that there was some synthesis suggests DHA may have a crucial role in astrocyte function (255). A study since, has shown that LA circulating in the plasma enters the brain of rats and is mainly oxidised, and very little is used to synthesise AA or DHA (256).

The liver is considered the main site for the synthesis of PUFAs; it is the tissue with the highest expression of all the enzymes involved in the pathway. Ablation of Elovl2 in the liver leads to a 90% reduction in serum levels of DHA in mice, demonstrating the liver is the major contributor of circulating DHA (227). Many studies have demonstrated that the brain obtains

most of its DHA from the liver. The liver produces enough DHA to maintain normal brain function and can increase its DHA synthesis during times of n-3 PUFA deprivation (257). In a mouse study using a radiolabelled 18:3n-3 have shown that it is taken up by the liver and it is there that it is elongated and desaturated and then supplied in the blood to the brain (258). However, the level of PUFA synthesis is different during pre-natal development, during this time the demand for PUFAs is very high for the brain and for the rest of the body. It has been shown in mice that the level of delta-6 desaturase reflects this demand for PUFA in the brain and in the liver during development. During pre-natal development the expression is a lot higher in both of these tissues to allow a greater synthesis of PUFAs to supply the fetus, especially for brain development. At post-natal day 21 the level of delta-6 expression in the brain decreases by 12-fold and remains constant thereafter, suggesting a much higher amount of PUFA synthesis during development. The level of expression reduces afterwards when the demand is a lot lower, consistent with the lower levels of synthesis in the brain. After this point the brain relies on the liver for PUFAs, which was discussed previously. The expression in the liver increases 9-fold between 3 days before birth and 9 fold after birth, this decreases slightly up to 4 months and then decreases more dramatically after that and continues decreasing with age (259).

The mammary glands are another example of tissue specific expression of the desaturase enzymes to meet functional requirements. As discussed, during pregnancy and lactation there is an increased demand for PUFA which are needed for the brain development of the new-born baby. It has been demonstrated that the rat mammary gland contains delta-5 and delta-6 desaturase and is able to synthesise DHA and AA and secrete it into the milk. This allows the mother to fulfil the developmental requirements of the baby. This synthesis is up-regulated when the PUFA content of the diet is low (260).

The testes have also been found to express all of the enzymes needed for the n-6 and n-3 PUFA pathway, this is another example of tissue specific expression of the enzymes in this pathways in relation to function. The testes have a high expression of the n-6 and n-3 pathway and very high levels of PUFAs. However, they are continuously drained of fatty acids as the spermatozoa are transported to the epididymis. Rat and human testes have been found to have high levels of 22:5n-6 and 22:6n-3. Therefore, the testes need to have a high expression of delta-5 and delta-6 desaturase enzyme to ensure the turnover of PUFAs to meet the demands of the testes. Therefore there is an even higher expression of these enzymes in the testes than seen in livers (261). PUFAs are essential membrane components of spermatocytes. Mice with knocked out Elovl2 demonstrated complete arrest of spermatogenesis and

spermatogonia and primary spermatocytes without further germinal cells (252). This clearly demonstrates the importance of Elovl2 in the testes and the importance of the expression of all of the enzymes within the pathway for normal function of the testes.

Synthesis of PUFAs has been shown to take place in human endothelial cells (262;263) and human smooth muscle cells (223). As described previously, eicosanoids have an important role in the regulation of vascular function and inflammation responses within the vasculature. These eicosanoids are produced from precursors such as AA, EPA and DGLA, which are all synthesised in the PUFA biosynthesis pathway (29). Therefore, the activity of the PUFA biosynthesis pathway in vascular tissue would meet the functional requirements for eicosanoid production and thus regulation of vascular function. However, the full extent of the pathway within the vasculature has not been shown. The activity of delta-6 desaturase has been demonstrated in human coronary artery smooth muscle by using the delta-6 desaturase inhibitor SC-26196 to inhibit the desaturation of 18 and 24 carbon PUFAs (223). Similarly, in human vascular endothelial cells only the activity of delta-6 desaturase has been demonstrated (262). The activity of the other enzymes involved in the pathway has not been determined. Therefore, in vasculature the PUFA biosynthesis pathway does happen to some extent, presumably to allow regulation of vascular function, but the full activity of the pathway needs to be determined.

1.6.7 FADS1 and FADS2 single nucleotide polymorphisms:

As discussed, FADS1 and FADS2 encode for delta-5 and delta-6 desaturase which are key enzymes within the n-3 and n-6 PUFA pathway. As shown by knock-out studies, any changes to the desaturation enzymes have significant effects on the synthesis of these PUFAs and thus impact the concentration within cell membranes and cell function. Single nucleotide polymorphisms (SNPs) are a genetic variation of one single nucleotide in a DNA sequence, there have been a number of studies to demonstrate that SNPs in FADS1 and FADS2 influence PUFA content of cells (264). The first study was very thorough and investigated a large cohort, consisting of 727 white adults and covering 18 SNPs in the FADS1 FADS2 gene cluster, changes in serum phospholipids were analysed. The region was chosen as it was a functional and positional candidate for having an important influence on fatty acid composition and disease development. They showed that carriers of 11 SNPs had higher serum concentrations of the PUFAs in the upper part of the biosynthesis pathway compared to non-carriers. There were also lower concentrations of gamma linolenic acid, AA and adrenic acid in the n-6 pathway and lower levels of EPA and DPA in the n-3 pathway. These are products of desaturation, so this demonstrates that the carriers of these SNPs had a lower expression of delta-5 and delta-6

desaturase. In this study to ensure that the data was as reliable as possible they ensured there were no low numbers to avoid inaccurate conclusions and provided highly significant associations. This therefore is a really reliable and clear study (265).

Another study which also used a large cohort and covered a number of SNPs, confirmed the results of the previous study. However, an additional benefit of this study was that it also extended the results of the previous study and showed associations with SNPs in FADS and PUFA content in human red blood cells. The previous study only looked at serum phospholipids. The most significant changes were seen to AA levels. As these are important for eicosanoid release, this suggests that these SNPs are useful to be studied in relation to disease (266). Kwak et al. investigated the associations of polymorphisms of FADS genes with PUFA concentrations in phospholipids and biomarkers for coronary artery disease in Korean people. They showed that the polymorphism rs174537T was associated with lower proportions of AA and higher proportions of LA in serum phospholipids. This is consistent with a reduction in the activity of delta-5 and delta-6 desaturase. Patients with this polymorphism also had a reduced risk of coronary artery disease. However, one key limitation of this study is that it looks at this SNP in a set population, which were Korean adults aged between 40-69. So the data may not represent other populations and cannot necessarily be extended to other populations. Therefore, other populations would need to be explored (267). The association with this polymorphism and lower risk of CAD was also shown in another study. In addition to this, the study also showed that carriers of another polymorphism; rs174460C had a higher risk of CAD, with higher levels of delta-6 desaturase (268). Again, this study also looked at a specific population of Chinese people, so similarly to the previous study discussed; more populations need to be investigated. However, within these populations the data clearly demonstrates that changes within the biosynthesis of PUFAs have an effect upon the susceptibility of CVD. This may also be the case in other populations.

1.7 Maternal diet and persistent changes in offspring membrane fatty acid composition and the effect on vascular function:

It has been suggested that maternal dietary fat and fat metabolism may programme endothelial dysfunction in the offspring. Napoli et al. showed that atherosclerotic –like lesions are present in human fetal aortas and that the occurrence and size of the lesions is enhanced by maternal hypercholesterolemia compared to normocholesterolaemic mothers. This shows that fatty streaks occur during development and are affected by maternal diet. This demonstrates that pathogenic events during fetal development cause persistent changes in the offspring which influence the progression of atherosclerosis later in life. As this study was carried out in humans this poses a number of obstacles which are limitations to this study (44). Human studies are difficult to investigate the mechanisms whereby these persistent changes are happening and are difficult to explore interventions which may alter the outcomes. The study was also unable to look at disease progression into adulthood and the samples came from aborted fetuses and premature new-borns that died. Finally, there are also many genetic differences which may play a role in the pathogenesis of disease and are therefore hard to control.

Therefore, the observations from human studies were necessary for the initial observations, but an animal study is invaluable to overcome the obstacles posed by human studies. Therefore, a very useful animal study was carried out to overcome these obstacles. This involved using an inbred rabbit and thus a homogeneous animal model to investigate whether maternal hypercholesterolemia causes enhanced fatty streak formation in offspring and whether intervention can reduce it. This study was able to confirm and extend the results from the study carried out in humans, showing that maternal hypercholesterolemia enhances fatty streak formation in offspring. The lesions were greatest in the group exposed to the highest cholesterol levels. The mechanism suggested to cause these lesions is due to an increase of LDL oxidation in the mother and fetus. The added benefit of the animal study was to investigate intervention. Intervention with antioxidants; vitamin E during pregnancy caused a reduction in lesion size. This gives additional evidence to the cause of these lesions being lipid oxidation. This study however showed a lack of progression to atherogenesis, which may be due to the postnatal diet being extremely low in cholesterol which is not consistent with a human diet (269). Therefore this needs to be explored in further studies utilising a higher cholesterol postnatal diet. However, overall this is a very good study which clearly strengthens the observations in humans and provides a good animal model for studying maternal hypercholesterolemia and outcomes in offspring.

Both of these are examples of studies where imbalanced diets lead to persistent changes in the composition by appearance of fatty streaks or atheroma and changes in function of offspring vasculature. It is clear that the developing fetus requires an appropriate supply of fatty acids to allow normal development of the cardiovascular system, but it is not clear the correct amount or the types of fatty acid needed and the effect that an inadequate supply will have on the offspring.

A lot of research has demonstrated that high fat diet during pregnancy leads to persistent vascular dysfunction in the offspring (5;190); it has been thought that changes in fatty acid composition could be responsible for the observed differences and that maternal dietary lipid and fat metabolism may be involved in programming of endothelial dysfunction (8). Defects in phospholipid synthesis can lead to changes in the cell function. Changes to fatty acid consumption from the diet may also cause changes to fatty acids incorporated into the membrane, which in turn may change the cell function. This may have physiological consequences and impact on the health, either advantageously or disadvantageously.

Many studies have shown changes to diet can have great effects on the cardiovascular system by altering the composition of fatty acids in aortae (270), adipocytes plasma membranes (271) and platelets (270); (272). These studies have looked at high fat diet during postnatal life and observed changes to fatty acid composition.

Very little work has been done into diet during pregnancy and the effect on the composition of offspring fatty acids, as these also demonstrate vascular dysfunction. One study that has looked at this is Ghosh et al. In this study female Sprague-Dawley rats were fed a diet high in saturated fat or a breeding diet during pregnancy and weaning. Vascular dysfunction was exhibited by the offspring with blunted responses to ACh in rats fed high fat diet. Fatty acid composition in the aortas of the adult offspring showed fatty acid abnormalities, including reduced AA and DHA content. Changes to saturated and monounsaturated fats were also seen, which may be responsible for inhibition of endothelial NO synthesis (8). This study provides evidence that maternal diet during pregnancy causes alterations in the fatty acid profile in adult female offspring and may provide some explanation for the vascular dysfunction reported. This is a really useful study; however, one disadvantage of this study was that it did not take blood pressure measurements. This would have been interesting to see if blood pressure was affected and thus whether the changes observed lead to systemic vascular problems and other risk factors of CVD.

Another similar study found that the intake of trans fatty acids in hydrogenated fat during pregnancy and lactation in pigs also caused a persistent change in the aortic phospholipid fatty acid composition in the offspring. It caused a change in the percentage of n-3 and n-6 PUFA metabolites. This was caused by the trans fatty acids that were incorporated into the cell membrane and caused a change in essential fatty acid metabolism, caused by inhibition of acyl-CoA desaturase. Decreases in n-3 PUFA in aortic phospholipids can result in a number of problems within the vasculature and can increase the risk of cardiovascular events (273).

1.8 Aims and rationale:

There is a breadth of data and information on the effect of diet on cardiovascular function in adults, including the different types of fats and the effects they have. This has led to recommendations on suggested intake of each different type of fat and their quantity. Research surrounding the developmental origins hypothesis has investigated the effect maternal nutrition during pregnancy has on the fetus, by causing physiological changes to aid survival. These changes can predispose the offspring to CVD later in life. However, there is very little research and recommendations on the different types and quantities of fat during pregnancy or the effects it has on the offspring. Equally, there is little research investigating the extent to which maternal total fat intake and dietary fatty acid composition interact. Nor is there knowledge of the mechanisms whereby these interactions lead to persistent changes in offspring vascular composition and function. The PUFA synthesis pathway may have a role in these changes. The importance of the n-3 and n-6 pathway has been discussed in great detail and the links between impaired delta-5 and delta-6 desaturase and CVD. This is thought to be due to altered synthesis of eicosanoids. It is assumed that changes in eicosanoids synthesis are due to changes in PUFA supply from the liver, as this is the main source of PUFAs within the body. However it may be due to changes in fatty acid supply from the vasculature itself. Synthesis of PUFAs has been shown to take place in endothelial cells (262) and smooth muscle cells (223), but the full extent and function of the pathway in vascular smooth muscle cells has not been determined. So therefore it is important to investigate this and to investigate the contribution of this pathway to vascular function. Therefore, this study will aim to address the following hypothesis:

***General hypothesis*:** Variation in quality and quantity of maternal fat intake before and during pregnancy will alter vascular function via persistent changes in the membrane fatty acid composition of the cardiovascular system of adult offspring. These changes within the composition of the membrane and altered function are linked to changes in polyunsaturated fatty acid biosynthesis *de-novo* in VSMCs.

To address this hypothesis the following questions will be investigated:

1. What is the effect of differences in the amount and type of fat consumed by the mother on vascular function in the offspring?
2. What are the biochemical and molecular processes that underlie the effects of maternal dietary fat on vascular function in the offspring?

1.8.1 Chapter-by-chapter hypotheses and thesis outline**:**

Chapter 3:

The aim of this chapter is to ascertain the effect of different types and quantities of maternal fat on adult offspring vascular function and membrane composition

To investigate this, dams will be fed diets enriched in different types of fat at an adequate fat level (7%) and at a high fat level (21%). Offspring blood pressure and vascular function will be measured. In addition to this, to determine whether alterations in membrane fatty acid composition could be responsible for changes in vascular function, the fatty acid composition of offspring aorta will be measured to see if there are physical changes in composition due to the quantity or quality of the maternal diet.

Chapter 3 hypotheses:

- Variations in maternal fat intake will lead to alterations in blood pressure and vascular function in adult offspring.
- The changes in vascular function are due to persistent changes to the membrane fatty acid composition of the adult offspring vascular system.
- The changes within the composition of the aorta membrane and altered function are linked to changes in polyunsaturated fatty acid biosynthesis *de-novo* in VSMCs (fig1.11).

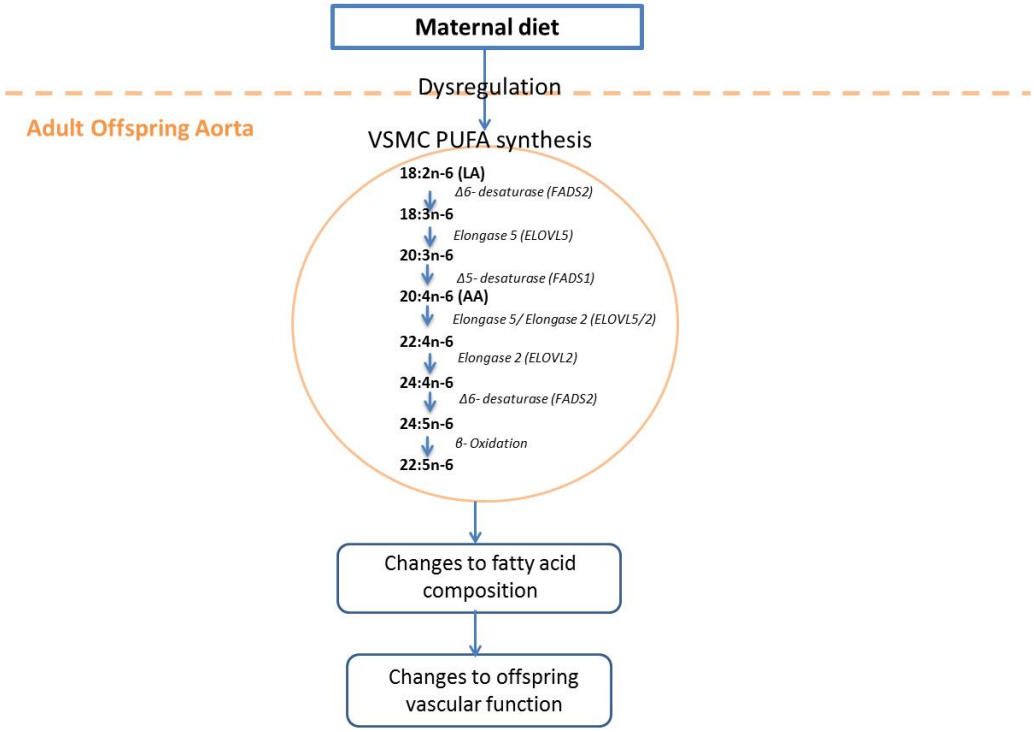


Figure 1.11: Hypothesis diagram which will be investigated throughout the progression of this thesis. The mechanism whereby the hypothesised changes happen will be investigated. LA: Linoleic acid, AA: Arachidonic acid, VSMC: vascular smooth muscle cell, PUFA: polyunsaturated fatty acid.

Chapter 4:

As a link between PUFA biosynthesis in VSMCs and α1-adrenergic receptor mediated vasoconstriction was shown in chapter 3, the aim of this chapter is to confirm that the PUFA biosynthesis *de-novo* does occur in VSMC's and to establish the full extent of the pathway in these cells (figure 1.11).

Molecular techniques will be used to study the activity of the PUFA biosynthesis pathway in VSMCs, this includes determining the expression of the key genes in the pathway and also by investigating the activity of the pathway by treating VSMCs with LA and measuring the fatty acid membrane composition or by direct measure using [U-^{13}C] labelled LA.

Chapter 4 hypothesis:

- VSMC are capable of PUFA biosynthesis *de-novo.*

Chapter 5:

Having established the extent of the PUFA biosynthesis pathway in VSMCs in chapter 4, the aim of this chapter is to determine the mechanism whereby PUFA biosynthesis *de-novo* is involved in vasoconstriction. Ascertaining the link between VSMC PUFA biosynthesis de-novo and α1-adrenergic receptor mediated vasoconstriction, will allow determination of a mechanism whereby maternal diet can cause dysregulation of the PUFA biosynthesis pathway in offspring VSMCs and cause changes in vascular function.

To ascertain the role of VSMC PUFA biosynthesis de-novo in α1-adrenergic receptor stimulation, changes in intracellular calcium release and eicosanoid production will be measured following phenylepherine (Pe) stimulation. Inhibition of specific points of the PUFA biosynthesis pathway will be carried out to determine the effect of the pathway on eicosanoid release and intracellular calcium release following Pe stimulation.

Chapter 5 hypotheses:

- PUFA biosynthesis *de-novo* in VSMC is involved in calcium release and vasoconstriction of VSMCs following stimulation by α1-adrenergic receptor agonist Pe.
- Inhibition of PUFA synthesis *de-novo* in VSMCs causes a decreased production of eicosanoid synthesis following α1-adrenergic stimulation. Demonstrating eicosanoid production is involved in α1-adrenergic mediated vasoconstriction in VSMCs.

Chapter 2-

Methods

2. Methods

2.1 Reagent composition:

Please see the appendices for a full list of chemicals, reagents and suppliers used throughout this thesis and for the composition of TNES and RIPA buffers.

2.1.1 Vascular studies:

Physiological salt soulution (PSS)- NaCl, 119; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.17; $NaHCO_3$, 25; KH_2PO_4, 1.18; EDTA, 0.026; and D-glucose, 5.5 mM.

Potassium Physiological salt solution (KPSS)- KCl, 125.00; $CaCl_2$, 2.5; $MgSO_4$, 1.17; $NaHCO_3$, 25; KH_2PO_4, 1.18; EDTA, 0.026; and D-glucose, 5.5 mM.

2.1.2 Fatty acid analysis:

Chloroform: methanol 2:1: Chloroform (2parts), Methanol (1part) and butylated hydroxytoluene ((BHT) 50 mg/l)

Methylating reagent: methanol containing 2% sulphuric acid (drop-wise addition and mixed by gentle inversion)

Neutralising reagent: (0.25M $KHCO_3$ (25.03 g/l), 0.5M K_2CO_3 (69.10 g/l))

2.2 Animals and diet protocol:

All animal procedures were performed in accordance with the regulations of the UK Home Office Animals (scientific procedures) Act, 1986 and were approved by the local ethical review process.

Virgin female wistar rats (Harlan U.K) (weighing approximately 200 g) were individually caged and randomly assigned to one of the experimental diets outlined in table 2.1 (see Table 2.2 for composition) for 14 days prior to mating. 14 days prior to mating was chosen to maximise the exposure of the offspring to the maternal diet. As this length of time covers at least two oestrous cycles and thus the maturation of the ovum, this ensures the ovum is exposed to the maternal diet. Each female was then placed with breeding males until conception was confirmed by the presence of a vaginal plug on the cage floor. The pregnant dams were returned to the cage and remained on the assigned diet from prior to conception and throughout pregnancy and lactation. Throughout pregnancy weekly food intake and body weight measurements were carried out. The chosen experimental diets include three diets which are associated with vascular dysfunction in non-pregnant adults, these are; butter (high saturated fat) (274), safflower oil (high linoleic acid with n-6: n-3, 76: 1) (136) and trans fatty acid-enriched hydrogenated soybean oil (122). In addition to these diets, menhaden fish oil was also used as an experimental diet, this is enriched in EPA and DHA which are fatty acids which have previously been shown to improve vascular function in non-pregnant adults (159). Therefore, the experimental diets were chosen because each diet has previously been shown to affect vascular function in non-pregnant adults. This allowed us to investigate whether the diets also had an effect on the vascular function of offspring of dams fed each diet and how the effects of the diets compare.

Table 2.1- Quality of diet and corresponding quantities with and abbreviations of the groups.

	7%	**21%**
Hydrogenated soybean oil (HSO)	7%HSO	21%HSO
Safflower oil (SAO)	7%SAO	21%SAO
Butter (SFA)	7%SFA	21%SFA
Menhaden Fish oil (FO)	7%FO	21%FO

At term, pregnant dams were allowed to deliver naturally. Two days after delivery, litters were weighed (to avoid rejection) and each litter was then standardised to eight pups. Excess

offspring were culled by cervical dislocation, leaving four males and four females where possible. Pups remained with the dam until they were weaned at 28 days. The offspring were then separated into cages of males and females for each diet and ear punched for identification, carried out by the Animal house staff. All offspring were placed on maintenance diet (AIN-93M) (TestDiet, MO, USA) (see Table 2.2) with *ad libitum* access to food and water until the end of the study. Immediately following weaning dams and a sub group of offspring were sacrificed and tissue was collected (see section 2.4). The remaining animals were taken to day 77 and during this time weights and food intake were measured. Within seven days prior to sampling at both time points, blood pressure was measured by tail cuff plethysmography (see section 2.3). A schematic of the protocol used is shown (fig 2.1).

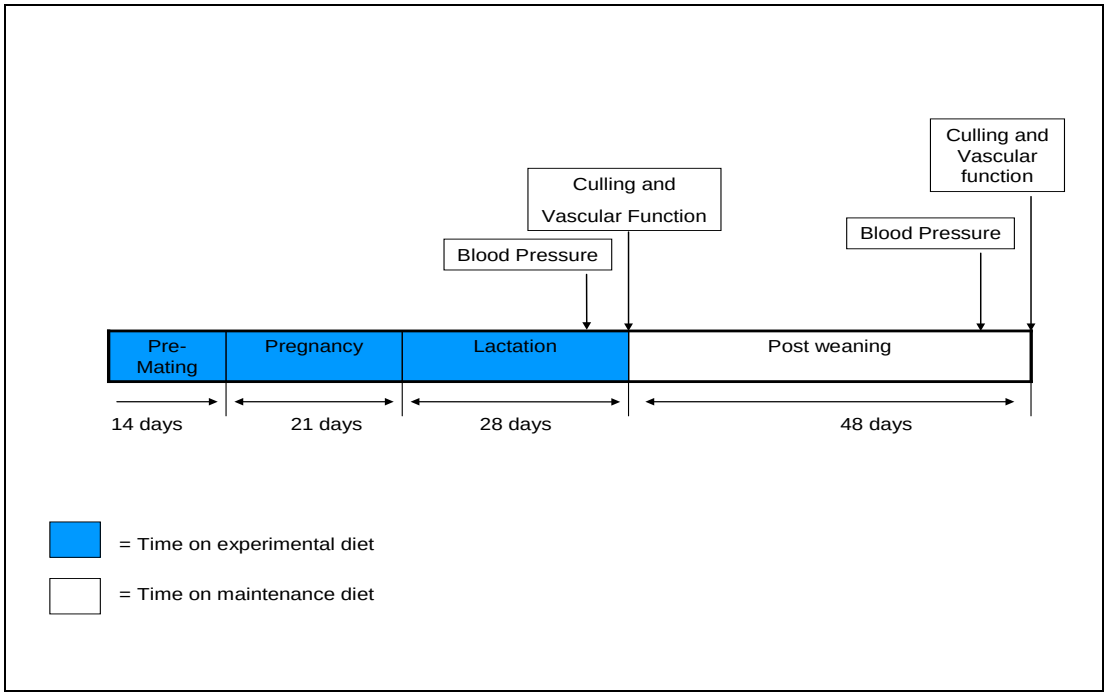


Figure 2.1- Timeline of breeding protocol and procedures carried out. (Culling point days 77 ±8 and 28 ±11)

Table 2.2: Diet composition. Total n-6 PUFA is the sum of 18:2n-6, 18:3n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6. Total n-3 PUFA is the sum of 18:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3. N = not detected. Diet formulated and supplied by TestDiet (MO, USA).

	Maternal diets								Post
	7% (w/w) Fat				21% (w/w) fat				Weaning (7%)
	SAO	HSO	Butter	FO	SAO	HSO	Butter	FO	AIN93M
Casein (g/100g)	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	14.0
Corn starch (g/100g)	39.7	39.7	39.7	39.7	25.7	25.7	25.7	25.7	46.6
Sucrose (g/100g)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Maltodextrin	13.2	13.2	13.2	13.2	13.2	13.2	13.2	13.2	15.5
Cellulose (g/100g)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
AIN93G/AIN93M mineral mix (g/100g)	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5 (AIN93M mix)
AIN93 vitamin mix (g/100g)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Choline (g/100g)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
α-Tocopheroyl acetate (mg/100g)	84	84	84	84	84	84	84	84	84
Total fatty acids (g/100g)	7.0	7.01	6.98	6.96	20.99	21.0	20.96	20.89	7.01
Cholesterol (g/100g)	0	0	0.0150	0.0364	0	0	0.0301	0.1092	0
Total metabolisable energy (MJ/100g)	1.6	1.4	1.6	1.6	1.9	1.1	1.8	1.9	1.6
	Fatty acid composition (g/100g)								
10:0	N	N	0.06	N	N	N	0.17	0.01	N
12:0	N	N	0.07	N	N	N	0.22	N	N
14:0	0.01	0.02	0.92	0.6	0.04	0.05	2.75	1.81	0.01
16:0	0.46	0.8	2.83	1.26	1.37	2.41	8.49	3.79	0.76
16:1n-7	0.01	N	0.12	0.75	0.02	0.01	0.35	2.26	0.01
18:0	0.19	0.89	0.88	0.24	0.57	2.68	2.64	0.73	0.29
18:1n-9	1.18	1.77	1.75	0.68	3.55	5.32	5.24	2.03	1.52
18:1n-7	0.04	0.2	0.07	0.25	0.13	0.60	0.2	0.74	0.1
Total TFA	N	3.05	N	N	N	9.14	N	N	N
18:2n-6	5.05	0.19	0.16	0.14	15.15	0.58	0.49	0.42	3.76
18:3n-6	N	N	N	0.03	N	N	N	0.08	0.02
18:3n-3	0.01	0.02	0.04	0.08	0.02	0.05	0.13	0.23	0.48
20:0	0.02	0.03	0.04	0.02	0.07	0.09	0.13	0.05	0.02
22:1n-9	N	N	0.01	0.24	N	N	0.02	0.71	N
20:1n-9	0.01	N	N	0.09	0.04	N	N	0.27	0.01
20:2n-9	N	N	0.01	0.02	N	N	0.03	0.05	N
20:3n-6	N	N	0.01	0.02	N	N	0.02	0.06	N
20:4n-6	N	N	0.01	0.1	N	N	0.02	0.29	N
22:0	0.02	0.03	0.01	00	0.05	0.08	0.02	00	0.02
20:5n-3	N	N	0.01	1.42	N	N	0.03	4.27	N
24:0	N	N	N	N	N	N	N	00	N
22:4n-6	N	N	N	0.01	N	N	N	0.04	N
24:1n-9	N	N	N	0.02	N	N	N	0.07	N
22:5n-6	N	N	0.01	0.02	N	N	0.02	0.06	N
22:5n-3	N	N	0.01	0.19	N	N	0.04	0.58	N
22:6n-3	N	N	0.01	0.83	N	N	0.02	2.48	N
SFA	0.7	1.77	4.80	2.13	2.09	5.30	14.41	6.40	1.1
MUFA	1.24	1.98	1.94	2.02	3.73	5.93	5.810	6.06	1.65
Total n-6 PUFA	5.05	0.19	0.17	0.29	15.15	0.58	0.52	0.88	3.78
Total n-3 PUFA	0.01	0.02	0.07	2.52	0.02	0.05	0.22	7.55	0.48
18:2n-6 : 18:3n-3	7.39	0.13	0.04	0.020	7.39	0.13	0.04	0.02	0.08

2.3 Blood Pressure measurement:

Systolic and diastolic blood pressure was recorded at 77 days of age in male and female offspring by tail cuff plethysmography (275), using a Columbus volumetric blood pressure monitor (NIBP-8, Linton Instruments, UK). Animals were placed in a room which had been heated to 29˚C for around 30 minutes to stimulate tail blood flow. Following acclimatisation to the temperature, rats were placed in appropriately sized restraint tube and a suitably sized tail cuff was placed around the tail and inflated. The cuff was inflated to 200 mmHg and the sensory cuff set at 50 mmHg. Blood pressures were recorded (see fig 2.2) and heart rate (HR) and mean arterial pressure (MAP) were calculated within the system. An example trace is shown below in figure 2.2. The trace shows the inflation of the tail cuff (fig 2.2 top trace) which causes occlusion of the blood flow in the tail of the rat. The occlusion cuff is slowly deflated, as the blood flow returns to the tail; the sensor cuff detects the swelling of the tail (fig 2.2 bottom trace). Systolic blood pressure is measured at the first sign of swelling and diastolic blood pressure is measured at the point when the swelling in the tail stops. To reduce stress the animals were handled by trained handlers and were familiarised with the equipment and the procedure before measurements were taken. Blood pressure measurements of offspring were carried out by Dr Kelsall.

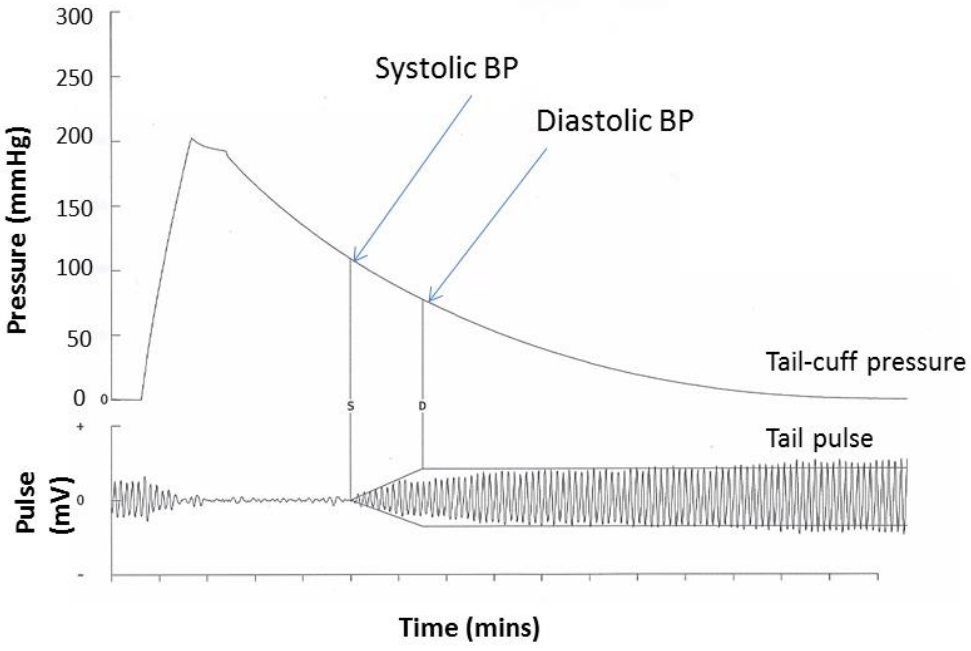


Figure 2.2: An example trace of a blood pressure recording using tail-cuff plethysmography. Trace shows tail cuff pressure during inflation and deflation and tail pulse in a rat. Systolic blood pressure and diastolic blood pressure are labelled on the trace. mmHg: millimetres of mercury, mV: millivolts, BP: blood pressure, mins: minutes.

2.4 Tissue collection

Tissues were collected from offspring at approximately day 28 (range ±11) and 77 (range ±8). All animals were fasted for at least 12 hours prior to sacrifice by CO_2 inhalation and cervical dislocation. Body weights were recorded and blood was collected immediately by cardiac puncture and stored in lithium heparin lined vacuotainers on ice (4˚C). The mesenteric arcade and the thoracic aorta were removed and placed into cold physiological salt solution (4˚C) (PSS, for composition see section 2.1) and stored on ice. Other organs which were collected were; heart, liver and gonadal fat, these were all weighed, snap frozen in liquid nitrogen and stored at -80˚C for molecular analysis and fatty acid analysis. The blood was separated into plasma and red blood cells by centrifugation at 3000 x g for 15 minutes, the plasma was removed and both fractions were snap frozen and stored at -80˚C. Tissue collection from offspring of dams fed 7% diet were carried out by Dr Kelsall and 21% collections were carried out in partnership with Dr Kelsall.

2.5 Vascular reactivity:

The aorta was cleaned of fat and connective tissue, and cut into 2 mm segments. Segments were mounted on a wire myograph (Danish Myo Technology A/S, Denmark) and bathed in warm PSS at 37° C and continuously gassed with 95% O_2 and 5% CO_2. The vessels were normalised by putting under a resting tension of 1 g for 30 minutes. To test the viability of the segment, 125mM KPSS (equimolar substitution of K^+ for Na^+, for composition see section 2.1) was added to the chamber and the contraction in response to the K^+ was allowed to plateau, once a plateau was reached the chamber was drained and replaced with warmed PSS. Vascular reactivity measurements of offspring of dams fed 7% diet were carried out by Dr Kelsall.

2.5.1 Assessment of vasoconstriction:

To assess vasoconstriction in the aortae, a cumulative concentration-response curve (CRC) to the α_1-adrenoceptor agonist phenylephrine (Pe) (1 nM-100 µM) was carried out by adding cumulative doses of Pe and incubating for 2 minutes between doses (fig 2.3).

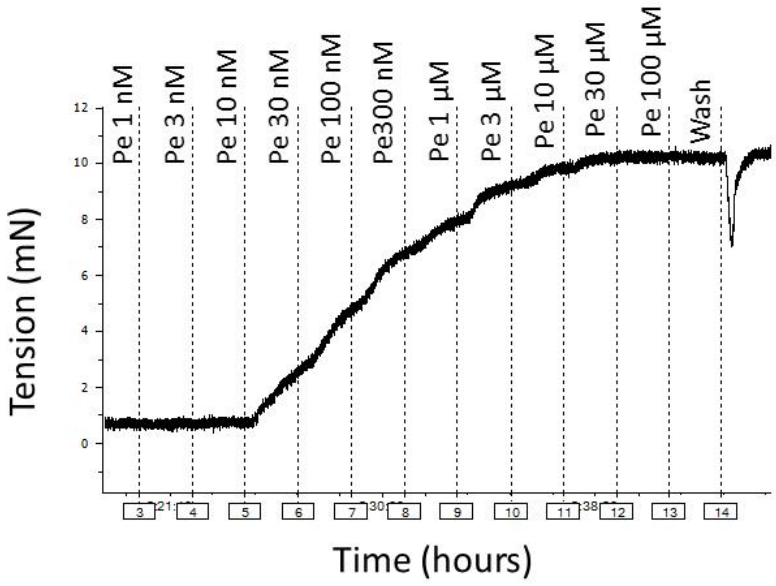


Figure 2.3: Representative trace of cumulative concentration-response curve to PE. mN: millinewton, Pe: phenylephrine.

2.5.2 Assessment of vasorelaxation:

The aorta segments were pre-constricted to pEC_{80} (negative logarithm of the concentration that produced 80% of the maximum response) Pe and CRCs to the endothelium-dependent vasodilator acetylcholine (ACh) (0.1 nM-10 µM) were performed to test endothelium-dependent relaxation (fig 2.4). To investigate the endothelium-independent relaxation the segment was again pre-constricted to pEC_{80} Pe and a CRC to the nitric oxide donor sodium nitroprusside (SNP 0.1 nM-10 µM) was carried out.

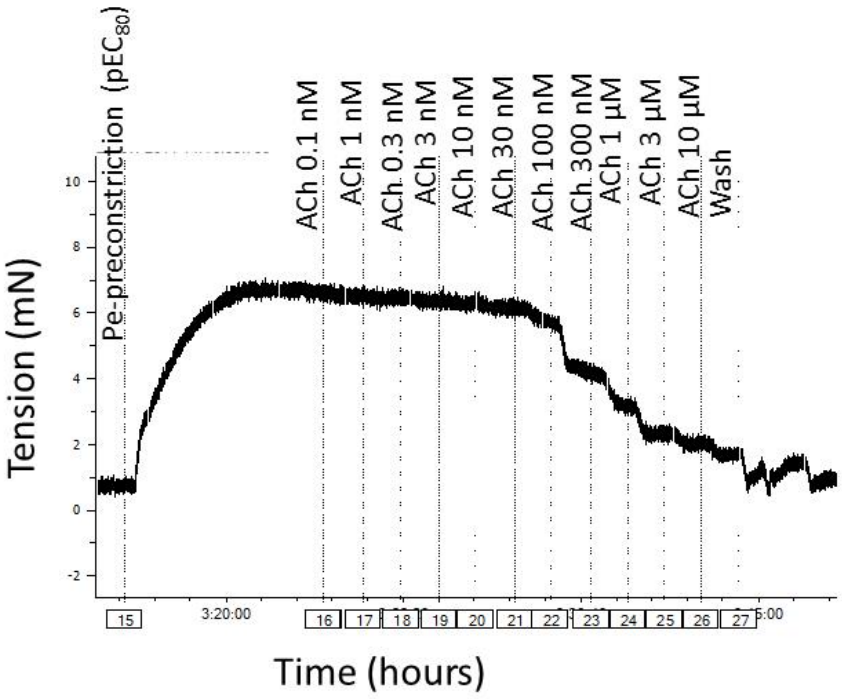


Figure 2.4: A representative trace showing pre-constriction by Pe and a cumulative concentration-response curve to ACh. mN: millinewton, Pe: phenylephrine, ACh: acetylcholine.

2.5.3 Nitric oxide contribution to endothelium-dependent relaxation:

In order to assess the contribution of nitric oxide to the relaxation of the aorta, CRC responses to ACh were repeated (described in section 2.5.2) in the presence of the NOS inhibitor L-Nitro arginine methyl ester (L-NAME) at a range of concentrations (0.1 nM, 100 nM, 1 µM and 100 µM). To allow incubation of the aortas with the inhibitor, L-NAME was added to the myograph 30 minutes prior to the preconstruction with pEC_{80} PE and the ACh CRC.

2.5.4 The role of PUFA biosynthesis in Pe-mediated vasoconstriction:

The following experiment was carried out by Dr Chris Kelsall. In order to determine whether PUFA biosynthesis *de-novo* is involved in the regulation of vascular tone, aorta from adult male rats, which had not been exposed to altered nutrition during development, were treated with the delta-6 or delta-5 desaturase inhibitiors; SC-26196 (10^{-4}) or sesamin (10^{-4}) respectively. Segments were incubated with the inhibitors for 30 minutes before Pe treatment. Control vessels were not incubated with the inhibitors. All vessels were then treated with Pe as described in section 2.5.1. In order to determine whether PUFA biosynthesis was active in the endothelial or smooth muscle cells, constriction to Pe was determined in whole aorta or endothelium denuded aorta. The endothelium was removed from sections of aorta by sliding a cotton thread through the vessel.

2.5.5 Analysis of vasoconstriction in human femoral arteries:

The following experiment was carried out by Dr Chris Kelsall. Human femoral arteries were collected from two subjects undergoing leg amputation for atherosclerotic disease. Regions of artery, which appeared to be uninvolved in atherosclerosis, were dissected, cleaned and placed in ice cold PSS (4ºC). Myography was performed using the method discussed in section 2.5.1.

2.6 Fatty acid analysis:

2.6.1 Sample preparation:

Aorta samples were dissected clean of surrounding fat and tissue and snap frozen following post mortem. Frozen aorta sample were prepared by crushing using a pestle and mortar in liquid nitrogen to keep the samples frozen. Aorta (approximately 100 mg) was used for fatty acid analysis, 0.9% NaCl solution (800 µl) was added to all aorta samples. For analysis of plasma samples, 800 µl of plasma was used; if this was not available the maximum amount was used and made up to 800 µl by the addition of 0.9% NaCl.

2.6.2 Total lipid extraction:

Extraction of the lipids from the aorta and plasma was carried out using the Folch et al. (1957) method (276). The samples of aorta were transferred into a labelled screw capped tube with of 0.9% NaCl solution (800 µl), chloroform:methanol (2:1) solution (containing the antioxidant butylated hydroxytoluene, see section 2.1for composition) (5 ml) was added to each sample and the tubes were vortexed. 1 M NaCl (1 ml) was added and the samples were thoroughly

mixed by vortex. The samples were then centrifuged for 10 mins at 900 x g (low brake and room temp) (Thermo Scientific Heraeus Megafuge 40R). Following centrifugation the lower phase of sample was collected by aspiration using a pasteur pipette and transferred into a clean screw capped tube. Samples were dried under nitrogen gas at 40˚C leaving the total lipid extract at the bottom.

2.6.3 Separation of lipid classes by solid phase extraction (SPE):

Plasma samples were separated into lipid classes by SPE (277). An SPE tank was connected to a vacuum pump. Aminopropylsilica SPE cartridges were placed on the SPE tank. Chloroform (2 ml) was added to each cartridge and allowed to drip through. The total lipid extract (prepared in section 2.6.2) was dissolved in dry chloroform (1 ml) and vortexed. The sample was then applied to the cartridge and allowed to drip through under gravity. The remaining liquid was collected by vacuum and the column was washed with chloroform (2 x 1 ml) under vacuum. The resulting product is a solution which contains both TAG and cholesterol ester (CE) fractions. These were then dried under nitrogen at 40 ºC. New collection tubes were then put in the SPE tank and chloroform: methanol (60:40, v/v) (2 ml) was added to the cartridges under vacuum, this elutes the phosphatidylcholine (PC) fractions which could be discarded. The NEFA fraction was then eluted by adding chloroform: methanol: glacial acetic acid (100:2:2. v/v/v) under vacuum. Once collected this was dried under nitrogen at 40 ºC.

To separate the dried TAG and CE fractions, new SPE cartridges were used and clean collection tubes. The cartridges were washed with hexane (4 x 1 ml), the first three washes were under vacuum and the final was allowed to drip through under gravity. The TAG and CE fraction was dissolved in dry hexane (1 ml) and vortex mixed. The samples were applied to the cartridges and allowed to drip through and washed with dry hexane (2 x 1 ml) under vacuum. This elutes the CE fraction which was not required. The TAG fraction was then eluted by the addition of dry hexane: chloroform: ethyl acetate (100:5:5) under vacuum. The TAG fraction was dried under nitrogen at 40ºC.

2.6.4 Preparation of fatty acid methyl esters (FAMEs):

From the total lipid extracts of aorta and the plasma lipid classes separated with SPE, FAMEs were prepared (277). Toluene (0.5 ml) was added to each of the dried purified lipid samples and vortexed. The methylation reagent (1 ml) was prepared (see section 2.1) and was added to each sample and gently mixed. Following this, the samples were heated for two hours at 50ºC before being allowed to cool. The neutralising reagent (see section 2.1 for composition) (1 ml) was made and added to each sample, followed by hexane (1 ml) and vortexed. Each tube was

centrifuged for 2 minutes at 200 x g (low brake and room temperature). The samples separated into two phases and the upper phase (FAME) was removed and transferred to a clean tube and dried under nitrogen gas at 40ºC. Hexane (75 µl) was added to each tube, vortexed and transferred to a GC autosampler vial containing a glass insert, this was repeated to make sure as much of the sample is transferred. The vial was either stored in the freezer or run on the GC. The proportions of individual fatty acids measured by gas chromatography using BPX70 fused silica capillary column (30 m x 0.25 mm x 0.25 µm) on an Agilent 6890 gas chromatograph equipped with flame ionisation detection. FAMEs were identified by their retention times relative to the standards; 37FAMEs, Menhaden oil and 22:4n-6 with 22:5n-6. FAMEs were quantified using Chemstation software (Agilent).

2.7 Cell culture:

For all cell culture protocols aseptic techniques were used to prevent any contamination of cells. All work using cells was carried out in a cell culture hood, which was regularly cleaned with bactericide and Vircon. Before each use the hood was thoroughly sterilised with 70% ethanol sprayed over all surfaces and equipment within the hood, including all pipettes. Gloves and cell culture lab coats were worn at all times and gloves were sprayed with 70% ethanol before touching flasks or working inside the hood. All flasks, plates, Stripettes and any containers including reagents and media were thoroughly sprayed with 70% ethanol before they entered the hood. Disposable Stripettes were used and then disposed of to avoid any cross contamination. All experiments were carried out as quickly as possible and caps on flasks and media were only removed immediately as required and replaced as soon as possible to prevent any contamination.

