The Impact of Different Sources of Fatty Acids on the Fetal Programming of Atherosclerosis

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ABSTRACT

Maternal hypercholesterolaemia has been associated with atherosclerosis in the offspring of humans and animal models. Partially hydrogenated vegetable oil (P) and ruminant milk fat (R) contain *trans* fatty acids (TFA) that differ in isomer distribution and cause changes to cholesterol metabolism. TFAs are passed to the offspring across the placenta during fetal development and via the mother's milk. The study aimed to assess whether maternal consumption of two types of TFA diet (P and R) and a diet rich in saturated fatty acids ("Western" W diet) would differentially alter maternal lipoprotein metabolism causing changes to the offspring's lipoprotein metabolism and increasing their susceptibility to atherosclerosis in adulthood. Experimental fat diets were fed to female C57BLJ6 mice during pregnancy (PC, RC, or WC), or throughout pregnancy and lactation (PP, RR, WW). Female offspring carrying the human ApoE*3 Leiden gene (AEL) were weaned onto post-natal diets for 12 weeks: (i) Chow (CCC, PCC, RCC); (ii) Atherogenic (CCA, PCA, PPA, RCA, RRA, WCA, WW); (iii) or remained on their dams' allocated fat diet (PPP, RRR or WWW). Maternal and offspring serum lipoprotein concentrations were measured, and offspring atherosclerosis assessed by lipid staining in cross sections of aorta. At day 17 gestation, dams consuming P diet had increased serum total cholesterol and triacylglycerol concentrations compared to R dams. Dams that had consumed P or R during pregnancy and C during lactation had similar serum cholesterol concentrations. However, continuing the fat diet throughout lactation caused R dams to have significantly greater serum cholesterol compared to P, W, and C dams. Dams consuming P and R had diet specific *trans* isomers in their adipose tissue, indicating the developing fetus and neonate were exposed to different TFA isomers. Maternal TFA consumption during pregnancy appeared to protect offspring from atherosclerosis in later life, irrespective of isomeric distribution of the TFA, however this effect was lost if the TFA diet was continued to be fed during lactation and early development periods. In conclusion, maternal consumption of TFA and SFA diets did not increase susceptibility of offspring to atherosclerosis. Both R and P TFA diets during pregnancy had an athero-protective effect. There was no effect of maternal W diet on offspring atherosclerosis.

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PUBLICATIONS

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COMMONLY USED ABBREVIATIONS

A	Atherogenic post-natal diet
AA	Arachidonic Acid
ABCA1	ATP Binding Cassette Transporter A1
AEL	ApoE*3 Leiden (in context to topic, mouse, or gene)
ALA	α-Linoleic Acid
ApoLP	Apolipoprotein
ASPA	The Animal (Scientific Procedures) Act 1986
C57	C57(Black)J6 Wild Type Mouse
C	Chow diet
CCA	Chow Pregnancy diet / Chow lactation diet / Atherogenic post-natal diet
CCC	Chow Pregnancy diet / Chow lactation diet / Chow post-natal diet
CoA	Co-enzyme A
CE	Cholesteryl Ester
Chol	Cholesterol
CLA	Conjugated Linoleic Acid
CRP	C-reactive protein
CVD	Cardiovascular disease
DHA	Docosahexaenoic Acid
EA	Elaidic Acid *trans 9*
EC	Endothelial Cell
ECM	Extracellular Matrix
EPA	Eicosapentaenoic acid
FA	Fatty Acid
FAME	Fatty Acid Methyl Ester
FFAs	Free Fatty Acids (or Non-esterified fatty acids)
HDL	High Density Lipoprotein
ICAM	Intercellular Adhesion Molecule-1
IDL	Intermediate Density Lipoprotein
IL	Interleukin
IUGR	Intrauterine growth restriction
LA	Linoleic Acid
LC	Long Chain Fatty Acid
LDL	Low Density Lipoprotein
LDLr	LDL Receptor
LP	Lipoprotein
MLP	Maternal Low Protein diet
MUFA	Mono-unsaturated Fatty Acid
NAFLD	Non-alcoholic fatty acid liver disease
NCD	Non-Communicable Diseases
NDNS	National Diet and Nutrition Survey 2008-2012