2.7.1 Human aortic smooth muscle cells:

Human aortic smooth muscle cells (HASMCs) were obtained from Life Technologies (Paisely, UK) at passage two. These are adherent cells. HASMCs arrived frozen on dry ice. They were immediately seeded as recommended by the supplier; the vial was dipped into a water bath at 37ºC to thaw and the contents of the vial were diluted in standard medium (5 ml) and counted using a haemocytometer. The standard growth medium used for HASMCs was Medium 231 supplemented with smooth muscle growth supplement (SMGS) and containing 1% (v/v) penicillin/streptomycin (pen/strep) antibiotic. Cells were seeded at a density of $2.5x10^3$ viable cells/cm^2 in three 75 cm^2 tissue culture flasks containing and contained a total of 15 ml of medium. The cultures were incubated in a humidified cell culture incubator at 37ºC, 5%

CO_2/95% air. Cells were allowed to attach for 24 hours. Once the culture was initiated, the medium was changed every 48 hours until the culture was 80% confluent and then the medium was changed daily.

The cells were allowed to grow to confluency which took approximately seven days from seeding. Once confluent, cells were passaged, as follows. The medium was removed and discarded and the cells were washed twice with Hanks buffered saline solution (HBSS) to remove any traces of medium. To detach the cells, Trypsin- EDTA (0.05% w/v) (1 ml) was added to each 75 cm^2 flask and incubated for 5 minutes. Once all the cells had detached standard medium (9 ml) was added to the flask and then split 1:2, detached cells (5 ml) were removed and put into a fresh 75 cm^2 with fresh medium (10 ml). This was repeated until passage 7, at which point cells exhibited changes in morphology.

At each passage cells were also frozen. As with splitting cells, the cells were rinsed in HBSS and trypsin was added, once all the cells were detached, fresh medium was added as described previously. The medium containing the cells (10 ml) was transferred to a falcon tube and was centrifuged at 180xg for 7 minutes, producing a pellet of cells at the bottom of the tube. The pellet was resuspended in standard growth medium with 10% (v/v) dimethyl sulfoxide (DMSO), and $1x10^6$ cells (1 ml) were aliquotted into cryovials. The cryovials were then stored in a passive freezer at -80ºC for 24 hours before being archived in the vapour phase of a liquid nitrogen storage vessel. HASMCs were used at passage 5 for all experiments.

2.7.2 MOVAS cells:

MOVAS are aorta, smooth muscle cells derived from mouse C57BL6 and immortalised with SV40 large T antigen. These cells are adherent. They were obtained from ATCC (CRL-2797, LGC standards) and arrived at passage 16. Cells arrived frozen on dry ice and culture was immediately initiated. The vial containing the cells was thawed in a water bath at 37ºC, once thawed, the contents was transferred to a falcon tube containing of medium (9 ml). The standard medium for MOVAS cells was Dulbecco's modified Eagle medium (DMEM) (containing high glucose and pyruvate) with 10% (v/v) Fetal bovine serum and 0.2 mg/ml G-418 and 1% (v/v) pen/strep. The falcon tube was centrifuged at 125xg for 5 minutes to produce a pellet of cells. The supernatant was discarded and the cell pellet was resuspended in medium (5 ml), this was then put in a pre-warmed 75 cm^2 flask containing of medium (10 ml. The cultures were incubated in a humidified cell culture incubator at 37ºC, 5% CO_2/95% air. The cells were allowed to attach for 24 hours. Once the culture was initiated and the medium

was changed every 48 hours until the culture was 80% confluent and then the medium was changed every day.

The cells were allowed to grow to confluency which took approximately four days from seeding. Once confluency was reached, cells were passaged; medium was removed and discarded, the cells were washed twice with HBSS to remove any traces of medium. To detach the cells, Trypsin-EDTA (0.05% w/v) (1ml) was added to each 75 cm^2 flask and incubated for 5 minutes and the sides of the flask were tapped to dislodge any attached cells. Once all the cells had detached, medium (9 ml) was added to the flask and then passaged 1:8, detached cells were removed (1.25 ml) and put into a fresh 75cm^2 with fresh medium (13.75 ml).

Cells went through multiple passages up to passage 32. At each passage cells were also frozen. As with passaging cells, the cells were rinsed in HBSS and trypsin-EDTA (0.05% w/v) was added, once all the cells were detached, fresh medium was added as described previously. The medium containing the cells (10 ml) was transferred to a falcon tube and was centrifuged at 125xg for 5 minutes, producing a pellet of cells at the bottom of the tube. The pellet was resuspended in standard growth medium with 5% (v/v) DMSO. The cryovials were then stored in a passive freezer at -80ºC for 24 hours before being archived in the vapour phase of liquid nitrogen storage vessel.

2.7.3 Mouse Hepa1-6:

Hepa1-6 cells are mouse hepatoma cells, derived from BW7756 tumour in C57L mouse. These cells are adherent. They were obtained from European collection of cell cultures (ECACC) and arrived at passage 5. Cells arrived frozen on dry ice and culture was immediately initiated. The vial containing the cells was thawed in a water bath at 37ºC, and the contents were transferred to a falcon tube containing medium (9 ml). The medium used for Hepa1-6 cells was DMEM (containing high glucose and pyruvate) with 10% (v/v) fetal bovine serum and 1% (v/v) pen/strep. The falcon tube was centrifuged at 125xg for 5 minutes to produce a pellet of cells. The supernatant was discarded and the cell pellet was resuspended in medium (5 ml), this was then transferred to a pre-warmed 75 cm^2 flask containing medium (10 ml). The cultures were incubated in a humidified cell culture incubator at 37ºC, 5% CO_2/95% air. The cells were allowed to attach for 24 hours. Once the culture was initiated, the medium was changed every 48 hours until the culture was 80% confluent and then the medium was changed daily.

The cells were allowed to grow to confluency which took approximately four days from seeding. Once confluency was reached, cells were passaged; medium was removed and discarded, the cells were washed twice with HBSS to remove any traces of medium. To detach

the cells trypsin-EDTA (0.05% w/v) (1ml) was added to each 75 cm^2 flask and incubated for 5 minutes and the sides of the flask were tapped to dislodge any attached cells. Once all the cells had detached medium (9 ml) was added to the flask and then passaged 1:8, detached cells (1.25 ml) were removed and put into a fresh 75cm^2 with fresh medium (13.75 ml).

Cells went through multiple passages up to passage 14. At each passage cells were also frozen. As with passaging cells, the cells were rinsed in HBSS and trypsin-EDTA (0.05% w/v) was added, once all the cells were detached, fresh medium was added as described previously. The medium containing the cells (10 ml) was transferred to a falcon tube and was centrifuged at 125xg for 5 minutes, producing a pellet of cells at the bottom of the tube. The pellet was resuspended in standard growth medium with 5% (v/v) DMSO and 1x10^6 cells (1 ml) were aliquotted into cryovials. The cryovials were then stored in a passive freezer at -80ºC for 24 hours before being archived in the vapour phase of liquid nitrogen storage vessel.

2.8 Real-time PCR:

2.8.1 RNA extraction:

Extraction of RNA was carried out using the mirVana miRNA isolation kit (Life Technologies) as instructed by the manufacturer see section 2.8.1.3. The samples were prepared for RNA extraction as detailed in 2.8.1.1 and 2.8.1.2.

2.8.1.1 Tissue sample preparation for RNA extraction:

Upon removal from the -80ºC freezer, the samples were stored on dry ice to prevent any thawing. Samples were crushed using a pestle and mortar under liquid nitrogen. The weight of each sample was measured; approximately 100 mg of tissue was used per sample, although this was not always possible with aorta samples. 10 volumes of lysis/binding buffer per tissue mass was pipetted onto the sample and vortexed to produce a homogenous mixture.

2.8.1.2 Cultured cells preparation for RNA extraction:

Cells were scraped and collected in PBS (1 ml), centrifuged for 5 minutes at 125x g and the PBS removed. The pellet was then stored at -80ºC until ready to use. Lysis/binding solution was added to the pellet depending on the number of cells, such that for 100-10^7 cells, 300-600 µl of solution was added respectively. The sample was then vigorously vortexed or sheared by pipetting to completely lyse the cells and to produce a homogenous lysate.

2.8.1.3 RNA extraction using the mirVana miRNA isolation kit:

The mirVana miRNA isolation procedure involves lysing the sample in a denaturing lysis solution which stabilises the RNA and inactivates RNases and removes most DNA. Acid-Phenol:Chloroform is then used to extract the lysate to leave a semi-pure RNA sample. A glass-fibre filter is used to purify RNA in combination with solutions which contain ethanol and are formulated to avoid loss of small RNAs. The glass filter immobilises the RNA. Increasing concentrations of ethanol are used to precipitate the RNA and provide a highly enriched RNA fraction. This extraction method allows a high yield of ultra-pure, high quality, small RNA. The high yield or RNA was particularly important for the aorta samples as the amount of tissue available was very small, so a sensitive method was needed to ensure the highest yield from the tissue available.

Once the cells or tissue were in a homogenous mixture, the volume of the lysate was noted. miRNA homogenate additive was added to the lysate (1:10) and then mixed by inversion and vortex. The mixture was left on ice for 10 minutes. An equal volume of acidified phenol: chloroform to that of the original lysate was added to the sample. This mixture was then vortexed for 30-60 seconds. The aqueous and organic phases were separated by centrifugation for 5 minutes at a speed of 10,000 x g at room temperature. The aqueous phase was removed and put into a fresh tube and the volume noted, the organic phase was discarded. To the aqueous phase, 1.25 volumes of room temperature 100% ethanol were added.

Filter cartridges were placed in collection tubes and the sample (700 µl) was applied to the filter cartridge. This was centrifuged for 15 seconds at 10,000 x g to pass the mixture through the filter. The flow through was discarded and the remaining sample was applied to the cartridge and this was repeated. Once the entire sample had passed through the filter, Wash Solution 1 (700 µl) was applied to the cartridge and centrifuged as before, to pass the sample through the filter. The flow-through was discarded. Wash Solution 2/3 (500 µl) was added to the filter and centrifuged. This was repeated. After the last centrifugation, the solution was discarded and the filter was spun in the collection tube for 1 minute to remove any residual fluid from the filter.

The filter cartridge was then transferred to a clean collection tube and RNAse free water (50 µl for cell and aorta RNA and 100 µl for liver) was applied to the cartridge and then centrifuged for 20-30 seconds to elute the RNA. The concentration of the RNA was measured using a Nanodrop1000 spectrophotometer (Labtech, East Sussex, UK). All RNA had a 260/280 ratio

(assessment of protein contamination) of above 1.8 and a 260/230 ratio (assessment of phenol contamination) of above 1.4. The RNA was stored at -80ºC until required.

2.8.2 DNase treatment:

Samples were treated with DNase in order to remove any residual DNA from RNA preparations. This was performed using the Amplification Grade DNase1 kit (Sigma-Aldrich). Each RNA sample (1 µg) was diluted using RNase-free water, to a total volume of 8 µl in an RNase-free PCR tube. 10x concentrated Reaction Buffer (1 µl) was added followed by Amplification Grade DNase 1 (1 µl). The mixture was gently mixed and incubated at room temperature for 15 minutes. Following incubation, Stop Solution (1 µl) was added to inactivate the DNase 1 and the preparation was then heated at 70ºC for 10 minutes to denature both the DNase 1 and the RNA. Samples were then cooled on wet ice (4ºC) prior to preparation of cDNA.

2.8.3 Preparation of cDNA:

To the DNase treatment product (1 µg), 10mM dNTP mix (1 µl) was added. This was followed by 5 µM random nanomers (1 µl). The preparation was mixed gently, pulse-centrifuged and then incubated at 70ºC for 10 minutes. Following incubation, samples were cooled on wet ice (4ºC).

cDNA was synthesised using the M-MLV Reverse Transcriptase kit. A master mix was prepared by adding 10X M-MLV Reverse Transcriptase Buffer (4 µl) to M-MLV Reverse Transcriptase (1 µl), followed by RNAse-free water (5 µl) and mixed using a pipette. The master mix solution was added to each sample (10 µl). The samples were incubated on the Veriti Thermal Cycler (Applied Biosciences, CA, USA) to complete the reverse transcription, the machine incubated the samples at 37 ºC for 50 minutes to produce the cDNA strand and then heated the samples at 90ºC for 10 minutes, in order to denature the M-MLV reverse transcriptase. This produces 1 µg of cDNA in 20 µl.

2.8.4 Primer design and optimisation:

Real Time RT-PCR primer sequences for fatty acid desaturase 1 (FADS1), Elongase 2 (ELOVL 2), Elongase 5 (ELOVL5) and peptidylprolyl isomerase A (PPIA,cyclophillin) (housekeeping gene) were supplied by Qiagen as a mix of forward and reverse primers (table 2.3). The company does not disclose the primer sequences. Human Fatty acid desaturase 2 (FADS2) primer sequences were designed using the Primer3 website (http://frodo.wi.mit.edu/). All primers were diluted to a concentration of 100µM in DNase free, RNase free H_2O upon arrival and stored at -20ºC. Primers were optimised by carrying out a temperature gradient between 51ºC

and 59ºC and assessing which annealing temperature gave the best product. This was done by performing electrophoreisis (as described in section 2.9.4) on the PCR product from each temperature and assessing which temperature produced a single strong band of correct size.

Table 2.3: Details of primers used for RT-PCR

Primers	**Primer Sequences/ Primer Assays**
Human PPIA (Cyclophillin A)	Qiagen Hs_PPIA_1_SG QuantiTect primer Assay (QT00052311)
Human Fatty acid desaturase 1	Qiagen Hs_FADS1_1_SG QuantiTect primer Assay (QT01003800)
Human Fatty acid desaturase 2	Forward primer: 5'-CGCAAGGTTTACAACATCAC-3' Reverse primer: 5'-GCCAGTTCACCATCAGC-3'
Human Elongase 2	Qiagen Hs_ELOVL2_1_SG QuantiTect primer Assay (QT00059017)
Human Elongase 5	Qiagen Hs_ELOVL5_1_SG QuantiTect primer Assay (QT00096334)
Mouse PPIA (Cyclophillin A)	Qiagen Mm_Ppia_1_SG QuantiTect primer assay (QT00247709)
Mouse Fatty acid desaturase 1	Qiagen Mm_Fads1_1_SG QuantiTect primer assay (QT00114184)
Mouse Fatty acid desaturase 2	Qiagen Mm_Fads2_1_SG QuantiTect primer assay (QT00130018)
Mouse Elongase 2	Qiagen Mm_Elovl2_1_SG QuantiTect primer assay (QT00091644)
Mouse Elongase 5	Qiagen Mm_Elovl5_1_SG QuantiTect primer assay (QT00117705)
Rat PPIA (Cyclophillin A)	Qiagen Rn_Ppia_1_SG QuantiTect Primer Assay (QT00177394)
Rat Fatty acid	Qiagen Rn_Fads1_1_SG QuantiTect primer assay

desaturase 1	(QT00188664)
Rat Fatty acid desaturase 2	Qiagen Rn_Fads2_1_SG QuantiTect primer assay (QT00186739)
Rat Elongase 2	Qiagen Rn_Elovl2_2_SG QuantiTect primer assay (QT01683899)
Rat Elongase 5	Qiagen Rn_Elovl5_va.1_SG QuantiTect primer assay (QT02466653)

2.8.5 Real-time PCR:

cDNA was diluted in DNase and RNase free water (200 µl) to produce a concentration of 5 ng/µl. Standards were made up by serial dilution using RNase/DNase free water and cDNA extracted from either mouse, human or rat liver cells or tissue. Standards (0.01 ng-200 ng) were prepared for each gene primer set. A master mix was prepared for each primer set containing; RNase/DNase free water (4.5 µl), each Qiagen primer set (2.5 µl), ROX (0.5 µl) and SYBR green (12.5 µl). The master mix for human FADS2 contained RNase/DNase free water (6.0 µl), forward primer (0.5 µl) and reverse primer (0.5 µl), ROX (0.5 µl) and SYBR green (12.5 µl). cDNA (5 µl) for each sample and control was aliquotted in duplicate into a 96-well plate with the specific master mix (20 µl). Two negative controls were used for each primer set, where cDNA was replaced with RNase/DNase free water (5 µl). All samples were measured using a Step One Plus PCR machine (Applied Bioscience, CA, USA). The parameters were 95 ºC for 2 minutes then 40 cycles of 95 ºC for 30 seconds followed by 1 minute at an annealing temperature of 55ºC (for FADS1, ELOVL2, ELOVL5, cyclophilin and Qiagen FADS2) and 52 ºC for Human FADS2, followed 72 ºC for 1 minute. Relative mRNA expression was determined by standard curve and normalised to the housekeeping gene; Cyclophilin A (198). Housekeeping genes are reference genes which are expressed in a wide variety of tissues and cells types and show minimal changes in expression levels between samples and experimental conditions (278). Cyclophilin A is found in all tissues in mammals, it is a gene that encodes a ubiquitous cytoplasmic protein that plays a role in protein folding through isomerisation of peptide bonds (279). Cyclophilin A has previously been shown to show a stable expression between tissue types and thus a reliable housekeeping gene (280). Comparing the cycle threshold (Ct) value which is number of cycle amplifications required for the fluorescent signal to exceed the background level allows stability to be determined. Comparing the Ct value in rat tissue

(P=0.932), mouse tissue (P=0.8776) and mouse cells (P=0.0600) shows that there is no significant difference between the expression of cyclophilin in aorta and liver, see figure 2.5. This demonstrates that the expression of cyclophilin in aorta and liver in both species and in the mouse cell lines is stable and is a suitable housekeeping gene.

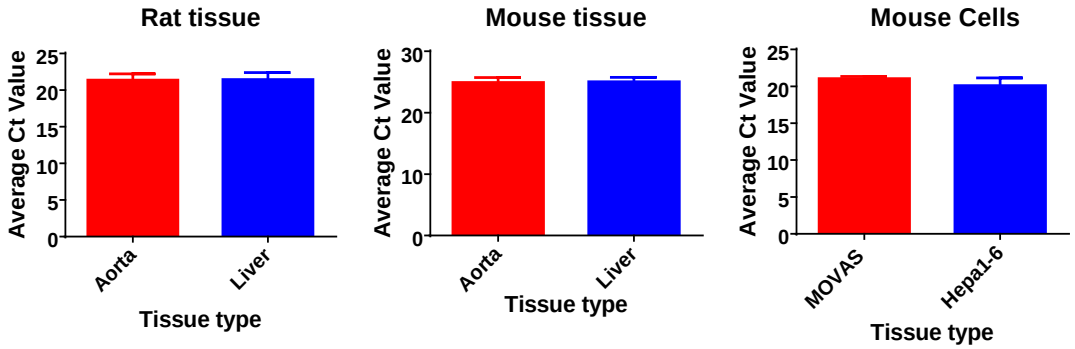


Fig 2.5: Stability of Cyclophilin expression between aorta and liver tissue in rat tissue, mouse tissue and mouse cells. Data shown as mean Ct value ±SD (n=6).

2.9 Analysis of DNA methylation:

2.9.1 DNA extraction:

2.9.1.1 Liver DNA extraction:

Mouse livers were crushed using a pestle and mortar in the presence of liquid nitrogen. Liver (approximately 0.5cm^2) was placed in an Eppendorf and TNES buffer (for composition see appendices) (500 µl) was added to the sample, followed by of (20 mg/ml) Proteinase K (2.5 µl). The tube was then mixed by inversion and incubated overnight at 55ºC.

Following the overnight incubation, 2.6 M NaCl (500 µl) was added to the sample and shaken for 20 seconds. The samples were centrifuged for 10 minutes at 16,200xg at room temperature. The supernatant was removed and placed in a clean Eppendorf, and an equal volume of cold 100% ethanol was added to the sample and mixed by inversion. This produces a white DNA precipitate. The samples were then frozen for 10-30 minutes at -20ºC and then centrifuged for 20 minutes at 16,200xg at room temp. The supernatant was removed and the sample was air dried for around 15 minutes, if DNA precipitated out of solution, a spool was used to remove the DNA. Once dried, the DNA was re-suspended in sterile distilled water (500 µl). To this, RNase A (5 µl) was added and incubated at 37ºC for 1-2 hours. Following the incubation, phenol:chloroform (5:1) (500 µl) was added and mixed by inversion. The samples were then centrifuged for 5 minutes at 16,000xg. The aqueous phase was then transferred into

a fresh Eppendorf containing cold 100% ethanol (1 ml) and sodium acetate (50 µl) was added. The samples were then cooled at -20ºC for a minimum of 10 minutes. Following freezing, the samples were centrifuged for 10 minutes at 16,000 x g and the supernatant removed, or the DNA spooled if present in larger quantities. The DNA pellets were air dried and then resuspended in RNase and DNase free water (10 µl) and frozen overnight. DNA extracted was quantified using a Nanodrop spectrophotometer (NanoDrop1000, Labtech, East Sussex, UK). All DNA had a 260/280 ratio (assessment of protein contamination) of above 1.8 and a 260/230 ratio (assessment of phenol contamination) of above 1.4. The DNA was stored at -80ºC until required.

2.9.2 Preparation of cell and aorta samples for DNA extraction:

DNA extraction of cell culture samples and aorta samples were carried out using the QiAamp DNA mini kit (Qiagen). This was due to a very low yield of DNA using the high salt method. All reagents were made up according to the manufacturer's instructions.

2.9.2.1 Cell culture samples:

Upon collection, cell culture samples were pelleted and resuspended in PBS (200 µl). Proteinase K (20 µl) and RNase (4 µl) was added to each sample. Buffer AL (200 µl) was added to each sample and mixed for 15 seconds by pulse vortexing to yield a homogenous solution. The samples were incubated at 56 ºC for 10 minutes. Following incubation the samples were centrifuged to remove any sample droplets on the lid and 100% ethanol (200 µl) was added to each sample. Samples were again mixed by pulse vortexing for 15 seconds and briefly centrifuged to remove any droplets. The spin column protocol on the next page was then followed.

2.9.2.2 Aorta samples:

Aorta samples were crushed using a pestle and mortar in the presence of liquid nitrogen. The tissue was placed in an Eppendorf whilst still frozen and Buffer ATL (180 µl) was added to the samples followed by proteinase K (20 µl). The samples were mixed by vortexing and incubated at 56ºC until the tissue was completely lysed. Following incubation, the samples were centrifuged to remove any sample droplets on the lid. RNase A (100 mg/ml) (4 µl) was added and the sample mixed by pulse-vortexing for 15 seconds and incubated at room temperature for 2 minutes. Buffer AL (200 µl) was added to the sample, vortexed and incubated at 70ºC for 10 minutes. Following incubation, 100% ethanol (200 µl) was added to the sample and mixed

by pulse vortexing for 15 seconds and briefly centrifuged to remove any droplets. The spin column protocol on the next page was then followed.

2.9.3 DNA extraction using the spin column protocol:

The sample mixture prepared from aorta or cell culture was then applied to the QIAamp mini spin column in a collection tube. This was then centrifuged at 6000xg (Heraeus, Biofuge, Pico) for 1 minute. The spin column was then placed in a clean collection tube. To the column buffer AW1 (500 µl) was added and the sample centrifuged as before. The column was put into a clean tube and buffer AW2 (500 µl) was added to the column and centrifuged at 16,000xg for 3 minutes. The flow-through was discarded and the tube was centrifuged again at 16,000xg for 1 minute to remove any residual solution. The column was then transferred into a clean collection tube and RNase and DNase free water (200 µl) was added to the column and allowed to stand for 10 minutes at room temperature. The column and collection tube were then centrifuged 6000xg for 1 minute to elute the DNA. The DNA was quantified by nano-dropping. The DNA samples were stored at -20ºC, all DNA had a 260/280 ratio (assessment of protein contamination) of above 1.8 and a 260/230 ratio (assessment of phenol contamination) of above 1.4. The RNA was stored at -80ºC until required.

2.9.4 Gel electrophoresis:

To check the quality of the isolated DNA, the samples were analysed by agarose gel electrophoresis. Agarose gels were prepared as follows. For a 0.8% gel, agarose (0.8 g) was dissolved in tris-acetate buffer (TAE) (100 ml) by heating in a microwave oven until the solution became clear. The gel frame was prepared by taping both ends. Once the agarose and TAE solution had cooled slightly, gel red (7 µl) was added to the solution to enable visualisation of DNA bands. The mixture was poured into the gel casting frame, a comb was put in to produce the wells. Any bubbles were removed and the gel was allowed to set. The tape was then removed from the ends of the frame, the gel and frame were placed into the gel electrophoresis tank and the comb was then removed. Each sample (10 µl) was then mixed with 1 µl of loading dye (containing Bromophenol blue) and pipetted into each well. 1 kb DNA ladder (5 µl) was also put into the first well of the gel. The gel was then run for 20-30 minutes at 120 volts. DNA bands were visualised under UV light, wavelength 312/302 nm.

2.9.5 Sodium bisulphite conversion:

Bisulphite conversion of the DNA previously extracted was carried out using the Zymo Research EZ-96 DNA methylation-Gold kit. All DNA samples were diluted to give 1 µg of DNA in

20 µl sterile water. CT conversion reagent was prepared as instructed in the kit; water (9 ml), Dilution buffer (3 ml) and M-Dissolving buffer (500 µl). DNA (1 µg) was aliquotted into each well of a 96 well plate, to which CT conversion reagent (180 µl) was then added. The mixture was shaken vigorously for 15 minutes and the plate was then placed on a Veriti Thermal Cycler (Applied Biosciences, CA, USA). The reaction conditions were as follows: 98ºC for 10 minutes, 64ºC for 2.5 hours and then a hold at 4ºC. M-Binding buffer (400 µl) was added to each well of silicon-A binding plate. Samples from the incubation were added to the binding plate and mixed by pipetting. The plate was centrifuged at 1000xg for 5 minutes and the flow-through was discarded. M-Wash buffer (400 µl) was then added to the binding plate and the centrifugation repeated. Following this, M-Desulphonation buffer (200 µl) was added to each well and incubated at room temperature for 15-20 minutes. The centrifugation was repeated and M-Wash Buffer (400 µl) was added to each well and centrifugation was repeated. The wash step was then repeated and followed by centrifugation for 10 minutes. The binding plate was placed on an elution plate and M-Elution buffer (30 µl) was added to each well. The plates were then centrifuged for 3 minutes to elute the bisulphite DNA. The eluted bisulphite DNA was stored at -20ºC.

2.9.6 Primer design and optimization:

PCR primer sequences for mouse ELOVL2 (table 2.4) were designed using Pyro Q CpG software (Biotage, Sweden). Upon arrival, all primers were diluted to 100mM in RNase/DNase free water and stored at -20ºC. Primers were optimised using an annealing temperature gradient and observing which temperature gave the best product by performing electrophoresis on each product, this was determined by the presence of a single, strong band of correct size.

Table 2.4: Details of each of the primers used for the four regions of methylation analysis of the mouse ELOVL2 promoter region. Regions include a CPG Island which covers -212 to +284 bp from transcription start site (TSS).

Primer name/ number of CpGs measured	CPG covered/ position (distance from (TSS))	Forward primer	Reverse primer	Amplicon length (bp)	Biotin Labelled strand	Sequencing Primer
1-6	-386, -346,-343, -341, -337, -313	AGTGTTGGGATTATATAGGTGTATTAATT	AAACCACCAACAACTCCT	141	Rev	GATTAGTTTTTGGTTATTTTTTTAA
7-10	-266, -262, -260, -239	AGTGTTGGGATTATATAGGTGTATTA	AAACCCCCCAAAAACCTA	297	Rev	GAGTTTGATGTTGTGATAGA
11-24	-212, -208, -198, -195, -193, -187, -184, -184, -181, -173, -171, -169, -164, -158, -156	AGTGTTGGGATTATATAGGTGTATTA	AAACCCCCCAAAAACCTA	297	Rev	GTTAGTTGTGTTTGTAAATTTTA
25-30	-135, -129, -127, 123, -120, -117	GTAGGTTTTTGGGGGGTTT	CCCCCACTCACTCACCAT	272	Rev	GGTTTTTGGGGGGTTTA

2.9.7 PCR of bisulphite converted DNA:

For PCR each primer was diluted to 10 mM in RNase/DNase free water. A master mix was made up for each amplicon. The master mix contained RNase/DNase free water (10.5 µl), forward primer (0.5 µl), reverse primer (0.5 µl) and Kapa Taq (12.5 µl). Bisulphite converted DNA (1 µl) was added to a 96-well PCR plate. Master mix (24 µl) was added to each sample. No

template controls (NTCs) were run in duplicate for each amplicon and these contained RNase/DNase free water (1 µl) instead of DNA. Plates were run on the Veriti Thermal Cycler (Applied Biosciences, CA, USA) with the optimised annealing temperatures for each amplicon as shown in table 2.5. The parameters for the machine were as follows; 95 ºC for 3 minutes, 45 cycles of; 95ºC for 15 seconds, annealing temperature for 15 seconds and 72ºC for 15 seconds. Following the 45 cycles, the samples were incubated at 72ºC for 15 seconds and then held at 4ºC.

Table 2.5: Optimum annealing temperatures for primers

Primer name/ number of CpGs measured	Annealing Temperature (ºC)
1-6	52
7-10	54
11-24	54
25-30	58

2.9.8 Pyrosequencing:

DNA methylation of 30 CpG dinucleotides were measured in the mouse Elovl2 promoter in a region between -117 and -386 upstream from the transcription start site (TSS). This was done by sodium bisulphite pyrosequencing.

Immobilisation of PCR product to beads:

PCR product (10 µl) from each amplicon was aliquotted into to a 96-well plate for each sample. High-purity water (30 µl) was also added. A master mix of streptavidin-coated sephorose beads was made up containing; PyroMark Binding Buffer (38 µl) and streptavidin-coated sepharose beads (2 µl). Master mix (40 µl) was dispensed into each well. The plate was sealed and shaken for 5 minutes at room temperature.

Strand separation:

Sequencing primers were diluted to 10 ng/µl from the stock. For each sequencing primer, a master mix was prepared containing; sequencing primer (0.5 µl) and annealing buffer (11.5 µl). The master mix (12 µl) was then dispensed into the appropriate well of a PSQ HS 96 plate, shaken and placed onto the PyroMark Q96 Vacuum Workstation (Biotage). The vacuum was

prepared by washing with Washing Buffer. The Vacuum Prep Tool was used to capture the streptavidin-coated sephorose beads by placing the tool into the PCR plate. The vacuum tool with the beads attached was then moved into 70% ethanol and allowed to flush through for 5 seconds. The tool was then moved into the denaturation solution and allowed to flush through for 5 seconds, this causes the double stranded PCR products to break into single strands. Finally, the tool was placed in the Washing buffer and allowed to flush through for 5 seconds. Once all the liquid was removed, the vacuum was turned off. The beads were released into the PSQ plate by shaking the tool whilst allowing the probes to rest on the bottom of the wells.

Primer annealing and pyrosequencing:

The PSQ plate containing the samples and sequencing primers was then heated to 80 ºC for 2 minutes and then allowed to cool down; this allows the sequencing primers to anneal. The plate was then left at room temperature for 5 minutes and then run on the PyroMark PSQ 96MA machine (Biotage). Percentage methylation was then calculated using Pyro Q CpG software (Biotage). Quality controls were included to ensure complete bisulphite conversion of the DNA had been successful; a C not followed by a G should be a T (100%), which can be used as a control for full conversion of un-methylated C to T by bisulphite treatment.

2.10 Protein extraction:

Cells which had been collected and pelleted were used for protein extraction; these samples had been stored at -80 ºC. An aliquot of RIPA buffer (for composition in appendices) was put into an eppendorf and protease inhibitor cocktail (PIC) (10 µl per ml of RIPA) was added to it. RIPA buffer containing PIC (200 µl) was added to each cell pellet, the samples were mixed using a pipette and vortexed. The samples were then incubated on ice for 20 minutes, this was extended for larger pellets. After incubation on ice, samples were centrifuged at 16,000xg for 10 minutes. The supernatant was then transferred into a clean eppendorf. Samples were then stored on ice.

To measure the concentration of protein in each sample the Pierce BCA protein assay kit (Fisher Scientific) was used and reagents were made up according to the manufacturer's instructions. Standards were made up at concentrations 2000, 1500, 1000, 750, 500, 250, 125, 25 and 0 µg/ml. This was done by serial dilutions of Albumin standard (BSA) provided, in RIPA buffer. The working reagent was then made up by making a 50:1 solution, by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. This solution is light resistant so was kept in the

dark until it was used. Each standard (10 µl) (in duplicate) and sample (10 µl) was then added to a flat bottomed plate. RIPA buffer (10 µl) was also added to two wells, as a blank. To all wells, working reagent (200 µl) was added. The plate was then sealed and incubated at 37ºC for 30 minutes. For samples where lower concentrations of protein were present this incubation time was extended to allow better colour development. The plate was then read with a spectrophotometer (Thermo labsystems multiskan Ex) set to 562 nm. A standard curve was plotted and the protein concentration of the samples was calculated using the standard curve.

2.11 PUFA biosynthesis pathway analysis:

2.11.1 Cell treatment with LA and ALA:

Hepa1-6 and MOVAS cells were grown as described in section 2.7. Liver cells were used as a positive control as the activity of the pathway is known in these cells. Once the cells had reached confluence in 75 cm^2 flasks, the cells were passaged as described in the methods chapter, section 2.8. Cells were plated onto 10 cm^2 plates and allowed to attach for 24 hours in their normal culture medium. LA was dissolved in ethanol and a LA stock (1 mM) was made up in the appropriate culture medium, ensuring that the solvent concentration did not exceed 0.1%. Culture medium was removed from the cultured cells and replaced with either culture medium (10 ml) without fatty acid supplement for control samples or culture medium containing LA (final concentration 100 µM). Cells were incubated for 48 hours in a humidified cell culture incubator at 37ºC, 5% CO_2/95% air. The medium was removed and the cells were washed three times with ice cold HBSS. 0.9% (w/v) NaCl (0.8 ml) was added to each plate and the cells collected using a cell scraper. The cell suspension was transferred to a glass screw top tube and placed on ice. Total lipid extraction and FAMEs preparation was then carried out as described in sections 2.6.2 and 2.6.4 respectively. This protocol was repeated using an ALA stock (1mM to give a working concentration of 100 µM) instead of LA in order to measure the activity of the n-3 PUFA pathway. A final concentration of 100 µM was chosen for both LA and ALA as this was a high concentration which guarantees a visible effect on the whole PUFA pathway. This concentration was also low enough to ensure it did not have a toxic effect on the cells. Viability was tested by staining the cells with trypan blue and the cells were checked daily to ensure no changes in morphology.

2.11.2 Cell treatment with LA in the presence of SC-26196 or sesamin:

MOVAS cells were grown and allowed to reach confluence in a 75 cm^2 flasks; the cells were split as described in section 2.7. Cells were plated at a density of 2.8. $1x10^6$ onto 10 cm^2 plates and allowed to attach for 24 hours in their standard culture medium. A LA stock (100 µM) was made up in standard culture medium. SC-26196 and sesamin were both dissolved in DMSO, as instructed by Sigma. A SC-26196 inhibitor stock solution containing LA (100 µM) and SC-26196 (200 nM) in culture medium was also prepared. A Sesamin inhibitor stock solution containing LA (100 µM) and sesamin (1 µM, 10 µM and 20 µM) in culture medium was prepared. Culture medium was removed from the cells and replaced with culture medium containing LA (100 µM). These were the control samples. To SC-26196 inhibitor treated cells, the SC-26196 inhibitor stock solution (containing LA) was added at a final concentration of 200nM. To sesamin inhibitor treated cells, the sesamin inhibitor stock solution (containing LA) was added to provide final concentrations of 1 µM, 10 µM and 20 µM. Cells were incubated for 48 hours in a humidified cell culture incubator at 37ºC, 5% CO_2/95% air. Following the incubation, the medium was removed from all the plates. All plates were washed three times with ice cold HBSS. 0.9% w/v NaCl (0.8 ml) was added to each plate and the cells were removed from the plate using a cell scraper. The NaCl solution containing the cells was transferred to a glass screw top tube and placed on ice. Total lipid extraction and FAMEs preparation was then carried out on each sample as described in sections 2.6.2 and 2.6.4 respectively.

2.11.3 N-6 PUFA Pathway analysis using [U-^{13}C]-LA:

The conversion of [U-^{13}C]-LA to its longer chain metabolites was measured in mouse liver and VSMC by GC-isotope-ratio mass spectrometry. Hepa1-6 and MOVAS cells were grown as described (section 2.7). Once the cells had reached confluence in 75 cm^2 flasks, the cells were passaged (section 2.7.) and $1x10^6$ cells were plated onto 10 cm^2 plates and allowed to attach for 24 hours in standard culture medium. A mixture of [U-^{13}C]-LA ethyl ester (10 µM) and unlabelled LA (90 µM) was prepared in standard culture medium. Culture medium was removed from the cells and replaced with either untreated medium for control samples or with medium containing [U-^{13}C]-LA ethyl ester with unlabelled LA. Cells were incubated for 48 hours in a humidified cell culture incubator at 37ºC, 5% CO_2/95% air.

In order to investigate the effect of α1-adrenergic receptor-mediated stimulation by Pe on the activity of the PUFA pathway in VSMCS, in the last 30 minutes of the 48 hour incubation, Pe (100 µM) dissolved in appropriate medium, was added to six MOVAS plates

which had been previously treated with the LA mixture (containing [U-^{13}C]-LA ethyl ester and unlabelled LA). This was allowed to incubate for 30 minutes at 37ºC, 5% CO_2/95% air.

The medium was then removed from all samples and the cells were washed three times with ice cold HBSS. 0.9% w/v NaCl (0.8 ml) was added to each plate and the cells were removed from the plate using a cell scraper. The cell suspension was transferred to a glass screw top tube and placed on ice. An aliquot of cells (20 µl) was reserved for protein analysis from each sample and frozen on dry ice. Total cell protein was measured using the BCA protein assay kit (Fisher Scientific) (section 2.10).

Total lipid extracts and FAMEs were prepared as described (sections 2.6.2 and 2.6.4). An internal standard of heptadecanoic acid (C17:0) (10 µg) was added to every sample. The mass of individual fatty acids was determined by gas chromatography (section 2.6.4) The incorporation of [^{13}C] into PUFA was determined by measuring the ^{13}C-to-^{12}C ratio in [U-^{13}C] LA in the aorta total lipid extracts using a Delta Plus XP GC-combustion-isotope ratio mass spectrometry (GC-C-IRMS; Thermo Electron, Bremen, Germany). Enrichment measurements and integration of data was kindly carried out by Dr Barabara Fielding (University of Surrey). The ^{13}C enrichment results (from FAME derivatives) expressed as delta values were converted to tracer-tracee ratio using the formula shown in table 2.6 and enrichment calculated using the ratio calculated.

Table 2.6: Calculation for determining the enrichment of MOVAS and Hepa1-6 cells (281).

The following equations were used to calculate the stable isotopic enrichment of the cells:
(Area of fatty acid peak on GC chromatograph/Area of internal standard)*Mass of standard added
=Mass or Fatty acid
Mass of Fatty acid/Mass of protein
The concentration of tracer was then calculated by calculating the tracer:tracee ratio
((Delta value of tracer/1000)+1)*0.0112372*19/18
Using the tracer:trace ratio the enrichment was calculated:
(tracer:trace ratio*mass of fatty acid)*1000

2.12 Measuring Intracellular calcium release:

2.12.1 Measuring intracellular calcium release in the presence of SC-26196 and sesamin:

To measure changes in intracellular calcium release in response to delta-5 and delta-6 desaturase inhibition, the Screen Quest Fluo-8 Medium removal assay kit (AAT Bioquest, CA) was used. MOVAS cells were plated in a black 96-well black sided, clear bottomed culture plate. Approximately 40,000 cells were plated per well. Inhibitor stock solutions were made up for SC-26196 (100 nM, 200 nM, 500 nM and 1 µM) and sesamin (10 µM and 20 µM) in normal culture medium and were added (100 µM) to the appropriate wells of the plate. Control cells were incubated with normal culture medium containing the vehicle (0.1% DMSO). The cells were incubated for 24 hours and allowed to attach. After 24 hours, the solutions from the kit were reconstituted as instructed in the kit; Fluo-8 was reconstituted with DMSO. The buffer solution was prepared by mixing HHBS buffer (Hanks buffer with 20 mM hepes) with 10X Pluronic F127 plus. The buffer solution containing 10X pluronic F127 was then mixed with Fluo-8 to form the dye-loading solution. The growth medium was then removed from the 96-well plate. Dye loading solution (100 µl) was then added to each culture well, followed by the SC-26196 (100 nM, 200 nM, 500 nM and 1 µM) and sesamin (10 µM and 20 µM) or vehicle for control samples. The plate was then incubated at 37ºC, 5% CO_2/95% air for 30 minutes, followed by 30 minutes at room temperature in darkness. After the incubation, Pe (10 µM) was then added to the relevant wells and gently shaken for 30 seconds. The fluorescence was then read with the Thermo Labsystems fluoroskan ascent FL with filter set of Ex/Em 485/538. As a positive control, the ionophore A23187 (500 nM) was used and added to positive control wells at the same time as Pe was added.

2.13 Eicosanoid production measurement:

2.13.1 Determination of eicosanoid production:

To determine the effect of inhibition of the PUFA biosynthesis pathway on the production of eicosanoids, MOVAS cells were treated with either the SC-26196 inhibitor or sesamin as described in section 5.2.3. Eicosanoid secretion was measured in response to stimulation by Pe as follows; MOVAS cells were passaged and approximately 60,000 plated in each well of a 6-well dish in normal culture medium. Cells were allowed to attach overnight. Cells were then treated with inhibitor stock solution SC-26196 (200 nM and 1 µM) and sesamin (10 µM and 20 µM) and incubated for 48 hours as described. After incubation all medium was removed and

the cells were washed with HBSS. New treatment media was made up for SC-26196 (200 nM and 1 µM) and for sesamin (10 µM and 20 µM) in serum free medium containing Pe (10 µM). The control cells were treated with serum free medium containing the vehicle (0.1% DMSO). All other wells were treated with either the SC-26196 or sesamin serum free treatment medium. Cells were incubated for 30 minutes at 37ºC, 5% CO_2/95% air. After incubation, medium was quickly collected for each sample, immediately frozen on dry ice and then stored at -80ºC. The concentrations of eicosanoids were analysed using ELISA kits for HETE, PGE_2, $PGF_{2\alpha}$ and TXB_2 following the methods described in section 2.13.2-2.13.5. HETE was used as a negative control as this remained unchanged in the study by Kelsall et al. (282).

2.13.2 Prostaglandin E_2 (PGE_2) production:

PGE_2 production was measured using enzyme linked immunosorbent assay (ELISA) kit; PGE_2 express EIA kit (Cayman Chemical Company). The ELISA was carried out according to the manufacturer's instructions; all reagents and standards were supplied by the kit and prepared following the manufacturers guidelines. PGE_2 production was measured in cell culture medium, which was frozen immediately upon collection. The samples were thawed on ice, to ensure no degradation of PGE_2. As culture medium was used, all standards were diluted in the same culture medium.

The 96 well plate provided in the kit was pre-coated with goat polyclonal anti-mouse IgG and blocked with a proprietary formulation of proteins. To the plate culture medium (50 µl) was added to non-specific binding (NSB) wells and to maximum binding wells (B_0) and EIA buffer (50 µl) was added to the NSB wells. Standards (50 µl) and samples (50 µl) were added to the appropriate wells. To all wells, except the blank and total activity (TA) wells, PGE_2 Express AChE Tracer (50 µl) was added. PGE_2 Express Monoclonal Antibody (50 µl) was then added to all wells except the TA, NSB and the blank wells. The plate was then covered and incubated at room temperature on an orbital shaker for 60 minutes.

Following incubation, all wells were emptied and washed five times with Wash Buffer. Ellman's Reagent (200 µl) was added to each well followed by PGE_2 Express AChE Tracer (5 µl) to the TA wells. The plate was covered and incubated for 90 minutes at room temperature, in the dark on an orbital shaker. Following incubation the plate was read on a plate reader (Thermo labsystems multiskan Ex) at wavelength 405 nm and the arbitrary units (A.U.) for each well was measured.

To analyse the data, the absorbance value for the blank wells were subtracted from the absorbance values for the readings of the rest of the plate. The average absorbance

readings from the NSB wells and B_0 wells were averaged. The NSB average was then subtracted from the B0 average to produce the corrected maximum binding value. Sample or standard bound/Maximum bound value (B/ B_0) was then calculated by subtracting the average NSB from standards or samples and dividing by the corrected B0 value. The standard B/B_0 values were then multiplied by 100 to produce the percentage B/B_0 (%B/B_0) and a logistic four parameter fit standard curve was created using GraphPad Prism. Then the %B/B_0 value for each sample was calculated. The concentration of PGE_2 in each sample was determined by interpolation of the sample %B/B_0 value from the standard curve and the data obtained transformed.

2.13.3 Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production:

$PGF_{2\alpha}$ production was measured using enzyme linked immunosorbent assay (ELISA) kit; $PGF_{2\alpha}$ EIA kit (Cayman Chemical Company). The ELISA was carried out according to the manufacturer's instructions; all reagents and standards were supplied by the kit and prepared following the manufacturers guidelines. $PGF_{2\alpha}$ production was measured in cell culture medium, which was frozen immediately upon collection. The samples were thawed on ice, to ensure no degradation of $PGF_{2\alpha}$. As culture medium was used, all standards were diluted in the same culture medium.

The 96 well plate provided in the kit was pre-coated with mouse anti-rabbit IgG and blocked with a proprietary formulation of proteins. To the plate, culture medium (50 µl) was added to non-specific binding (NSB) wells and to maximum binding wells (B_0) and EIA buffer (50 µl) was added to the NSB wells. Standards (50 µl) and samples (50 µl) were then added to the appropriate wells. To all wells except the blank and total activity (TA) wells, $PGF_{2\alpha}$ AChE Tracer (50 µl) was added. $PGF_{2\alpha}$ EIA Antiserum (50 µl) was added to all wells except the TA, NSB and the blank wells. The plate was then covered and incubated at 4ºC for 18 hours.

Following incubation, all wells were emptied and washed five times with Wash Buffer. Ellman's Reagent (200 µl) was added to each well followed by $PGF_{2\alpha}$ AChE Tracer (5 µl) to the TA wells. The plate was covered and incubated for 120 minutes at room temperature, in the dark on an orbital shaker. Following incubation the plate was read on a plate reader (Thermo labsystems multiskan Ex) at wavelength 405 nm and the arbitrary units (A.U.) for each well was measured. The same calculations as described for the PGE_2 kit were used to calculate the concentration of $PGF_{2\alpha}$ produced (see section 2.13.2).

2.13.4 Thromboxane B_2 (TXB_2) production:

TXB_2 is an inactive metabolite of TXA_2. TXA_2 is rapidly hydrolysed and is therefore difficult to accurately measure, so TXB_2 is used as an indirect measure of TXA_2 production. TXB_2 production was measured using enzyme linked immunosorbent assay (ELISA) kit; TXB_2 EIA kit (Cayman Chemical Company). The ELISA was carried out according to the manufacturer's instructions; all reagents and standards were supplied by the kit and prepared following the manufacturers guidelines. TXB_2 production was measured in cell culture medium, which was frozen immediately upon collection. The samples were thawed on ice, to ensure no degradation of TXB_2. As culture medium was used, all standards were diluted in the same culture medium.

The 96 well plate provided in the kit was pre-coated with mouse anti-rabbit IgG and blocked with a proprietary formulation of proteins. To the plate, culture medium (50 µl) was added to non-specific binding (NSB) wells and to maximum binding wells (B_0). EIA buffer (50 µl) was added to the NSB wells. Standards (50 µl) and samples (50 µl) were then added to the appropriate wells. To all wells except the blank and total activity (TA) wells, TXB_2 AChE Tracer (50 µl) was added. TXB_2 EIA Antiserum (50 µl) was then added to all wells except the TA, NSB and the blank wells. The plate was then covered and incubated at room temperature for 18 hours.

Following incubation, all wells were emptied and washed five times with Wash Buffer. Ellman's Reagent (200 µl) was added to each well followed by TXB_2 AChE Tracer (5 µl) to the TA wells. The plate was covered and incubated for 120 minutes at room temperature, in the dark on an orbital shaker. Following incubation the plate was read on a plate reader (Thermo labsystems multiskan Ex) at wavelength 405 nm and the arbitrary units (A.U.) for each well was measured. The same calculations as described for the PGE_2 kit were used to calculate the concentration of TXB_2 produced (see section 2.13.2).

2.13.5 12(s)- Hydroxyeicosatetraenoic acid (HETE) production:

HETE production was measured using enzyme linked immunosorbent assay (ELISA) kit; 12(S)-HETE ELISA Kit (Abcam, Cambridge, UK). The ELISA was carried out according to the manufacturer's instructions; all reagents and standards were supplied by the kit and prepared following the manufacturers guidelines. HETE production was measured in cell culture medium, which was frozen immediately upon collection. The samples were thawed on ice, to ensure no degradation of HETE. As culture medium was used, all standards were diluted in the same culture medium.

The 96 well plate provided was pre-treated with goat anti-rabbit IgG. To the plate culture medium (100 µl) was added to non-specific binding (NSB) wells and to maximum binding wells (B_0). Assay buffer (50 µl) was added to the NSB wells. Standards (100 µl) and samples (100 µl) were then added to the appropriate wells. To all wells, except the blank and total activity (TA) wells, HETE conjugate (50 µl) was added. HETE antibody (50 µl) was then added to all wells except the TA, NSB and the blank wells. The plate was then sealed and incubated at room temperature for 2 hours.

Following incubation, all wells were emptied and washed three times with Wash Buffer (400 µl). HETE conjugate (5 µl) was then pipetted into the TA wells followed by pNpp substrate (200 µl) to every well. The plate was covered and incubated at 37ºC for 3 hours without shaking. Following incubation, Stop Solution (50 µl) was added to every well and the plate was read on a plate reader (Thermo labsystems multiskan Ex) at wavelength 405 nm and the arbitrary units (A.U.) for each well was measured. The same calculations as described for the PGE_2 kit were used to calculate the concentration of HETE produced (see section 2.13.2).

2.14 Statistical analysis:

All data are expressed as mean ± SEM. Most statistical analysis was carried out using GraphPad Prism 6. Except where a repeated measures analysis with a general linear model was used with sex, total maternal dietary fat and fat type as between subject factors with Tukey's post-hoc test, in these cases, analysis was carried out with IBM SPSS Statistics21. Significance was considered when $P<0.05$. Normality was confirmed by use of SPSS and the Kolmogorov-Smirnov Test. Any data that were not normally distributed were transformed, this included the vascular reactivity data, dose response curves were log transformed to ensure they were log-normally distributed in order to derive the pEC50 values. In addition to this, the data obtained from the eicosanoid assay experiments were log transformed in order to ensure linearity and to allow the concentrations of eicosanoid produced in each assay to be determined.

For the analysis of multiple independent variables including diet type, quality and sex, ANOVA with Tukey's post hoc test was used. This was used to analyse the aorta and plasma composition data in addition to the analysis of the pEC50 and maximum response vascular reactivity data. During the measurement of the vascular reactivity data, the experiments were carried out by two people. As multiple investigators can lead to potential inter-observer variability which can confound comparisons of dietary groups, the observer was included as a factor in the ANOVA statistical test to see if this had a significant effect on the observations. Growth, food intake and blood pressure data were analysed by the repeated measures general

linear model with Tukey's post hoc analysis. For comparing two groups of data, as seen with the methylation data, Hepa and MOVAS fatty acid compositions, the [U-^{13}C] Linoleic acid enrichment data and SC-26196 treated MOVAS cells; an unpaired student T test was used. Sesamin treatment of MOVAS cells, calcium release and Eicosanoid measurement data were analysed by one-way ANOVA with a Dunnett's post hoc test, allowing the comparison of treatment groups to the control.

Calculation of pEC50 was calculated by constructing dose response curve, using non-linear regression curve fit with sigmoidal dose response in GraphPad Prism 6. This produced maximum response (top of the curve) and EC50 data. EC50 is the concentration of agonist which produced 50% of the maximum response. pEC50 which is potency, was calculated by the negative logarithm of the EC50.

2.14.1 Sample size calculations:

2.14.1.1 Retrospective sample size calculations for vascular reactivity and fatty acid composition in rats:

Based on retrospective power analysis of the data reported in this thesis:

Using either comparisons between groups fed saturated or polyunsaturated fatty acids for type of fat analyses or between 7% and 21% fat diets for amount of fat analyses n=7 offspring provided:-

90% statistical power for detecting a 5% difference in pEC50 between groups fed different types or amounts of fat with alpha = 0.05.

95% statistical power for detecting a 5% difference in maximum response to Pe between groups fed different types or amounts of fat with alpha = 0.05.

80% statistical power for detecting a 20% difference in blood pressure between groups fed different types or amounts of fat with alpha = 0.05.

85% statistical power for detecting a 20% difference in the proportion of 20:4n-6 or 22:6n-3 between groups fed different types or amounts of fat with alpha = 0.05.

2.14.1.2 Retrospective sample size calculations for experiments carried out in chapter 4 and 5:

Sample size calculations were carried out using the Statistical Solutions, LLC sample size calculator software: http://www.statisticalsolutions.net/pss_calc.php

Calculations were carried out for each type of data collected and were based on mean control data with standard deviation from this thesis and an expected 10% significant difference in effect. Examples of the power calculations are shown below:

Fatty acid Enrichment data:

MOVAS enrichment with [U-^{13}C] Linoleic acid:

Concentration of [U-^{13}C] labelled fatty acid: 1.313, SD: 0.266 and expected mean value: 1.4443. Two sided test.

A sample size of 33 per experimental group was sufficient to provide statistical power of 70% with a $p<0.05$.

Fatty acid synthesis:

Linoleic acid treatment of MOVAS cells:

Control mean proportion of linoleic acid= 1.270%, SD= 0.120 and expected mean value: 1.397%. Two-sided test.

A sample size of 6 per group was sufficient to provide statistical power of 70% with a $p<0.05$.

mRNA expression:

Mouse Aorta Fads1 expression:

Mean expression relative to cyclophillin: 0.421, SD: 0.067 and expected mean expression relative to cyclophillin: 0.4631. Two sided test.

A sample size of 16 per experimental group was needed to provide statistical power of 70% with a $p<0.05$.

Methylation analysis:

Methylation of aorta tissue:

Mean % methylation of aorta tissue at position -337 from TSS: 16.466, SD: 1.225 and expected mean value: 18.1126.

A sample size of 7 per experimental group was needed to provide statistical power of 70% with a $p<0.05$

Calcium Assay:

Calcium release from MOVAS cells:

Control/untreated cells mean calcium release (RFU): 1.32, SD: 0.02764 and expected mean value: 1.452. Two sided test.

A sample size of 3 per experimental group was sufficient to provide statistical power of 80% with a $p<0.05$.

Eicosanoid production:

PGF2a production in MOVAS cells:

Control concentration of PGF2a: 26.712 pg/ml, SD: 3.140 and expected mean value: 29.382 pg/ml. Two sided test.

A sample size of 22 per experimental group was sufficient to provide statistical power of 70% with a $p<0.05$.

Chapter 3-

The effect of different types and quantities of maternal fat on offspring vascular function and composition

3.1. Introduction:

There is substantial evidence linking dietary intake to CVD. High intakes of trans fats (120;122) and saturated fat (111) in post-natal life have both been linked to an increased risk of cardiovascular disease. Classically it was thought to relate to current dietary intake. However, the developmental origins hypothesis proposes that the prenatal environment is also important and the risk of developing CVD is established during early development (176).

Endothelial dysfunction is a recognised marker and an important factor in the progression of CVD. It has been well documented that maternal under nutrition during pregnancy and/or a postnatal high fat diet results in blunted endothelial response to ACh; which demonstrated endothelial dysfunction (284). It has also been shown that high fat diets during pregnancy leads to raised blood pressure in the rat offspring (5;189). This outcome has been demonstrated in offspring from undernourished dams (7) and also in rat offspring of dams fed a high saturated fat diet (285). Both studies demonstrated an enhanced constrictor response in addition to impaired endothelium-dependent vasorelaxation. Much of the previous work has focussed on diets rich in saturated fats, but fat intake typically covers a wide range of fats. N-3 PUFAs are of particular significance as they are considered to be cardioprotective (159). Rat offspring of n-3 deficient dams demonstrate increased blood pressure. Therefore, supply of n-3 PUFA in early life may reduce the risk of cardiovascular dysfunction, such as hypertension later in life (6).

Changes in fatty acid consumption can alter the fatty acid composition of the cell membrane which, in turn may alter cell function (106). In endothelial cells, membrane fatty acids provide substrates for signalling pathways, including those involved in vasoconstriction and vasorelaxation (286). Changes to fatty acid composition may be responsible for changes in NO production; another study has demonstrated that changes to the fatty acid composition causes disruption to eNOS activity in human endothelial cells. It was postulated that this may be due to changes in binding stability of eNOS to the membrane which involves caveolae (287). Caveolae are flask-shaped invaginations in the plasma membrane. Caveolae are classified as a subset of lipid rafts and are identifiable by the presence of the protein caveolin (288). Caveolin-1 is a negative regulator of eNOS and caveolin-1 directly binds eNOs and therefore regulates NO production (289). Caveolae are very abundant in the cardiovascular system, including in the endothelial cells, smooth muscle cells, cardiac myocytes and fibroblasts (290). Changes to the structure of these caveolae may have an impact on NO production as it changes the ability of the eNOS to bind to the membrane. As previously discussed, NO plays a key role in the cardiovascular system, in addition to causing vasorelaxation it also has the ability to reduce vascular permeability, platelet aggregation, tissue oxidation and activation of

thrombogenic factors (15). AA and DHA are key constituents of VSMCs and are therefore of particular importance. Changes in these fatty may provide another mechanism whereby changes in fatty acid composition result in impaired regulation of vascular tone.

Many studies have shown that changes to a postnatal diet can have great effects on the cardiovascular system by altering the composition of fatty acids in aortae (270), adipocytes plasma membranes (271) and platelets (272), (270). Less research has been done into the effect of a maternal diet on the composition of the offspring's cardiovascular system. Maternal dietary fat and fat metabolism may programme endothelial dysfunction in offspring. In rabbits, hypercholesterolaemic dams produced offspring with atherosclerotic-like lesions, with increased oxidised fatty acids (269). In rats a maternal high fat diet during pregnancy leads to abnormal vascular function accompanied by a change in aorta composition (8), (291).

Throughout the animal part of this study the aorta will be the vessel investigated to determine offspring vascular function. As discussed in chapter 1, section 1.1.2, the aorta is a conduit artery and therefore has a lower amount of smooth muscle and a greater amount of elastic tissue (9). Resistance arteries such as mesenteric arteries have a greater amount of vascular smooth muscle and for this reason they provide a greater determination of blood pressure (7). However, the mesenteric arteries are very small and would not provide enough tissue to extract fatty acids from. As this study is investigating how maternal diet effects vascular function and composition of the offspring vascular system, investigating changes in the aorta allows consistency. Aorta allows the measure of both vascular function and is large enough to extract fatty acids from. The aorta is still susceptible to CVD and is well characterised, it also demonstrates endothelial dysfunction (190). Therefore, the aorta is still representative of altered vascular function, due to its lower amount of vascular smooth muscle, it may not reflect changes seen in blood pressure, but it is still able to reflect endothelial dysfunction which precedes CVD as the endothelium is important in the control of vascular tone (292). However, aortic stiffness is a pathological problem that happens in aging and can lead to reduced contractility of VSMCs and reduced compliance (65) which can lead to increased blood pressure. This shows that changes in VSMCs in the aorta can affect blood pressure (190). In this thesis, the rats used were relatively young adults at day 77 and therefore, ensures there are minimal alterations to VSMCs due to aging.

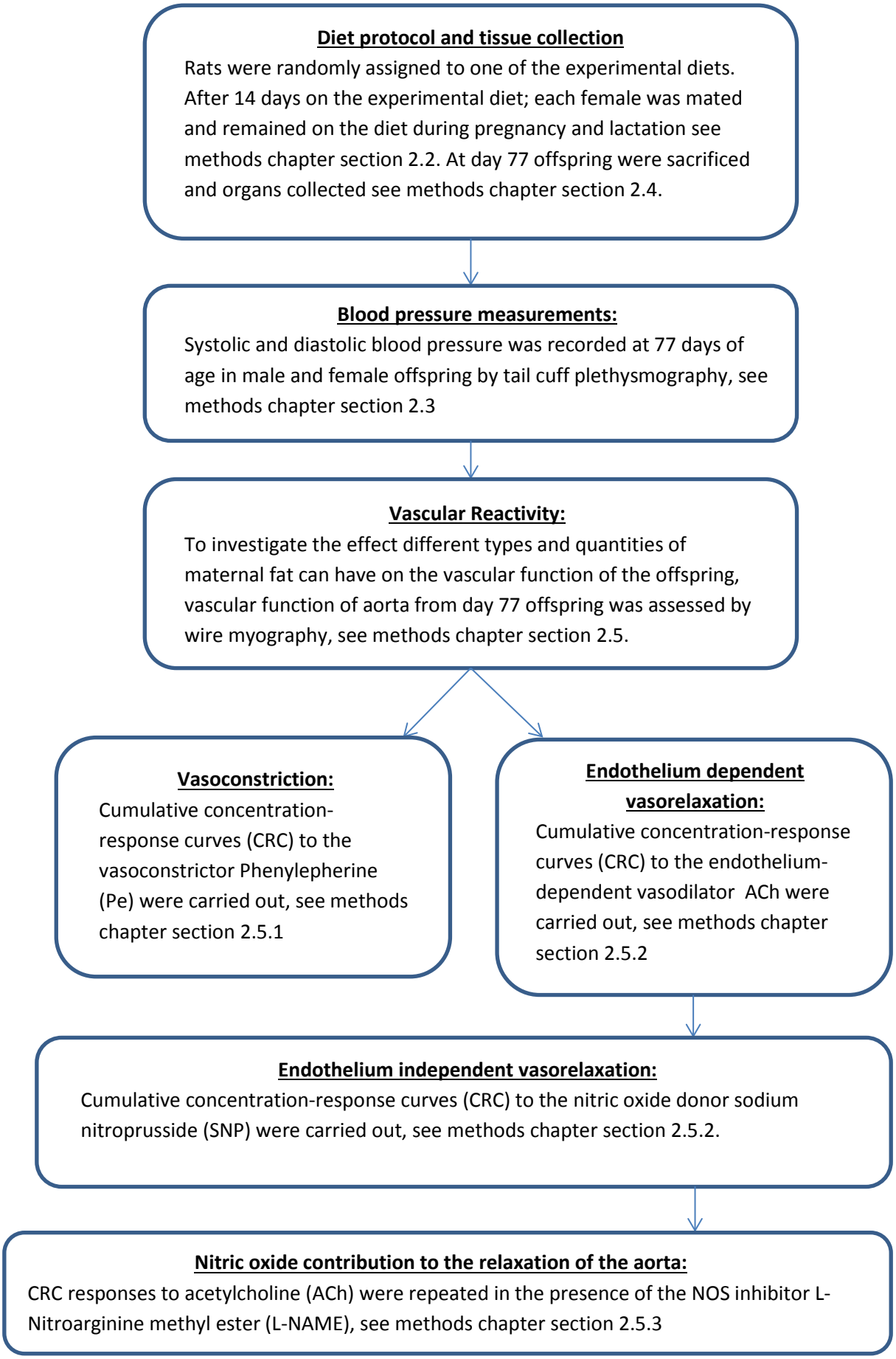
Relatively little is known about the effects of differences in the type and amount of maternal dietary fat on cardiovascular function or composition in the offspring. Most studies look at one specific type of fat, high fat or under-nourishment. However, no other study has looked at different types and amount of fat and the effect on vascular function and risk of CVD. Therefore, this chapter will address the following hypotheses and investigate the effect of

feeding maternal diets enriched with either n-6 PUFAs, n-3 PUFAs, trans-fats or saturated fats at a high fat and normal intake level on vascular function in the adult rat offspring. In addition to this, it will address whether these changes in the maternal fat content lead to alterations in the fatty acid compositions of the aorta and plasma.

Chapter hypotheses:

- Variations in maternal fat intake will lead to alterations in blood pressure and vascular function in adult offspring.
- The changes in vascular function are due to persistent changes to the membrane fatty acid composition of the adult offspring vascular system.
- The changes within the composition of the aorta membrane and altered function are linked to changes in polyunsaturated fatty acid biosynthesis *de-novo* in VSMCs.

3.2 Methods:


Diet protocol and tissue collection
Rats were randomly assigned to one of the experimental diets. After 14 days on the experimental diet; each female was mated and remained on the diet during pregnancy and lactation see methods chapter section 2.2. At day 77 offspring were sacrificed and organs collected see methods chapter section 2.4.
Blood pressure measurements:
Systolic and diastolic blood pressure was recorded at 77 days of age in male and female offspring by tail cuff plethysmography, see methods chapter section 2.3
Vascular Reactivity:
To investigate the effect different types and quantities of maternal fat can have on the vascular function of the offspring, vascular function of aorta from day 77 offspring was assessed by wire myography, see methods chapter section 2.5.
Vasoconstriction:
Cumulative concentration-response curves (CRC) to the vasoconstrictor Phenylepherine (Pe) were carried out, see methods chapter section 2.5.1
Endothelium dependent vasorelaxation:
Cumulative concentration-response curves (CRC) to the endothelium-dependent vasodilator ACh were carried out, see methods chapter section 2.5.2
Endothelium independent vasorelaxation:
Cumulative concentration-response curves (CRC) to the nitric oxide donor sodium nitroprusside (SNP) were carried out, see methods chapter section 2.5.2.
Nitric oxide contribution to the relaxation of the aorta:
CRC responses to acetylcholine (ACh) were repeated in the presence of the NOS inhibitor L-Nitroarginine methyl ester (L-NAME), see methods chapter section 2.5.3

Role of PUFA biosynthesis in Pe-mediated vasoconstriction

To determine whether PUFA biosynthesis de novo is involved in the regulation of vascular tone, aorta were treated with delta-6 or delta-5 desaturase inhibitors and a CRC to Pe was carried out then treated with Pe, see methods chapter section 2.5.4

Fatty acid analysis of plasma and aorta samples of day 77 offspring

To determine the effect maternal diet has on the fatty acid composition of offspring aorta and plasma, see methods chapter section 2.6. Total lipid analysis and FAMEs was carried out on aorta samples, see methods chapter section 2.6.2 and 2.6.4. TAG and NEFA fractions were investigated in plasma samples by total lipid extraction, SPE and FAMEs see methods chapter section 2.6.2-2.6.4

3.3 Results:

3.3.1 Maternal diet and growth:

The maternal diet had no significant effect upon the size of the litters, nor the ratio of males to females, as shown in figure 3.1.

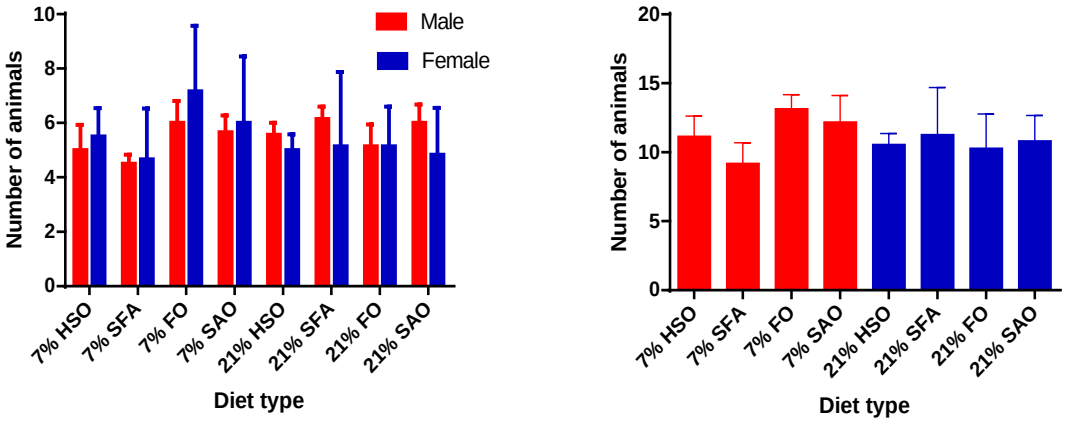


Figure 3.1: Left: Average number of male and female offspring for each diet type. Right: Average total litter size for each diet type. Data shown as mean ±SEM. (n=6/7 per dietary group)

3.3.2 Dam energy intake:

In the 14 days prior to pregnancy no changes in the dam weights were observed between the different dietary groups. The energy intake of the dams during pregnancy did not significantly differ between dietary types for the 7% diet (table 3.1). In the 21% fat fed dams there was a significantly lower intake of HSO compared to the other groups within the 21% group ($P<0.0001$) at every time point.

Table 3.1: Dam energy intake (kJ / 100g body weight / 24 hours) for each diet group during pregnancy and lactation. Data shown as mean ± SEM (n=6/7 per dietary group). Different superscripted letters indicate values significantly different ($P < 0.05$) between types of fat within either the 7% or 21% total dietary fat groups by a general linear model with Tukey's *post hoc test*. †Indicates values significantly different ($P < 0.05$) between 7% and 21% total fat intakes within a dietary fat type $^{a/b}$ indicates values significantly different ($P<0.05$) between diet types.

	7% (w/w) total dietary fat				**21% (w/w) total dietary fat**				**Statistical significance**		
	SAO	**HSO**	**Butter**	**FO**	**SAO**	**HSO**	**Butter**	**FO**	**Total Fat (TF)**	**Fatty acid Content (FA)**	**TF*FA**
Pregnancy - 7 days	91±19	89±14	113±21	110±11	115±22^{a}	62±13^{b†}	102±21^{a}	94±12^{a†}	NS	<0.0001	0.005
Pregnancy day 14	107±36	82±13	105±14	116±14	100±14^{a}	69±9^{b}	102±18^{a}	114±11^{a}	NS	<0.0001	NS
Lactation day 14	141±16	115±17	138±38	130±21	176±7^{a†}	95±7^{b†}	180±24^{b†}	172±11^{a†}	<0.0001	<0.0001	0.001

3.3.3 Offspring diet and growth:

There was a significant interactive effect of time and sex ($P < 0.0001$) and time and total maternal fat intake ($P < 0.0001$) on offspring weight gain after weaning.

Male offspring weight:

In all male offspring there was no significant difference between weight gain from birth until weaning at day 28. After weaning all male offspring in all diet groups gained weight at a steady rate regardless of type of dietary change (fig 3.2). In the FO ($P=0.0001$), HSO ($P=0.001$) and SFA ($P=0.02$) there is a significant difference between the weights of offspring on a 21% and 7% fat diet at day 35, after this point they converge. In offspring of SAO, the offspring of the 21% SAO are significantly heavier ($P=0.027$) than 7% SAO offspring at day 56.

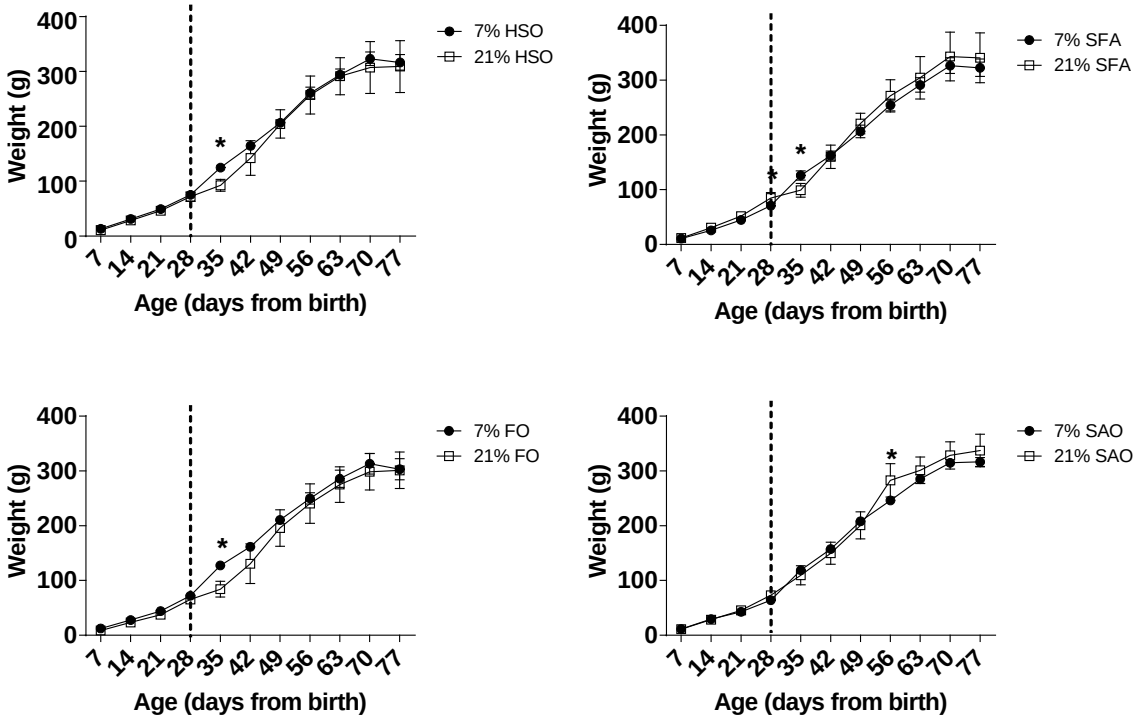


Figure 3.2: Male offspring postnatal body weights for each diet at 7% and 21% quantity. Data shown as mean ± SEM (n=6/7 per dietary group). Statistical significance calculated using general linear model with Tukey's post hoc analysis. Values significantly different ($P<0.05$) between quantity of fat is shown by *. Dashed line shows point at which offspring were weaned.

Female offspring weight:

All female offspring gained weight at a steady rate from birth until sacrifice at day 77 (fig 3.3). At all points the females were lighter than the male offspring (P<0.0001). Like the male offspring, the type of diet had no effect on the weight of the offspring. However, the quantity of fat did have a significant effect on the offspring weight (P<0.0001). There were no significant differences between the weight gain from birth until weaning at day 28. After weaning, there is a significant difference between the weights of offspring on a 21% and 7% fat diet at day 35 in the FO (P=0.0006), HSO (P=0.001) and SFA (P=0.01) offspring, this is the same as seen in the male offspring. In the SAO the 21% offspring weighed more than the 7% offspring, at day 56 (P=0.024). At day 63 the 21% SAO offspring weighed 50g more than the 7% offspring (P=0.03). The 7% offspring in the FO group were also significantly heavier than the 21% offspring at day 42 (P=0.006).

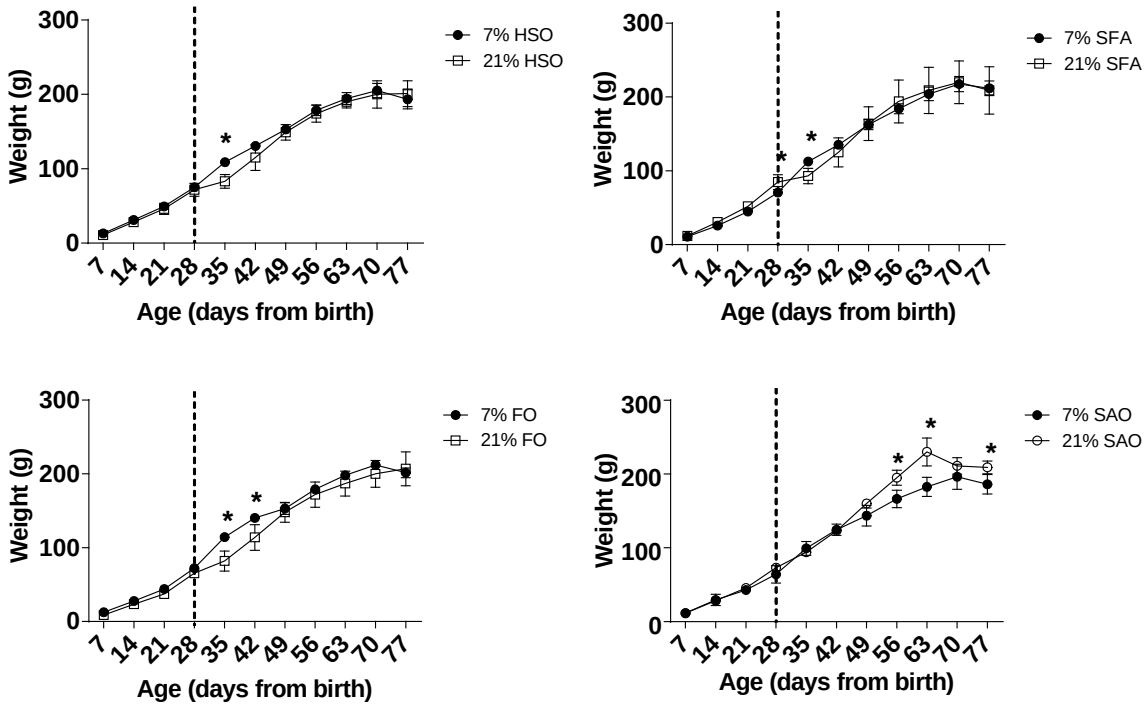


Figure 3.3: Female offspring postnatal body weights for each diet at 7% and 21% quantity. Data shown as mean ± SEM (n=6/7 per dietary group). Statistical significance calculated using general linear model with Tukey's post hoc analysis. Values significantly different (P<0.05) between quantity of fat is shown by *. Dashed line shows point at which offspring were weaned.

3.3.4 Offspring energy intake:

For any given diet the 24 hour energy intake of the 21% offspring was significantly greater in both males and females compared to the 7% offspring at day 52 (table 3.2). There was a significant effect of total maternal dietary fat ($P < 0.0001$), and interactive effects of sex and maternal total fat intake ($P = 0.02$), and of total and type of maternal dietary fat ($P = 0.007$) on energy intake over 24 hours on postnatal day 52, but there was no difference between groups on day 70.

Table 3.2: Average energy consumed per 100g body weight on post-weaning days 52 and 70 for males and females for each diet type at 7% and 21% fat. Values are means ± SEM (n=6/7 per dietary group). Statistical significance calculated using general linear model with Tukey's post hoc analysis. †Indicates values significantly different ($P < 0.05$) between 7% and 21% total fat intakes within a dietary fat type.

	Energy intake (kJ / 100g body weight / 24 hours)							
	7% (w/w) total dietary fat				**21% (w/w) total dietary fat**			
	SAO	**HSO**	**SFA**	**FO**	**SAO**	**HSO**	**SFA**	**FO**
	Males							
Day 52	112 ± 7	109± 9	111± 5	106± 19	176± 28†	180± 28†	171± 26†	200±12†
Day 70	103± 12	115± 6	117± 5	108± 5	101±14	101± 11	102± 13	109± 17
	Females							
Day 52	102± 32	104± 28	107± 36	106± 21	184± 21†	173±22†	149± 42†	185±13†
Day 70	117± 8	113± 20	130± 21	122± 12	104± 12	128±16	125± 16	115± 34

3.3.5 Offspring blood pressure:

Blood pressure is measured by determining the systolic blood pressure (SBP) and diastolic blood pressure (DBP). Systolic blood pressure is the maximum pressure exerted upon the arteries by the blood during a contraction of the ventricles. Diastolic pressure is the minimum pressure exerted upon the arteries by the blood just prior to the next contraction of the heart (293). There was a significant single factor effect of the amount of maternal dietary fat ($P = 0.006$) and a significant interactive effect of amount and type of maternal dietary fat ($P < 0.0001$) on systolic blood pressure in the offspring on day 77. The systolic blood pressure in 7% male offspring was raised in offspring of HSO fed dams compared to the other three diet types ($P<0.0001$). Increasing fat quantity to 21% caused an increase in the systolic blood pressure of the offspring of dams fed SAO. A reduction in systolic blood pressure of the offspring of HSO fed dams was observed (see fig 3.4).

The female offspring of dams fed 7% fat SAO had lower systolic blood pressure compared to the other dietary groups (fig 3.4). Increasing the quantity of fat to 21% caused a decrease in systolic blood pressure in offspring of dams fed HSO, SFA and FO (P=0.006) and an increase in the offspring of dams fed SAO, as seen in male offspring.

There was a significant single factor effect of the amount of maternal dietary fat (*P* = 0.003), and significant interactions between sex and type of dietary fat (*P* = 0.026) and amount and type of dietary fat (*P* < 0.0001) in diastolic pressure. In males, the pattern of effects of maternal total and type of dietary fat on diastolic pressure followed systolic pressure. However, in females the maternal 21% SAO diet offspring demonstrated the opposite change in diastolic pressure compared to systolic pressure.

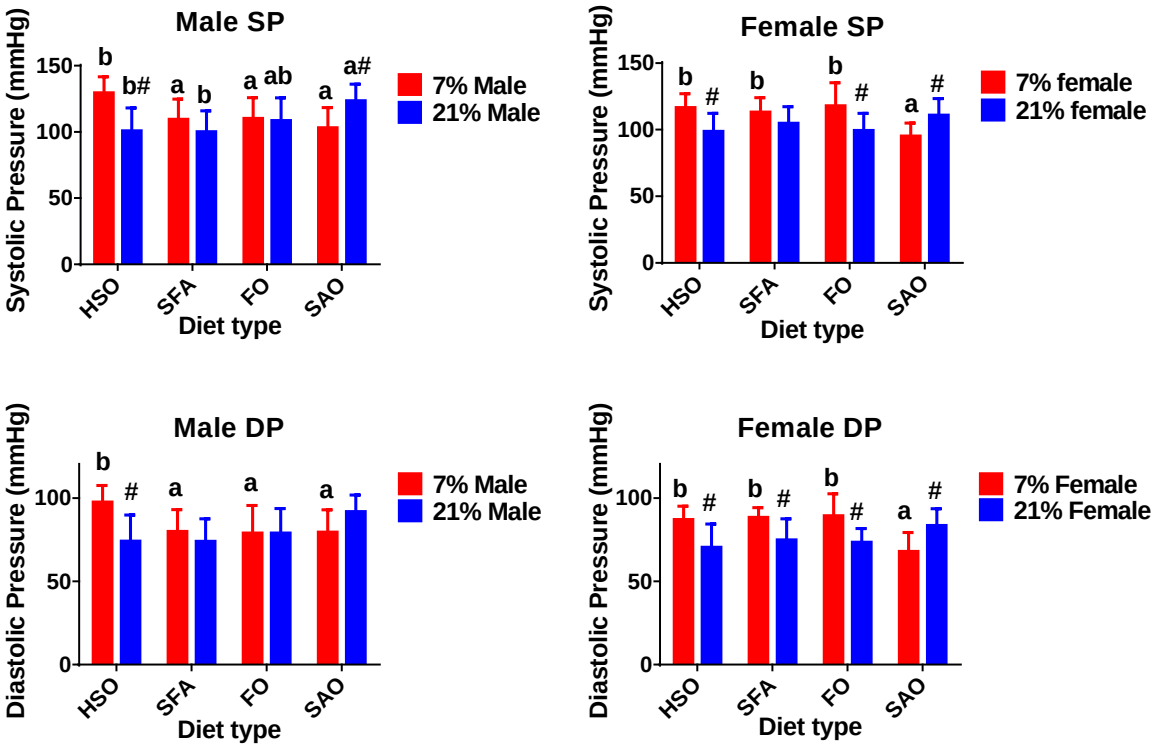


Figure 3.4: Top: Male and Female systolic blood pressure (SP). Bottom: Male and Female diastolic blood pressure (DP). Values are mean ± SEM (n=6/7 per dietary group). Statistical comparisons were by ANOVA with Tukey's post hoc analysis. Values significantly different within a sex and maternal total fat intake are indicated by different superscripts. # indicates values significantly different between maternal total fat intakes according to the type of fat consumed by dams. Values significantly different (P<0.05) within a sex between maternal dietary groups are indicated by different letters.

3.3.6 Aortic Reactivity- Vasoconstriction to Pe:

Male aorta- fat quality:

The male aortae of offspring of dams fed the 7% SFA and SAO diet produced a significantly ($P<0.05$) greater contraction to Pe compared to those fed the 7% HSO ($P=<0.0001$ and $P=<0.0001$ respectively) and FO ($P=<0.0001$ and $P=0.0003$ respectively) (fig 3.5). However, type of fat at the 7% level did not have a significant effect on the pEC50 ($P>0.05$). Pe- induced constriction was greatest in offspring of 21% FO ($P=0.0232$ and $P=<0.0001$ respectively) and 21% HSO ($P=0.0006$ and $P=<0.0001$ respectively) compared to 21% SAO and 21% SFA (fig 3.5).

Male- fat quantity:

Comparing the male 7% and 21% diets (fig 3.5), the maximum response was significantly greater in 21% HSO compared to 7% HSO ($P=<0.0001$). Whilst 21% SAO and SFA demonstrated a significantly decreased maximum response to PE compared to 7% SAO ($P=<0.0001$) and SFA ($P=<0.0001$). Vasoconstriction of offspring of dams fed the 21% FO diet remained unaffected by the increase in quantity of this fat type. Irrespective of type of diet, increasing the quantity of fat to 21% caused a significant increase in pEC50, demonstrating an increased sensitivity to Pe when maternal fat quantity is increased.

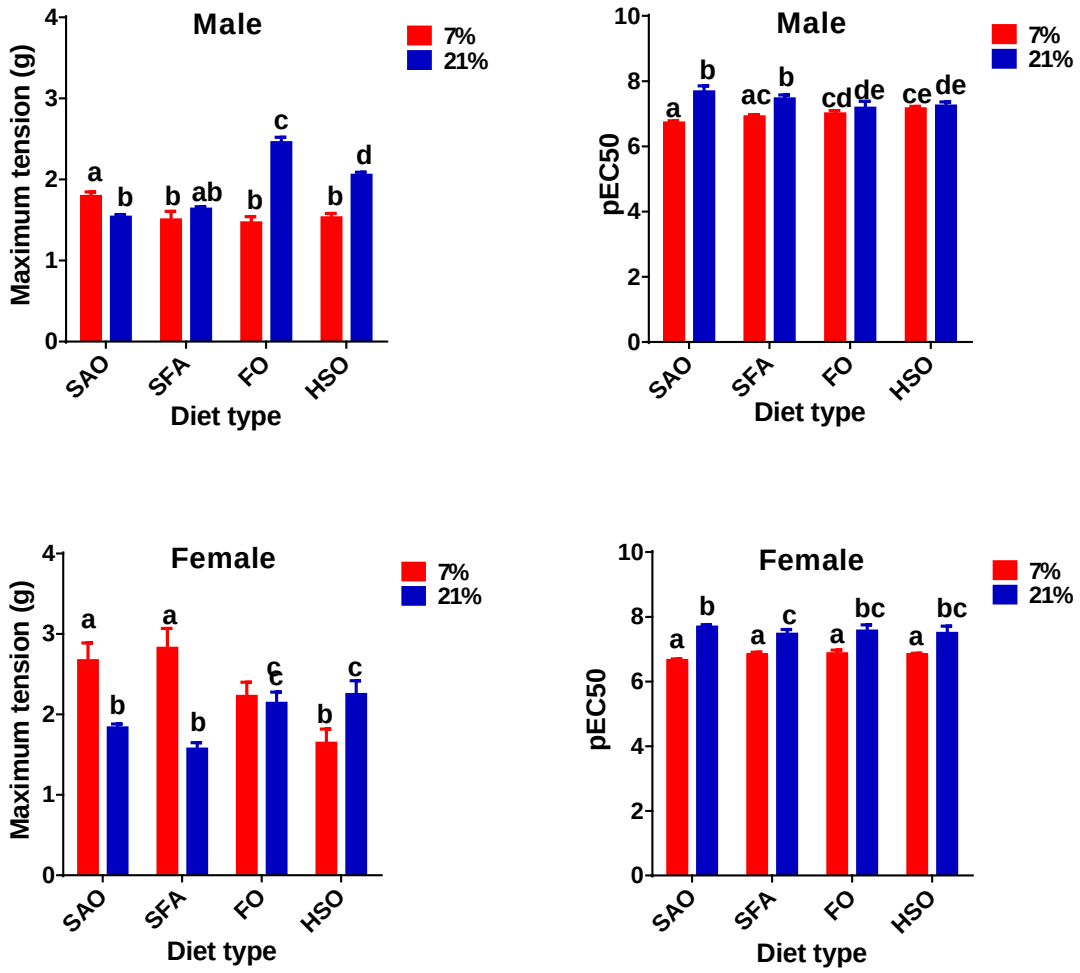


Figure 3.5: Top left: Male maximum vasoconstriction response to Pe and Top right: Male pEC50 values for male aorta treated with Pe. Bottom left: Female maximum vasoconstriction response to Pe and Bottom right: Female pEC50 values for male aorta treated with Pe. Values are mean ±SEM (n=5/6). Statistical comparisons made by ANOVA with Tukey's post-hoc analysis. Values significantly different (P<0.05) between maternal dietary groups within a sex are indicated by letters.

Female aorta- fat quality:

The aortae of female offspring of dams fed a 7% SAO diet produced a significantly greater maximum response to Pe than offspring of dams fed 7% SFA (P=0.0191), FO (P=0.0029) or HSO (P=0.0177) (fig 3.5). The pEC50 was also significantly greater in those fed 7% FO (P=0.0211) and HSO (P<0.0001) than SAO. Female offspring of dams fed 21% fat diet showed a significantly greater maximum response in FO (P<0.0001 and P<0.0001 respectively) and HSO (P<0.0001 and P<0.0001 respectively) compared to those exposed to 21% SAO and SFA. However, the

pEC50 of the 21% FO ($P<0.0001$ and $P=0.0016$ respectively) and HSO ($P<0.0001$ and 0.0247 respectively) was significantly lower than the 21% SAO and SFA offspring (fig 3.6).

Female aorta- fat quantity:

Comparing the quantity of maternal fat, the 21% FO and HSO produced a greater maximum response to Pe compared to all 7% diet types. An increase in pEC50 was also seen in 21% SAO and SFA offspring (fig 3.5). These changes seen in the maximum response in FO and HSO and the increase in pEC50 demonstrate an increased vasoconstriction and sensitivity to Pe with a higher quantity of fat.

3.3.7 Aortic reactivity- endothelial dependent:

In all offspring of dams fed a 7% and 21% diet the aorta produced a concentration-dependent relaxation of the Pe induced tone in response to ACh, regardless of sex or diet type (fig 3.6). There was a significant single factor effects effect of sex ($P<0.05$) and amount ($P < 0.0001$), but not type, of maternal dietary fat, and an interactive effect of sex and amount of maternal dietary fat *($P = 0.04$)* on ACh-induced vasorelaxation in aorta.

Male aorta-

To compare changes in response to ACh caused by diet type and fat quantity, the maximum response and pEC50 (negative log of the EC50) was studied. In males the 7% diet type did not affect the pEC50 but the aorta of offspring of 7% SFA ($P=0.0045$) maternal diet showed a significant reduction in the maximum response elicited by ACh compared to 7% FO (fig 3.6). A 21% diet however, caused a significantly blunted maximum response to ACh compared to the 7% diets in all diet types. The lowest maximum response was observed in offspring of 21% SAO ($P<0.0001$) and SFA ($P<0.0001$) maternal diets. ACh sensitivity was also reduced in 21% FO ($P=0.0001$) and HSO ($P=0.0011$) diet at the 21% fat level, demonstrated by a significantly lower pEC50 compared to the 7% level of these types of fat.

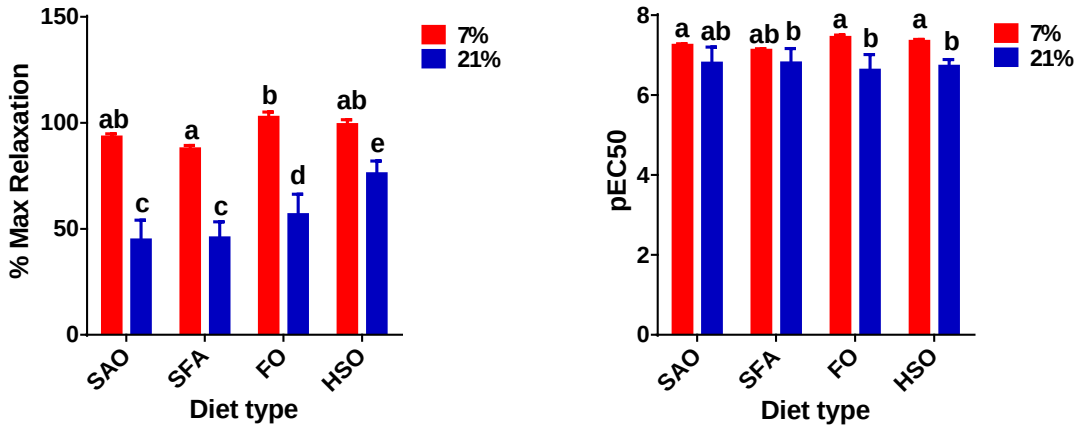


Figure 3.6: Left: Male maximum vaso-relaxation response to ACh and Right: Male pEC50 values for aorta treated with ACh. Values are mean ±SEM (n=5/6). Statistical comparisons made by ANOVA with Tukey's post-hoc analysis. Values significantly different ($P<0.05$) between maternal dietary groups within a sex are indicated by letters.