OA	Oleic Acid *cis* 9
P or PHVO	Partially Hydrogenated Vegetable Oil diet
PCA	PHVO Pregnancy diet / Chow Lactation diet / Atherogenic post-natal diet.
PCC	PHVO Pregnancy diet / Chow Lactation diet / Chow post-natal diet
PPA	PHVO Pregnancy diet / PHVO Lactation diet / Atherogenic post-natal diet
PPP	PHVO Pregnancy diet / PHVO Lactation diet / PHVO post-natal diet
PPARγ	Peroxisome proliferation activated receptor-γ
PPL	Phospholipid
PUFA	Poly-Unsaturated Fatty Acid
RA	Rumenic Acid
R or RTFA	Ruminant Trans Fatty Acid diet
RCA	RTFA pregnancy diet / Chow lactation diet / Atherogenic post-natal diet
RCC	RTFA pregnancy diet / Chow lactation diet / chow post-natal diet
RRA	RTFA pregnancy diet / RTFA lactation diet / Atherogenic post-natal diet
RRR	RTFA pregnancy diet / RTFA lactation diet / RTFA post-natal diet
SFA	Saturated Fatty Acid
SMC	Smooth Muscle Cell
TAG	Triglyceride
TFA	*Trans* Fatty Acid
VA	Vaccenic Acid *trans* 11
VCAM-1	Vascular Cellular Adhesion Molecule-1
VLDL	Very Low Density Lipoprotein
W or Western	'Western style' diet which is high in saturated and contains mono and poly-unsaturated fatty acids
WCA	Western pregnancy diet / Chow lactation diet / Atherogenic post-natal diet
WWA	Western pregnancy diet / Western lactation diet / Atherogenic post-natal diet
WWW	Western pregnancy diet / Western lactation diet / Western post-natal diet

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CHAPTER 1 INTRODUCTION

1. GENERAL INTRODUCTION

The fetal and developmental programming of atherosclerosis forms the basis of this thesis. The following paragraphs of the introduction outline the impact of nutrition on the health of populations and their link to the high rate of death through cardiovascular diseases such as atherosclerosis (paragraph 1.1). The aetiology and physiology of atherosclerosis (paragraph 1.3) is described followed by the impact of different sources of dietary fats on human lipid metabolism (paragraph 1.4) and the cholesterol-atherosclerosis link (paragraph 1.5). These paragraphs set the scene of how different dietary fats impact on lipid metabolism and atherosclerosis. It goes onto review the origins of fetal and developmental programming in both humans and animal models of disease giving examples of maternal under nutrition and over nutrition, with note to the impact of different maternal dietary fats on the fetal programming of atherosclerosis.

1.1 NATIONAL AND GLOBAL HEALTH INITIATIVESAND RECOMMENDATIONS

An unhealthy diet and lifestyle choice can cause serious health problems such as obesity, type 2 diabetes and cardiovascular diseases which together increase the possibility of premature mortality worldwide (WHO, 2013a, WHO, 2015). In the following paragraphs, global and national (UK) nutrition and health initiatives are described in order to understand the ever-increasing rise in premature mortality from cardiovascular diseases (CVDs) and their risk factors. Thereafter it outlines the UK government's strategy to educate and empower its population into making healthier choices and changing their lifestyles to try and resolve these endemic health problems (DH, 2014b, DH, 2015, PHE, 2010). It is recognised an excess of energy provided by a variety of nutrients such as added sugars, saturated fat and salt all contribute to health problems throughout an individual's lifespan.

1.1.1. Global Health Initiatives for Action on Non-Communicable Diseases and Premature Mortality

The World Health Organisation (WHO) reports that over 36 million people die from non-communicable diseases (NCD) annually; including 14 million who die young (aged 30-70). NCDs include CVDs, chronic respiratory diseases, diabetes, and cancers. CVDs, including conditions such as coronary heart disease (CHD) and cerebrovascular disease, are a major cause of death globally. In 2008, the WHO reported that by 2030, 23 million people worldwide will die annually from CVDs (WHO, 2008) with populations from developing, low or middle income countries seeing a rise in CVD related deaths. In 2012 an estimated 17.5m (31%) deaths worldwide were caused by CVD related diseases (WHO, 2015). The WHO are seeking to prevent and control CVDs worldwide, working through local governments and their public health initiatives, to educate populations in the management of their own CVD risk. Inherent CVD risk factors include genetics, age, gender, and ethnicity. It is recognised that prevalence of CVDs are also heavily influenced by socio-economic factors such as geographic region, poverty, poor diet, physical inactivity, smoking and harmful alcohol consumption (Townsend et al., 2012b, WHO, 2013a).