Female aorta:

Diet type at the 7% fat level had no effect on the maximum response or pEC50 elicited by the female offspring aorta in the presence of ACh (fig 3.7). Increasing the quantity of fat to 21% caused a significantly attenuated response of the offspring aorta in all diet types, compared to the 7% fat diets; SAO (P<0.0001), SFA (P=0.0011), FO (P<0.0001) and HSO (P<0.0001). The aorta of offspring of dams fed 21% FO (P<0.0001) and HSO (P<0.0001) produced the lowest maximum response compared to offspring from a 21% SAO and SFA maternal diet. Sensitivity to ACh was also reduced in 21% FO (P= 0.0001) and SFA (P=0.0237), demonstrated by a significantly reduced pEC50 compared to 7% FO and SFA.

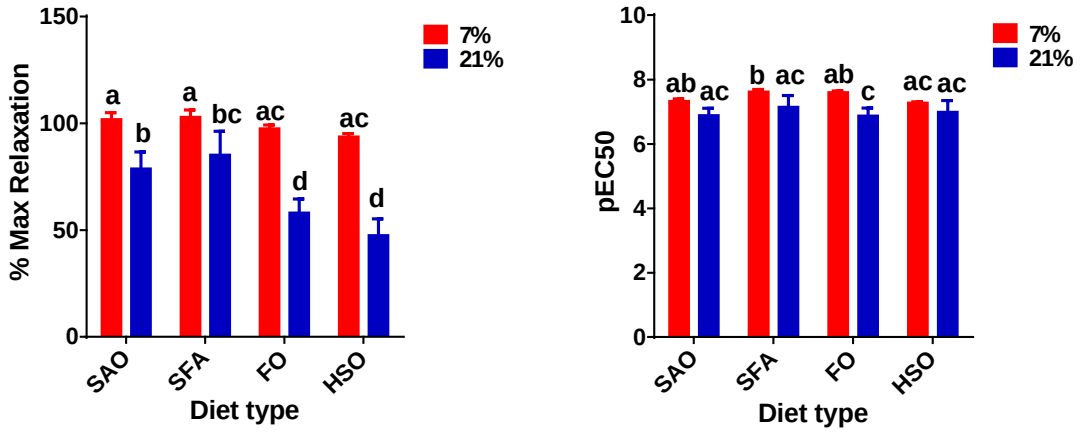


Figure 3.7: Left: Female maximum vasorelaxation response to ACh and Right: Female pEC50 values for aorta treated with ACh. Values are mean ±SEM (n=5/6). Statistical comparisons made by ANOVA with Tukey's post-hoc analysis. Values significantly different (P<0.05) between maternal dietary groups within a sex are indicated by letters.

3.3.8 Aortic Reactivity- Endothelial Function:

To investigate the role of eNOS activity in ACh induced dilatation. The ACh dose response curve was carried out in the presence of the eNOS inhibitor L-NAME. In all of the offspring an abolished response to ACh was observed in the presence of L-NAME 100 nM, this is regardless of the sex, diet type or quantity. Most responses also produced a gradual contraction on the aorta when with the increasing concentrations of ACh (fig 3.8).

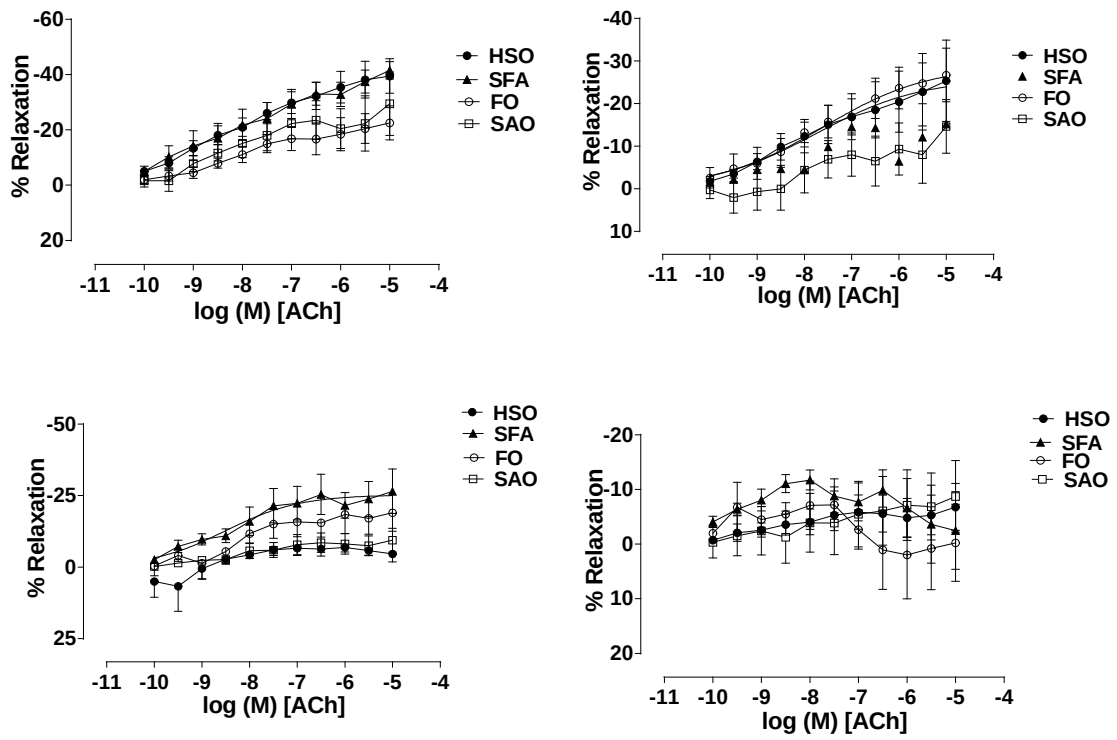


Figure 3.8: ACh response of male rat aorta on a 7% fat diet pre-constricted with Pe and incubated with L-NAME 100 µM. Top left: Male 7%, Top Female 7%, Bottom left: Male 21%, Bottom right: Female 21%. Data shown as mean ± SEM. (n=5/6).

3.3.9 Aorta Reactivity- Endothelial independent:

To confirm that the changes in response to ACh were due to only endothelial dysfunction and not due to changes in smooth muscle function, aorta were treated with SNP to cause an endothelial independent relaxation. SNP caused a concentration dependent relaxation of all aorta regardless of sex, diet type or diet quantity. There was no significant difference between any of the responses (fig 3.9).

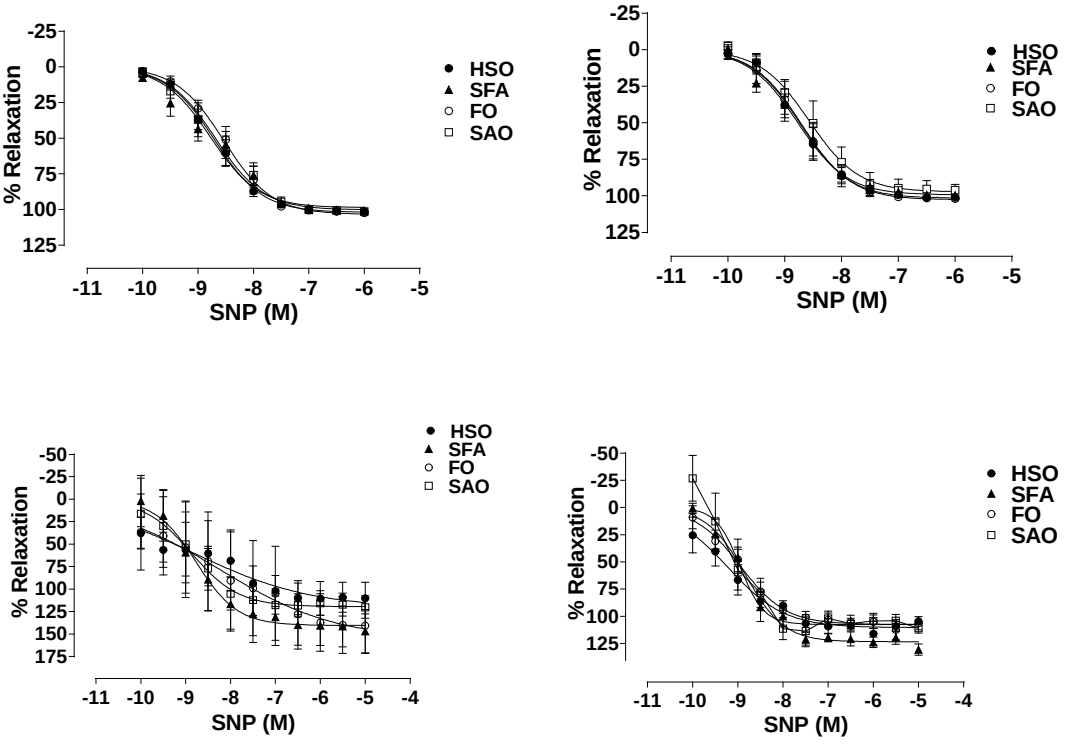


Figure 3.9: Relaxation of aorta in response to sodium nitroprusside (SNP). Top left: Male 7%, Top right: Female 7%, Bottom left: Male 21%, Bottom right: Female 21%. Data shown as mean ± SEM. (n=5-6).

3.3.10 The effect of delta-5 and delta-6 desaturase inhibition on Pe-mediated vasoconstriction:

To assess the potential role of PUFA biosynthesis in regulation of vascular tone the delta-5 and delta-6 desaturase inhibitors were incubated with rat aorta and human femoral arteries. Aorta were treated with Pe and demonstrated a dose-dependent increase in vasoconstriction. Treatment of endothelium-denuded aorta also demonstrated a dose-dependent increase in vasoconstriction and caused a small but non-significant ($P=0.3129$) increase in Pe-mediated vasoconstriction compared to aorta with intact endothelium (fig 3.10 top). Pre-treating rat aorta with both delta-5 and delta-6 desaturase inhibitors caused approximately a 75% reduction in vasoconstriction to Pe in both intact aorta ($P<0.0001$) and endothelium denuded aorta ($P<0.0001$) compared to the appropriate control (fig 3.10, top left and right).

The same experiment was carried out on human arteries to determine the consistency of the response between species. As seen in rat aorta, treatment with Pe caused a dose-dependent vasoconstriction in intact and denuded arteries (fig 3.10 bottom left). However, in human arteries, the magnitude of response between the two individual arteries in response to Pe was different. As seen in the rat aorta, pre-treatment with SC-26196 and sesamin reduced vasoconstriction in femoral arteries irrespective of the presence of endothelium (figure 3.10, bottom left and right). Unfortunately only two human samples were available and data as shown as each individual response so statistical analysis was not carried out on these samples.

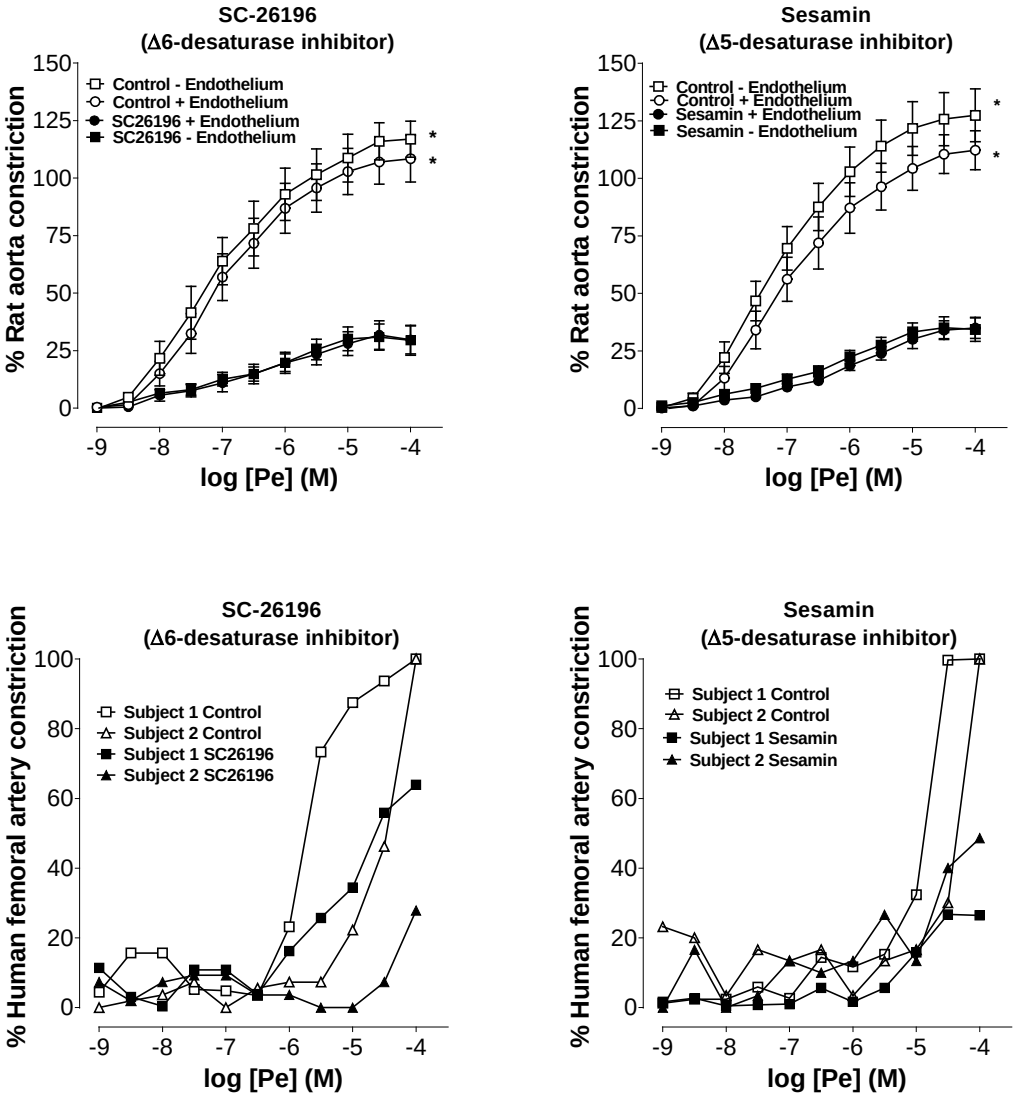


Figure 3.10: The effect of Delta-6 (SC-26196) and Delta-5 desaturase (sesamin) inhibition in rat aorta and human femoral artery on Pe-mediated vasoconstriction. Top left: SC-26196 in Pe treated rat aorta, Top right: Sesamin in Pe treated rat aorta. Values are mean ±SEM (n=6). * shows significantly different (P<0.05) maximum responses in control vs inhibitor treated vessels by ANOVA with Tukey's post hoc analysis. **Bottom left: Human femoral artery treated with SC-26196. Bottom right: Human femoral artery treated with sesamin** (n=2)**.** Values are percent constriction relative to maximum Pe-induced constriction.

3.4.1 Aorta fatty acid composition:

Male offspring:

The offspring of the 21% SFA diet caused significant increase in the 16:0 (P=0.0274) and 18:1n-9 (P=0.0453) fatty acids compared to the offspring of the 7% SFA diet. The aorta of the offspring from 7% FO group had a significantly greater (P<0.0001) proportion of 18:0 and a significantly lower proportion of 16:1n-7 (P=0.0293) and 18:2n-6 (P=0.0016) compared to offspring of the 21% group as shown in table 3.3.

In aortae of male offspring, the proportion of fatty acids varied in relation to the type of diet. A maternal diet of 7% SAO caused an increase in the 18:2n-6 compared to a maternal diet rich in 7% FO (P=0.005). Conversely a maternal diet rich in 7% HSO caused a significant increase in the saturated fatty acid 18:0 compared to 7% SAO and FO (P=0.005).

Female offspring:

The 21% SAO diet produced an increase in the proportion of 18:1n-6 (P=0.0049) and 18:1n-9 (P=0.0203) in the aorta compared to the offspring of the 7% SAO diet. The proportion of 18:1n-9 was also significantly greater (P=0.0323) in the aorta of offspring fed the 21% SFA diet compared to the 7% SFA diet.

Sex differences:

Sex differences were seen between the males and females fed the same type of fat 7% maternal diets (see table 3.3). Significant differences were seen in the SFA group, as female show a significantly (P<0.0001) greater proportion of 16:1n-7. Females in the FO group show a significantly lower proportion of 16:1n-7 compared to males (P<0.0001). The SAO groups also showed sex differences with females having a greater proportion of 18:0 (P=0.001) and males having a larger proportion of 16:1n-7 (P<0.0001). However, there was no sex differences observed in the HSO group. Furthermore, trans fatty acids were not detected in the aorta from 7% HSO nor in aorta from any other diet type in either males nor females.

There were also significant differences (P<0.05) observed between the fatty acid composition of the aorta in males and females at 21% fat for each fat type (fig 3.14). The greatest differences were seen in the female offspring from the 21% SFA diet, males showed an increase proportion of 16:0 (P=0.002), 16:1n-7(P=0.028) and 18:1n-7 (P<0.0001) compared to the female offspring. The males also demonstrated a higher proportion of 16:1n-7 (P<0.0001) acid and 18:1n-7 (P<0.0001) in the FO fed offspring compared to females. Like the

FO and SFA diets, the SAO diet also showed a decrease in 16:1n-7 ($P<0.0001$). There was no significant difference between males and females fed the HSO diet at 21%. As with the 7% diets, trans fatty acids were not detected in any diet type at the 21% fat level.

Table 3.3: Fatty acid composition of male and female offspring aorta. Values are mean ± SEM (n=5/6). Different superscripts indicate values which were significantly different ($P< 0.05$) by a general linear model with Tukey's *post hoc* analysis. †Shows values significantly different between offspring of dams fed 7% or 21% fat diets of the same fat type. ‡ Shows values significantly different between sexes within a maternal dietary group.

Male Aorta Fatty acid concentration (% proportion of total fatty acids)								
Maternal dietary group								
	SAO		**HSO**		**SFA**		**FO**	
Fatty Acid	**7%**	**21%**	**7%**	**21%**	**7%**	**21%**	**7%**	**21%**
16:0	28.0±2.2	29.7±1.4	29.5±2.6	31.7±1.1	29.7±0.8	31.5±0.8†	30.2±1.2	31.2±1.5
18:0	10.0±2.2[a]	9.4±2.7	14.0±2.9[b]	11.2±5.9	12.1±1.2[ab]	9.2±3.4	13.6±2.6[a]	9.8±3.4†
16:1n-7	7.9±1.2	7.1±2.3	8.0±1.1	7.6±2.9	7.4±1.0	9.5±1.7†	6.4±1.4	8.9±1.4†
18:1n-9	25.3±1.5	26.3±1.9	23.6±3.3	28.0±5.3	26.1±2.0	30.0±3.2†	24.4±2.2	27.3±2.3
18:1n-7	3.4±0.3	3.5±0.8	3.3±0.3	2.9±0.6	3.3±0.3	3.3±0.4	3.3±0.5	3.0±0.3
22:1n-9	0.2±0.1	0.2±0.6	0.2±0.1	0.5±0.6	0.2±0.1	0.3±0.1	0.3±0.1	0.3±0.2
18:2n-6	13.6±2.9[a]	15.1±2.5	11.7±2.4[ab]	11.4±2.0	12.2±0.7[ab]	10.3±2.0	9.4±0.9[b]	12.5±3.1†
20:3n-6	0.5±0.2	0.3±0.1†	0.4±0.4	0.2±0.8	0.3±0.2	0.2±0.0†	0.5±0.3	0.3±0.1
22:4n-6	0.2±0.1	0.8±0.4†	0.9±0.7	0.4±0.2	0.3±0.1	0.5±0.4	0.4±0.3	0.4±0.1
18:3n-3	0.1±0.5	1.0±0.9†	0.3±0.2	1.1±0.3†	0.2±0.2	1.0±0.3†	0.2±0.1	1.2±0.2†
20:5n-3	0.2±0.1	0.2±0.1	0.2±0.1	0.1±0.8	0.2±0.1	0.2±0.2	0.2±0.1	0.2±0.5
22:5n-3	0.5±0.1	0.4±0.2	0.5±0.2	0.6±0.9	0.3±0.2	0.2±0.1†	0.6±0.2	0.4±0.1
Female Aorta Fatty acid concentration (% proportion of total fatty acids)								
Maternal dietary group								
	SAO		**HSO**		**SFA**		**FO**	
Fatty Acid	7%	21%	7%	21%	7%	21%	7%	21%
16:0	29.1±2.4	29.4±1.0	29.2±1.2	29.7±1.5	13.1±1.7	27.5±4.9	28.5±2.0	30.4±2.6
18:0	15.2±2.9‡	14.1±4.1	14.5±3.4	14.2±9.4	4.2±1.5‡	15.0±4.7‡	13.2±3.6	13.9±5.6
16:1n-7	3.4±0.9‡	4.4±1.0‡	5.2±1.6	6.0±3.2	22.4±1.9‡	3.8±1.0‡	3.8±0.5‡	5.3±1.9‡
18:1n-9	22.3±4.0	25.5±3.6	24.3±2.8	26.6±5.7	3.1±0.5	26.2±3.9	25.4±2.1	24.6±4.7
18:1n-7	2.9±0.7	2.4±0.2‡	3.2±0.5	2.5±0.6†	0.2±0.1	2.6±0.4‡	2.9±0.3	2.5±0.4‡
22:1n-9	0.1±0.1	0.6±0.7	0.2±0.1	0.4±0.4	11.1±1.1	0.9±0.8	0.2±0.1	0.5±0.3†
18:2n-6	11.1±1.5	14.7±2.8†	9.6±1.4	12.5±3.6	0.7±0.3‡	12.5±3.6	11.0±2.5	12.9±3.3
20:3n-6	0.7±0.3	0.4±0.1	0.6±0.2	0.3±0.1†	0.5±0.3	0.3±0.1†	0.7±0.3	0.5±0.1
22:4n-6	0.3±0.2	0.8±0.3†	0.6±0.1	0.6±0.4	0.2±0.1	2.4±3.9	0.2±0.1	0.5±0.2†
18:3n-3	0.2±0.1	1.2±0.5†	0.2±0.1	1.2±0.3†	0.1±0.1[a]	1.9±1.3†	0.1±0.1	1.0±0.2†
20:5n-3	0.1±0.1[a]	0.2±0.1[ab]	0.1±0.0[a]	0.1±0.1[b]	0.7±0.3‡	0.2±0.1[ab]	0.3±0.2[b]	0.2±0.0[a]
22:5n-3	0.4±0.2	0.3±0.1	0.5±0.2	0.6±1.0	0.7±0.3‡	0.3±0.1†	0.9±0.5	1.0±0.8

3.4.2 Aorta AA and DHA content:

The composition of AA (20:4n-6) and DHA (22:6n-3) in offspring aorta were shown to consistently change in all dietary groups when the amount of fat was increased from 7% to 21%. This was observed in both male and female offspring.

AA:

There were significant effects on the proportion of AA in the aorta of type (P=0.007) and amount of fat (P<0.0001) and sex (P=0.00014) with interactions in sex*fat type P=0.002, fat amount*fat type (P=0.036). In male offspring of dams fed the 7% diet, the proportion of AA was significantly lower in those from dams fed HSO and SFA compared to those fed SAO or fish oil. There was no significant difference between the AA content of female aorta from offspring of dams fed any of the 7% fat diets (fig 3.11). The offspring of dams fed 21% fat, exhibited a significant decrease in the content of AA in both male and female offspring, irrespective of diet type.

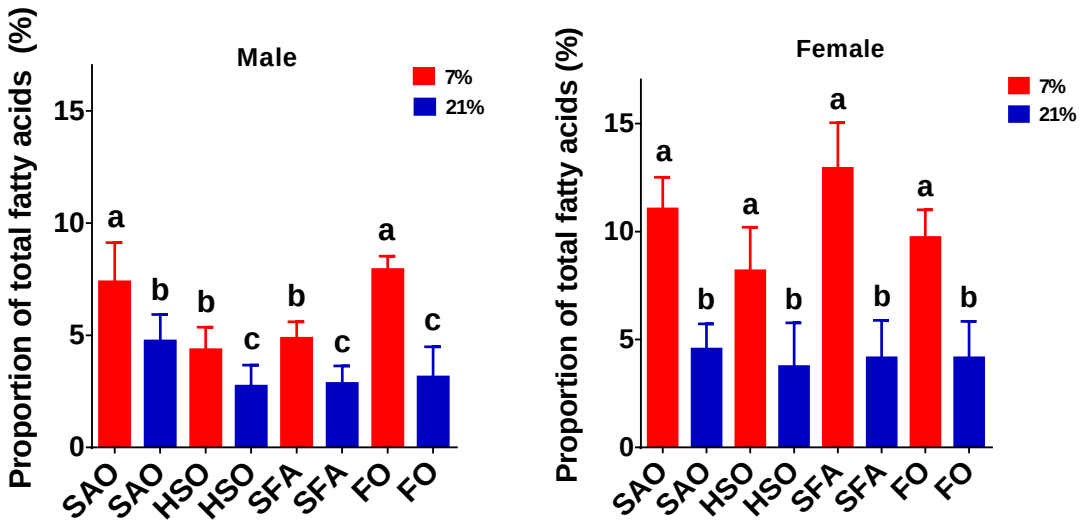


Figure 3.11: Left: Male proportion of AA content in aorta and Right: Female proportion of AA content in Aorta. Values are mean ± SEM. Statistical comparisons were by ANOVA with Tukey's post-hoc analysis. Values significantly (P<0.05) different within sex are indicated by different letters (n=6/group).

DHA:

There were significant effects of type (P=0.02) and amount (P=0.0004) of maternal dietary fat and sex (P=0.003) on the proportion of DHA in offspring aortae. With the following interactions; sex*fat amount P=0.001, sex*fat type P=0.007 and fat amount*fat type P=0.04). As seen with AA content, increasing the fat content of maternal diet to 21% caused a

significant decrease in DHA content in male offspring irrespective of diet type and all female offspring with the exception of offspring from dams fed fish oil (fig 3.12).

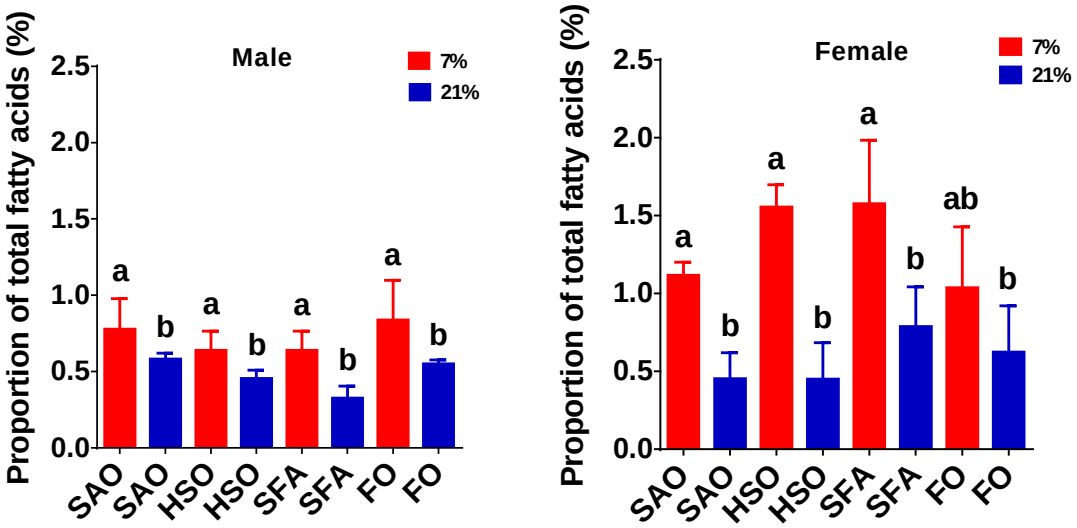


Figure 3.12: Left: Male proportion of DHA content in the aorta. Right: Female proportion of DHA content in Aorta. Values are mean ± SEM. Statistical comparison were by ANOVA with Tukey's post-hoc analysis. Values significantly ($P<0.05$) different within sex are indicated by different letters (n=6/group).

3.4.3 Plasma fatty acid composition:

To investigate whether the differences in aorta fatty acid composition between different maternal dietary groups were due to changes in the supply from the plasma to the aorta, the fatty acid composition of the plasma was measured. The composition of plasma triacylglycerol (TAG) and non-esterified fatty acids (NEFA) were measured; these are the major transport pools that supply PUFAs to blood vessels.

Male offspring plasma composition:

TAG:

The differences in offspring TAG composition were significantly affected by type of maternal diet (table 3.4). A 7% SAO diet had significantly greater SAO ($P<0.0001$) than SFA. 21% SAO offspring had significantly higher 18:3n-6 compared to 21% HSO ($P=0.0344$) and 21% SFA ($P<0.0001$). 21%FO offspring also had significantly more 18:3n-6 compared to HSO ($P<0.0001$) and 21% SFA ($P<0.0001$). Increasing the 7% SAO to 21% SAO ($P<0.0001$) and 7% FO to 21% ($P<0.0001$) caused a significant increase in 20:3n-6 content.

NEFA:

There were differences in the NEFA composition of male offspring plasma fed different fat types at the 7% level of fat (table 3.4). The content of 16:0 was significantly greater in offspring of 7% SFA ($P<0.0001$) and FO ($P=0.007$) than those of 7% SAO. The offspring of 7% SAO exhibited a higher proportion of 18:2n-6 compared to offspring of a 7% SFA diet ($P<0.0001$). Comparing the plasma composition of offspring of 7% and 21% fat maternal diet shows significant increases in 18:3n-3 in 21% FO, SFA, HSO and SAO compared to 7% ($P<0.0001$). A decrease in the proportion of 16:0 was also seen in 21% FO compared to 7% FO ($P=0.0248$).

Table 3.4: Fatty acid composition of male offspring plasma. **Top: Triacylglycerol and Bottom: non-esterified fatty acid.** Values are mean ± SEM (n=5/6). Different superscripts indicate values which were significantly different (P< 0.05) by a general linear model with Tukey's *post hoc* analysis. †Shows values significantly different between offspring of dams fed 7% or 21% fat diets of the same fat type. ‡ Shows values significantly different between sexes within a maternal dietary group.

Male Fatty acid concentration (% proportion of total fatty acids)								
Maternal dietary group								
	SAO		HSO		SFA		FO	
Fatty Acid	7%	21%	7%	21%	7%	21%	7%	21%
	Plasma Triacylglycerol (TAG)							
16:0	21.4±1.9	19.4±3.2	21.1±2.1	20.9±2.1	23.4±1.9	24.2±1.5	23.9±2.7	19.1±6.1
18:0	2.4±1.0	3.7±0.8	2.6±1.0	2.7±0.7	2.4±0.4	2.5±1.8	2.6±0.6	2.9±0.8
20:0	0.2±0.1	0.7±0.2†	0.2±0.1	0.6±0.3	0.2±0.1	0.4±0.1	0.1±0.0	0.7±0.3†
16:1n-7	2.3±0.8	1.9±0.5	2.6±0.5	2.7±0.5	3.2±0.3	1.2±1.4	3.0±0.8	2.1±1.0
18:1n-9	17.8±1.8	15.9±1.1^{b}	20.0±1.1	20.3±2.1^{ab}	20.2±1.5	23.5±2.8^{a}	18.7±1.3	16.6±5.2^{b}
18:1n-7	2.7±0.3	2.3±0.4	2.8±0.4	2.7±0.4	2.6±0.2	3.0±0.5	2.6±0.2	2.1±0.4
20:1n-9	0.4±0.2	0.5±0.2	0.5±0.2	0.5±0.4	0.4±0.1	0.3±0.1	0.4±0.2	0.6±0.3
18:2n-6	37.3±2.1^{a}	35.7±3.8	35.4±2.4^{ab}	33.6±1.8	30.5±1.1^{b}	31.0±1.5	32.9±3.5^{ab}	28.1±5.0
20:2n-6	0.2±0.1	0.7±0.3†	0.2±0.1	1.0±0.5	0.2±0.0	0.6±0.1†	0.2±0.0	1.1±0.5†
20:3n-6	0.3±0.1	10.8±3a†	0.3±0.1	5.3±1.3b†	0.2±0.0	4.7±1.8b†	0.3±0.1	12.2±17^{a}
22:4n-6	0.3±0.2	0.6±0.2	0.5±0.1	0.6±0.6	0.5±0.1	0.2±0.1	0.2±0.1	0.7±0.3†
18:3n-3	2.3±0.3	2.1±0.3	2.3±0.5	2.4±0.3	2.2±0.2	2.4±0.2	2.3±0.3	2.0±0.6
20:5n-3	0.2±0.0	0.7±0.2†	0.1±0.0	0.8±0.3†	0.3±0.1	0.5±0.1	0.3±0.1	2.2±3.7
22:5n-3	0.9±0.2	1.1±0.5	0.8±0.1	1.2±0.4	1.0±0.2	1.2±0.2	1.2±0.4	1.9±1.1
Male Fatty acid concentration (% proportion of total fatty acids)								
Maternal dietary group								
	SAO		HSO		SFA		FO	
Fatty Acid	7%	21%	7%	21%	7%	21%	7%	21%
	Plasma Non esterified fatty acids (NEFA)							
16:0	26.5±2.2^{a}	24.1±4.1	27.6±1^{ab}	26.3±2.0	31.2±1.5^{b}	26.5±4†	30.2±1.5^{b}	27.0±2†
18:0	6.9±1.6	8.0±2.4	8.3±1.1	8.2±1.8	8.5±1.8	7.9±1.3	7.8±1.0	9.5±0.6
16:1n-7	7.0±1.8^{a}	5.7±1.6	7.5±2.2^{a}	6.6±1.3	10.2±0.5^{b}	8.4±2.6	8.6±1.5^{ab}	6.6±1.3†
18:1n-9	19.1±1.3	15.8±2^{a}†	19.0±2.0	19.3±1^{b}	20.1±0.9	20.8±2.2^{b}	17.9±1.4	17.2±1.7^{a}
18:1n-7	3.0±0.3	2.6±0.3^{a}†	3.2±0.4	3.2±0.2^{b}	2.8±0.3	3.3±0.4^{b}†	3.5±1.1	2.8±0.3^{a}
18:2n-6	28.7±4.6^{a}	29.0±4.5^{a}	25.2±3.9^{a}	24±1.5^{b}	19.6±2.5^{b}	20.8±2.4^{b}	22.8±3.5^{ab}	22.7±2.0^{b}
20:3n-6	0.3±0.1	0.5±0.5	0.2±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.0
18:3n-3	0.1±0.1	2.3±0.7†	0.2±0.1	2.8±0.2†	0.2±0.1	2.5±0.2†	0.1±0.1	2.7±0.2†
20:5n-3	0.3±0.1	0.7±0.5	0.3±0.2	0.4±0.1	0.3±0.1	0.5±0.2†	0.7±0.3	1.1±0.5
22:5n-3	0.6±0.4	0.6±0.3	0.3±0.1	0.4±0.0	0.6±0.3	0.7±0.2	0.6±0.3	0.9±0.2

Female offspring plasma composition:

TAG:

Higher proportions of 18:2n-6 were seen in offspring of 7% SAO compared to offspring of a 7%FO (P=0.0068) and 7%SFA (P=0.0004) diet (table 3.5). Increasing the fat content from 7% to 21% caused increases in 20:2n-6 (P=0.008), 20:5n-3 (0.0064) and 20:3n-6 (P<0.0001) in all diet types. Sex differences were only seen in 21% HSO offspring with significantly higher 18:2n-6 (P=0.0253) in males than females.

NEFA:

The proportion of 18:2n-6 was significantly higher in offspring of a 7% (P<0.0001) and 21% (P=0.0064) SAO diet compared to all other diet types (table 3.5). Offspring of 21% FO demonstrated a significantly higher proportion of 20:5n-3 (P=0.0002) compared to all other diet types. Differences within diet type were observed in offspring fed a 7% and 21% fat. Offspring of 21% FO demonstrated increased proportions of 18:3n-3 (P<0.0001) and 20:5n-3 (P=0.0032) compared to offspring of 7% FO. Sex differences were only seen in offspring of 7% SFA maternal diet with significantly higher 16:2n-7 in males than females (P=0.0348) .

Table 3.5: Fatty acid composition of female offspring plasma. **Top: Triacylglycerol and Bottom: non-esterified fatty acid.** Values are mean ± SEM (n=5/6). Different superscripts indicate values which were significantly different (P< 0.05) by a general linear model with Tukey's *post hoc* analysis. †Shows values significantly different between offspring of dams fed 7% or 21% fat diets of the same fat type. ‡ Shows values significantly different between sexes within a maternal dietary group.

Female Fatty acid concentration (% proportion of total fatty acids)								
Maternal dietary group								
	SAO		HSO		SFA		FO	
Fatty Acid	7%	21%	7%	21%	7%	21%	7%	21%
	Plasma Triacylglycerol (TAG)							
16:0	21.4±1.5	20.2±3.2	21.4±2.5	19.2±3.4	23.4±1.1	21.6±2.2‡	24.1±0.9†	19.5±2.7
18:0	3.7±1.1	3.9±0.9	3.4±0.9	4.4±1.8	3.5±0.9	3.3±0.8	3.3±0.9	3.6±0.9
20:0	0.3±0.1	0.9±0.4	0.2±0.0	1.1±0.5	0.2±0.0	0.7±0.4	0.3±0.1	1.0±0.5
16:1n-7	1.5±0.1	1.8±0.6	2.2±0.9	1.6±0.8	2.3±1.0	2.5±0.8	2.5±0.5	2.0±0.6
18:1n-9	20.1±1.4	17.8±1.9	22.7±3.2	21.0±3.3	23.9±3.0	22.3±3.9	21.4±2.4	19.3±1.9
18:1n-7	2.3±0.2	1.8±0.1	2.6±0.3	2.1±0.8	2.3±0.4	2.1±0.2	2.1±0.2	1.8±0.3
20:1n-9	0.5±0.2	0.8±0.5	0.7±0.8	1.0±0.8	0.5±0.3	0.6±0.5	0.5±0.2	0.8±0.4
18:2n-6	34.1±2.5^{a}	30.2±4.5	31.3±4.5^{ab}	26.9±1.6‡	28.9±1.8^{b}	26.3±2.1	29.2±3.0^{b}	25.0±3.4
20:2n-6	0.3±0.2	1.1±0.3†	0.2±0.1	1.9±2.4†	0.2±0.1	0.9±0.8†	0.2±0.1	1.0±0.3†
20:3n-6	0.3±0.2	13.4±3.2†	0.2±0.0	10.9±3.4†	0.2±0.0	9.2±3.5†	0.2±0.0	9.1±3.3†
22:4n-6	0.3±0.1	0.7±0.5	0.4±0.2	1.1±0.9	0.6±0.5	0.6±0.4	0.4±0.2	0.9±0.4
18:3n-3	1.5±0.3	1.6±0.5	2.0±0.4	1.7±0.5	1.9±0.6	1.7±0.2	1.8±0.4	2.0±0.5
20:5n-3	0.2±0.1	1.0±0.5†	0.1±0.0	1.3±0.5†	0.1±0.0	1.0±0.7†	0.2±0.1	1.0±0.5†
22:5n-3	0.9±0.3	1.3±0.8	0.7±0.1	1.3±0.5	0.6±0.1	1.5±0.9†	1.0±0.2	1.6±0.3†
Female Fatty acid concentration (% proportion of total fatty acids)								
Maternal dietary group								
	SAO		HSO		SFA		FO	
Fatty Acid	7%	21%	7%	21%	7%	21%	7%	21%
	Plasma Non esterified fatty acids (NEFA)							
16:0	27.2±2.3	24.2±1.8	28.8±1.4	26.5±2.9	29.0±1.2	26.1±3.7	29.8±1.4	26.9±1.6
18:0	9.5±1.8	9.8±1.3	9.1±2.2	9.4±1.7	9.9±1.5	10.3±2.9	9.6±3.7	9.8±1.6
16:1n-7	4.9±0.7	4.6±0.9	7.6±2.2	5.9±2.0	7.4±1.6‡	5.9±2.5	7.2±1.7	6.5±1.7
18:1n-9	17.1±3.5	17.9±1.7^{a}	21.6±2.6	20.4±1.9^{a}	20.6±1.7	20.7±1.3^{b}	19.8±2.4	18.7±1.7^{a}
18:1n-7	2.2±0.1	2.0±0.2	2.4±0.4	2.3±0.2	2.2±0.2	2.3±0.2	2.2±0.4	2.2±0.2
18:2n-6	29.3±3.0^{a}	27.7±1.6^{a}	23.2±1.9^{b}	22.9±1.7^{b}	22.8±2.3^{b}	21.4±2.0^{b}	23.0±3.1^{b}	21.4±2.4^{b}
20:3n-6	0.3±0.1	0.3±0.1	0.1±0.1	0.2±0.1	0.2±0.1	0.3±0.3	0.4±0.3	0.3±0.1
18:3n-3	0.1±0.1	2.3±0.2†	0.5±0.5	2.3±0.2†	0.2±0.1	2.2±0.2	0.2±0.2	2.5±0.2†
20:5n-3	0.3±0.1‡	0.3±0.1^{a}	0.1±0.0	0.3±0.2^{a}†	0.6±0.8	0.4±0.3^{a}	0.5±0.2	1.1±0.3^{b}†
22:5n-3	0.5±0.2	0.5±0.2	0.3±0.2	0.4±0.2	0.4±0.3	0.6±0.3‡	0.5±0.2‡	0.9±0.2†

3.4.4 AA and DHA content of offspring TAG fraction:

There was a significant effect of the amount of fat (P<0.0001) but not type of fat or sex on the proportions of AA in TAG. The proportion on AA was significantly lower in plasma TAG of male and female offspring of dams fed 21% fat compared to those fed 7% fat, the type of diet had no significant effect (fig 3.13). However, the proportion of DHA in the plasma TAG was not affected by either the type or quantity of fat in the maternal diet of male or female offspring, with no significant effect observed. This is not consistent with the DHA composition of the offspring aorta.

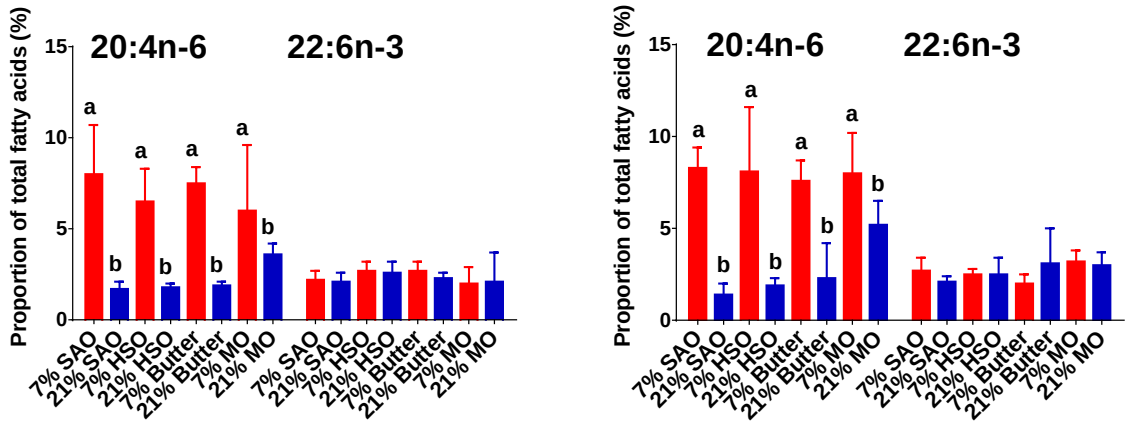


Figure 3.13: Left: Male proportion of AA and DHA content in TAG fraction of plasma. Right: Female proportion of AA and DHA content in TAG fraction in plasma. Values are mean ± SEM. Statistical comparisons were by ANOVA with Tukey's post-hoc analysis. Values significantly (P<0.05) different within sex are indicated by different letters (n=6/group).

3.4.5 AA and DHA content of offspring NEFA fraction:

There was no significant difference between male or female offspring of the different maternal dietary groups in the proportion of AA and DHA in plasma NEFA composition (fig 3.14). This does not reflect the aorta composition of either AA or DHA in the offspring.

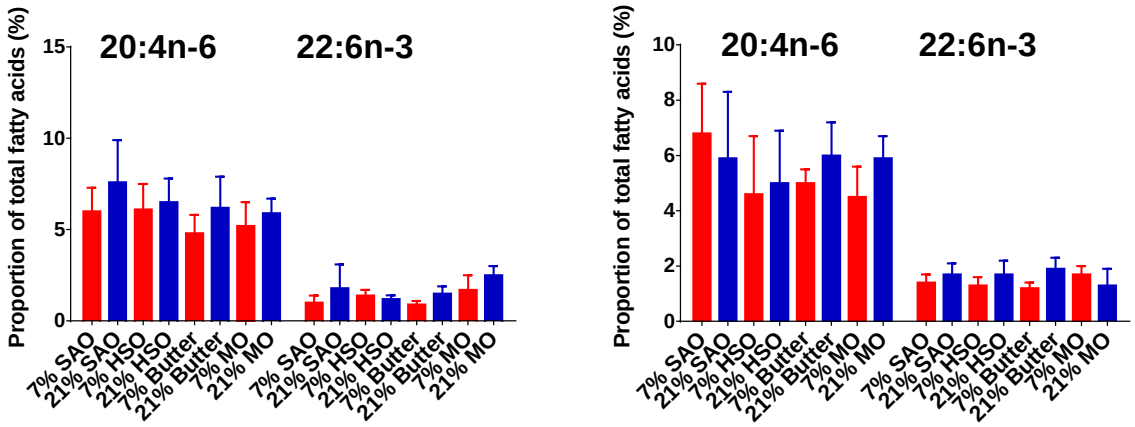


Figure 3.14: Left: Male proportion of AA and DHA content in NEFA fraction of plasma. Right: Female proportion of AA and DHA content in NEFA fraction in plasma. Values are mean ± SEM. Statistical comparisons were by ANOVA with Tukey's post-hoc analysis. Values significantly ($P<0.05$) different within sex are indicated by different letters (n=6/group).

3.5 Discussion:

The present study demonstrates that feeding dams a diet of differing compositions and quantity of fat results in alterations in the offspring blood pressure, vascular function and the aorta composition, with amount of maternal fat having the greatest effect. The data showed that both amount and type of maternal dietary fat consumed during pregnancy and lactation induced persistent changes in vascular function and the fatty acid composition of the aorta. A summary table of the key observations is shown in table 3.6.

Table 3.6: Summary table showing the key changes observed in 21% offspring compared to 7% offspring. ↑shows increase in outcome in offspring of 21% dams compared to offspring of 7% dams, ↓shows decrease in outcome in 21% offspring compared to offspring of 7% dams and = shows no significant difference between outcome in offspring of 7% and 21% dams. BP: Blood pressure, Pe: phenylephrine, ACh: acetylcholine, AA: arachidonic acid and DHA: docosahexaenoic acid.

	Male				Female			
	SAO	**SFA**	**FO**	**HSO**	**SAO**	**SFA**	**FO**	**HSO**
Dam energy intake during lactation					↑	↑	↑	↓
Offspring energy intake day 52	↑	↑	↑	↑	↑	↑	↑	↑
Systolic BP	↑	=	=	↓	↑	=	↓	↓
Diastolic BP	=	=	=	↓	↑	↓	↓	↓
Max response to Pe	↓	↓	=	↑	↓	=	↑	↑
pEC50 to Pe	↑	↑	↑	↑	↑	↑	=	=
Max response to ACh	↓	↓	↓	↓	↓	↓	↓	↓
pEC50 to ACh	=	=	↓	↓	=	↓	↓	=
Proportion of AA	↓	↓	↓	↓	↓	↓	↓	↓
Proportion of DHA	↓	↓	↓	↓	↓	↓	=	↓

Growth and energy intake:

The maternal diet fed had no effect on the litter size, nor the proportions of males and females. There was also no effect on weight gained during pregnancy. However, the energy intake in the dams was affected by diet at the high fat level; the SAO, FO and SFA diets caused an increase in energy intake for the 21% diet during lactation, this is supported by other

studies where high fat diet causes increased energy intake (294). This may be explained by the higher calorific content of the food. The HSO diet caused the opposite effect and resulted in a decrease in energy intake at the 21% level and also resulted in a lower energy intake compared to the other types of fat. HSO contains less metabolisable trans-fatty acid compared to the other fatty acid types and therefore results in a lower energy intake compared to the other groups. Intake of 21% HSO contains more HSO compared to the 7% HSO diet, as more of the diet consists of the low metabolisable HSO, it results in a lower energy intake. Dams fed the 21% diet demonstrated an increased energy intake during lactation compared to during pregnancy; this was seen in all diet types. This was not seen as clearly in the 7% data as the data was very variable with large standard error of the mean in all diet types. Increased energy intake during lactation occurs to meet the energy cost of producing milk; this has previously been observed in humans (295).

The offspring of the dams fed a 21% diet demonstrated an increase in food intake compared to the 7% diet during the juvenile-pubertal period. Hyperphagia has previously been observed in offspring from dams fed a high fat diet, albeit this was observed with obesogenic diet which had changes in the carbohydrate content. However, hyperphagia may be due to changes in the appetite-controlling pathway during development (296). It has been suggested that leptin may be responsible for these changes. During neonatal development leptin was found to cause neuronal outgrowth to the paraventricular nucleus and thus hard-wiring the hypothalamic appetite-regulatory system. Leptin affects two opposing pathways and it appears to favour the development of the appetite stimulatory NPY and agouti-related protein over appetite inhibitory containing neurones (297), thus leading to the hyperphagia observed.

Blood pressure observations:

The current study also shows that there were changes in the blood pressure of the offspring dependent on the sex and type of diet fed at the 7% fat quantity, this amount of fat is considered adequate. This is the first time that these observations have been made. Male offspring of dams fed a 7% HSO diet produced a raised blood pressure, but there was no difference in the offspring of other types of 7% diets. Increasing the maternal fat content to 21% caused a decrease in systolic and diastolic blood pressure in offspring of dams fed a 21% HSO diet compared to those of a 7% HSO diet. An increase in systolic blood pressure was also observed in offspring of dams fed a 21% SAO compared to offspring of a 7% SAO maternal diet. However, no changes in blood pressure were observed in offspring of dams fed a FO and SFA diet. In female offspring of 7% HSO, SFA and FO demonstrated a higher systolic and diastolic blood pressure compared to offspring of 7% SAO. Increasing the fat content of the maternal

diet to 21% fat caused decreases in systolic and diastolic blood pressure in offspring of dams fed HSO, SFA and FO. However, offspring of dams fed 21% SAO demonstrated an increase in systolic and diastolic blood pressure compared to offspring of dams fed a 7% SAO diet. Previous observations have also shown sex differences and that a high fat diet during pregnancy leads to a raised blood pressure in female offspring. However, the diet used in the study contained 25.7% fat which is higher than used in the current study (5).

As discussed, some changes to blood pressure were seen between the offspring of dams fed different amounts and types of fat. It has previously been demonstrated that trans fats increase the risk of CVD, as they are associated with endothelial dysfunction and increases in inflammatory markers, which could contribute to high blood pressure (123). The link between trans fat and endothelial dysfunction could provide an explanation for the higher blood pressure seen in male offspring of 7% HSO fed dams. However, increasing the quantity of trans fat to 21% in dams caused a decrease in blood pressure in male and female offspring, which was unexpected and the reason for this remains unclear. Like trans-fat, a maternal diet rich in saturated fat has previously been shown to lead to hypertension in mice offspring and would be expected to cause a raise in offspring blood pressure in this thesis (71). However, in male offspring, a 21% saturated fat maternal diet had no effect and in females, it led to a decrease in diastolic blood pressure. Both of these observations were unexpected and the reasons for these observations remain unclear. Previous studies have proposed that hypertension following a high saturated fat diet is due to endothelial dysfunction (71). As will be discussed, 21% SFA offspring demonstrated endothelial dysfunction but did not demonstrate higher blood pressure.

The current animals were exposed and primed to the test diet during in-utero development, but after weaning the offspring rats were placed on a standard chow diet, which produces a mismatch in diet exposure. According to the predictive adaptive response theory, the fetus makes adaptations based on the prenatal signals about the environment from the mother; this enables the fetus to prepare for postnatal life. These adaptations are postnatally advantageous if the prediction is correct and the postnatal nutrition matches the in-utero nutrition. If the predictions are incorrect and the postnatal nutrition does not match the in-utero nutrition, then the adaptations are detrimental and increase the risk of disease (298). Therefore, it was expected that the offspring of the 21% fat fed dams would show signs of cardiovascular disease, including hypertension (71) due to the mismatched diet. However, the only diet which appeared to have this affect was SAO, as a maternal diet of 21% caused an increase in blood pressure in males and females compared to 7% offspring. The 21% fat diet

creates a large mismatch between the standard chow diet which is a 7% balanced fat diet. This may be the reason for the increase in blood pressure in SAO offspring but it would have been expected in all dietary types and this did not happen. In contrast, offspring of HSO, SFA and FO fed dams demonstrated decreases in blood pressure in 21% fat female offspring compared to 7% offspring and the higher fat quantity had no effect on male offspring of SFA and FO fed dams. The increase in blood pressure observed in the offspring of 21% SAO fed dams may be due to changes in eicosanoids produced from the n-6 PUFAs, including pro-constrictor eicosanoids, such as TXA_2. A previous study has shown that a diet high in n-6 PUFAs causes an increase in TXA_2 production. The level of TXA_2 production was higher in n-6 fed people compared to those fed a high saturated fat diet (299). The increase in AA derived pro-constrictor eicosanoids, as a result of increased linoleic acid in the 21% diet provides a potential mechanism whereby a higher n-6 PUFA diet may lead to increased blood pressure in the offspring of 21% SAO fed dams compared to the offspring of 7% SAO dams. The 21% FO female offspring demonstrated a decrease in blood pressure compared to the 7% FO offspring. This could be expected as n-3 PUFAs are also found to have favourable effects on blood pressure. A study on females with metabolic syndrome showed that intake of fish oil displayed a reduced blood pressure. However, this was coupled with an increase in NO (153). The increase in NO was presumed to be responsible for the reduced blood pressure as it has a vasodilatory effect in the study, but in this thesis there was reduction in NO-mediated vasodilation in 21% offspring, this will be discussed later in this discussion, so increased NO production cannot be responsible for the changes in blood pressure observed in this thesis. Therefore, different types of fat were shown to produce different blood pressure responses, this demonstrates that the developing vascular system is clearly sensitive to the type of fatty acid it is exposed to and may be difficult to predict.

The blood pressure data does not provide conclusive information and does not follow what previous studies have shown. Potential reasons for the inconclusive data may be due to the method of measurement. The optimal method for measuring blood pressure in conscious and unrestrained rats is by using radiotelemetry. Tail cuff plethysmography is known to induce a rise in blood pressure due to the use of a restraint and also due to heating, this may have led to inaccurate data for the blood pressure measurements and may have masked greater differences between the groups (300). In addition to this, due to the scale of the study, the 7% and 21% aspects of the study were carried out at different times and this is a variable. It has been shown that seasonal variation may play an essential role in the metabolism and activity of laboratory animals. Therefore this is a limitation of this study. Had it been possible to carry

out the experiments at the same time, clearer blood pressure data may have been produced (301) .

Vascular function observations:

Vascular function analysis of the aorta showed that increasing the quantity of fat to 21% caused increased reactivity to the alpha-1 adrenergic receptor agonist Pe. In all male offspring, increasing the fat quantity caused an increase in sensitivity to Pe, demonstrated by an increase in pEC50, irrespective of diet type. In females, similar effects were seen, increasing the quantity of fat to 21% caused increased sensitivity in aorta of offspring of SAO and SFA and an increase in the contraction in FO and HSO, which as observed with the males demonstrates increased vasoconstriction. Changes to the vasoconstriction of the blood vessel causes abnormal vascular tone and can lead to disorders in blood pressure regulation, such as hypertension and ultimately CVD (26;302). A similar outcome of an enhanced constrictor response has been demonstrated previously in offspring from undernourished dams (7) and also in rat offspring of dams fed a high saturated fat diet (285). However, the results from our study extend these findings and show that increased vasoconstriction and increased sensitivity and vasoconstriction to agonists is seen in high fat diet irrespective of the fat type. The changes in vasoconstriction did not represent changes in blood pressure, so cannot be responsible for these changes. However, the data from this study shows aorta data and mesenteric data is more representative of peripheral resistance and thus determination of blood pressure (284).

In addition to changes in vasoconstriction, maternal diet also had an effect on the vasorelaxation of the offspring aorta. Endothelial dysfunction can be caused by poor diet, leading to a poor intrauterine environment. Many studies have shown that feeding a high saturated fat diet during pregnancy and lactation can cause endothelial dysfunction in offspring (71;189). Endothelial dysfunction is a common factor of CVD and is an important step in the development of atherosclerosis, (303). In this study endothelial dysfunction was observed in the offspring, this was dependent on type of fat, quantity of fat and gender of the offspring. In male offspring of dams fed the 7% fat diet, SFA caused a blunted maximum response to ACh compared to FO, this shows evidence that the endothelium is impaired in SFA. Responses to SNP were unaffected by diet confirming that changes in ACh response was not due to dysfunction in the smooth muscle cells. The response to ACh was abolished in the presence of L-NAME and thus demonstrates that the relaxation to ACh was solely due to endothelial NO release. Taken together the blunted response to ACh in SFA is due to a decrease in NO bioavailability and is due to endothelial dysfunction. Increasing the quantity of

fat to 21% caused a further blunted response in SFA and blunted response in all types of fat, but to a lesser extent in 21% HSO. The pEC50 was also reduced in FO and HSO, demonstrating a reduced sensitivity to ACh which is another hallmark of endothelial dysfunction.

Females also showed signs of endothelial dysfunction in those fed 7% HSO compared to SFA, this was shown by a decreased pEC50 and thus sensitivity to ACh. As seen with the male offspring increasing the fat quantity to 21%, caused blunting to the ACh response irrespective of diet type in females. FO and HSO experienced the greatest endothelial dysfunction with the greatest attenuation in response to a 21% fat maternal diet. Like the males there was no change in SNP response, so smooth muscle was not responsible for the blunted response and again the ACh response could be abolished with L-NAME, confirming the sole role of NO in the ACh response.

Implications of vascular function changes:

These findings show for the first time that consuming different types and amounts of fat during pregnancy and lactation induce differences in the regulation of vascular tone in the offspring, which resembled endothelial dysfunction. This was demonstrated with decreased sensitivity to ACh and decreased level of relaxation in response to ACh. The effects were different between sexes, type of diet and quantity of fat. The biggest effect was with quantity of fat, increasing maternal fat quantity to 21% caused large decreases in the maximum relaxation in all dietary groups irrespective of diet. However, some fats caused a greater attenuation of response compared to other types of fat, for example 21% HSO demonstrated less attenuation compared to 7% HSO than other fat types, such as that between 7% and 21% SAO in males. The SAO diet is enriched in n-6 PUFAs and has a high concentration of LA which is a potent activator of nuclear factor kappa light chain enhancer of activated B cells (NFkB). LA is a source of oxidative stress, diets enriched with LA lead to LA-enriched LDL particles which have been shown to be more susceptible to oxidation (304). NFkB is an oxidative stress sensitive transcription factor that plays a role in regulating the production of cell adhesion molecules and inflammatory cytokines and reduces the production of NO (305). In females 21% HSO caused the greatest attenuation of maximum relaxation. Trans fatty acids (306) and saturated fatty acids (117) have also been shown to activate NFκB, and cause attenuation of NO production and thus endothelial dysfunction. However, n-3 PUFAs are associated with lower levels of inflammation and endothelial activation (307) and therefore endothelial dysfunction wouldn't be expected in offspring of FO fed dams, however the level of fat, irrespective of the type of fat may outweigh the beneficial effects of n-3 on endothelial function. In addition to this, n-3 PUFAs are highly susceptible to oxidation and therefore could

increase lipid peroxidation of the endothelium and cause endothelial damage (308), as it has been shown that lipid peroxidation is linked to endothelial dysfunction (309). It has also been shown that n-3 fatty acids produce the greatest monocyte adhesion to the endothelium (310). This shows that there may be specificity in the response of the developing vasculature to certain fatty acids and certain fatty acids may have a more detrimental effect on endothelial dysfunction than others. However, the mechanism for this cannot be deduced from this study. Blood pressure and endothelial dysfunction did not correlate as expected. Endothelial dysfunction was seen in all offspring of dams fed a 21% diet but blood pressure was not raised in all groups as might be expected. This may suggest that endothelial dysfunction may precede more significant blood pressure changes and this may have been observed at an older age in these rats (311). In addition to this, the endothelial dysfunction is observed in aorta and therefore does not necessarily represent blood pressure as it is a conduit artery.

ACh-induced vasorelaxation in the aorta is dependent upon NO release. The use of the L-NAME to inhibit nitric oxide production and thus inhibit the vaso-relaxation of the aorta in response to ACh confirms the role of NO in endothelial dependent vaso-relaxation seen in the rat aorta. Previous studies have shown that dietary interventions during pregnancy in rats, such as protein restriction led to a reduction in NO activity and eNOS mRNA expression (312). This shows that regulation of NO biosynthesis is sensitive to early life environment. This is clearly demonstrated by the effect of the 21% fat maternal diet on the reduced vaso-relaxation to ACh. However, in this study maternal fat intake had no effect on eNOS mRNA expression (282) and therefore cannot explain the reduction in NO bioavailability. However, there may be changes to other enzymes within the NO pathway which could account for reduced NO bioavailability, however this was not investigated.

As demonstrated in this study, there was a reduction in NO dependent vasorelaxation to ACh, as ACh-induced vasorelaxation in the aorta is dependent primarily on NO (313), a blunted response to ACh suggests a reduction in NO availability. Therefore, the change in fatty acid composition may play a role in the production of NO and be responsible for the endothelial dysfunction observed in this study. The mechanism for changes in fatty acid composition reducing NO availability may be due to changes in the structure of caveolae. Changes to the fatty acid composition of the caveolae may change the interaction of eNOS with the membrane and result in a reduction of NO production. To deduce the exact mechanism for the change in fatty acid composition and the endothelial dysfunction more work needs to be done in the future.

Parts of the vascular function data shown in this thesis were carried out by two investigators. Although training was carried out to ensure consistency of the performance of experiments and the observations between the two investigators, there may still be inter-observer variability which could confound the comparisons between the dietary groups. The measurements for the 7% myography data and all blood pressure measurements were carried out by Dr Chris Kelsall. Analysis of all myography and blood pressure traces were carried out by the same person for all dietary groups to ensure consistency and to stop differences in the subjectivity of the analysis. Factoring the observer into the ANOVA for the vascular reactivity data did not have an effect on the significance of the data, this allows any uncertainty of the interpretation of the data due to inter-observer differences to be ruled out. As discussed, seasonal effects may have an impact on the data presented in this chapter. Due to time constraints, it was not possible to run the whole study at the same time. Therefore, the study had to be split into two parts; the 7% arm and the 21% arm. Mating and therefore birth was also staggered across all groups, this was to ensure that myography could be carried out on the vessels at the correct developmental time point, as myography could only be carried out on 2 animals per day due to time and equipment constraints.

Changes to membrane fatty acid composition:

Key changes in the membrane composition were observed within the offspring of dams fed different types and quantities of fat during pregnancy and lactation. The most consistent changes were in the amount of AA and DHA present in the membrane of the aorta. Increasing the quantity of fat in the diet to 21% caused decreases in the amount of AA and DHA present in the membrane, irrespective of the type of diet. This is the case for both male and female offspring. These findings are consistent with those seen in a previous study, which showed that feeding a maternal high saturated fat diet caused lower proportions of AA and DHA in rat aorta (8). However, this study extends the finding from Ghosh et al because we have shown that a high fat diet, irrespective of type of diet causes decreases in these key fatty acids. The decreases in the proportion of DHA in the aorta also provide further explanation for the endothelial dysfunction observed in the 21% fat fed offspring. A previous study has shown that DHA displaces caveolin-1 from the caveolae, which is responsible for securing eNOS and thus resulting in increased eNOS activity. Therefore, a reduction in DHA may cause a reduction in eNOS activity and cause endothelial dysfunction as observed in all offspring of 21% fat fed dams (314).

Vascular function analysis of the aorta showed that maternal diet had an effect on the offspring vasoconstriction. The data showed that increasing the quantity of fat to 21% caused

increased reactivity to the alpha-1 adrenergic receptor agonist Pe. In all male offspring, increasing the fat quantity caused an increase in sensitivity to Pe, demonstrated by an increase in pEC50, this observation happened irrespective of type of diet. In female offspring, similar effects were seen, increasing the quantity of fat to 21% caused increased sensitivity in aorta of offspring of SAO and SFA fed dams and an increase in the contraction in FO and HSO, which as observed with the males, demonstrates increased vasoconstriction.

As previously discussed, increases in fat content of the maternal diet lead to increased vasoconstriction and sensitivity to Pe, which does not correlate with the changes in AA content. Many pro-constrictor eicosanoids are produced from AA, these include PGE_2, PGF_{2a} and TXA_2. These eicosanoids are synthesised *de-novo* from AA released from phospholipids in the cell membrane. Eicosanoids are not stored but synthesised when cells are activated by medical trauma, cytokines or growth factors (29). Upon stimulation, AA is released from the membrane by $cPLA_2$ (230). With a lower proportion of AA present in the membrane of the aorta it would be expected that a decrease in constriction would be observed due to a decrease in synthesis of pro-constrictor eicosanoids, however the opposite was seen.

Analysis of the offspring plasma TAG and NEFA fractions showed evidence that the maternal diet modified the offspring plasma composition, this was apparent by higher levels of LA (18:2n-6) in the 7% SAO fed offspring compared to the offspring of the other dietary groups, this would be expected as SAO is enriched in LA. The amount of LA was also shown to be higher in the 21% SAO group compared to the 7% SAO group. Other n-6 fatty acids were also increased in the offspring of 21% SAO compared to the 7% SAO, such as, 20:3n-6. In addition to this, higher levels of n-3 fatty acids were seen in offspring of FO fed dams. Also, the levels of n-3 were higher in offspring of 21% FO fed dams compared to the offspring of 7% FO fed dams, which would be expected with greater enrichment of the maternal diet with n-3 fatty acids. Other changes were observed in all dietary groups such as increases in certain fatty acids in the offspring of 21% dams compared to 7% dams which is due to higher enrichment of those fatty acids in the maternal diets. However, no trans fats were detected in the plasma or the aorta, even in the offspring of dams fed the HSO enriched diet, it is not known what the reason for this observation is, the lack of persistence of trans fats is not explored in this thesis.

The differences observed in the proportions of AA and DHA in the offspring aorta were not due to the persistence of the fatty acids derived from the maternal diet. Firstly, AA and DHA fatty acid composition of the TAG and NEFA fractions did not reflect the fatty acid composition of the aorta. Although, the male and female TAG fatty acid proportions reduced as quantity of fat increased, the same did not happen for the AA proportions of the TAG

fraction. There was no difference in AA and DHA content of the vessels between offspring of different dietary groups for the NEFA fraction. As the TAG and NEFA fractions are the major fatty acid transport pools that supply fatty acids to blood vessels, it would be expected that changes in the aorta would reflect changes in these pools if it was due to what was provided to the aorta. Therefore, this suggests that difference in the fatty acid composition of the aorta in the offspring were not due to persistence of fatty acids derived from the maternal diet and must reflect changes in the regulation of fatty acids synthesis that supplies the aorta with fatty acids.

Membrane composition and vascular function:

Synthesis of AA and DHA happens via a number of desaturation and elongation reactions which take place in the endoplasmic reticulum. Delta-5 and Delta-6 desaturase are the enzymes responsible for the main regulation of the n-6 and n-3 PUFA pathway, they are encoded for by the genes FADS1 and FADS2 respectively (109). Synthesis of PUFA has been shown to take place in isolated endothelial cells (262) and smooth muscle cells (223), but the extent and function of the pathway has not been investigated in VSMCs. In addition to vascular function and composition studies, gene expression analysis was carried out on samples from this study. It was observed that offspring of dams fed 21% fat diets had higher expression of fads1 and lower expression of fads2 in the aorta compared to offspring of dams fed the 7% fat diets, irrespective of the type of fat in the diet (282). This demonstrated dysregulation of the synthesis of AA and DHA. The increase in Fads1 expression is consistent with increases in vasoconstriction and increased sensitivity, as observed in response to Pe; this is because it would result in an increase in AA production and thus greater production of pro-constrictor eicosanoids. However, the down-regulation of fads2 does not support this and therefore suggests dysregulation of the pathway. These data however may suggest that synthesis of AA is involved in vasoconstriction. The reduction in AA observed in the aorta of the offspring of dams fed 21% may be due to it being channelled into eicosanoid synthesis rather than being incorporated into the cell membrane. Even a maternal diet with a high LA content such as the 21% SAO, which would be expected to provide a higher AA content, would result in a decrease in the proportion of AA in the aorta. This is because the expression of fads1 is higher in the 21% offspring compared to the 7% offspring. This would result in an increased ability to produce newly synthesised AA in the PUFA biosynthesis pathway in the offspring of dams fed 21% fat compared to the 7% offspring. If newly synthesised AA is channelled into eicosanoid production, more newly synthesised AA produced in 21% offspring would be released from the membrane and used for the production of eicosanoids, rather than being stored in the

membrane of the aorta. Therefore, this would result in a decrease in the proportion of AA in the aorta of offspring of 21% fat fed dams compared to 7% offspring, as observed. The higher quantity of fat would result in a higher production of newly synthesised AA and an increase in pro-constrictor eicosanoids and thus greater vasoconstriction. If linked to Pe stimulation, it would result in a greater AA production at a lower concentration of Pe, as demonstrated by the increased sensitivity, shown by an increased pEC50. Measuring eicosanoid production from the aorta vessels of offspring rats would have allowed investigation of whether a lower proportion of AA in the aorta is due to the increased production of pro-constrictor eicosanoids. This would also explain the increased vasoconstriction observed. Unfortunately, one limitation of this study is that eicosanoid production was not measured in the aorta from the rat offspring as the fatty acid composition was not measured until the end of the study. In addition to this, the observations made from the myography data were also not made until all the myography experiments had been completed, so it was too late to measure eicosanoid production from these vessels.

PUFA synthesis and Pe-mediated vasoconstriction:

Further experiments were carried out to investigate the effect of the PUFA biosynthesis pathway on vasoconstriction. These experiments demonstrated that inhibition of and delta-6 desaturase caused a reduction in vasoconstriction in aorta treated with Pe in both rat aorta and in human femoral artery. Removal of the endothelium from the rat aorta did not alter the effect of the delta-5 or delta-6 inhibitor on the Pe-mediated vasoconstriction. This suggests that PUFA synthesis *de-novo* plays a role in α1-adrenergic receptor mediated vasoconstriction and is localised to the VSMCs. As the data was consistent between the rat aorta and the human femoral artery, it suggests that the same mechanism for *de-novo* PUFA synthesis and α1-adrenergic receptor mediated vasoconstriction is present in both species. This demonstrates the rat model is representative of human physiology. Therefore, the findings from this chapter and the investigation into the mechanism for this observation in chapters 4 and 5 can therefore be extended into humans. In addition to this the levels of pro-constrictor eicosanoids were measured in aorta treated with the delta-6 desaturase inhibitor. In the presence of the inhibitors there was a decrease in the synthesis of PGE_2, PGF_{2a} and TXA_2 (282). This therefore supports the role of *de-novo* synthesis of PUFAs in vasoconstriction.

In conclusion, this study has shown that both amount and type of maternal diet cause some changes to vascular tone in the offspring, with amount of fat having the most significant effect. These changes include increased vasoconstriction, increased sensitivity to α1-adrenergic receptor stimulation and changes to vasorelaxation which are indicative of

endothelial dysfunction. Changes to the maternal diet also results in altered fatty acid composition of the aorta which is not purely due to the persistence of the maternal diet and is a result of altered PUFA biosynthesis (figure 3.15). The changes to the PUFA biosynthesis of the aorta may play a role in α_1-adrenergic receptor mediated vasoconstriction in both rat and human blood vessels. The remaining sections of this thesis describe the characteristics of the role of PUFA biosynthesis in Pe-mediated vasoconstriction.

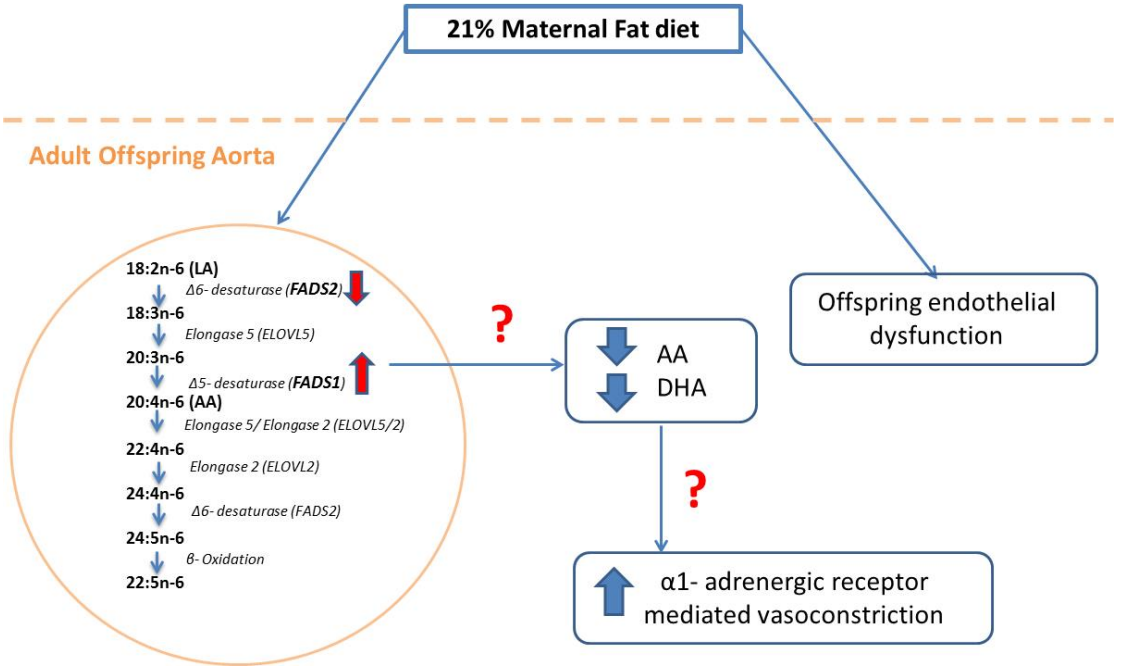


Figure 3.15: Potential pathway linking maternal fat to changes in offspring vasoconstriction, showing dysregulation in PUFA biosynthesis in offspring aorta to increased a1-adrenergic receptor mediated vasoconstriction. LA: linoleic acid, AA: arachidonic acid, DHA: docosahexanoic acid.

Chapter 4-

Synthesis of polyunsaturated fatty acids *de-novo* in vascular smooth muscle cells

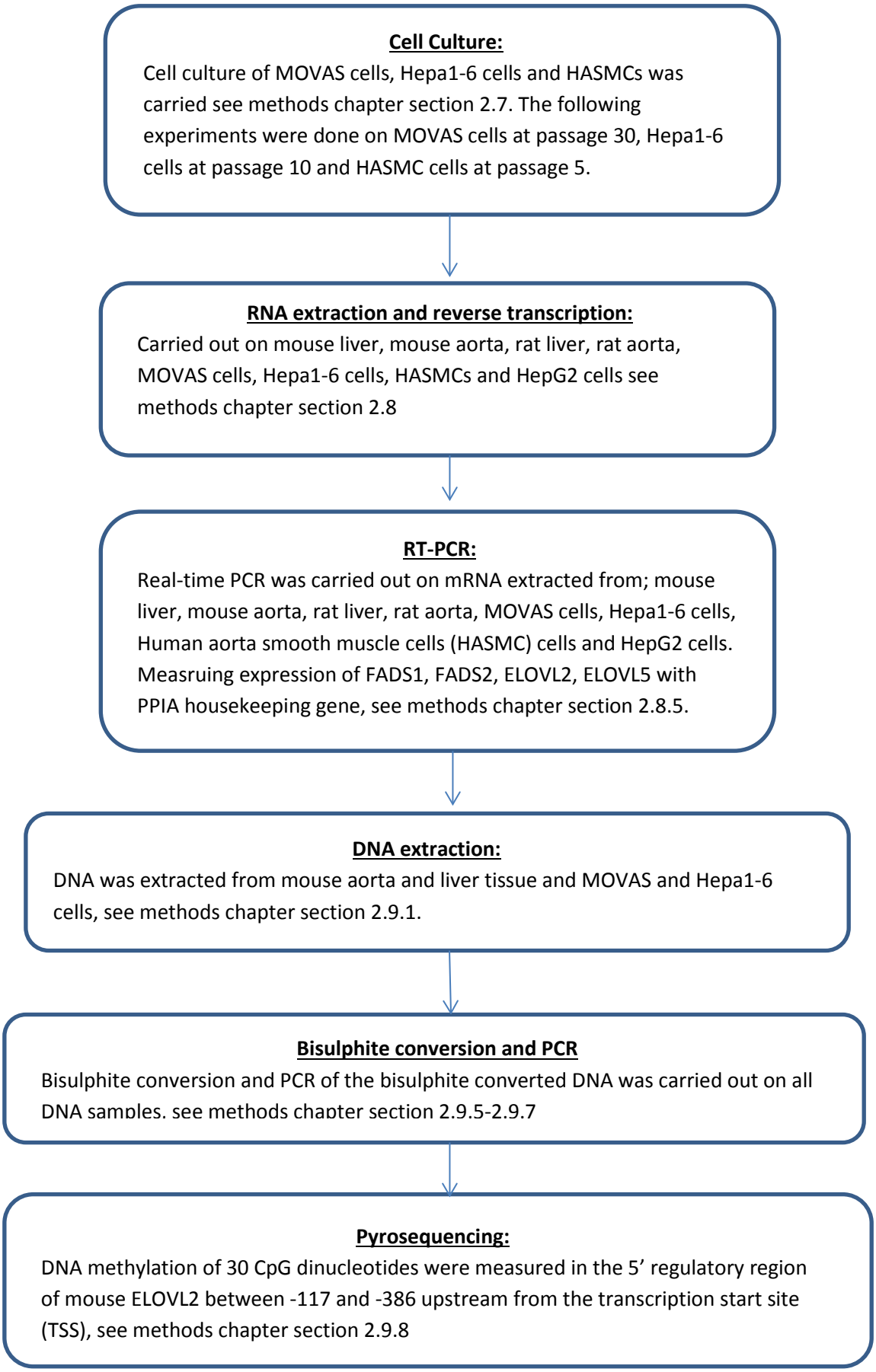
4.1 Introduction:

In mammals, the synthesis of long chain PUFAs involves the conversion of the essential fatty acids, LA and ALA to longer chain n-6 PUFAs and n-3 PUFAs respectively, through a series of desaturation and elongation reactions (109). Delta-5 and delta-6 desaturase are the enzymes responsible for the main regulation of the n-6 and n-3 PUFA pathway, with delta-6 desaturase as the rate determining step and the Elovl5 and Elovl2 involved in elongation reactions in the pathway (227). The data from Chapter 3 showed that PUFA biosynthesis appears to be involved in α1-adrenergic receptor-mediated vasoconstriction of aortae. PUFA biosynthesis has been demonstrated previously in arterial endothelial cells (263) (262), however, very little is known about the activity of this pathway in vascular smooth muscle. A previous study has demonstrated the activity of delta-6 desaturase in human coronary artery smooth muscle cells, by using the delta-6 desaturase inhibitor SC-26196 to inhibit desaturation of 18 and 24 carbon PUFA (223). However, there has been no direct evidence of PUFA biosynthesis *de novo* in VSMCs and the full extent of the pathway has also never been demonstrated. Therefore, to establish the role of PUFA biosynthesis in vasoconstriction of vascular smooth muscle the activity of the PUFA biosynthesis pathway in these cells needs to be determined.

Chapter hypothesis: VSMC are capable of PUFA biosynthesis *de-novo.*

In order to characterise the PUFA biosynthesis pathway in vascular smooth muscle and address this hypothesis, the expression of the key genes involved in the pathway; Fads1, Fads2, Elovl2 and Elovl5 were measured in aorta and VSMCs. The activity of the biosynthesis pathway was measured by treating VSMCs with linoleic acid (LA) or α-linolenic acid (ALA) and measuring membrane composition or by direct measure of synthesis using [U-^{13}C] LA. As it is hypothesised that Pe-stimulation may lead to the channelling of AA into eicosanoids, the effect of Pe stimulation on the PUFA biosynthesis pathway was assessed. If Pe stimulation causes the release of AA for eicosanoid production, it would be expected that a decrease in the proportion of AA present in the membrane of VSMCs would decrease.

4.2 Methods:


Cell Culture:
Cell culture of MOVAS cells, Hepa1-6 cells and HASMCs was carried see methods chapter section 2.7. The following experiments were done on MOVAS cells at passage 30, Hepa1-6 cells at passage 10 and HASMC cells at passage 5.
RNA extraction and reverse transcription:
Carried out on mouse liver, mouse aorta, rat liver, rat aorta, MOVAS cells, Hepa1-6 cells, HASMCs and HepG2 cells see methods chapter section 2.8
RT-PCR:
Real-time PCR was carried out on mRNA extracted from; mouse liver, mouse aorta, rat liver, rat aorta, MOVAS cells, Hepa1-6 cells, Human aorta smooth muscle cells (HASMC) cells and HepG2 cells. Measruing expression of FADS1, FADS2, ELOVL2, ELOVL5 with PPIA housekeeping gene, see methods chapter section 2.8.5.
DNA extraction:
DNA was extracted from mouse aorta and liver tissue and MOVAS and Hepa1-6 cells, see methods chapter section 2.9.1.
Bisulphite conversion and PCR
Bisulphite conversion and PCR of the bisulphite converted DNA was carried out on all DNA samples, see methods chapter section 2.9.5-2.9.7
Pyrosequencing:
DNA methylation of 30 CpG dinucleotides were measured in the 5' regulatory region of mouse ELOVL2 between -117 and -386 upstream from the transcription start site (TSS), see methods chapter section 2.9.8

Cell treatment with LA and ALA:

The activity of the n-3 and n-6 PUFA biosynthesis pathway was determined by treating MOVAS and Hepa1-6 cells with LA and ALA, see methods chapter section 2.11.1

N-6 PUFA Pathway analysis using [U-^{13}C]-LA

The conversion of [U-^{13}C]-LA to its longer chain metabolites was measured in MOVAS and Hepa1-6 cells to confirm pathway activity. This was also carried out following stimulation of Pe in MOVAS cells to investigate the effect of α1-adrenergic receptor-mediated stimulation by Pe on the activity of the PUFA pathway in VSMCS, see methods chapter section 2.11.3

4.3 Results:

4.3.1 Gene expression:

Fads1, Fads2, Elovl2 and Elovl5 expression were measured in tissues from three species; mouse, rat and human. These genes encode the four enzymes involved in PUFA synthesis. The data from this study confirms that all genes are present in both liver tissue and cells in all species as shown in fig 4.1. Fads1, Fads2 and Elovl5 were detected in mouse and rat aortae and human (HASMC) and mouse vascular smooth muscle (MOVAS). However, Elovl2 was not detected in aortae or VSMCs from any of the species tested. In the rat liver, the expression of Fads1 ($P<0.0001$), Fads2 ($P<0.0001$) and Elovl5 ($P=0.0184$) were all significantly greater than the expression in the aorta. In mouse tissue, Fads1 ($P=0.9942$) and Fads2 ($P=0.09948$) expression is not significantly different between liver and aorta. However, Elovl5 has a significantly greater expression in mouse liver than mouse aorta ($P<0.0001$). There were no significant differences in expression of Fads1, Fads2 or Elovl5 in MOVAS cells.

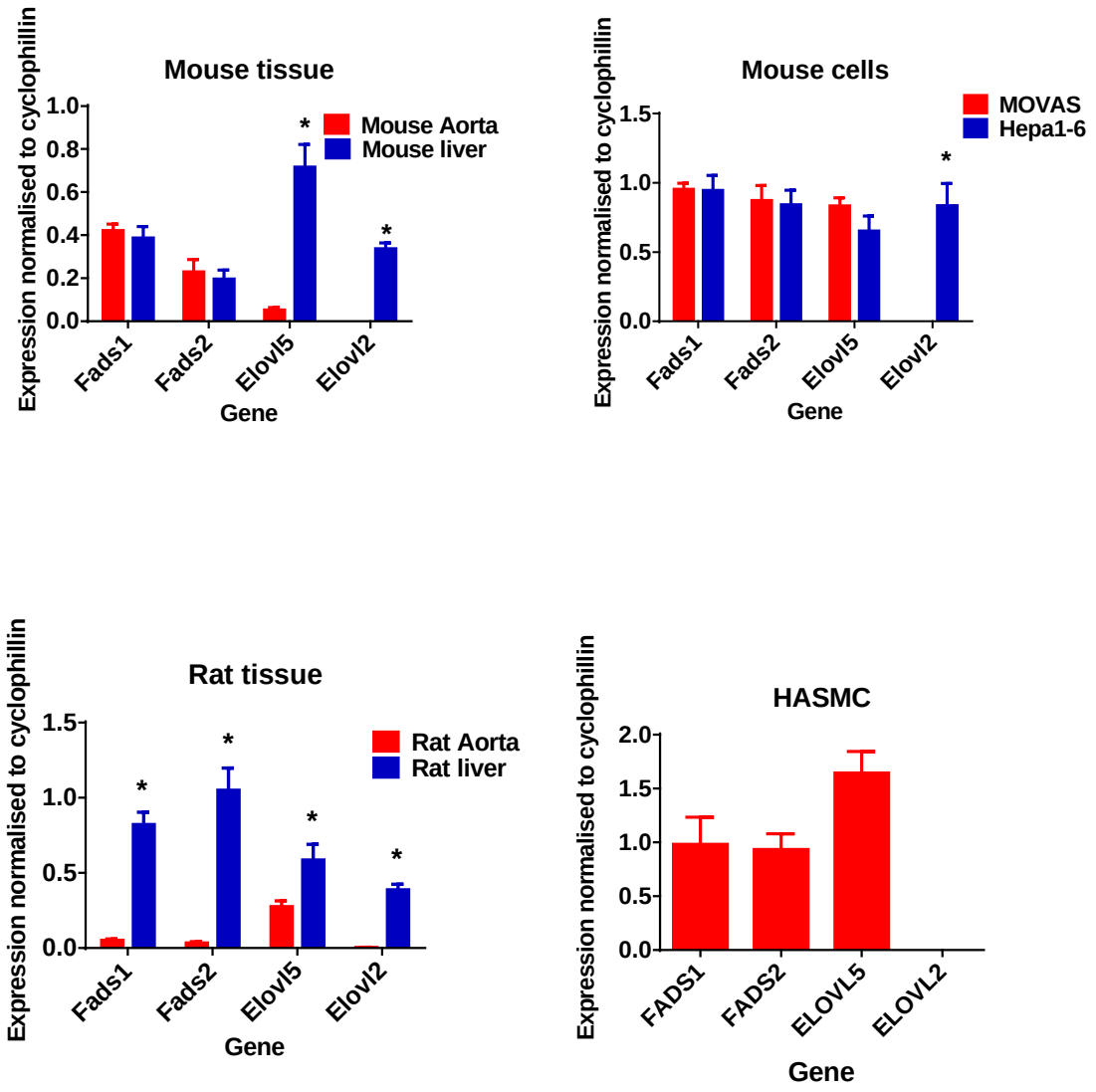


Figure 4.1: Gene expression of Fads1, Fads2, Elovl2 and Elovl5 in: Top left: mouse aorta and liver. Top right: MOVAS cells and Hepa1-6 cells. Bottom left: Rat aorta, rat liver. Bottom right: HASMC cells. Values are mean expression normalised to cyclophillin ± SEM (n=6/group). Statistical comparisons were ANOVA with Bonferroni post hoc test. Values significantly different (P<0.05) between tissue/cell type are indicated by *.

4.3.2 Elovl2 promoter methylation analysis:

In order to determine whether increased DNA methylation could explain the absence of Elovl2 expression in arterial samples, the methylation status of individual CpG loci was measured in the 5' regulatory region of the Elovl2 gene in mouse aorta tissue and in MOVAS cells. Liver was used as a positive control. The methylation status of the region that was measured was relatively low in aorta tissue (Fig. 4.2), with nearly all CpG loci less than 15% methylated. Only three CpG loci differed significantly between liver and aorta. These were located at -346, -337

and -313 base pairs relative to the transcription start site (TSS). Methylation of these CpG loci was significantly higher in the aorta than in the liver; -346 (P=0.004), -337 (P=0.006) and -313 (P=<0.0001).

More CpG loci differed significantly between MOVAS and Hepa1-6 (Fig 4.2) than between aorta and liver. Comparing MOVAS and Hepa1-6 cells show that Hepa1-6 methylation is significantly higher (P=<0.0001) than the MOVAS at CpG -368 to -212. Methylation then drops at -212 for both cell types, which is where the CpG island begins. Methylation remains low in both cell types until -135 where MOVAS methylation increases significantly compared to the HEPA1-6, with MOVAS having significantly higher (P=<0.0001) methylation at all 6 remaining CpGs -135 to -117. There was no consistency in patterns of methylation in aorta and liver compared with MOVAS and Hepa1-6. Although there were sites where aorta methylation was higher than liver methylation, these same differences were not seen when comparing MOVAS to Hepa1-6 cells.

Tissue vs. Cells

Comparing corresponding tissue and cells, shows that cells are more highly methylated than tissue samples (shown in fig 4.3). In Liver cells, all CpG's between -368 to -239 are very highly methylated (P=<0.0001) compared to liver tissue. Methylation then drops at -212 but Hepa1-6 cells still remains significantly more highly methylated than liver tissue, for example -184 (P=0.0005) and -195 (P=<0.0001). However, closer to the TSS, there is no significant difference between cells and tissue, at CpG site -135 (P=0.947).

As expression of Elovl2 is silenced in both aorta tissue and cells, it is important to see if the methylation patterns are consistent to confirm whether the silenced Elovl2 expression is due to methylation changes. The methylation states of the vascular tissue and cell lines is not comparable, the MOVAS cells are more highly methylated than the aorta tissue. The highest methylation in cells compared to tissue is seen between -346 to -239 (P=<0.0001). There is no significant difference between -212 to -155, for example CpG -184 (P=0.1878). Methylation then increases closer to the TSS and is significantly greater than MOVAS cells at all measured CpG's between -135 to -117 (P=<0.0001).

A high level of methylation in aorta tissue and cells compared to liver would be expected, in order to explain the lack of expression of Elovl2 in the aorta. However, the difference in methylation patterns between aorta and liver were not consistent in tissue and cells and therefore cannot be used to explain the suppression of Elovl2 expression in aorta.

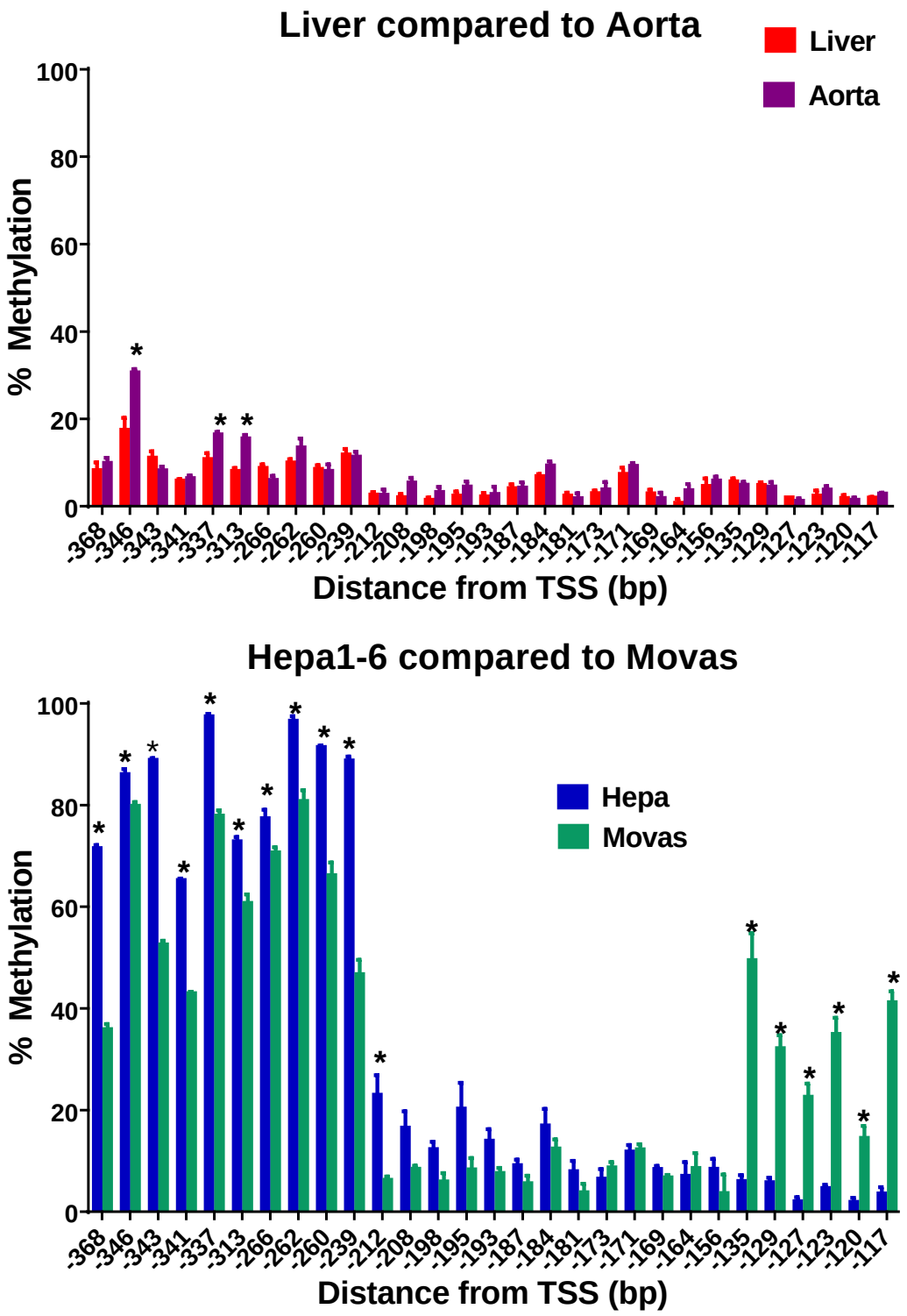


Figure 4.2: Mouse Elovl2 methylation. Top: Liver tissue compared to Aorta tissue. Bottom: Hepa1-6 cells vs MOVAS cells. Data shown as mean ± SEM (n=6/group). CpG island -212 to +284 (Distance from transcription start site). Statistical significance (P=<0.05) between sample type for each CpG were by T test and shown by *.