The WHO Global Action Plan 2013-2020 sets out 9 voluntary targets for its member states (including the USA and UK) in order to reduce premature death from NCDs, many of which are risk factors associated with CVDs. These targets include:

1. Halting the rise in diabetes and obesity.
2. Reducing activity insufficiency by 10%.
3. Reducing salt/sodium intake by 30%.
4. Increase the number of people receiving drug therapy to reduce heart attacks and stroke by 50%.
5. Reducing and controlling raised blood pressure in populations by 25%
6. Increase availability of medical equipment and aid to treat NCDs by 80%.

7. Reducing tobacco use by population over the age of 15 years, by 10%.
8. Reducing harmful alcohol consumption by 10%
9. Reducing premature deaths from CVDs, cancer, diabetes, and respiratory disease by 25%. (WHO, 2013a)

The WHO indicated that a healthy diet and lifestyle changes are the key to achieving these targets. They set out recommendations that governments should work nationally with food manufacturers and producers to assist populations in meeting these targets. The WHO Action Plan recommendations cover all age groups of populations, but also specifically apply these recommendations to maternal, infant, and young child nutrition. Their recommendations are that policies are developed to regulate and reduce portion sizes of energy dense products, reduce salt and added sugar content of products, and replace the use of *trans* fats (TFA) and saturated fats (SFA) with unsaturated fats (UFA) - ultimately reducing the use of energy dense products and increase the availability of affordable fruit and vegetables. They state that the marketing of energy dense food and beverages to populations and particularly to children should be carefully regulated. Additionally, they suggest that governments promote and support maternal policies such as breast feeding until 2 years of age.

The WHO goes onto suggest many other voluntary policies for implementation to address the 9 factors outlined in the paragraph above for example physical activity for health – for governments to provide the infrastructure for leisure activities such as walking and cycling and addressing physical education needs in schools from infant age upwards.

1.1.2. UK Governments' Health Initiatives and Recommendations

The UK Government's Department of Health (DH) has disseminated national public health strategies through its White Paper presented at parliament in 2010 (DH, 2010) and the development of Action Plans "Living Well for Longer" (DH, 2014b, DH, 2015). The Government's white paper and subsequent action plans reflect the targets set out by the WHO for reducing preventable deaths from NCDs including CVD risks and implementing

maternal, infant and child health initiatives. In particular, the Public Health Outcomes Framework 2013-2016 sets out key areas for improvement that address reducing premature mortality from CVDs through public health improvements. For example (i) reducing the number of low birth weight term babies; (ii) implementation and support for breastfeeding initiatives and, (iii) reducing excess body weight in adults and children through diet; (DH, 2012c).

Working alongside the National Health Service and other public health bodies such as Public Health England the strategy is to influence the UK's population's behaviour through promoting healthier living habits and earlier detection of NCDs. These areas of public health are encompassed through the introduction of health initiatives including: (i) providing NHS health checks for all; (ii) Reducing salt intake; (iii) Increasing physical activity and providing infrastructure for activities; (iv) using the "Eat Well Plate" which recommends the consumption of fruit and vegetables "5 portions a day", increasing oily fish consumption; (v) reducing smoking and harmful consumption of drugs and alcohol and, (vi) effectively reducing the prevalence of obesity and type 2 diabetes (NHS., 2014, Townsend et al., 2012a, DH, 2015, DH, 2010).