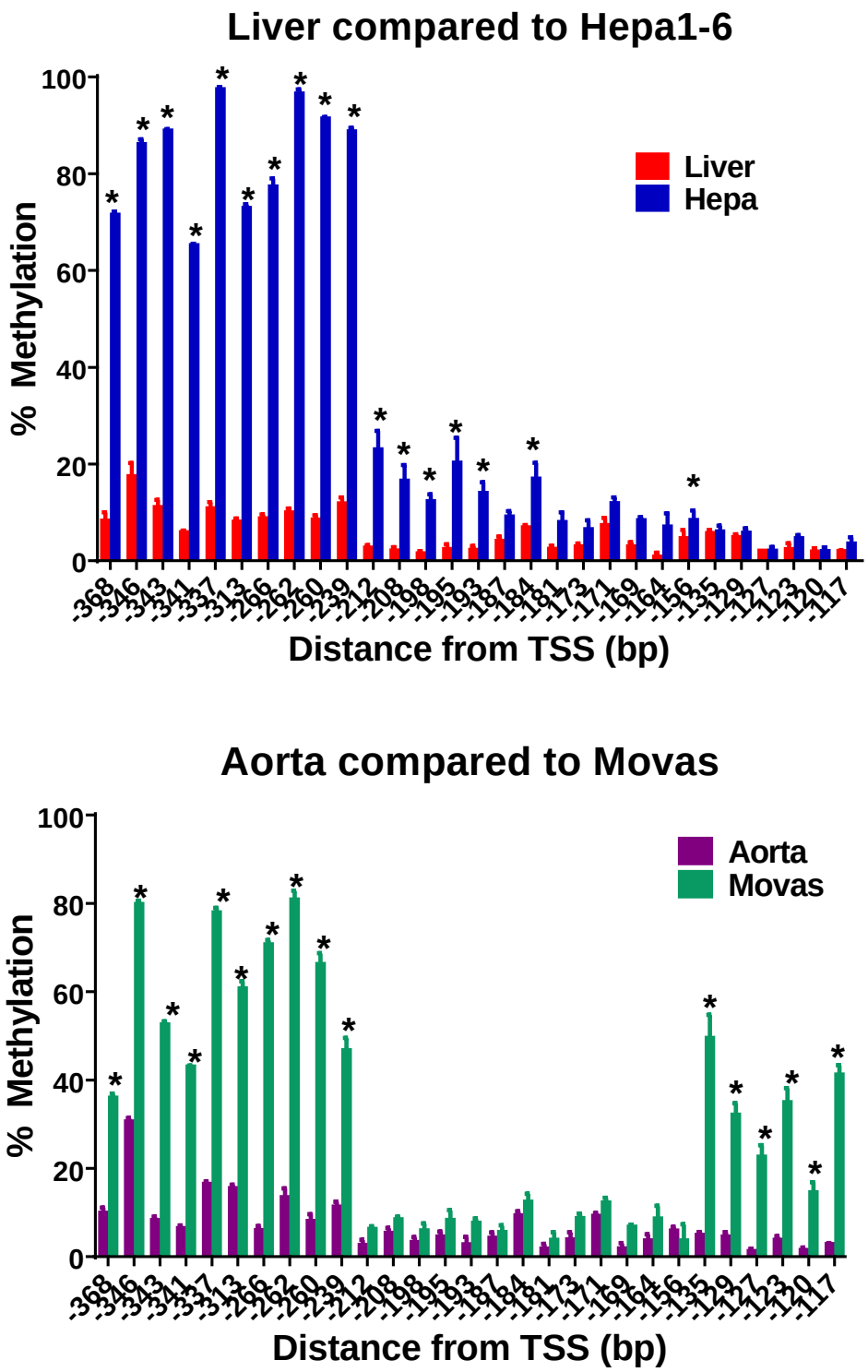


Figure 4.3: Mouse Elovl2 methylation. Top: Liver tissue compared to Liver cells. Bottom: Aorta tissue vs MOVAS cells. Data shown as mean ± SEM (n=6/group). CpG island -212 to +284 (Distance from transcription start site). Statistical significance (P=<0.05) between sample type for each CpG were by T test and shown by *.

4.3.3 N-6 PUFA synthesis pathway analysis:

Liver cells treated with LA confirm that the pathway does function in liver cells (shown in fig 4.4). The proportion of LA (18:2n-6) was significantly higher (P=<0.0001) in the LA treated cells (+LA) than in the control cells which were not treated with LA, confirming that the LA was taken up into the cells. The proportions of 18:3n-6, 20:3n-6, AA (20:4n-6) and 22:4n-6 were significantly higher (P<0.0001) in Hepa1-6 cells that were treated with LA than in untreated cells. However, there was no significant difference (P=0.6348) in the proportion of 22:5n-6 between those treated with LA and untreated cells.

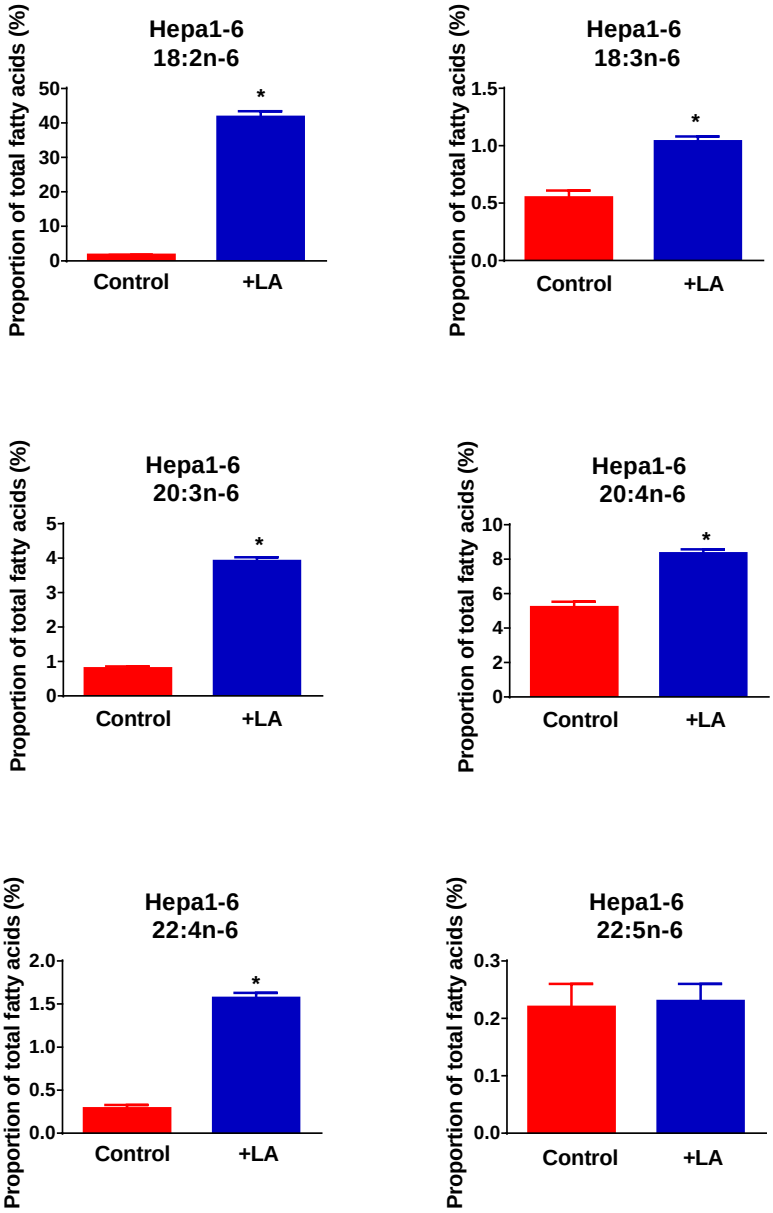


Figure 4.4. Proportion of n-6 PUFA's in Hepa1-6 cells following treatment with LA. +LA: LA treated. Values are mean ± SEM (n=6/group). Statistical comparisons were by T test. Values significantly different (P<0.05) between treated cells compared to control cells is indicated by *.

In VSMCs, like the liver, the proportion of LA (18:2n-6) in cells treated with LA (+LA) was significantly greater (P=<0.0001) than untreated cells (Fig 4.5). The proportions of 18:3n-6, 20:3n-6, AA (20:4n-6) and 22:4n-6 were significantly higher (P<0.0001) in MOVAS cells that were treated with LA than in untreated cells. However, there was no significant difference (P=0.8426) in the proportion of 22:5n-6 between those treated with LA and untreated cells. The data also shows that AA is the most abundant fatty acid in the pathway compared to the proportions of other downstream fatty acids.

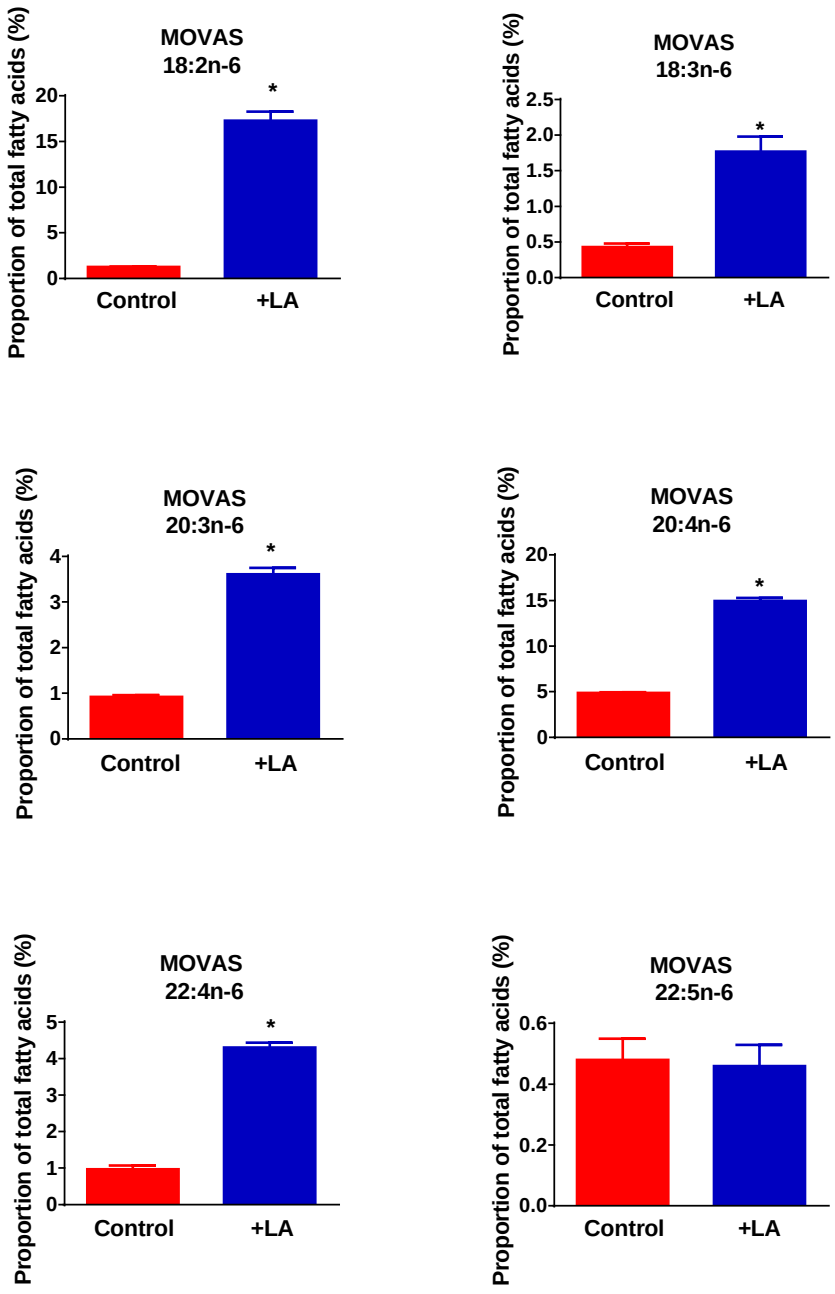


Figure 4.5: Proportion of n-6 PUFA's in MOVAS cells following treatment with LA. +LA: LA treated. Values are mean ± SEM (n=6/group). Statistical comparisons were by T test. Values significantly different (P<0.05) between treated cells compared to control cells is indicated by *.

4.3.4 Analysis of n-3 PUFA synthesis:

To further assess the function of the PUFA synthesis pathway in vascular smooth muscle cells, liver (Hepa1-6) and aorta vascular smooth muscle cells (MOVAS) were treated with ALA and the proportion of each n-3 fatty acid in the pathway was measured. Again, liver cells were used as a positive control as the pathway is known to function in these cells.

Liver cells treated with ALA confirmed that the n-3 pathway does function in liver cells (shown in fig 4.6). The proportion of ALA (18:3n-3) was significantly higher (P=<0.0001) in the treated cells (ALA) than in the control cells, this confirmed that the ALA was taken up into the cells. The proportions of 20:4n-3, 20:5n-3 and 22:5n-3 were significantly higher (P<0.0001) in Hepa1-6 cells that were treated with ALA than in untreated cells. However, the proportion of 22:6n-3 significantly reduced (P=<0.0001) compared to untreated cells.

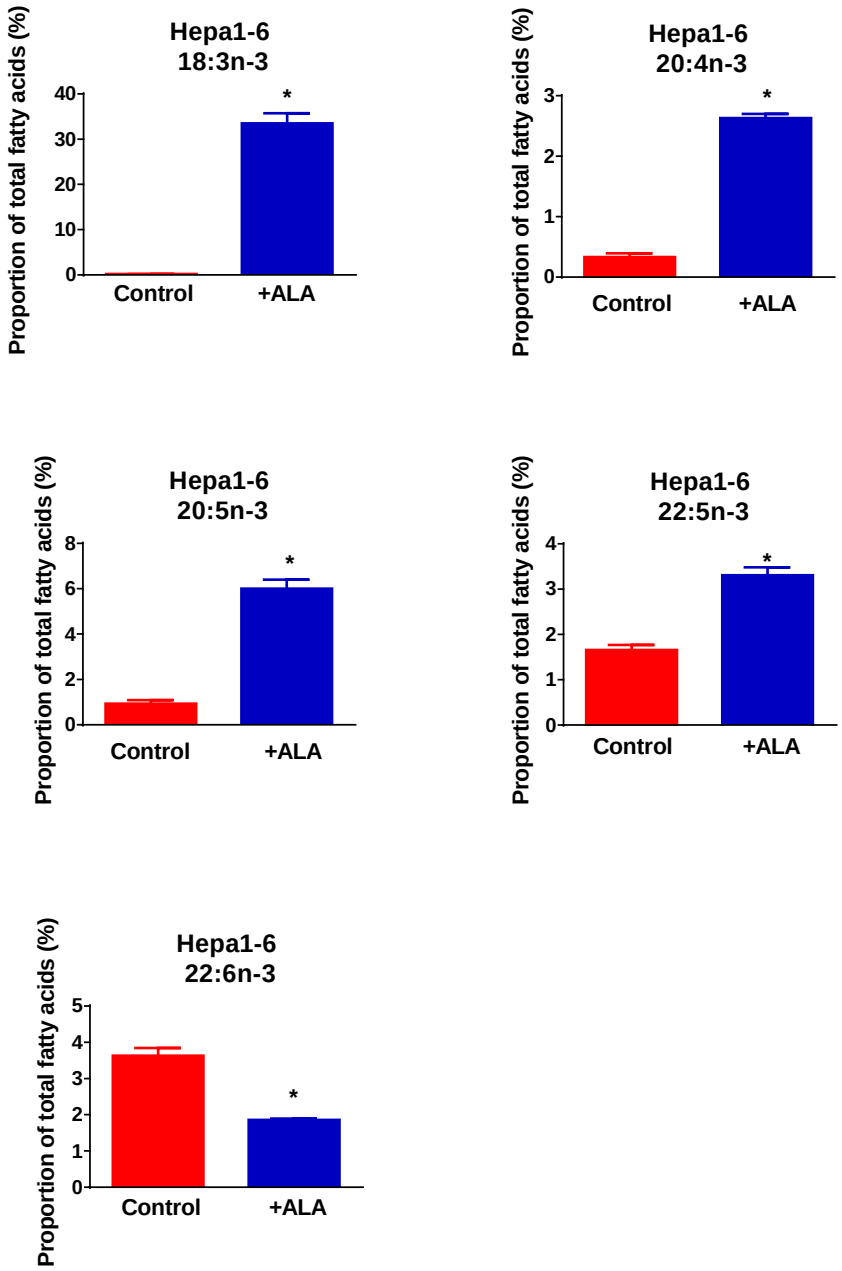


Figure 4.6: Proportion of n-3 PUFA's in Hepa1-6 cells following treatment with ALA. +ALA: linolenic acid treated. Values are mean ± SEM (n=6/group). Statistical comparisons were by T test. Values significantly different (P<0.05) between treated cells compared to control cells is indicated by *.

In VSMCs, like the liver cells, the proportion of ALA (18:3n-3) in cells treated with ALA was significantly greater (P=<0.0001) than control cells (shown in fig 4.7). This confirms the incorporation of ALA in the vascular smooth muscle cells. The proportions of 20:4n-3, 20:5n-3 and 22:5n-3 were significantly higher (P<0.0001) in VSMCs that were treated with ALA than in

untreated cells. However, as observed with the Hepa1-6 cells, the proportion of 22:6n-3 significantly reduces (P=<0.0001) in MOVAS cells compared to untreated cells.

As seen with the LA treated VSMCs, one specific fatty acid is present in a greater proportion (P=<0.0001) compared to other fatty acids in the n-3 pathway, excluding 18:3n-3. EPA (20:5n-3) is six-fold greater than 20:4n-3.

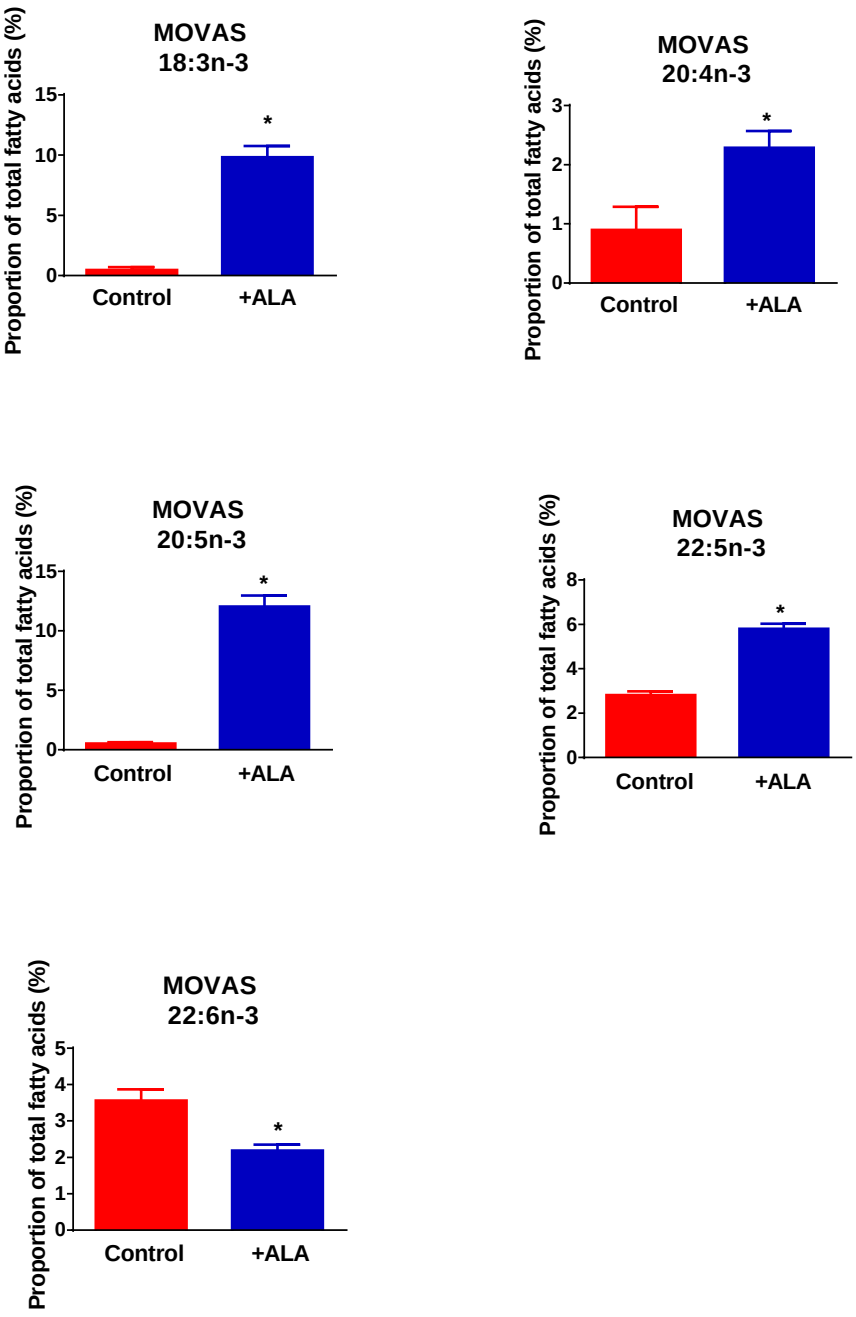


Figure 4.7: Proportion of n-3 PUFA's in MOVAS cells following treatment with ALA. +ALA: Linoleic acid treated. Values are mean ± SEM (n=6/group). Statistical comparisons were by T test. Values significantly different (P<0.05) between treated cells compared to control cells is indicated by *.

4.3.5 Determination of newly synthesised N-6 PUFA using [U-^{13}C] LA:

In order to measure the activity of the PUFA synthesis pathway directly, MOVAS and Hepa1-6 cells were incubated with [U-^{13}C] LA. In both MOVAS and Hepa1-6 cells there was enrichment throughout the pathway, except 22:5n-6 where the level of enrichment was below the level of detection (Fig 4.8). There was no significant difference between the enrichment of the Hepa1-6 cells compared to the MOVAS cells. However, as seen in the unlabelled LA treatment in section 4.3.3, the amount of enrichment in MOVAS cells for AA (20:4n-6) was greatest compared to the other fatty acids, (excluding LA), although this is not significant (22:4n-6, P=0.2329 and 20:3n-6, P=0.2238). The enrichment of AA (20:4n-6) shows a near significant trend towards being greater than that seen in the liver (P=0.0576). This pattern is also observed for the enrichment of 22:4n-6 which shows a trend towards being greater in the MOVAS cells than the liver cells (P=0.0553).

To investigate whether Pe had an effect on the activity of the n-6 PUFA synthesis pathway, cells were treated with Pe. However, there is no significant effect (P>0.05) seen between MOVAS cells treated with Pe and those treated with LA alone. The sample size for this experiment was lower than the post-hoc power calculations suggest, as n=33 are required. Therefore, if a greater sample size was used these data may be significant.

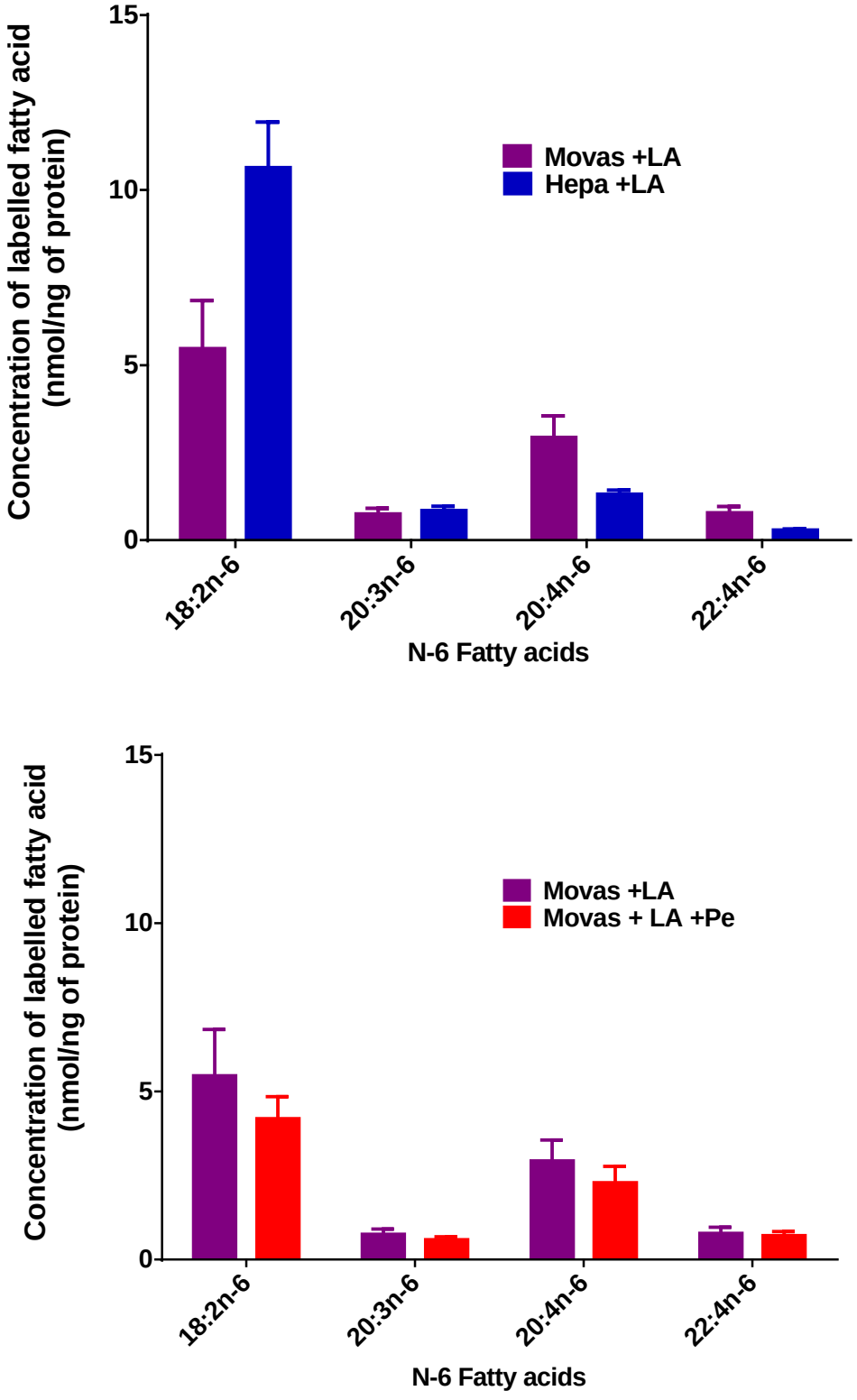


Figure 4.8: Enrichment of MOVAS and Hepa1-6 cells with [U-^{13}C] LA. Top: MOVAS vs Hepa1-6. Bottom: MOVAS LA vs MOVAS +LA+Pe. +LA+Pe: linoleic acid and phenylephrine treated. Values are mean ± SEM (n=6/group). Statistical comparisons were by T test. Statistical significance (P<0.05).

4.4 Discussion:

The findings from these data, for the first time, provide direct evidence for the activity of the PUFA biosynthesis pathway in VSMCs and the extent of the pathway in these cells. It is shown that the PUFA biosynthesis pathway is constrained by the absence of Elovl2 and therefore these cells may not be capable of producing all fatty acids in this pathway.

PUFA biosynthesis pathway activity:

Assessment of the expression of the genes present in the PUFA synthesis pathway showed that in rat aorta and mouse aorta there was expression of Fads1, Fads2 and Elovl5. This was also the case in the human and mouse VSMCs. As the mouse and rat aorta tissue also includes other cells such as endothelial cells. Using the MOVAS cells, which are mouse VSMCs, allowed gene expression to be specifically assessed in vascular smooth muscle and were consistent with that seen in the whole tissue. Although Fads1, Fads2 and Elovl5 were all expressed in both aorta tissue and VSMCs, there was no expression of Elovl2 in any of these samples. Liver tissue and cells were used as a positive control as expression of all the genes involved in the pathway has been shown previously (220), (221), (225). The data from the present study are consistent with previous studies and shows that all genes involved in the pathway were present. In rat tissue the expression of the genes involved in the pathway was shown to be greater in the liver than in the aorta, this is consistent with previous studies that have shown the activity of the pathway is greatest in the liver (220;221) as it is main site of PUFA biosynthesis in the body and required to supply PUFAs to other organs in the body. It can be concluded from the data that Elovl2 is not expressed in the PUFA synthesis pathway in the VSMCs. Elovl2 has been shown to be involved in the conversion of 22:4n-6 to 24:4n-6 and 22:5n-3 to 24:5n-3 (227). So, the absence of Elovl2 expression would suggest that the n-6 and n-3 PUFA synthesis may not happen beyond this step in VSMCs. The consistency between species in the absence of Elovl2 means that this constraint happens in all species and mouse cells can be used as a reliable model for assessing the function of the pathway.

Functional analysis of the n-6 and n-3 pathway confirmed that the pathway did occur in the VSMCs. Analysis of both the PUFA biosynthesis pathway in VSMCs showed that after treatment with LA and ALA, the proportion of fatty acids increased down the respective pathways (fig 4.9). This was confirmed with the [U-^{13}C] LA. In VSMCs, enrichment was seen from LA to 22:4n-6, confirming newly synthesised fatty acid production through the n-6 PUFA pathway. Analysis of the pathway using unlabelled LA showed that in the VSMCs, 22:5n-6 was not formed and was not significantly different to control cells. This is what would be expected

in the absence of Elovl2. This suggests that there is no elongase2 to elongate the fatty acid into 22:5n-6. In the n-3 pathway analysis the proportion of 22:6n-3 decreases compared to the control. This is also what would be expected in the absence of elovl2. These observations are confirmed in the isotope labelled LA analysis, as 22:5n-6 was not enriched and therefore was not detected. However, 22:5n-6 was also not detected in liver samples. To convert 22:4n-6 and 22:5n-3 to 22:5n-6 and 22:6n-3 respectively is a multi-step process which involves translocation to the peroxisome, where it undertakes one cycle of β-oxidation to produce 22 carbon PUFAs. The 22 carbon PUFAs are then transported back to the endoplasmic reticulum where they can then be used for membrane lipid biosynthesis (fig 4.9) (109). β-oxidation is affected by a number of internal factors such as, the expression of ligand-activated transcription factors and peroxisome proliferators. In addition to this, external factors are also important, such as nuclear receptor ligands and specific substrate concentrations (315). Therefore, it is much more complicated process and requires co-ordination and therefore may be one explanation for a lack of 22:5n-6 production in these cells.

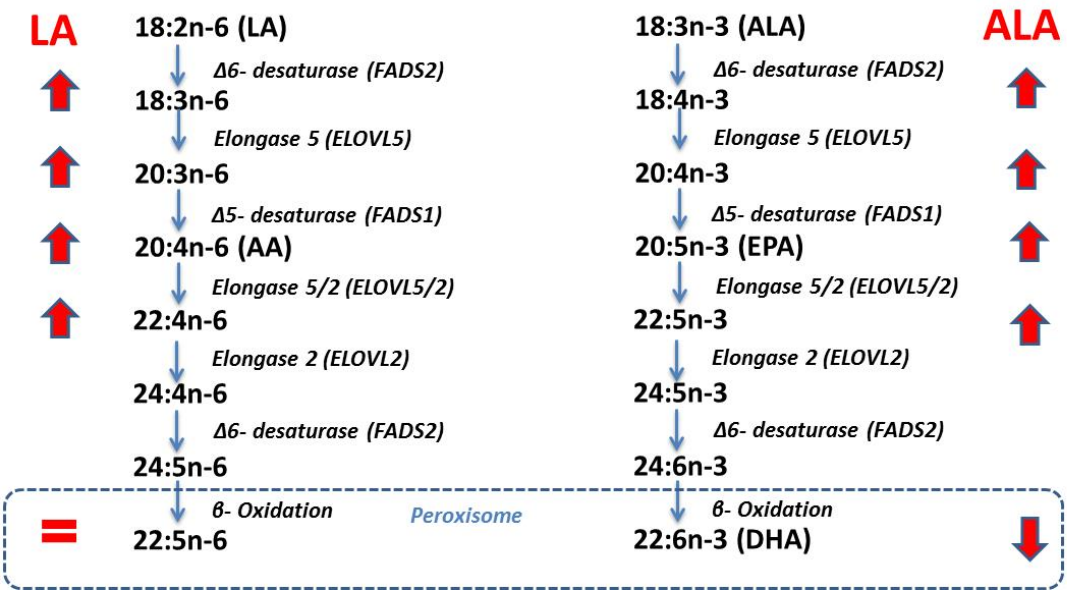


Figure 4.9: Diagram showing the activity of the PUFA biosynthesis *de-novo* pathway in VSMCs determined by functional analysis. The red arrows show the enrichment of each fatty acid following treatment with linoleic acid (LA) or alpha-linolenic acid (ALA). The equal sign shows where no change in enrichment was observed. AA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexanoic acid.

ELOVL2 Methylation:

Measurement of the DNA methylation status of Elovl2 showed differences in specific CpG loci between aorta and liver, such that the level of methylation was higher in aorta. Since DNA methylation is regarded generally as a repressive epigenetic mark (199), these findings

suggest that higher methylation of Elovl2 could explain suppression of transcription at these loci. However, although the same loci differed in methylation between MOVAS and Hepa1-6 cells, methylation was higher in the liver cell line than in the VSMCs. Furthermore, additional loci to those identified in tissues differed between MOVAS and Hepa1-6 cells, for example the 6 loci closest to the transcription start site were hypermethylated in MOVAS compared to Hepa1-6 cells. Overall, the lack of consistency in the pattern of differences in Elovl2 methylation between liver and aorta does not support the suggestion that DNA methylation is involved in suppression of its transcription. Post-hoc calculations of sample size shows that the numbers of samples per group were correct and show that the data produced here are reliable.

It may be that there are changes in the methylation further upstream, but the region covered in this analysis was only between -117 and -368 of the transcription start site. A possible alternative mechanism which may be responsible for the silencing of Elovl2 may be histone modifications, such as deacetylation which leads to chromatin being in a condensed state and thus inhibits the transcription machinery from interacting with the DNA and silencing gene expression (199). However, this is only a potential mechanism for silencing Elovl2 and was not investigated in this study, but could be investigated in future. Furthermore, histone modifications tend to be associated closely with patterns of DNA methylation. Alternatively, histone methylation may be responsible for the silencing of Elovl2, methylation of histone 3 lysine 9 (H3K9) is associated with transcriptional repression (316) and also methylation of histone 3 lysine 27 (H3K27) has also been associated with repression of gene expression and this may involve polycomb which maintains long term gene silencing (317).

Implications of pathway activity:

One important observation from the analysis of the PUFA biosynthesis pathway, was that in the VSMCs, the fatty acids present and synthesised in the greatest abundance was AA in the n-6 pathway and EPA (20:5n-3) in the n-3 pathway. AA was also the fatty acid most highly synthesised in the isotope enrichment data. AA and EPA are both precursors for eicosanoid synthesis. AA is considered the main eicosanoid precursor within mammals, as humans generally have a greater intake of LA. Therefore, more AA will be present in membranes compared to EPA, so AA is considered the major substrate for eicosanoid synthesis (142). Eicosanoids are synthesised *de-novo* from AA released from the phospholipids of the cell membrane, they are not stored but synthesised when cells are activated by medical trauma, cytokines or growth factors (29). Eicosanoids are signalling molecules which have diverse biological actions throughout the body. These actions include physiological functions such as regulation of smooth muscle tone, platelet aggregation and vascular permeability. They are

also very important for immune and inflammation function within the body (29). Many of the products of AA eicosanoid synthesis are considered pro-inflammatory, and have atherogenic and pro-thrombotic effects. However, the eicosanoid products from EPA produce anti-inflammatory actions and products which oppose the products of AA (30). Prostaglandin PGI_2 and PGE_2 and TXA_2 which are products of AA have been shown to have pro-arrhythmic and contractions of myocytes. TXB_3, PGI_3, PGF_{3a} and PGE_3 which are eicosanoid products of EPA have anti-arrhythmic actions (31). In addition to this 22:4n-6 is also a precursor of eicosanoids which have been found to play a role in endothelium-dependent relaxations (318), but has also been shown to be have pro-thrombotic actions (319).

As eicosanoids are important for vascular homeostasis, one explanation for the higher levels of AA and EPA is that the *de-novo* synthesis of the n-6 and n-3 PUFAs is channelled into making precursors for eicosanoid synthesis. This would also be an explanation for the pathway being constrained by the absence of Elovl2 in the pathway. In the pathway analysis using the tracer, Pe-stimulation caused a small but non-significant effect on AA content, if AA is channelled into eicosanoid synthesis a decrease in AA would have been expected. So it may show some AA is being channelled into eicosanoids and not stored in the membrane. However, the data does not confirm this; a larger decrease may have been seen with a larger sample size. The prospect of the PUFA synthesis pathway in VSMCs being channelled into making precursors for eicosanoid synthesis is something that needs to be further investigated as this may provide an explanation for how the PUFA synthesis pathway is linked to vasoconstriction. If the activity of the PUFA synthesis pathway increases and is channelled into making eicosanoid precursors, this would result in an increase in pro-constrictor eicosanoids and would produce a mechanism for increased vasoconstriction. The effect of Pe on the release of eicosanoids also needs to be assessed, as data from this thesis has shown that PUFA biosynthesis is involved in α1-adrenergic receptor stimulation. Therefore, this may occur through a mechanism of Pe stimulated release of eicosanoids. These important points will be addressed in the subsequent chapter.

Data from this chapter could suggest that the PUFA synthesis pathway may only function to make what the tissue requires, so therefore is turned off at lower parts of the pathway to ensure optimum synthesis of eicosanoid precursors (figure 4.10). However, the data from this study shows the same fatty acids were synthesised in the liver and the vascular smooth muscle. Therefore, it may be that the activity of another Elongase is involved in the conversion of 22:4n-6 to 24:4n-6 and 22:5n-3 to 24:5n-3, this may be Elongase 5. Elongase2 and Elongase 5 activities have been shown to overlap in the liver (225) so it may be happening

in VSMCs. However, Elongase 5 has lower activity against 22 carbon fatty acids than 20 carbon fatty acids (228). Furthermore, Elovl2 activity has been shown to be required for PUFA biosynthesis in liver (227) and in sperm (252). Thus it is unclear whether the elongation of 22 carbon PUFA in VSMCs involves Elongase5 instead of Elongase2, or whether another elongase is involved. Furthermore, it is not possible to speculate from current knowledge (320) why Elongase2 is suitable for catalysing the elongation of 22 carbon PUFA in liver, but not in VSMCs. Therefore, it is unclear whether this reaction is carried out by Elongase 5 and this needs to be further investigated. This could be done by silencing Elongase 5 using siRNA and treating the cells with 22:4n-6 to determine whether there is production of the 24:4n-6 and 22:5n-6. The sample size for isotope enrichment experiment was lower than the post-hoc power calculations suggest, as a sample size of 33 is required. Therefore, if a greater sample size was used, these data may be significant and more conclusive.

In conclusion, data from this chapter has confirmed that VSMCs are capable of *de-novo* synthesis of PUFA. However, the PUFA biosynthesis pathway may be constrained by the absence of Elovl2 and therefore VSMCs are not capable of synthesising 22:5n-6 or 22:6n-3. Alternatively, another Elongase may be involved in catalysing the conversion of longer chain metabolites to allow the production of 22:5n-6 or 22:6n-3, however, this remains unclear.

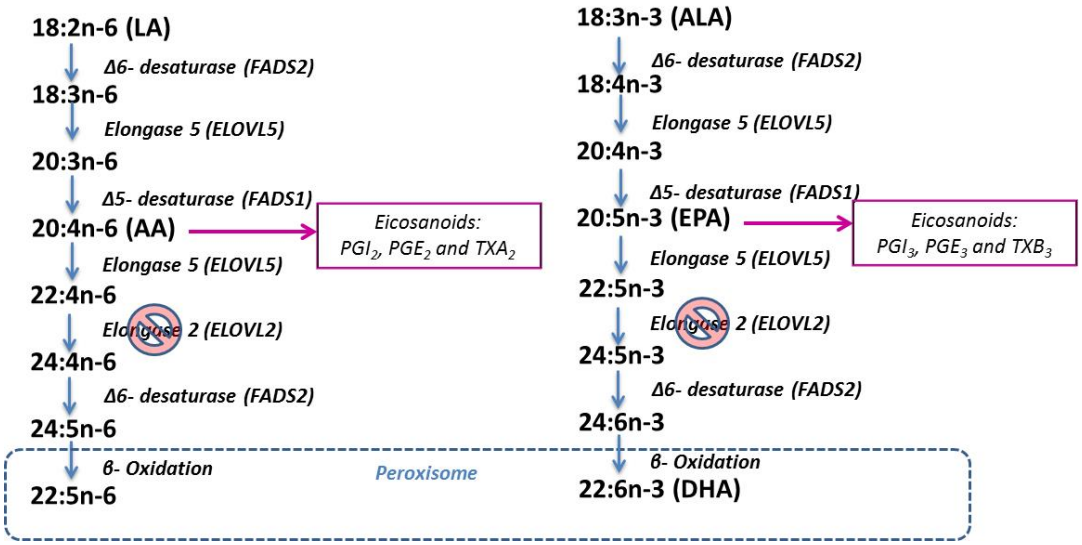


Figure 4.10: PUFA biosynthesis *de-novo* pathway in VSMCs. LA: linoleic acid, ALA- alpha-linolenic acid, AA: arachidonic acid, DHA: docosahexanoic acid, EPA: eicosapentaenoic acid, PGI_2: prostacyclin, PGE_2: prostaglandin E_2, TXA_2: thromboxane A_2, Prostaglandin I_3, prostaglandin E_3 and TXB_3: Thromboxane B_3.

Chapter 5-

The role of polyunsaturated fatty acid biosynthesis in Pe-mediated calcium release

5.1 Introduction:

The findings reported in Chapter 4 provide direct evidence that VSMCs are capable of PUFA synthesis *de novo* and confirmed the range of n-6 and n-3 PUFAs synthesised by these cells. These data also suggested that newly synthesised PUFA may be channelled into the production of eicosanoids.

VSMCs express both COX-1 and COX-2, and are able to synthesise a range of eicosanoid species (321). It has been proposed that eicosanoids secreted by VSMCs may act in an autocrine or paracrine manner to induce vasoconstriction and maintain vascular tone, although this process has not been investigated in detail (322). If newly synthesised PUFAs in VSMCs are used preferentially for the production of pro-constriction eicosanoids, then it may be expected that the activity of the PUFA biosynthesis pathway would be linked to intracellular calcium release. In addition to this, in chapter 3 these data presented showed that PUFA biosynthesis in VSMCs may be involved in vasoconstriction, however, the mechanism whereby this happens is unknown.

Chapter hypotheses:

- PUFA biosynthesis *de-novo* in VSMC is involved in calcium release and vasoconstriction of VSMCs following stimulation by α1-adrenergic receptor agonist Pe.
- Inhibition of PUFA synthesis *de-novo* in VSMCs causes a decreased production of eicosanoid synthesis following α1-adrenergic stimulation. Demonstrating eicosanoid production is involved in α1-adrenergic mediated vasoconstriction in VSMCs.

To address these hypotheses, an assay for intracellular calcium release in VSMCs was developed. The effect of inhibition of either delta-6 or delta-5 desaturase by SC-26196 and sesamin respectively, on calcium release was then determined. The effect of inhibition of these enzymes on the secretion of pro-constriction eicosanoids was also determined.

5.2 Methods:

Cell Culture:

Cell culture of MOVAS cells at passage 28 were used for the following experiments, see methods chapter section 2.7.