1.1.3. UK Population Nutrient Intake

The Scientific Advisory Committee on Nutrition (SACN) identified that 62% adults and 28% children (2-15 years) in the UK were overweight or obese and at increased risk of heart disease, stroke, Type-2 diabetes, liver disease and cancers due to consumption of energy dense foods such as sugar, TFA and SFA. The DH and Food Standards Agency state that the greatest proportion of food energy in a daily diet is obtained from fat. Current guidelines are that on average diets should contain <35% fat with < 2% fats obtained from TFAs, and 11% SFA (DH, 2014b, DH, 2015). In 2014, the UK Government released its findings from the National Diet and Nutrition Survey (NDNS) 2008-2012 which monitored the population's average nutrient intake across the UK. The NDNS results showed that average consumption exceeded the recommended daily intakes (RDI) for added (non-milk extrinsic sugars) sugars,

SFA and salt (see table 1.1). It was noted that although overall fat consumption was normal for dietary reference values across all age groups the majority of the saturated fat consumed came from dairy and meat products. They also reported that consumption of TFAs, including those TFA found in animal products, were also in keeping with dietary recommendations (DH, 2014a). However, it is acknowledged that self-reported energy intake observations such as those obtained by the NDNS can be skewed and misreported by participants and are therefore limited in their use due to the inaccuracy of measurements (Subar et al., 2015, Ashwell et al., 2006). Subar et al (2015) noted reasons for misreported nutrient intake include participants not wanting to appear unhealthy, they have difficulty recalling food and drink consumed and have difficulty in estimating quantities of nutrients when weighing food was not carried out. Subar et al (2015) and the European Commission's report on TFAs (EC, 2015) also noted that due to the lower cost of use of PHVOs in manufactured food products and therefore lower price at point of sale, that poorer socio-economic groups would consume greater quantities of fast food or high fat manufactured foods thereby consuming higher quantities of TFAs. It was acknowledged that this demographic of the population is under-represented in the population average nutrient surveys (Suber et al, 2015, EC, 2015).

Further issues to consider is the lack of labelling of TFAs on food products sold loose e.g. bakery items and those present in ruminant meat and dairy products. Additionally, manufactured foods do not need to declare PHVO TFAs on packaging if under 1% is present (EC, 2015). It is therefore recognised that content of total TFA in products sold and therefore quantity of TFAs consumed are underestimated (EC, 2015). The variation in quantities of TFAs consumed across populations is evident from maternal dietary studies. Desci and Boehm (2013) reported that between 0.5% TFA in Germany, and up to 13.8% TFA in Canada were present in breast milk samples of nursing mothers (see paragraph 1.6.7).

Table 1.1 Abridged Results of the National Diet and Nutrition Survey 2008-2012

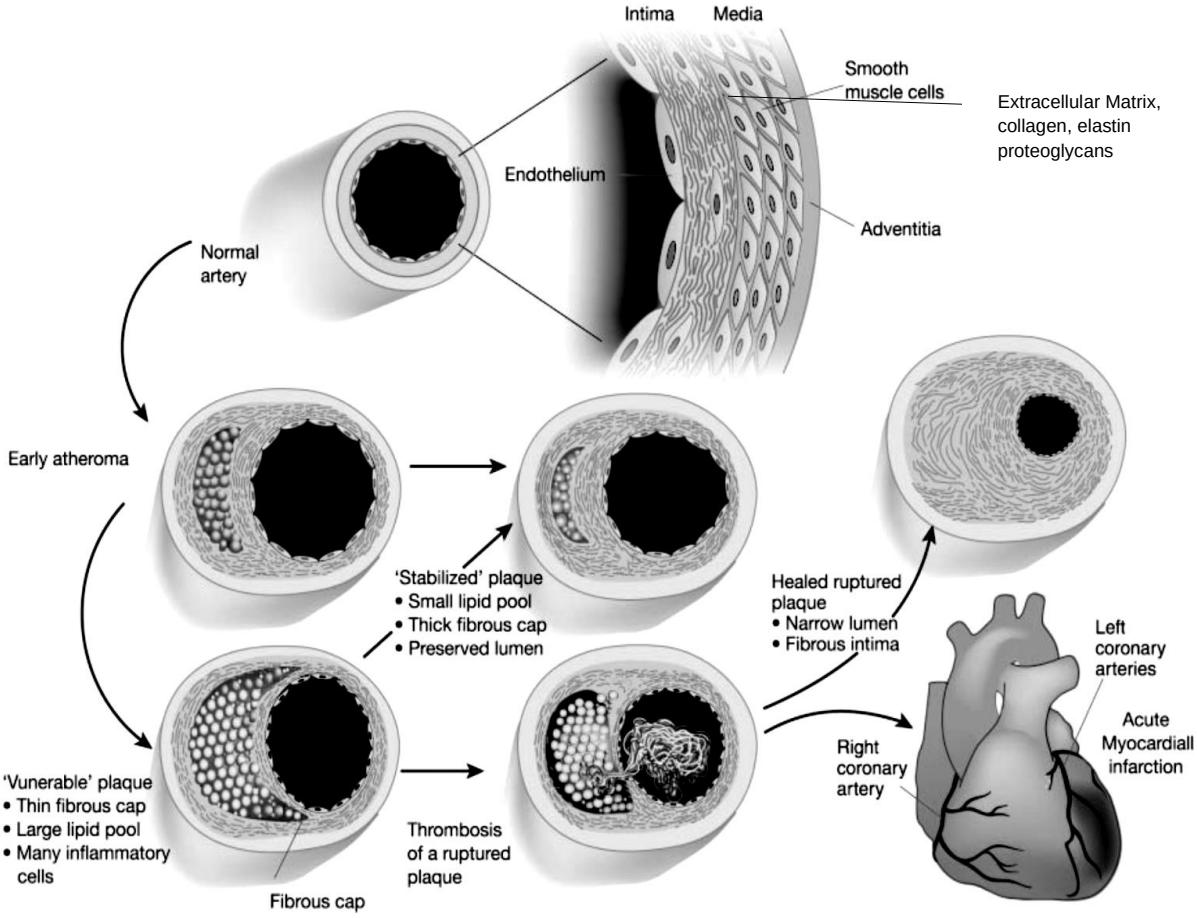
		Current Daily Intakes (Age Group)					
Nutrient Consumption	**Recommended Daily Intakes**	**Infants 0-18m**	**1.5y-3y**	**4y-10y**	**11y-18y**	**19y-64y**	**65y+**
Added sugars (non-milk extrinsic sugars) (%food energy)	<11% all age groups	4-8%	11.8%	14.7%	15.6%	12.7%	11.4%
Total Fat (% food energy)	<35% 4y-64y	N/A[1]	N/A[1]	<35%	<35%	<35%	36.2%
Cis Mono-unsaturated Fat (%food energy)	<13% all age groups	N/A[1]	N/A[1]	11-13%	11-13%	12-13%	12-13%
Saturated fat (%food energy)	<11% all age groups	15-18%	15-18%	13.2%	12.5%	12.6%	13.8%
Trans fat (% food energy)	<2% all age groups	0.5%	0.5%	0.7%	0.7%	0.8%	0.8%
Salt (g/day)	0-12m<1g/day 1y-3 <2g/day 4y+ 6g/day	2.3g	3.7g-5.1g	3.7g-5.1g	6.7g	6.9g	7.2g
Oily fish (% Participants)	Percentage of participants consuming 1 portion/week (140g)	N/A	N/A	8-12%	8-12%	23%	38%
Fruit & vegetables (portions/day)	Participants consuming x portions/day	N/A	N/A	N/A	2.9 portions /day	4.1 portions /day	4.6 portions /day

Results show mean intakes for combined male/female populations across each age group. [1]RDI do not apply to children under 5y age for Total Fat as children are unable to consume sufficient energy required in small volumes of food. Source: (DH, 2011, DH, 2012b, DH, 2012a, DH, 2014c)

1.2 CORONARY HEART DISEASE AND ATHEROSCLEROSIS

Coronary heart disease is described as the partial or complete blockage of the myocardial coronary arteries which occurs as a result of progressive atherosclerosis (Figure 1.1).

Figure 1.1 Progressive Atherosclerosis



Source: Adapted from Libby (2002)

It has been recognised that atherosclerosis is a vascular endothelium inflammatory disease, induced and exacerbated by risk factors such as high fat-high cholesterol diets, diabetes, obesity, hypertension and smoking (Hansson, 2005, Townsend et al., 2012b, Badimon and Vilahur, 2014). Atherosclerosis has been characterised by the accumulation and uptake and infiltration of oxidised LDL cholesterol which create fatty and fibrous lesions within the intima

of arteries and veins (Libby et al., 2009, Lusis, 2000, Libby et al., 2002). It is noted that atherosclerosis can also develop within limb and cerebral arteries with equally deleterious effects such as thrombosis, aneurism, vessel rupture and ischemic stroke (Daniels, 2008).