Cell treatment with LA in the presence of SC-26196 or sesamin::

To determine the optimum concentration of the delta-5 and delta-6 desaturase inhibitors; sesamin and SC-26196 respectively, the activity of the pathway n-6 PUFA biosynthesis pathway was measured by LA treatment in the presence of the inhibitors, see methods chapter section 2.11.2

The effect of SC-26196 and sesamin on intracellular calcium release:

To determine whether PUFA biosynthesis is involved in intracellular calcium release in VSMCs, intracellular calcium release was measured in response to PE in the presence of the delta-5 and delta-6 desaturase inhibitors SC-26196 (100 nM, 200 nM, 500 nM and 1 µM) and sesamin (10 µM and 20 µM). This was carried out in MOVAS cells , see methods chapter section 2.12.1

Determination of eicosanoid production:

To determine the effect of inhibition of the PUFA biosynthesis pathway on the production of eicosanoids in MOVAS cells following treatment with either the SC-26196 or sesamin. Eicosanoid secretion was measured in response to stimulation by Pe. The eicosanoids that were measure were; HETE, PGE_2, $PGF_{2\alpha}$ and TXB_2, see methods chapter section 2.13

5.3 Results:

5.3.1 Pathway analysis with delta-6 desaturase inhibition:

To confirm the presence of delta-6 desaturase and the activity of the delta-6 desaturase inhibitor; SC-26196, the PUFA pathway was investigated in the presence of SC-26196. A concentration of 200 nM of SC-26196 was used as recommended by the supplier. At 200 nM SC-26196 is a selective delta-6 desaturase inhibitor, at higher concentrations (>200 μM), SC-26196 displays selectivity over delta-5 and delta-9 desaturases. SC-26196 caused a significant increase in the proportion of LA (18:2n-6) compared to the control alone (shown in fig 5.1). The proportions of all down-stream metabolites; 18:3n-6 (P=<0.0001), 20:3n-6 (P=<0.0001), AA (20:4n-6) (P=<0.0001), 22:4n-6 (P=<0.0275) and 22:5n-6 (P=<0.0001), were all significantly reduced compared to the control treatment.

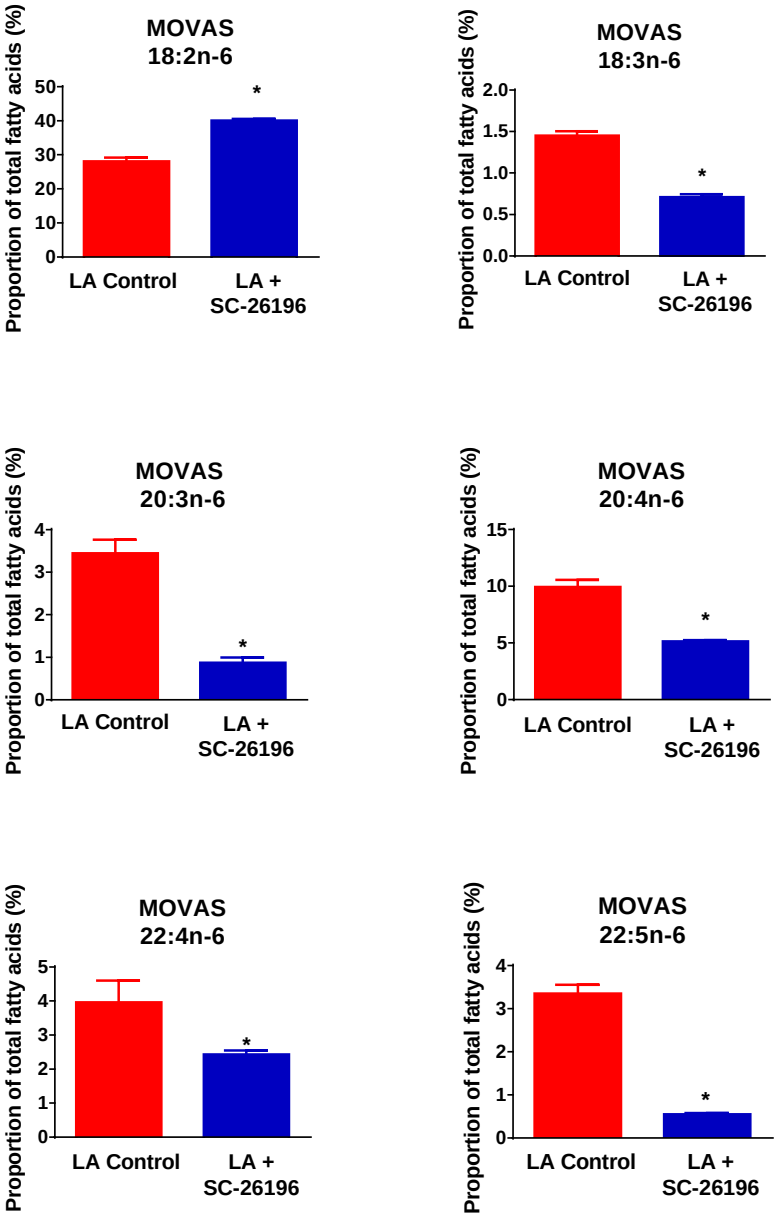


Figure 5.1: Proportion of n-6 PUFAs in MOVAS cells following treatment with linoleic acid (LA control) and linoleic acid in the presence of SC-26196 (200nM) (LA+SC-26196). Values are mean ± SEM (n=6/group). Statistical comparisons were by unpaired student T test. Values significantly different (P<0.05) between treated cells compared to control cells is indicated by *.

5.3.2 Pathway analysis with delta-5 desaturase inhibition:

Sesamin caused a concentration dependent increase in proportion of LA (18:2n-6) compared to LA treatment alone, with 20 µM sesamin producing a significant increase (P=0.0009) (shown in fig 5.2). The proportion of the downstream metabolites; 18:3n-6 and 20:3n-6 demonstrate the same pattern of increase. A concentration dependent decrease in the proportion of AA (20:4n-6), 22:4n-6 and 22:5n-6 is seen in the presence of sesamin. Out of the concentrations tested, 10 µM and 20 µM of sesamin were chosen to be used for all future experiments as these concentrations caused the greatest attenuation of the pathway compared to 1µM of sesamin, which had very little effect as shown in fig 5.2.

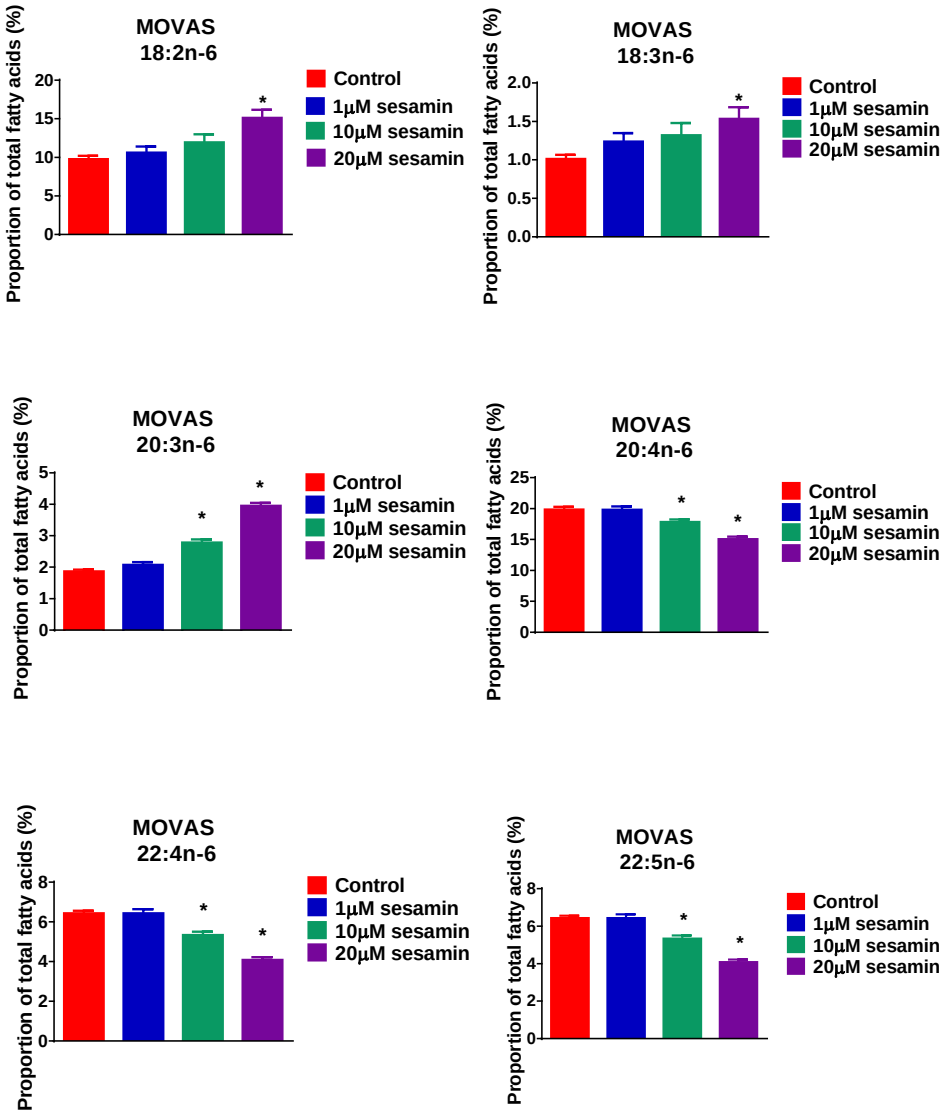


Figure 5.2: Proportion of n-6 PUFAs in MOVAS cells following treatment with linoleic acid (control) and linoleic acid in the presence of increasing concentrations of sesamin. Values are mean ± SEM (n=6/group). Statistical comparisons were by ANOVA with Dunnett's post hoc analysis. Values significantly different (P<0.05) are indicated by *.

5.3.3 Calcium assay optimisation:

The protocol included within the calcium assay was very basic, with very little explanation about how to use the kit, therefore to get the most reliable data, a number of experiments were carried out to develop a protocol, ensure the kit worked and also to optimise the kit. To ensure that the assay worked and to determine the best 96-well plate to use, the assay was carried out according to the assay preparation described in 5.2.3. This was carried out in both clear sided culture plates and black sided culture plates. Measuring fluorescence may be affected by high background fluorescence or by light scattering; both of these issues may happen in a clear sided culture plates, thus leading to erroneous readings. To ensure that the most accurate readings were obtained, the experiment was carried out in both black sided and clear sided plates to establish which plates give the most reliable results. The ionophore A23187 was used as this is known to cause a large increase in intracellular calcium.

The data confirms that the assay works, as addition of the ionophore causes an increase in intracellular calcium concentration, this occurs in both clear and black plates. However, the black plates cause a significantly larger increase in calcium release at all tested concentrations; at 200 nM ($P<0.0001$), 500 nM ($P=0.0009$) and 1 µM ($P=0.0077$). This demonstrates more sensitive detection of the fluorescence using the black plates. The lower fluorescence from samples in the clear sided plates may be due to light scattering and a diluted level of the emitted fluorescence reaching the sensor (fig 5.3). Therefore, black plates were used for all further calcium assay experiments.

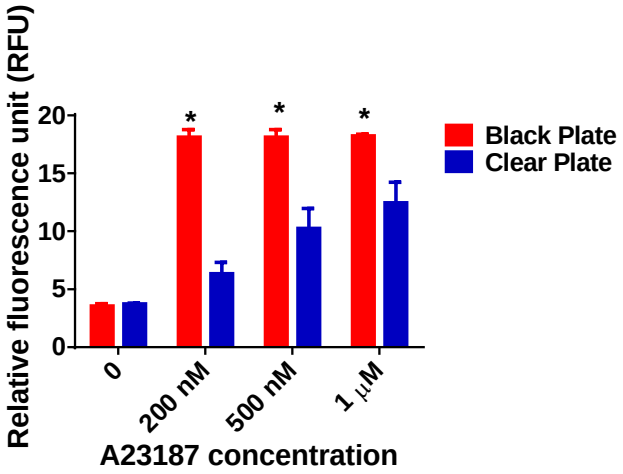


Figure 5.3: Intracellular calcium release at increasing concentration of A23187 in black and clear culture dishes. Data is shown as mean ±SEM. (n=8) Statistical analysis is by ANOVA with Bonferroni post-hoc test. Significance ($P=<0.05$) is shown by *.

To determine the optimum concentration of Pe to use for optimum release of intracellular calcium, the assay was carried out with two concentrations of Pe, these concentrations produce the maximum response of vasoconstriction in aorta. In addition to this, the plate was read over a time course of 30 minutes to establish the optimum point to read the plate, ensuring maximum release of intracellular calcium.

Both the A23187 and Pe cause the maximum intracellular calcium concentration after 1 minute of treatment. Following the A23187 treatment, the calcium concentration then reaches a plateau (fig 5.4). However, following Pe treatment, the calcium concentration decreases at the 5 minute time point and gradually increases. Therefore, the optimum time for reading the plate following treatment is 1 minute. The optimum concentration for Pe was 10 µM, as at 1 minute the calcium concentration was significantly greater than the untreated (P=0.0003) and 100 µM (P=0.0392).

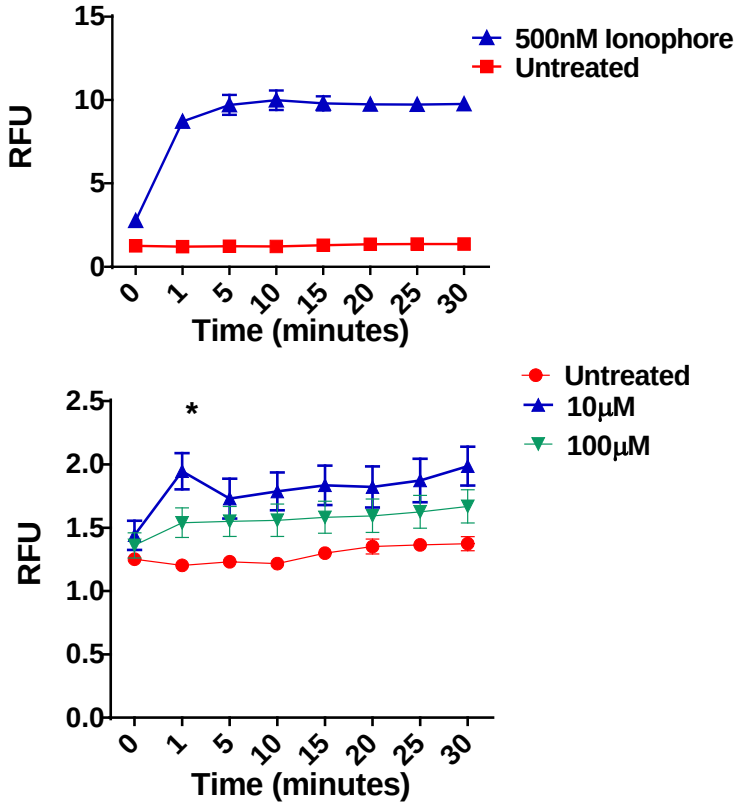


Figure 5.4: Intracellular calcium release in MOVAS cells over a time course of 30 minutes from treatment. Top: A23187. Bottom: Pe (10 µM and 100 µM). Data is shown as mean ±SEM relative fluorescence unit (RFU). (n=8) Statistical analysis is by ANOVA with Bonferroni post-hoc test. Significance (P=<0.05) is shown by *.

5.3.4 The effect of delta-6 desaturase inhibition on calcium release:

To investigate the affect PUFA synthesis inhibition has upon the calcium release, MOVAS cells were stimulated with Pe in the presence of the delta-6 desaturase inhibitor, SC-26196. Treatment with Pe alone caused a significant increase in calcium release compared to untreated cells (P<0.0001). Increasing SC-26196 induced a dose-dependent decrease in the release of intracellular calcium (Fig 5.5) such that there appeared to be a complete inhibition in cells treated with 500nM SC-26196.

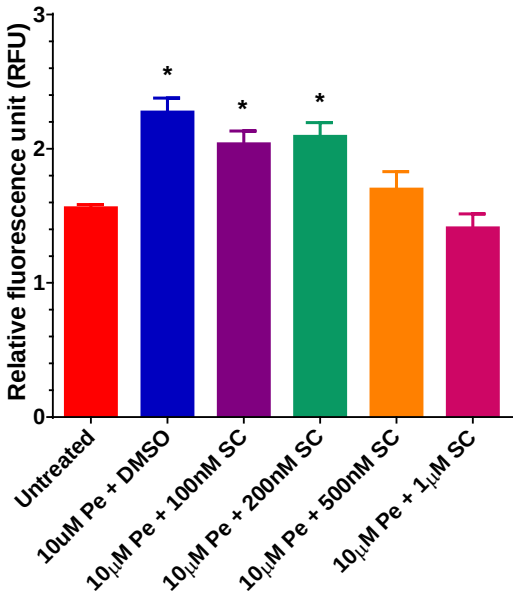


Figure 5.5: Calcium release in the presence of increasing concentrations of SC-26196. Data is shown as mean ±SEM. (n=8) Statistical analysis is by ANOVA with Dunnett's post-hoc test. Significant difference compared to the control group (untreated) (P=<0.05) is shown by *.

5.3.5 The effect of delta-5 desaturase inhibition on calcium release:

To investigate whether delta-5 desaturase inhibition affects calcium release, cells were stimulated with Pe in the presence of sesamin. Treatment with Pe caused a significant increase in calcium release compared to untreated cells (P=0.0189). Pe stimulation in the presence of the sesamin caused complete inhibition of intracellular calcium release (fig 5.6).

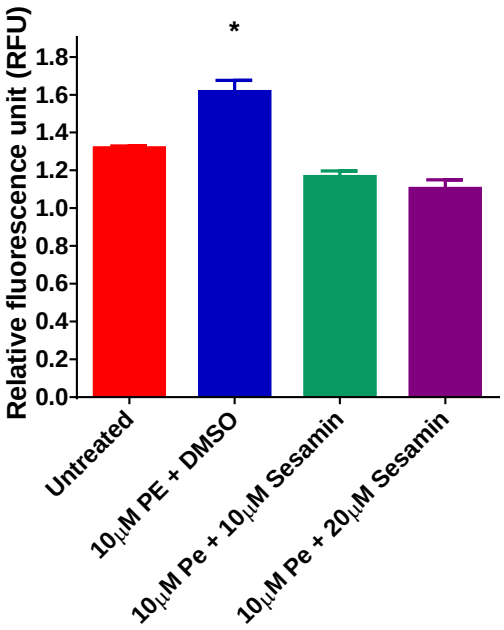


Figure 5.6: Calcium release in the presence of increasing concentrations of sesamin. Data is shown as mean ±SEM (n=8). Statistical analysis is by ANOVA with Dunnett's post-hoc test. Significant difference compared to the control group (untreated) (P=<0.05) is shown by *.

5.3.6 The effect of PUFA biosynthesis inhibiton on eicosanoid secretion:

PGE_2 and SC-26196:

To investigate changes in PUFA synthesis *de-novo* on the release of pro-constrictor eicosanoids, the production of PGE_2 was measured in the presence of SC-26196. This showed that upon stimulation of the cells with Pe, there was an increase in PGE_2 production (P=0.1797) (Fig 5.7). In the presence of SC-26196 there is a concentration- dependent decrease in PGE_2 production, and total inhibition of PGE_2, equal to the control (P=0.9687).

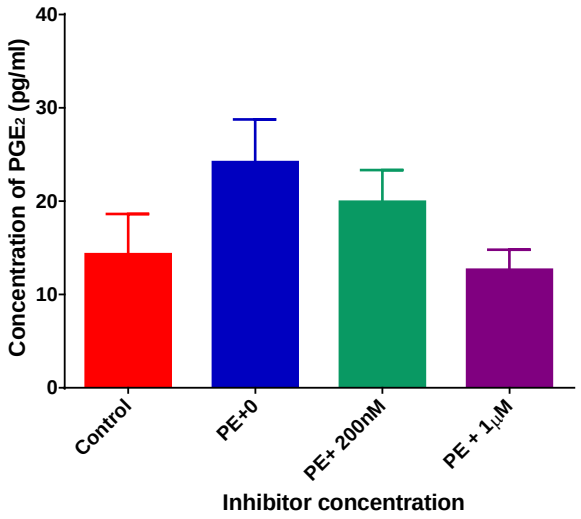


Figure 5.7: PGE_2 production following Pe stimulation in the presence of increasing concentrations of SC-26196. Data is shown as mean ±SEM. (N=8) Statistical analysis is by ANOVA with Dunnett's post-hoc test Significant difference compared to the control group (untreated) (P=<0.05) is shown by *.

PGE_2 and sesamin:

PGE_2 production in the presence of sesamin caused a significant change in PGE_2 production (Fig 5.8). Stimulation with Pe caused a small but non-significant increase in PGE_2 release (P=0.8068). When the cells were stimulated with Pe in the presence of 10 µM PE, there was complete abolition of PGE_2 release, significantly lower than control cells (P=0.0011). However, increasing the concentration of sesamin to 20 µM caused greater production of PGE_2 than observed at 10 µM of sesamin. The PGE_2 production at 20 µM is not significantly different (P= 0.1679) to the production of PGE_2 of the control cells.

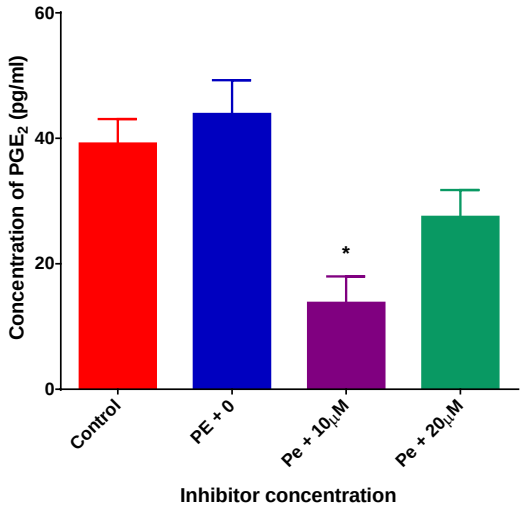


Figure 5.8: PGE_2 production following Pe stimulation in the presence of increasing concentrations of sesamin. Data is shown as mean ±SEM. (N=6) Statistical analysis is by ANOVA with Dunnett's post-hoc test. Significant difference compared to the control group (untreated) (P=<0.05) is shown by *.

$PGF_{2\alpha}$ and SC-26196:

Stimulating the cells with Pe caused a significant increase in the production of PGF_{2a} compared to the control cells (P=0.0019). Stimulating with Pe in the presence of 200 nM and 1 µM SC-26196 caused inhibition of PGF_{2a} production to a level not significantly different to control cells (P=0.1960) and (P=0.4933) (Fig 5.9).

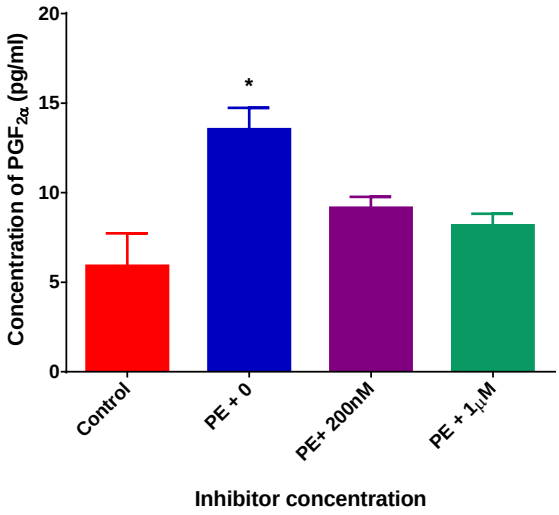


Figure 5.9: PGF_{2a} production following Pe stimulation in the presence of increasing concentrations of SC-26196. Data is shown as mean ±SEM. (N=6) Statistical analysis is by ANOVA with Dunnett's post-hoc test. Significant difference compared to the control group (untreated) (P=<0.05) is shown by *.

PGF_{2a} and sesamin:

Stimulation with Pe caused a large increase in PGF_{2a} compared to control cells (P=0.0020). Stimulation with Pe in the presence of sesamin (Fig 5.10) produced a similar pattern of PGF_{2a} production to that seen for PGE_2 production (Fig 5.8). At 10 µM of sesamin there was large decrease in PGF_{2a} release compared to Pe stimulation alone (P=0.0033). This produced PGF_{2a} production nearly equal to that in control cells (P=0.9953). Stimulating cells with Pe in the presence of a higher concentration of sesamin (20 µM) caused a smaller decrease in PGF_{2a} production compared to Pe but not significantly different to control cells (P=0.0997).

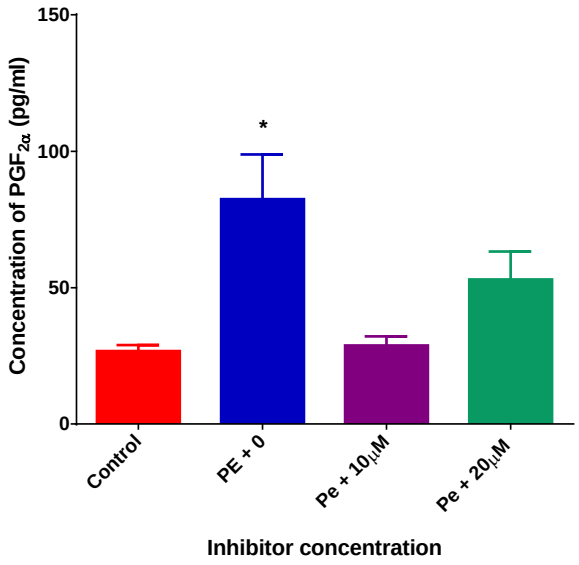


Figure 5.10: PGF_{2a} production following Pe stimulation in the presence of increasing concentrations of sesamin. Data is shown as mean ±SEM. (N=6) Statistical analysis is by ANOVA with Dunnett's post-hoc test. Significant difference compared to the control group (untreated) (P=<0.05) is shown by *.

TXB_2 and SC-26196:

The production of TXB_2 is very low in these cells compared to the production of the other eicosanoids investigated. TXB_2 production in the presence of Pe showed a significant increase compared to the control cells (P=0.0125). Pe stimulation in the presence of 200 µM SC-26196 causes a small decrease in TXB_2 production, to a level not significantly different to control cells (P=0.5210) (Fig 5.11). However, the TXB_2 production following Pe stimulation is not effected by 1 µM SC-26196, as it is nearly equal to the production of TXB_2 production following Pe stimulation with no inhibitor present and significantly greater than the control cells (P=0.0040).

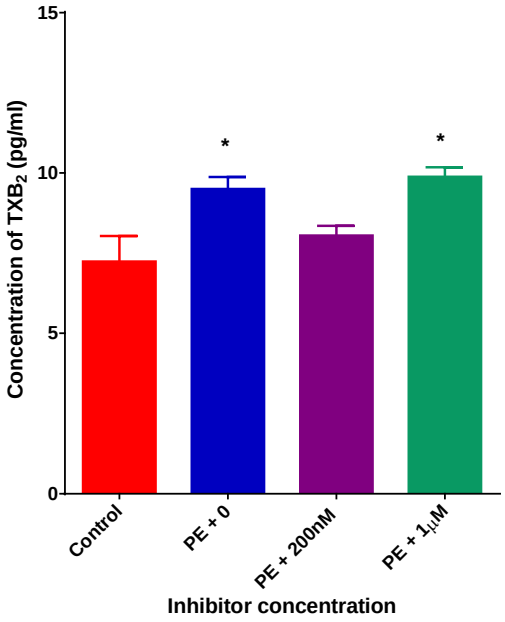


Figure 5.11: TXB_2 production following Pe stimulation in the presence of increasing concentrations of SC-26196. Data is shown as mean ±SEM. (N=6) Statistical analysis is by ANOVA with Dunnett's post-hoc test. Significant difference compared to the control group (untreated) (P=<0.05) is shown by *.

TXB_2 and sesamin:

In the experiment carried out investigating the effect of sesamin on TXB_2 production (Fig 5.12). Pe stimulation demonstrated only a very small and non-significant increase in TXB_2 production (P=0.6745). Stimulating with Pe in the presence of sesamin at 10 µM caused an increase in TXB_2 production compared to in the presence of Pe alone, this was a significant increase in TXB_2 production compared to the control (P=0.0489), at 20 µM there is a trend towards increased TXB_2 compared to the control cells (P=0.0974).

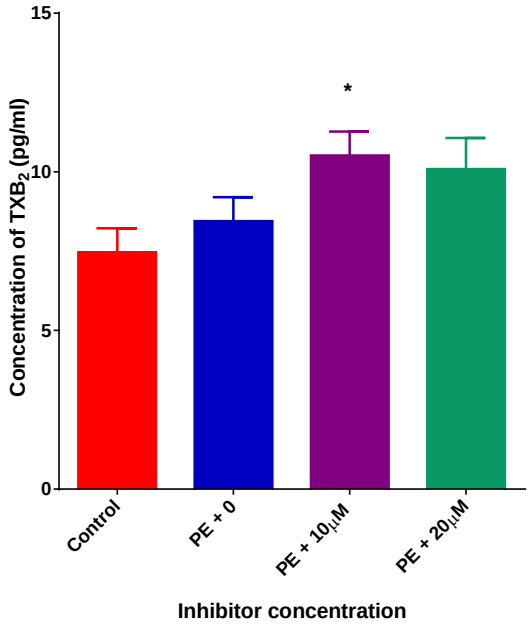


Figure 5.12: TXB_2 production following PE stimulation in the presence of increasing concentrations of sesamin. Data is shown as mean ±SEM. (N=6) Statistical analysis is by ANOVA with Dunnett's post-hoc test. Significant difference compared to the control group (untreated) (P=<0.05) is shown by *.

HETE and SC-26196:

Pe treatment had very little effect on HETE production, there was no significant difference between control cells and Pe treated cells (P=0.5073). The presence of 200 nM SC-26196 caused a small increase in HETE production compared to Pe and this was significantly different to control cells (P=0.0270). In the presence of 1 µM SC-26196, HETE production is not significantly different from the HETE production to in control cells (P=0.6456) (Fig 5.13). However, HETE production in the presence of the SC-26196 inhibitor is not significantly different (P=0.4273 and P=0.9928) from Pe stimulated cells with no inhibitor.

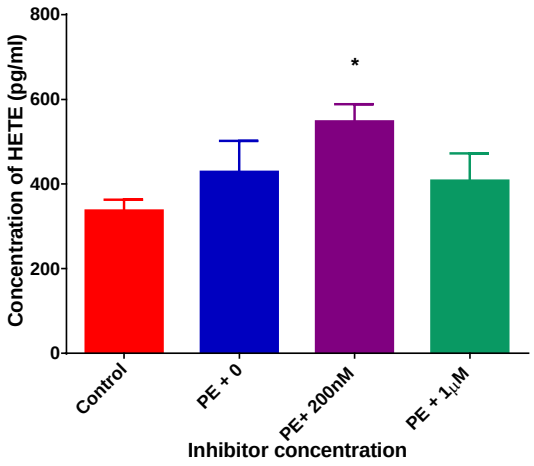


Figure 5.13: HETE production following PE stimulation in the presence of increasing concentrations of SC-26197. Data is shown as mean ±SEM. (N=6) Statistical analysis is by ANOVA with Dunnett's post-hoc test. Significant difference compared to the control group (untreated) (P=<0.05) is shown by *.

HETE and sesamin:

The experiment carried out to test HETE production in the presence of sesamin shows a similar pattern of results to that of SC-26196. Pe causes an increase trend in HETE production compared to control cells (P=0.0593). In the presence of 10 µM sesamin a similar increased trend in production of HETE production is observed compared to the control cells (P=0.0722) and at 20 µM there is a further increase in HETE production which is significantly different to the control (P=0.0296) (Fig 5.14). However, HETE production in the presence of the sesamin inhibitor is not significantly different (P=0.9842 and P=0.2982) compared to Pe stimulated cells with no inhibitor.

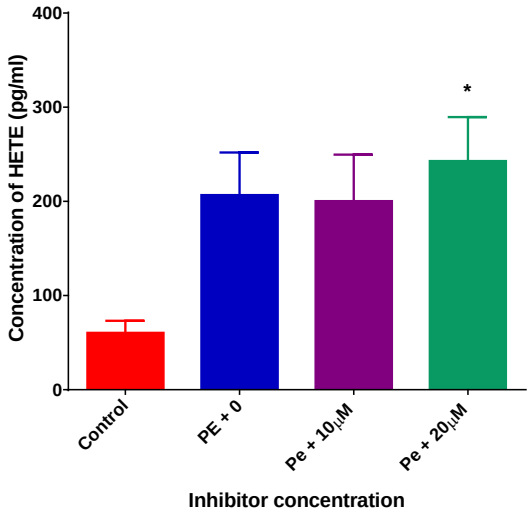


Figure 5.14: HETE production following PE stimulation in the presence of increasing concentrations of sesamin. Data is shown as mean ±SEM. (N=6) Statistical analysis is by ANOVA with Dunnett's post-hoc test. Significant difference compared to the control group (untreated) (P=<0.05) is shown by *.

5.4 Discussion:

The findings from these experiments show that inhibition of the PUFA biosynthesis pathway causes a decrease in calcium release and also a decrease in the release of pro-constrictor eicosanoids.

Treating cells with SC-26196 and sesamin and measuring the MOVAS fatty acid content allowed the optimum concentration of the inhibitors to be determined to ensure that the pathway was being inhibited as expected and at which concentration the most optimum inhibition occurred. For the sesamin the optimum concentration was confirmed by the concentration which caused the largest decrease in metabolite production compared to LA treated alone. The optimum concentration was 10 µM and 20 µM for sesamin. The 200 nM SC-26196 concentration was recommended by the supplier and the data confirmed its activity at that concentration. With the SC-26196 inhibitor there was also a pooling effect in LA, as less of the LA was being used for synthesis of n-6 fatty acids, so there was a build-up in the proportion of LA compared to the LA treated cells without the inhibitor. This was also seen in the sesamin treated cells, there was accumulation of the proportion of LA, 18:3n-6 and 20:3n-6 as they are not being converted into fatty acids further down the pathway. This level of accumulation was dose dependent on the concentration of the inhibitor.

VSMC PUFA biosynthesis and intracellular calcium release:

Inhibition of delta-5 or delta-6 desaturase in the PUFA biosynthesis pathway reduced Pe-medicated vasoconstriction in aortae and in human femoral arteries as shown in Chapter 3 (282). The α_1-adrenoreceptor is a Gq coupled receptor which acts by the stimulation of PLC and formation of IP3 and DAG. The eicosanoids PGE_2 and PGF_{2a} have been shown to activate the TP receptor and cause vascular smooth muscle contraction (33). The TP receptor is also a Gq coupled receptor which acts through the same pathway as a1-adrenergic receptor. Both receptors lead to release of calcium from the sarcoplasmic reticulum and then muscle contraction (24). Inhibition of delta-6 desaturase inhibited intracellular calcium release in a concentration-dependent manner, such that at the highest concentration of SC-26196, calcium release was inhibited completely. In addition, inhibition of delta-5 desaturase caused complete inhibition of calcium release in response to Pe stimulation. Together these findings suggest that PUFA biosynthesis *de-novo* is important for Pe-mediated release of intracellular calcium. Intracellular calcium release, acting through calmodulin is critical for activation of MLCK and hence the actin-myosin cross-bridge cycle, leading to muscle contraction (22). Thus these findings are consistent with previous observations in arteries (282) and so suggest a

mechanism by which inhibition of delta-6 or delta-5 desaturase may impair vasoconstriction. Post-hoc sample size analysis shows that the sample size for measurements of calcium releases are big enough to provide reliable and conclusive data.

VSMC PUFA biosynthesis and eicosanoid production:

Vasoconstriction involves the activities of specific eicosanoids, which include PGE_2, PGF_{2a} and TXA_2 (30). The present findings show that inhibition of delta-6 desaturase reduced PGE_2 and PGF_{2a} release, which was abolished at the highest concentration of the SC-26196. Furthermore, inhibition of delta-5 desaturase abolished PGE_2 and PGF_{2a} secretion at the highest concentration of sesamin. The pattern of PGE_2 and PGF_{2a} production in the presence of the delta-5 and delta-6 desaturase inhibitors was a similar pattern to the intracellular calcium data. Thus, inhibition of delta-5 and delta-6 desaturase caused inhibition of calcium release and inhibited the release of pro-constrictor eicosanoids. One possible explanation is that newly synthesised PUFA were used preferentially for the synthesis of specific eicosanoids. Thus inhibition of the delta-6 desaturase reduced synthesis of AA and DGLA leading to impaired production of PGE_2 and PGF_{2a}. While it is possible that SC-26196 or sesamin may have inhibited eicosanoid synthesis directly, there is no evidence to support this suggestion. This possibility is also reduced by using two inhibitors with different chemical structures (323;324). In the presence of sesamin at 20 µM, the concentration of PGE_2 and $PGF_{2\alpha}$ increases compared to 10 µM. As seen from fig 5.2, inhibition with sesamin causes accumulation in fatty acids above the point of pathway inhibition, such as 20:3n-6 (DGLA). DGLA is a precursor for series 1 eicosanoids, which include PGF_{1a} and PGE_1 (325). The ELISA kits used have cross reactivity of between 40-65% with these eicosanoids. Therefore, the increase in PGE_2 and PGF_{2a} production at the higher concentration may be due to detection of eicosanoids which are cross-reactive and the accumulation of DGLA exacerbates the affect. Differences were seen between the control concentrations of PGE_2, PGF_{2a} and HETE released between SC-26196 and sesamin experiments. The reason for this observation is unknown; however, it may also be due to cross reactivity of the ELISA kits. In addition to this, due to time constraints, a very low sample size was used for all of the eicosanoid experiments and the results are under-powered. Retrospective power calculations suggest a sample size of 22. Therefore, further experiments need to be done to get more reliable eicosanoid production data.

Secretion of TXA_2 is measured as its spontaneous degradation product; TXB_2. The level of TXB_2 production was almost below the level of detection of the ELISA assay. Thus, the lack of an effect on TXA_2 secretion may have reflected a limitation of the assay. A previous study described that in arterial smooth muscle cells PGE_2 is preferentially released and that there are

low levels of TXA_2 synthase present, thus resulting in less TXA_2 produced and so less breakdown to the TXB_2 metabolite which was measured (326). In a similar manner to the findings of Kelsall et al. (282), there was no effect of the desaturase inhibitors on the secretion of HETE in MOVAS cells compared to Pe stimulated cells. Although changes in HETE production were seen in inhibitor treated cells compared to control cells, inhibition of the PUFA biosynthesis pathway caused no significant change in HETE production compared to the Pe-only stimulated cells, this was as expected. This suggests that HETE production is not involved in newly synthesised PUFA.

Together the present findings suggest that PUFA biosynthesis *de-novo* is involved in calcium release and vasoconstriction of vascular smooth muscle following Pe stimulation. One suggested mechanism whereby PUFA biosynthesis may contribute to Pe-mediated calcium release is by providing substrate for the synthesis of pro-constriction eicosanoids from newly synthesised AA (figure 5.15).

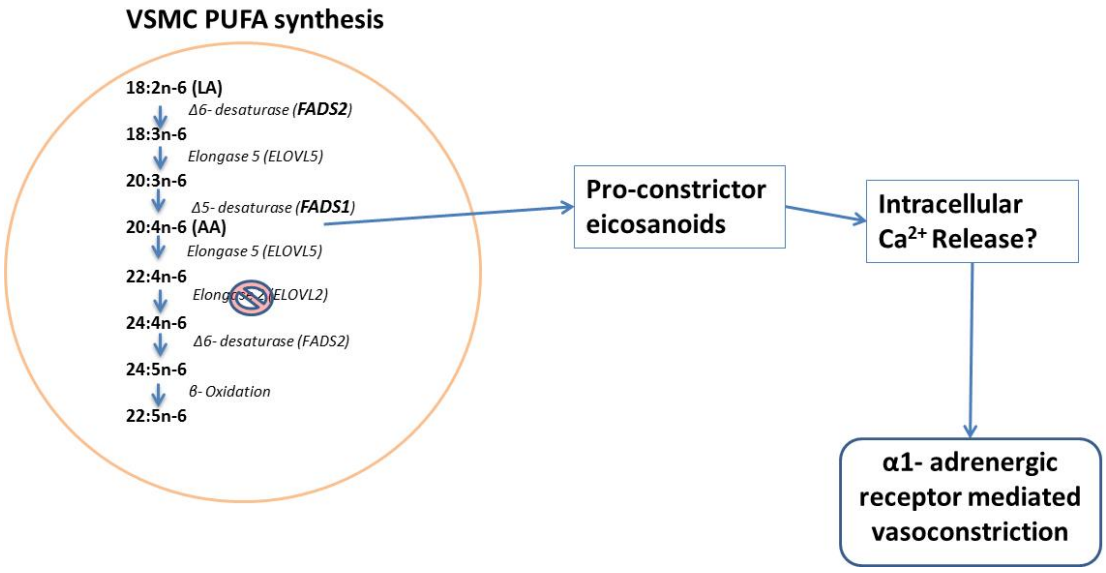


Figure 5.15: Pathway which links VSMC PUFA biosynthesis de-novo with α1-adrenrgic receptor mediated vasoconstriction. LA: Linoleic acid, AA: arachidonic acid, VSMC: vascular smooth muscle cell, PUFA: polyunsaturated fatty acid.

Chapter 6-

Discussion

The findings from this study show that both amount and type of maternal dietary fat consumed during pregnancy and lactation leads to persistent changes in offspring vasoconstriction and vasorelaxation, with the amount of maternal dietary fat having the greatest effect. This effect of maternal dietary fat appears to involve changes in the capacity of VSMCs to synthesise PUFA that are associated with altered epigenetic regulation of FADS2. Furthermore, the study also shows for the first time that AA biosynthesis *de-novo* in VSMCs is required for Pe-induced vasoconstriction in both human and rat arteries. This appears to involve a direct link between capacity for synthesis of PUFA, principally AA, and mobilisation of calcium stores in VSMCs. Together these findings suggest a novel pathway in the control of vascular smooth muscle function and a putative mechanism by which dietary fat can alter vascular tone (figure 6.1).

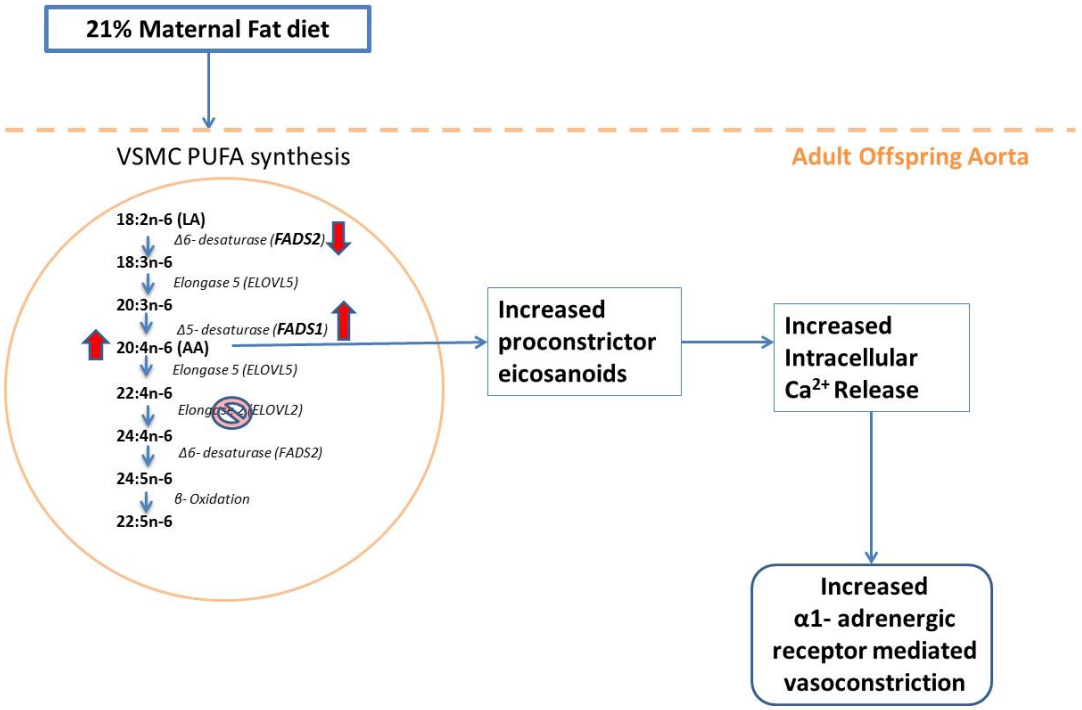


Figure 6.1: The mechanism which links changes in maternal diet to increased α1-adrenergic receptor mediated vasoconstriction in the aorta of adult offspring through dysregulation of VSMC PUFA biosynthesis *de-novo*. LA: linoleic acid, AA: arachidonic acid, PUFA: polyunsaturated fatty acid, VSMC: vascular smooth muscle cells.

The study by Ghosh et al is a highly relevant study to this thesis. The study has shown that a maternal high fat diet during pregnancy and weaning resulted in vascular dysfunction and abnormal aorta and lipid fatty acid composition in the offspring (8). The data presented in this thesis supports these findings but also extends the findings and provides further

contribution to this field of research. The Ghosh et al study demonstrated that a maternal high fat diet caused endothelial dysfunction in the aorta (8). The findings from this thesis demonstrate that a maternal high fat diet causes both endothelial dysfunction and also increased Pe-induced vasoconstriction, which is pinpointed to changes in vascular smooth muscle function. The Ghosh et al study also demonstrated reduced AA and DHA content in the rat offspring aorta following a maternal high fat diet (8). This thesis supported these finding, but also provides a mechanism whereby a maternal high fat diet leads to changes in offspring aorta fatty acid composition through changes to PUFA biosynthesis and provides a hypothesis of how this relates to vascular function.

6.1 The effect of maternal dietary fat on vascular function:

The prevalence of obesity has increased worldwide over the last few decades as a result of changes in food intake, including high fat diet and lifestyle factors, such as low physical activity. This poses a serious public health concern as it has a number of health implications including CVD (327). As a result of the increase in obesity there is also an increase in prevalence of obesity among women of childbearing age (4). This leads to complications such as pre-eclampsia and gestational diabetes through pregnancy and also leads to abnormal fetal growth (327). Maternal obesity has also been shown to have a number of long-term detrimental effects on offspring health, including obesity (328), insulin resistance (329) and increased blood pressure (330).

Previous studies in animal models have investigated the effect of maternal high fat diet (5;331), n-3 deficient diet (6;194) and a hypercholesterolaemic diet (332) on the cardiovascular system of the offspring. All of these studies have demonstrated decreased vascular function, including endothelial dysfunction, and changes in blood pressure. The present study supports these findings by showing that a maternal high fat diet during pregnancy induced decreased vasorelaxation in the offspring which is an indicator of endothelial dysfunction; L-NAME data confirmed that this change in response was due to changes in NO production from the endothelium. This study also extends these findings and shows that in addition to fat quantity, different types of maternal fat during pregnancy and lactation caused differences in the regulation of vascular tone in the offspring which resembled endothelial dysfunction.