In the first stages of atherosclerosis, arterial endothelial cells (ECs) become inflamed in response to modified circulating cholesterol and lipoprotein particles, e.g. oxidised LDL and phospholipids and short-chain aldehydes, and a vascular inflammatory response is activated (Libby, 2002, Sprague and Khalil, 2009). Sprague and Kahlil (2009) describe the vascular inflammatory response as including, vasodilation, increased permeability of the endothelium and in severe cases of atherosclerosis such as ischemia and thrombosis - blood stasis. The following paragraphs describe the molecular events which occur in the stages of atherosclerosis development and deterioration.

1.2.1. The Role of the Vascular Endothelium in Atherosclerosis

Endothelial cells (ECs) provide a semi-permeable barrier between the blood flow, extracellular matrix, and intima of the artery wall. A healthy endothelium is naturally athero-protective has anticoagulation properties e.g. negatively charged surface, nitrous oxide and prostaglandin production which inhibit platelet activation (Lusis, 2000, Sprague and Khalil, 2009, Sitia et al., 2010, Badimon et al., 2012, Petersen, 2007). The extracellular matrix (ECM) provides a connection between the Smooth Muscle Cells (SMC) and basement of ECs and contain amongst other molecules, collagens, elastin, and proteoglycans (Figure. 1.1) (Khalil et al., 2004, Badimon et al., 2012). Fluid shear stress, the frictional force of blood flow on ECs, is also a key factor in determining EC morphology and ergo their regulation of inflammation. For example, in cylindrical arterial regions of laminar blood flow without any branches or junctions, ECs lie parallel with the direction of blood flow, are elliptical in shape and show reduced permeability to particles e.g. LDL. These cells contain genes for superoxide dismutase, and nitric oxide synthase which produces nitric oxide which has vasorelaxation properties (Lusis, 2000, Chiu and Chien, 2011, Libby, 2002). Up-regulation of these genes inhibits Nuclear Factor $_{K}$B ($NF_{K}B$) that regulates expression of Vascular Cellular Adhesion

Molecule 1 (VCAM1) which is a binding site for monocytes - one the first leukocytes involved in atherogenesis (Lusis, 2000, Sprague and Khalil, 2009, Badimon et al., 2012, Libby, 2002). Subsequently less inflammation and atherosclerosis has been found to occur in these arterial regions. In contrast, regions of the arterial system that have branches and a greater disturbed blood flow have polygon-shaped ECs that do not align with blood flow (Lusis, 2000, Libby, 2002). The endothelial inflammatory response makes changes within the cytoskeleton of the ECs that modifies the cellular tight junctions and increases permeability. It is therefore recognised that the ECs themselves in their locale, morphology and genetic content either potentiate atherogenesis or confer athero-protective properties.

Platelets have been found to arrive at the site of inflammation prior to leukocytes and can recruit leukocytes themselves thus becoming mediators between ECs and leukocyte migration (Massberg et al., 2002). Platelet glycoproteins adhere to endothelial cells in tandem with vascular inflammatory gene activation e.g. up-regulation of NF_KB in response to increased levels of oxidised lipids and products, which in turn up regulates e- and p-selectins and adhesion molecules such as VCAM1 and Intracellular Adhesion Molecule 1 (ICAM1) on the EC wall (Massberg et al., 2002, Rainger et al., 2015, Badimon et al., 2012). These EC inflammatory molecules tether and roll monocytes and T-lymphocytes along the EC wall through the action of transient ligands such as integrin (Lusis, 2000, Rainger et al., 2015).