Vascular tone is regulated by the interaction between the endothelium and VSMCs which maintains a balance between vasoconstriction and vasorelaxation (48). There is substantial evidence that the function of the endothelium is impaired in vascular dysfunction

and CVD (333), (334), (53). Dietary fat is one factor which has been associated with endothelial dysfunction, studies have shown that a high saturated fat diet (115;274) (115)and high a trans-fat intake (123) leads to endothelial dysfunction and an increased risk of CVD. In contrast, it has also been shown that a high n-3 PUFA intake has a beneficial effect on endothelial dysfunction (159), (160). However, no study has compared the effect of different types of maternal fat and different quantities of these fats on offspring endothelial function. Therefore, the study presented in this thesis is the first study to show that both type and quantity of maternal fat have an effect on endothelial function in adult offspring. Fat quantity had the greatest effect, with endothelial dysfunction observed in offspring of 21% fat diet, irrespective of diet type.

VSMC and CVD:

Less is known about the role of VSMCs in vascular dysfunction. There is some evidence that vascular smooth muscle function is impaired in human patients with cardiovascular disease, such as those at risk of atherosclerosis (335) and in particular hypertension (54). It has been recently demonstrated that stiffness in individual vascular smooth muscle cells causes vascular stiffness in hypertension in rats (65). In addition to this, as shown in this thesis, maternal diet can lead to changes in offspring VSMC function. The offspring of rat dams fed a lard rich diet exhibited increased blood pressure, a reduction in the number of smooth muscle cells, increased aortic stiffness which results in reduced compliance and increased resistance which may be the cause or the result of increased blood pressure (190). However, the precise mechanism is not well understood. It has also been demonstrated that rat maternal protein restriction induces abnormalities in vascular smooth muscle function in the offspring (187). Therefore, it is not just the endothelium that is important in vascular dysfunction and vascular smooth muscle may play an important role. A mechanism whereby VSMCs play a role in vascular dysfunction is demonstrated in this thesis. In addition to the effect maternal fat diet had on endothelial dysfunction, a maternal high fat diet, irrespective of fat type was shown to cause dysregulation in VSMCs PUFA biosynthesis *de-novo* and increased sensitivity and vasoconstriction in response to α1-adrenergic receptor activation. These observations show that a maternal high fat diet leads to changes in offspring VSMCs which could result in CVD. Therefore, the role of vascular smooth muscle and its role within vascular dysfunction and CVD need to be better understood to allow the development of better therapies for treating it.

VSMCs and Eicosanoids:

Changes in eicosanoid production from the endothelium have been demonstrated to play a role in increased vasoconstriction. TXA_2, PGH_2, $PGF_{2\alpha}$, PGE_2, PGD_2 and PGI_2 have all been shown to stimulate the TP receptor and cause vasoconstriction (33). Most studies have explored changes in production of eicosanoids and other vasoactive factors from the endothelium in arterial dysfunction (292). However, the function of eicosanoids synthesised in the vascular smooth muscle is less understood and very little is known about the role this may play in vascular dysfunction and may be responsible for the changes in vasoconstriction observed in offspring of dams fed a 21% fat diet shown in this thesis. It has been shown that VSMCs are capable of eicosanoid synthesis, as COX-1 and COX-2 are both expressed in VSMCs (321;336). The ability for VSMCs to synthesise eicosanoids has been demonstrated in this thesis, as inhibition of the PUFA synthesis pathway decreased eicosanoid production. Thus eicosanoids synthesised in the VSMCs may also be involved in the regulation of vascular tone, possibly acting in a paracrine manner. One possible explanation for the induction of altered vascular function by dietary fat is changes to the fatty acid composition of cells in the artery wall leading to altered production of lipid mediators including eicosanoids.

The findings of the present study show that differences in the fat content of the maternal diet induced changes in the fatty acid composition of aortae in the adult offspring. The data demonstrated that a high maternal fat intake decreased the proportion of AA and DHA in the aortae of the offspring irrespective of the type of fat consumed by the dams. This is consistent with the findings of Ghosh et al. which showed that a maternal high saturated fat diet decreased the proportions of AA and DHA in aortae and they also exhibited blunted endothelium-dependent relaxation induced by ACh in rats (8). The present study extends these observations by showing that the amount of dietary fat rather than the fatty acid composition of the diet was the main factor in inducing reduced AA and DHA in the aorta. Since the offspring in the different maternal dietary groups were fed the same diet from weaning, one possible explanation for persistent changes in the fatty acid composition of arteries is that the maternal diet induced changes in fatty acid metabolism. Comparison of the fatty acid composition of the plasma, from which fatty acids in blood vessels are generally considered to be derived, with aorta fatty acid composition showed decreases in the proportions of AA plasma TAG in offspring of dams fed a 21% diet, but there were no differences in the proportions of DHA in plasma TAG or NEFA. Therefore, the difference in aorta fatty acid composition of the aorta in offspring of dams fed a 21% diet is not due to altered supply of

fatty acid from the blood but potentially due to the release of AA and DHA for the synthesis of eicosanoids in the VSMCs.

6.2 Polyunsaturated fatty acid biosynthesis in arterial cells:

Since there was a discrepancy between the induced changes in the fatty acid composition of plasma, which suggested impaired hepatic PUFA biosynthesis, and those in aorta, the PUFA biosynthesis pathway was investigated in isolated aortae. Previous studies have shown the PUFA biosynthesis pathway to be active in arterial endothelial (262) and smooth muscle cells (223). These studies showed the activity of delta-5 and delta-6 desaturase within these cells. However, the biological role of PUFA biosynthesis in these cells has not been reported. The offspring of dams fed a 21% diet had a higher expression of Fads1 and a lower expression of Fads2 in the aorta compared to the offspring of dams fed a 7% diet (282). The increase in Fads1 expression would result in an increase in AA synthesis and thus an increase in pro-constriction eicosanoids, which is consistent with the increased vasoconstriction in response to Pe. As Fads2 expression is down regulated, this suggests that PUFA metabolism in response to 21% fat is dysregulated. This implies dysregulation of the PUFA biosynthesis pathway at the level of the transcription of these genes. This also suggests that synthesis of AA in the vascular wall is involved in vasoconstriction and that as there was no increase in AA proportions in the offspring aorta of dams fed 21% fat diets, this suggests that PUFA synthesised in the vascular wall is channelled into eicosanoids.

PUFA biosynthesis in VSMCs:

The findings of the present study suggest that, in addition to the role of maintaining the fatty acid composition of cell membranes, PUFA biosynthesis plays a fundamental role in the regulation of vascular tone. Inhibition of delta-5 and delta-6 desaturase enzymes reduced vasoconstriction in response to Pe, in rat aortae and mesenteric arteries, and in human femoral arteries by approximately 50%. These findings suggest that the products of the PUFA biosynthesis, such as AA, are linked directly with the pathway by which α_1-adrenergic receptor signalling induces vasoconstriction. Furthermore, such association requires newly synthesised PUFA rather than PUFA obtained preformed from plasma or cellular pools. Since the effect of the desaturase inhibitors was not altered significantly by removal of the arterial endothelium, these findings suggest that the PUFA biosynthesis activity that is involved in vasoconstriction is present in VSMCs rather than in the endothelium. This is consistent with the report that PUFA biosynthesis activity is present in vascular smooth muscle cells (223). Although PUFA biosynthesis has also been detected in vascular endothelial cells (262), the present findings

suggest that either this activity is substantially lower than in VSMCs and/or that it is involved in other biological processes other than α_1-adrenergic receptor-mediated vasoconstriction.

PUFA biosynthesis has been shown in a small number of previous studies to be involved in cellular functions other than just supplying PUFA for membrane synthesis. Specifically, some reports have suggested that PUFA biosynthesis is involved in macrophage function as they have been shown to synthesize and secrete a large number of eicosanoids derived from AA. The details of the mechanism have not been elucidated (337).

One possible mechanism by which PUFA biosynthesis could contribute to the regulation of vasoconstriction could be modulating the availability of substrates for eicosanoid biosynthesis. Treatment of rat aorta *in-vitro* with the delta-6 desaturase inhibitor SC-26196 significantly reduced the production of the pro-constriction eicosanoids PGF_{2a}, PGE_2 and TXA_2 (282). One possible explanation is that newly synthesised AA is required for the synthesis of these specific eicosanoids, possibly as the result of a form of metabolic channelling of AA synthesized *de-novo* into the COX pathway, which is regulated separately from eicosanoid production from the bulk of the cellular AA. However, to date there is no direct evidence to support this suggestion.

6.3 Polyunsaturated fatty acid biosynthesis is involved in regulating intracellular calcium release:

Previous studies have focussed on activities of delta-6 and delta-5 desaturases (223). Further characterisation of the mRNA expression of the four key genes involved in PUFA biosynthesis showed that only Fads1, Fads2 and Elovl5 are expressed in VSMCs from mice, rats and humans and that Elovl2 is not expressed in VSMCs. One possible interpretation is that the pathway is truncated after the reaction that converts AA (20:4n-6) to 22:4n-6 and EPA (20:5n-3) to 22:5n-4. Thus 24:4n-6 and 24:5n-5 and their downstream metabolites are not synthesised. However, treating VSMCs with LA or ALA showed that VSMCs are able to convert the 18 carbon essential fatty acids into 24:4n-6 and 24:5n-3 respectively. This suggests that the activity of another elongase, such as Elongase 5 is involved. The activities of Elovl5 and Elovl2 have been shown to overlap in liver (225), although Elovl5 has lower activity against 22 carbon fatty acids than 20 carbon fatty acids (228). Furthermore, Elovl2 activity has been shown to be required for PUFA biosynthesis in liver (227) and in sperm (252). Thus it is unclear whether the elongation of 22 carbon PUFA in VSMCs involves Elongase5 instead of Elongase2, or whether another elongase is involved. Furthermore, it is not possible to speculate from current knowledge (320) why Elongase2 is suitable for catalysing the elongation of 22 carbon PUFA in liver, but not in VSMCs,

although such silencing was found in two rodent species and in humans in this study. This suggests selection against Elongase-2 activity in VSMCs, although the present findings do not support methylation of the Elovl2 promoter as the silencing mechanism. Overall, the PUFA biosynthesis pathway in VSMCs may differ from other tissues, such as the liver, which may be consistent with their role in the production of vasoactive eicosanoids.

6.4 A model for polyunsaturated fatty acid biosynthesis in vasoconstriction in vascular smooth muscle cells:

Inhibition of delta-5 or delta-6 desaturase activity resulted in abolition of intracellular calcium concentrations following stimulation by Pe. This suggests that PUFA biosynthesis *de-novo* is required for calcium release from intracellular stores and, in turn, vasoconstriction. As in isolated rat aortae, inhibition of delta-5 and delta-6 desaturase also decreased pro-constriction eicosanoid production (282). Thus, one possible mechanism by which PUFA biosynthesis *de-novo* may contribute to Pe mediated calcium release is by providing substrates for the synthesis of specific eicosanoids.

The findings of the present study suggest the following model for the role of PUFA biosynthesis in α_1-adrenergic receptor activation in vascular smooth muscle. One possible aspect of the mechanism could involve activation of PLA_2 in response to Pe stimulation leading to the release of AA from membrane phospholipids (230), followed by synthesis of pro-constriction eicosanoids, TP receptor activation and release of calcium from calcium stores via the activity of DAG/IP3. In turn, the increase in cellular free calcium would activate MLCK kinase thus leading to vasoconstriction (32) as shown in fig 6.2.

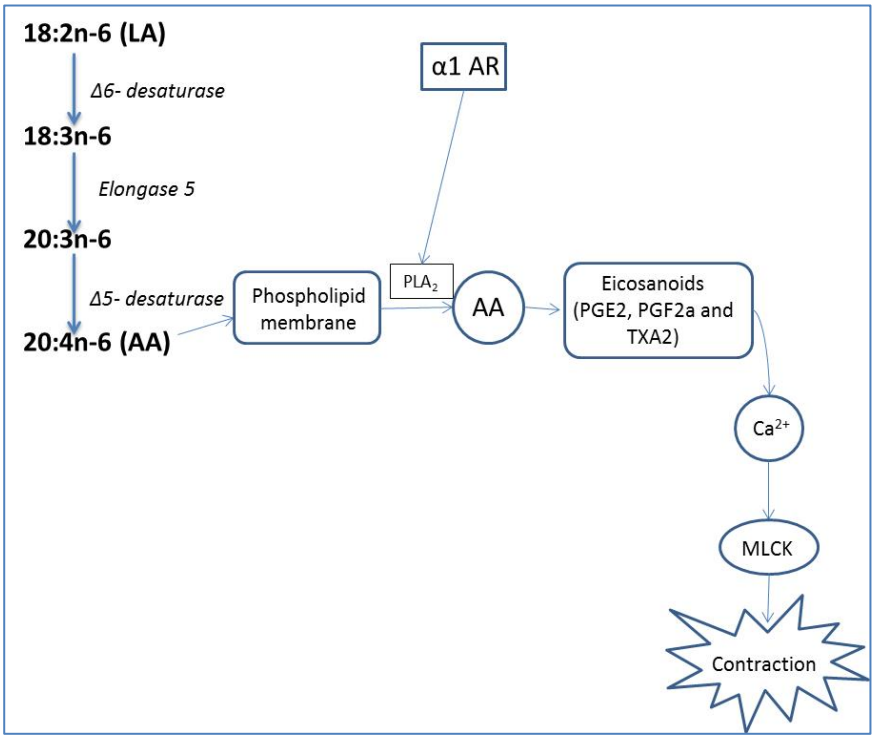


Figure 6.2: Model showing the interaction between PUFA biosynthesis *de-novo* and the interaction with a1-adrenergic receptor stimulation. (AA: Arachidonic acid, LA: Linoleic acid, PLA_2: Phospholipase A2, α1 AR: α_1-adrenergic receptor, PGE_2: Prostaglandin E2, PGF_{2a}: Prostaglandin F_{2a}, TXA_2: Thromboxane A_2, Ca^{2+}: Calcium, MLCK: Myosing light chain kinase.)

This model does not explain how newly synthesised AA specifically is channelled into eicosanoid synthesis and therefore calcium release, as this would require PLA_2 activity that differentiates between AA synthesised *de-novo* and the main pool of AA in the cell membrane. It is currently unknown how PLA_2 activity differentiates between AA synthesised *de-novo* and bulk membrane AA, this current study does not show how this happens. If PLA_2 does select newly synthesised AA it explains how inhibition of the PUFA biosynthesis pathway leads to both a decrease in eicosanoid release and a decrease in calcium release as shown in the data from this study.

AA directly causes calcium sensitisation:

However, inhibition of the PUFA biosynthesis pathway *de-novo* completely inhibited calcium release; the vasoconstriction of aorta and human femoral artery in the presence of the inhibitors was dramatically decreased but not completely inhibited (282). This suggests that there may be more than one mechanism controlling the vasoconstriction. As calcium release was completely inhibited by inhibition of PUFA biosynthesis, but the vasoconstriction was not completely inhibited, another mechanism may cause a contraction without the increase in

calcium. It has been shown that Pe-mediated increase in non-membrane AA causes calcium sensitisation (338). Calcium sensitisation occurs when myosin phosphatase is inhibited, thus preventing the dephosphorylation of MLCK (339). This causes an increase in contraction without an increase in calcium release (340). Calcium sensitisation by AA has been shown to occur by a number of mechanisms. It has previously been shown that AA can act directly on MLC by directly inhibiting dephosphorylation of the MLC leading to an increase in contraction without a change in calcium concentration (339). Therefore, newly synthesised AA can be directly released into the cytoplasm and cause calcium sensitisation as shown in figure 6.3. Calcium sensitisation through AA and direct action on MLC phosphorylation and eicosanoid production from newly synthesised AA may act synergistically to produce PUFA biosynthesis induced vasoconstriction.

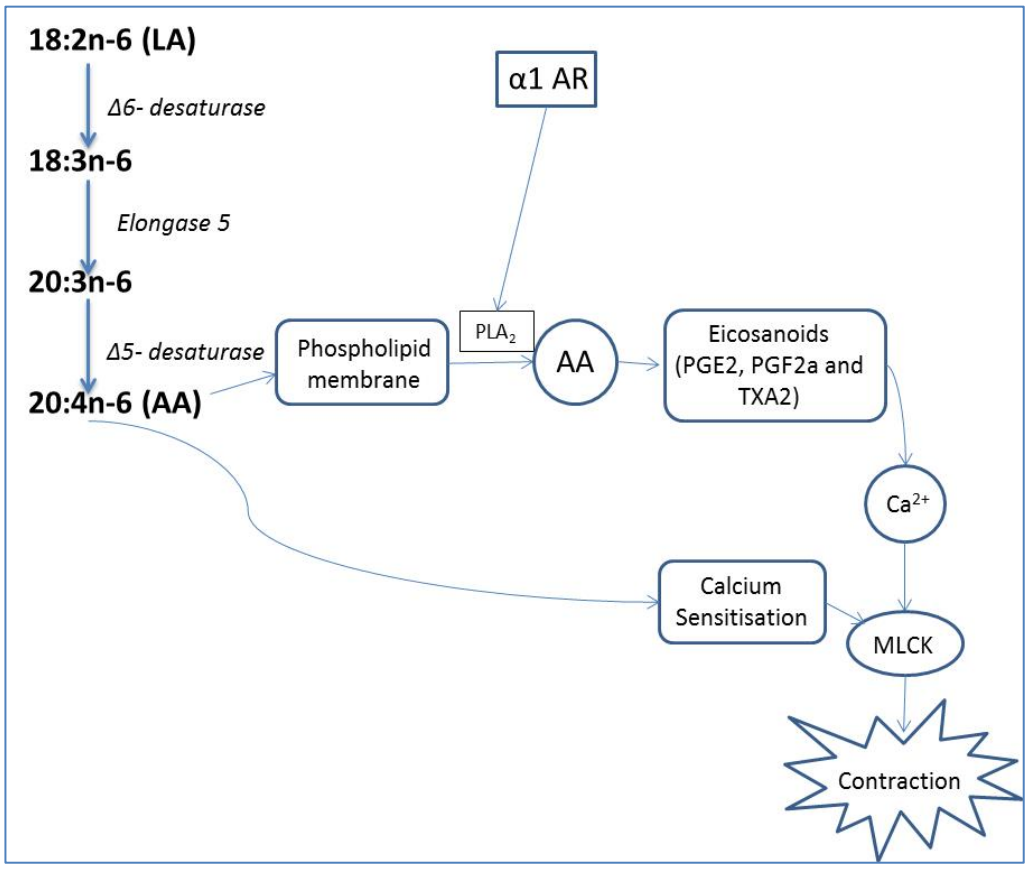


Figure 6.3: Model showing the interaction between PUFA biosynthesis *de-novo* and the interaction with a1-adrenergic receptor stimulation involving AA mediated calcium sensitisation. (AA: Arachidonic acid, LA: Linoleic acid, PLA_2: Phospholipase A2, α1 AR: α_1-adrenergic receptor, PGE_2: Prostaglandin E2, PGF_{2a}: Prostaglandin F_{2a}, TXA_2: Thromboxane A_2, Ca^{2+}: Calcium, MLCK: Myosing light chain kinase.)

Calcium sensitisation through atypical protein kinase C:

However, calcium sensitisation by AA is part of a dual mechanism. In addition to calcium sensitisation through the direct action of AA on the MLC it has also been shown that AA causes calcium sensitisation through activation of atypical protein kinase C (aPKCζ), that is not activated by DAG or calcium. This mechanism of calcium sensitisation has been shown to happen through a PLA_2-AA-aPKC pathway (341). This could be the mechanism which accounts for the residual vasoconstriction following inhibition of PUFA biosynthesis, as Pe may stimulate AA release from the cell membrane and cause vasoconstriction without the release of calcium, as seen in the data from this study, shown in fig 6.4.

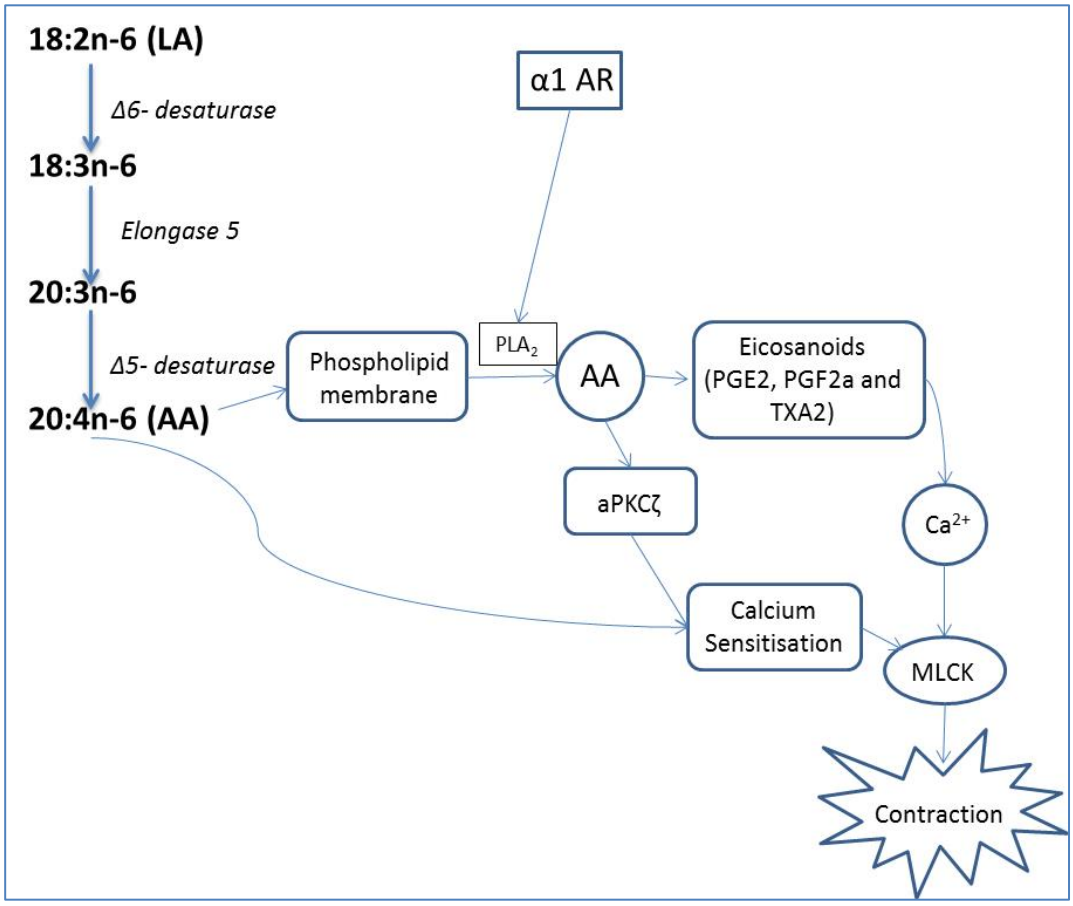


Figure 6.4: Model showing the interaction between PUFA biosynthesis *de-novo* and the interaction with a1-adrenergic receptor stimulation involving AA mediated calcium sensitisation and aPKCζ mediated calcium sensitisation. (AA: Arachidonic acid, LA: Linoleic acid, PLA_2: Phospholipase A2, α1 AR: α_1-adrenergic receptor, PGE_2: Prostaglandin E2, PGF_{2a}: Prostaglandin F_{2a}, TXA_2: Thromboxane A_2, Ca^{2+}: Calcium, MLCK: Myosing light chain kinase, aPKCζ: atypical protein kinase Cζ.)

Calcium sensitisation through novel and conventional PKCs:

In addition, to these mechanisms, calcium sensitisation can happen through a third mechanism. The increase in calcium and DAG as a result of AA and eicosanoid action could also lead to activation of novel and conventional PKCs (nPKC and cPKC) which also promote calcium sensitisation and maintenance of vasoconstriction, although this is a minor pathway in vascular smooth muscle cells and a secondary pathway, as it requires release of calcium and DAG (341), shown in figure 6.5.

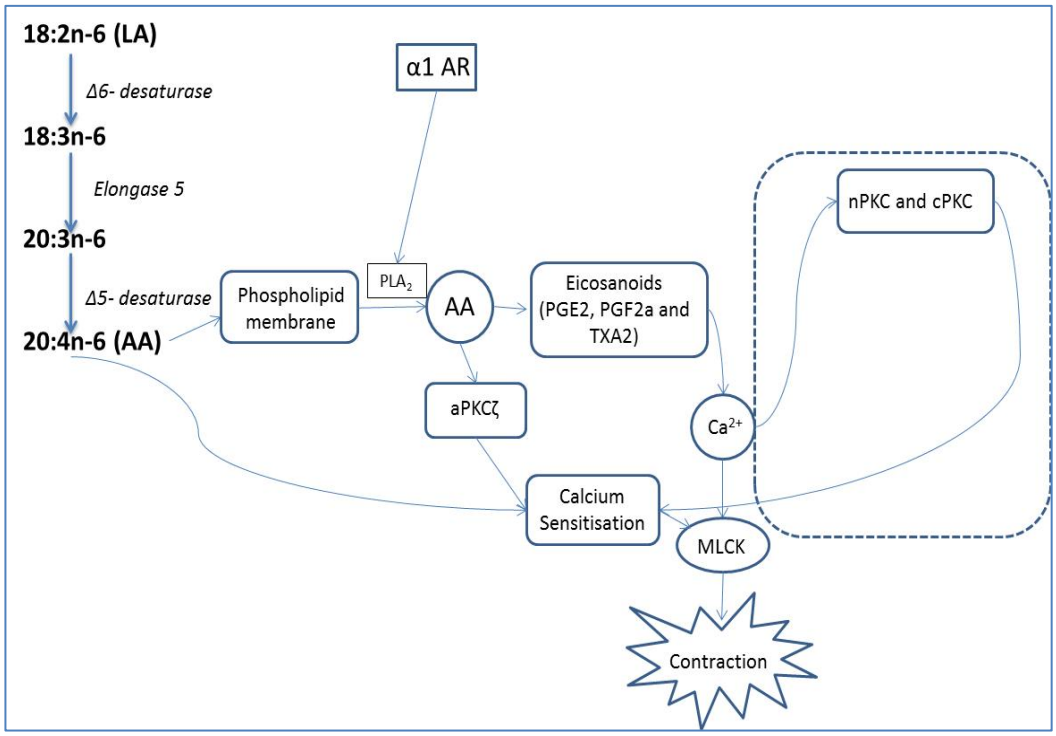


Figure 6.5: Model showing the interaction between PUFA biosynthesis *de-novo* and the interaction with a1-adrenergic receptor stimulation involving AA mediated calcium sensitisation and aPKCζ mediated calcium sensitisation, with the minor role of nPKC and cPKC activity. (AA: Arachidonic acid, LA: Linoleic acid, PLA_2: Phospholipase A2, α1 AR: α_1-adrenergic receptor, PGE_2: Prostaglandin E2, PGF_{2a}: Prostaglandin F_{2a}, TXA_2: Thromboxane A_2, Ca^{2+}: Calcium, MLCK: Myosing light chain kinase, aPKCζ: atypical protein kinase Cζ, nPKC: novel protein kinase C and cPKC: conventional protein kinase C.)

6.5 The role of maternal diet on offspring VSMC PUFA biosynthesis and vasoconstriction:

In the context of the data from the present study, the offspring of dams fed a 21% diet experienced increased vasoconstriction and increased sensitivity in response to Pe, they also had an increase in fads1 expression (282) compared to offspring of 7% dams. This would result

in an increase in AA production in these animals through PUFA biosynthesis *de-novo*. Upon stimulation of the α_1-adrenergic receptor by Pe, there would be a greater release of AA and activity of the pathway shown in fig 6.4, compared to the offspring of dams fed a 7% diet. This would lead to greater vasoconstriction and also a greater sensitivity to Pe stimulation which was observed in the study. The aorta composition data showed that the offspring of dams fed 21% fat diets had a lower proportion of AA. This is because the AA is being channelled into eicosanoid synthesis for vasoconstriction rather than being stored in the cell membrane.

This proposed model is also consistent with the PUFA biosynthesis *de-novo* inhibition experiments. Inhibition of the pathway caused a decrease in eicosanoid and calcium production in response to Pe, suggesting that newly synthesised AA is needed for the production of eicosanoids. Inhibition of eicosanoid production would lead to the inhibition of calcium release, as observed. However, as previously mentioned, inhibition of the PUFA biosynthesis *de-novo* pathway did not completely inhibit vasoconstriction; the remaining vasoconstriction could represent calcium sensitisation through aPKC.

The data from this thesis support the hypothesis that variations in quality and quantity of maternal fat intake before and during pregnancy cause persistent changes in membrane composition and vascular function in the offspring. These persistent changes are due to changes in PUFA biosynthesis de-novo in VSMCs. It has also been possible to suggest a potential mechanism whereby changes in PUFA biosynthesis *de-novo* may be responsible for the changes in vascular function observed.

6.6 Possible experiments to further develop this work:

The model in this thesis is a potential mechanism and further experiments need to be carried out to confirm it. To assess whether PLA_2 is needed for the effect of newly synthesised PUFA on calcium release, knocking down PLA_2 in MOVAS cells by siRNA and calcium release measurements could be carried out. Alternatively, PLA_2 could be inhibited and the vessel treated with Pe to confirm whether PLA_2 inhibition causes inhibition of vasoconstriction. In addition to this, eicosanoid production measurements could also be carried out to confirm the role of PLA_2 in eicosanoid production. To assess whether newly synthesised AA is used for eicosanoid synthesis, MOVAS cells could be treated with [U-^{13}]-LA as demonstrated in Chapter 4 of this thesis. Enrichment of the eicosanoids with isotope can be determined following Pe stimulation. High levels of enrichment would confirm that the eicosanoids are from newly synthesised PUFA. Alternatively, to assess whether newly synthesised PUFA are needed for eicosanoid synthesis, MOVAS cells could be treated with the Fads1 and Fads2 inhibitors. The

cells could then be treated with AA and stimulated with Pe. The production of eicosanoids could be measured using the ELISA kits used in chapter 5. If eicosanoid production is inhibited, it would confirm the need for newly synthesised PUFAs for eicosanoid production. To confirm the presence of calcium sensitisation within the vasoconstriction following Pe stimulation, MLCK phosphorylation needs to be measured. If there was an increase in MLCK phosphorylation in the MOVAS cells following Pe stimulation calcium sensitisation would be confirmed.

6.7 Vascular smooth muscle PUFA biosynthesis *de-novo* and CVD:

Polymorphisms of Fads1 and Fads2:

Previous reports have shown an association between polymorphisms in Fads1 and Fads2 and an increased risk of developing CVD. One study showed that polymorphisms associated with a reduction in the activity of delta-5 and delta-6 desaturase led to a reduction in the risk of CHD (267). These studies are therefore consistent with the data from this thesis, showing that changes in the PUFA biosynthesis pathway that result in an increase in AA production, increase the risk of CVD. However, both of these studies assume that the underlying mechanism for the polymorphisms and increased risk of CVD is dependent on impaired hepatic PUFA biosynthesis and supply to blood vessels. However, from the findings from this thesis an alternative explanation can be proposed. Polymorphisms which result in changes in Fads expression cause alterations in PUFA biosynthesis *de-novo* in the VSMCs which results in impaired local control of vascular tone, such as increased vasoconstriction. Another recent study which is consistent with our findings has shown that inhibition of delta-6 desaturase results in attenuation of contractile dysfunction in mice. In addition to this they also show that inhibition leads to attenuation of cardiac hypertrophy in aged mice. This shows that inhibition of the PUFA-biosynthesis pathway with delta-6 desaturase has a number of potently protective effects on the aged heart (342). Therefore, the information from this thesis along with the information from the aforementioned studies suggests that delta-5 and delta-6 desaturase inhibitors could be a potential therapeutic agent for the prevention or treatment of cardiovascular disease.

Knockout models:

A Fads2 or Fads1 knockout mouse model could be developed. Using this knockout model the vascular reactivity of the aorta and mesenteric arteries could be explored to confirm the findings from this study and show that changes to PUFA biosynthesis *de-novo* does have a detrimental effect on vasoconstriction of these arteries. Blood pressure could also be measured to see if changes in vascular reactivity have an effect on the blood pressure of these

animals. As a lot of the work involving the role of PUFA biosynthesis *de-novo* and its role in vasoconstriction has been done in cells it would be useful to investigate it in animals in a whole body system. It would provide more information about the effects of polymorphisms within these genes. As shown in a previous study, inhibition of delta-6 desaturase can also cause hypertrophy attenuation (342). Therefore, using an animal model will provide a way of specifically eliminating these genes to gain a greater insight into PUFA biosynthesis within vascular smooth muscle cells and vasoconstriction.

6.8 Implications for future therapeutic targets:

Increased vasoconstriction is a causal process in a number of pathological conditions including hypertension. This thesis has provided a novel pathway in which VSMC PUFA biosynthesis *de-novo* is involved in increased vasoconstriction following α1-adrenergic receptor stimulation. Therefore, the data from this thesis could provide evidence for the development of future therapeutic targets. One potential therapeutic target could involve targeting the PUFA biosynthesis pathway and inhibiting of delta-5 desaturase. This would result in a decrease in AA production and thus a decrease in eicosanoid production, resulting in a reduction in vasoconstriction due to a reduction in pro-constrictor eicosanoids. As shown in this thesis, inhibition of delta-5 desaturase led to a decrease in calcium release and eicosanoid release, so would lead to a decrease in vasoconstriction. This would need to be a targeted therapy to VSMCs and not cause total inhibition of AA production as complete depletion of AA would cause a number of biological problems throughout the body (247). Another potential therapeutic target is to inhibit calcium sensitisation. Impaired regulation of calcium sensitisation has been shown to play a causal role in a number of vascular disorders (343). Calcium sensitisation is part of the potential mechanism shown in figure 6.5, whereby AA release leads to calcium sensitisation either directly or through aPKCζ activation and leads to increased vasoconstriction. Inhibiting calcium sensitisation would inhibit the increased vasoconstriction in VSMCs caused by increased AA production as a result of dysregulation in PUFA biosynthesis pathway and therefore provide a therapy for vascular disease.

6.9 Limitations of thesis:

Animal data:

There were a number of limitations of this study. Parts of the animal work shown in this thesis were carried out by two investigators. Although training was carried out to ensure consistency of the performance of experiments and the observations between the two investigators, there may still be inter-observer variability which could confound the comparisons between the

dietary groups. Analysis of all myography and blood pressure traces were carried out by the same person to ensure consistency and to stop differences in the subjectivity of the analysis. Therefore, lengths were taken to prevent any variability in the data caused by different investigators but this may still have had an effect. Where possible the inter-observer variability was factored into the statistical analysis and did not have an effect on the significance or interpretation of the data. Another limitation of the animal work, was that due to the size of the study, including; the number of measurements taken, the number of animals required and the number of dietary groups, it was not possible to run the whole study at the same time. Therefore, the study had to be split into two parts; the 7% arm and the 21% arm. Mating and therefore birth were staggered, this was to ensure that myography could be carried out on the vessels at the correct time point, as myography could only be carried out on 2 animals per day due to time and equipment constraints. Other than the differences in diet intake, the animals were kept in the same environment and maintained in the same way, but there may have been some variability on the data due to seasonal fluctuations (301). Seasonal fluctuation have been shown to have an impact on rat metabolism (344). However, this was difficult to take into account due to the staggered breeding within dietary groups. Another limitation in the measurements for the animal aspect of the project was the blood pressure measurement; the tail cuff plethysmography method is not perfect and is susceptible to a number of factors affecting the readings, including stress, due to restraint and heating. Although the animals had been exposed to the restraint and heating a number of times to reduce the effect of stress, it still may have had an effect on the readings. Therefore, another method could be used in future, such as radiotelemetry which is considered a more accurate method and allows measurement in unrestrained animals (300). However, this method has its disadvantages as it is a more expensive method.

Molecular data:

In addition to the animal work, there were also some limitations with the molecular work. HASMCs would have been the preferred cell line to carry out a majority of the experiments in as they are human cells and are therefore better for translational purposes. However, unfortunately the cells were no longer suitable for experiments after passage 7; there were changes in morphology of the cells and cell death. For this reason, MOVAS cells were used for a majority of the molecular experiments as these were an immortalised cell line and were able to undergo multiple passages and still remain reliable. Some experiments were carried out in HASMC cells, including the gene expressions data, this was consistent with the MOVAS cell data and so it was decided that MOVAS cells would be representative of the human cells. A

further limitation of the data collected from molecular studies includes data obtained from the eicosanoid production measurement assays. Due to time constraints this limited the achievable sample size for each condition. The data was variable between the kits and the post-hoc power calculations show the requirement of a greater sample size for each experiment in order to provide conclusive and reliable data. Therefore, had there been more time available the assays would have been repeated a number of times. This is the same for the enrichment data as samples were sent away and the price of the tracer was so high, a limited sample size was used. To provide more significant and conclusive data, a greater sample size would be needed. Another limitation of the eicosanoid assays was that there was cross reactivity in the binding of certain eicosanoids which may have affected the results and given false readings, this is a limitation of the assay. Using mass spectrometry to measure the eicosanoid production would provide more reliable data and provide a more exact determination of the specific eicosanoids being measured. However, the use of this technology was not possible during this study. In addition to this, it would have also been useful to measure the eicosanoid production in the offspring rat aorta. As changes in vasoconstriction were observed, measuring the eicosanoid production would have allowed determination of whether the increased vasoconstriction was due to changes in eicosanoid production and would have provided more conclusive information. However, the changes in fatty acid content were not measured until after the study and the myography data also was not analysed until after the study was complete, so it was not possible to collect the supernatant for eicosanoid production analysis.

Chapter 7-

Glossary

7. Glossary:

Autocrine: When a hormone or chemical messenger is secreted and binds to autocrine receptor on the same cell leading to changes in the cell.

Cardiovascular disease: A range of diseases of the heart and blood vessels, including coronary heart disease and peripheral vascular disease.

Caveolae: Flask-shaped invaginations in the plasma membrane

EC50: The concentration of agonist that produces 50% of the maximal response.

Eicosanoid: Signalling molecules produced through the oxidative pathway from 20-carbon fatty acids. These include prostaglandins, thromboxanes and leukotrienes.

Endothelial dysfunction: A shift from normal endothelial function towards a reduced vasodilation, pro-inflammatory and pro-thrombotic state.

Endothelium: Monolayer of endothelial cells that form the inner wall of a blood vessel, they are the in direct contact with circulating blood.

Epigenetics: Chemical alterations to DNA or histone proteins which lead to heritable changes in the structure of the chromatin and alter the readability of the DNA, without altering the DNA sequence.

Essential fatty acids: A fatty acid that cannot be synthesised by the body and must be obtained from dietary sources.

Fatty acids: Hydrocarbon chains with a methyl group at one end and a carboxylic acid group at the other.

Hypertension: Elevated blood pressure

Hypertrophy: An increase in the volume of an organ or tissue due to enlargement of its cells.

Lignans: A group of chemical compounds found in plants

DNA Methylation: An epigenetic process where a methyl group is added to the 5-carbon of the cytosine ring

Monounsaturated fatty acids: Fatty acids containing one double bond

Non esterified fatty acids: A free fatty acid and not esterified with glycerol

Paracrine: When a hormone or chemical messenger is released and binds to a nearby cell and affects their function

pEC50: The negative logarithm of the EC50

Polyunsaturated fatty acids: Fatty acids that contains more than one double bond

Saturated fatty acids: Fatty acid containing no double bonds

Single nucleotide polymorphism (SNP): A genetic variation of one single nucleotide in a DNA sequence

Trans fatty acids: Unsaturated fatty acids where the hydrogen atoms are on opposite sides of the double bond

Vasoconstriction: Narrowing of the blood vessels resulting from smooth muscle contraction of the blood vessel.

Vasodilation: The widening of blood vessels resulting from smooth muscle relaxation.

Chapter 8-

Reference List

8. Reference List

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Chapter 9-

Appendices

9. Appendices:

Table: 9. 1: Kits, Chemicals and Reagents used:

Reagents/Chemicals	Supplier
12(S)-HETE ELISA Kit	Abcam, Cambridge, UK
Acetylcholine chloride	Sigma Aldrich, Dorset, UK
Agarose Technical Grade	Melford, Suffolk, UK
Amplification Grade Deoxyribonuclease I (DNase I) Kit	Sigma-Aldrich, Dorset, UK
Blue Juice loading buffer	Life Technologies, Paisley, UK
Butylated hydroxytoluene	Sigma Aldrich, Dorset, UK
Calcium Chloride	Sigma Aldrich, Dorset, UK
Chloroform	Fisher Scientific, Leicestershire, UK
Deoxynucleotide (dNTPs) set (dATP, dCTP, dGTP, and dTTP)	Sigma-Aldrich, Dorset, UK
D-glucose	Sigma Aldrich, Dorset, UK
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich, Dorset, UK
DMEM (containing high glucose and pyruvate)	Life Technologies, Paisley, UK
EDTA	Sigma Aldrich, Dorset, UK
Ethanol, absolute	Fisher Scientific, Leicestershire, UK
EZ-DNA-Methylation Gold Kit	Zymo Research, CA, USA
FADS2 Pyrosequencer Primers	Biomers, Söflinger, Germany
Fatty Acid Desaturase 2 (FADS2) PCR Primers	Biomers, Söflinger, Germany

Fatty Acid Desaturase 2 (FADS2) PCR Primers	Biomers, Söflinger, Germany
G-418 Sulfate	PAA Ltd, Somerset, UK
Gel red	Biotium, CA, US
Gene Ruler 1kb DNA ladder	Thermo-Scientific, UK
Hanks buffered saline solution	PAA Ltd, Somerset, UK
HASMCs	Life Technologies, Paisley, UK
Heat Inactivated Foetal Bovine Serum	PAA Ltd, Somerset, UK
Hepa1-6 cells	European collection of cell cultures (ECACC), Porton Down, UK
Hexane	Fisher Scientific, Leicestershire, UK
Human Elongase 2	Qiagen, West Sussex, UK
Human Elongase 5	Qiagen, West Sussex, UK
Human Fatty acid desaturase 1	Qiagen, West Sussex, UK
Human Fatty acid desaturase 2	Biomers, Söflinger, Germany
Human PPIA (Cyclophillin A)	Qiagen, West Sussex, UK
Kapa Taq Hotstart DNA polymerase	Kapa Biosystems, Wilmington, US
L-Nitroarginine methyl ester	Sigma Aldrich, Dorset, UK
Magnesium Sulphate	Sigma Aldrich, Dorset, UK
Medium 231	Life Technologies, Paisley, UK
Methanol HPLC Grade	Fisher Scientific, Leicestershire, UK
mirVana miRNA isolation kit	Life Technologies, Paisley, UK
M-MLV Reverse Transcriptase kit	Promega, Southampton, UK

Mouse Elongase 2	Qiagen, West Sussex, UK
Mouse Elongase 5	Qiagen, West Sussex, UK
Mouse Fatty acid desaturase 1	Qiagen, West Sussex, UK
Mouse Fatty acid desaturase 2	Qiagen, West Sussex, UK
Mouse PPIA (Cyclophillin A)	Qiagen, West Sussex, UK
MOVAS (ATCC- CRL-2797)	LGC Standards, Middlesex, UK
NP-40 Solution	Fisher Scientific, Leicestershire, UK
Penicillin / Streptomycin	PAA Ltd, Somerset, UK
PGE2 express EIA kit	(Cayman Chemical Company, Michigan, USA
PGF2α EIA kit	Cayman Chemical Company, Michigan, USA
Phenol:chloroform	Sigma-Aldrich, Dorset, UK
Phenylepherine hydrochloride	Sigma Aldrich, Dorset, UK
Phosphate Buffered Saline Solution (PBS)	Sigma-Aldrich, Dorset, UK
Pierce BCA protein assay kit	Fisher Scientific, Leicestershire, UK
Potassium bicarbonate	Sigma Aldrich, Dorset, UK
Potassium carbonate	Sigma Aldrich, Dorset, UK
Potassium Chloride	Sigma Aldrich, Dorset, UK
Potassium dihydrogen phosphate	Sigma Aldrich, Dorset, UK
Proteinase Inhibitor Cocktail	Sigma-Aldrich, Dorset, UK
Proteinase K	Promega, Southampton, UK
Pyro Mark binding buffer	Qiagen, West Sussex, UK

Pyro Mark Gold Q96 CDT reagent	Qiagen, West Sussex, UK
Pyrosequencing primers	Biomers, Söflinger, Germany
QiAmp DNA mini kit	Qiagen, West Sussex, UK
Random Nanomers	Sigma-Aldrich, Dorset, UK
Rat Elongase 2	Qiagen, West Sussex, UK
Rat Elongase 5	Qiagen, West Sussex, UK
Rat Fatty acid desaturase 1	Qiagen, West Sussex, UK
Rat Fatty acid desaturase 2	Qiagen, West Sussex, UK
Rat PPIA (Cyclophillin A)	Qiagen, West Sussex, UK
Reagents/Chemicals	Supplier
Reference dye for quantitative PCR (ROX)	Sigma-Aldrich, Dorset, UK
RNase A	Promega, Southampton, UK
RNase and DNase free water	Fisher Scientific, Leicestershire, UK
SC-26196	R&D Systems, Abingdon, UK
SDS	Sigma Aldrich, Dorset, UK
Sesamin	Sigma-Aldrich, Dorset, UK
Smooth muscle growth supplement	Life Technologies, Paisley, UK
Sodium Acetate	Sigma Aldrich, Dorset, UK
Sodium Bicarbonate	Sigma Aldrich, Dorset, UK
Sodium Chloride	Sigma Aldrich, Dorset, UK
Sodium deoxycholate	Sigma Aldrich, Dorset, UK
Sodium nitroprusside	Sigma Aldrich, Dorset, UK
Streptavidin sepharose beads	GE Health Care, Chalfont, UK

Sulfuric acid (H2SO4)	Sigma-Aldrich, Dorset, UK
SYBR GREEN Jump Start Taq Ready Mix for Quantitative PCR (QPCR)	Sigma-Aldrich, Dorset, UK
Toluene Analytical Grade	Fisher Scientific, Leicestershire, UK
Tris-Acetate-EDTA	Fisher Scientific, Leicestershire, UK
Trizma Base	Sigma-Aldrich, Dorset, UK
Trypsin-EDTA (0.05%)	PAA Ltd, Somerset, UK
TXB2 EIA kit	Cayman Chemical Company, Michigan, USA
U44619	Sigma Aldrich, Dorset, UK

Table 9.2: TNES buffer composition:

	Volume (ml)	Final Concentration
1M Tris pH 7.5	2.5	50mM
4M NaCl	5	400mM
0.5M EDTA	10	100mM
10% SDS	2.5	0.5%
Distilled water	30	

Table 9.3: RIPA buffer composition:

	Final Conc	Amount added for 20 ml
Tris (pH 8.0)	50mM	660 µl of 1.5 M
NaCl	150mM	750 µl of 4M
NP40	1%	2 ml of 10%
Sodium deoxycholate	0.5%	1 ml of 10%
SDS	0.1%	200 µl of 10%

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