Endothelial cells express a cascade of inflammatory factors such as C-reactive Protein (CRP), and cytokines such as Interleukins (IL1β), Tumour Necrosis Factor-α (TNFα) and Interferon-γ (INFγ), (Lusis, 2000, Rainger et al., 2015, Sprague and Khalil, 2009). They activate signal transduction cascades that alter the cytoskeleton structure and ECM cellular adhesiveness. Tethered leukocytes migrate through the altered EC junctions into the sub-endothelial space. Once in the intima monocytes undergo proliferation and differentiation into macrophages through the action of Macrophage Colony Stimulating Factor which also regulates expression of their surface scavenger receptors, e.g. SRA Types I and II (Badimon et al., 2012). Scavenger receptors are further regulated by the transcription factor Peroxisome Proliferation Activated

Receptor-γ (PPARγ) whose ligands include oxidised FAs, TNFα and IFNγ (Libby, 2002, Lusis, 2000, Sprague and Khalil, 2009). It is the action of these cells and their receptors which bind to the modified lipoprotein (LP) ligands and Cholesteryl Esters (CEs) internalising them, creating lipid laden "foam" cells. Additionally, it has been found that LDL passively diffuses through the EC junctions and becomes retained in the intima via the action of LDL ApoB and the proteoglycans within the ECM (Khalil et al., 2004). Oxidised LDL is able to be taken up by macrophages, increasing the ability of macrophages to bind with oxidised LDL and products – all of which increase accumulation of lipid within nascent intima lesions or "fatty streak" – the first stage of atherosclerosis (Lusis, 2000, Libby, 2002, Sprague and Khalil, 2009, Badimon et al., 2012).

1.2.2. Plaque Development

Platelets and SMCs play vital roles in endothelium regeneration or plaque growth and development. It is reported that, dependent on conditions, - chemokine Stromal Derived Factor-1 regulates progenitor cell differentiation into foam cells or endothelial cells (Badimon et al., 2012). A recent study (Allahverdian et al., 2014) concluded that SMCs in human atherosclerosis play a greater role in atherosclerosis lipid retention and lesion development. SMCs can become foam cells in the presence of LPs due to the action of their membrane receptors ATP Binding Cassette Transporter A1 (ABCA1). In the intima layer SMC ABCA1 have reduced expression and become unable to efflux cholesterol from the cell, retaining the lipid and becoming foam cells that contribute to the lipid burden of the lesion (Choi et al., 2009, Allahverdian et al., 2014).

Both the body's immune response and CHD risk factors have also been found to influence SMC proliferation. For example, hypertension has been found to regulate expression of platelet-derived growth factor which stimulates SMC production. Furthermore, hypertension regulates angiotensin II in the renin-angiotensin pathway which stimulates SMC and ECM production (Sitia et al., 2010, Lusis, 2000, Libby, 2002). In addition to these risk factors, the body's immune system continues its cellular chemotactic response - macrophages, B and T

lymphocytes initiate downstream cytokines e.g. IL6 and CRP which direct migration and proliferation of SMCs and promote fibrous plaque development (Sprague and Khalil, 2009, Lusis, 2000). As foam cell, ECM and SMC proliferation continue, the fatty lesion increases into a bulky fibrous lesion that contains increasing levels of thrombin generating SMC microparticles. Over time, these microparticles add fuel to the thrombotic nature of the plaque, the plaque increases in size and can cause arterial stenosis, limiting blood flow and reducing oxygen supply to the heart, thereby inducing clinical presentation of CHD conditions such as angina or ischemic events (Lusis, 2000, Libby, 2002, Badimon and Vilahur, 2014).

1.2.3. Atherothrombosis

When a plaque ruptures, it exposes the underlying ECM vasculature and brings the plaque contents into contact with the circulating blood, instigating the blood coagulation cascade simultaneously with platelet aggregation. Platelet aggregation and the coagulation cascade enhance and chemoattract more platelets and leucocytes to the injury site which perpetuates the growth of the thrombus leading to occlusion of the artery (Badimon et al., 2012, Badimon and Vilahur, 2014, Petersen, 2007). In the best of circumstances, the endothelium in repairing itself through the formation of a thrombus can also instruct dissolution of the clot through fibrinolysis and be resolved without incident. However, thrombus formation during sudden rupture or disruption can have fatal outcomes: (i) The thrombus grows in the arterial lumen and occludes it bringing about blood stasis, ergo reduced oxygen flow to the heart, ischemia and acute myocardial infarction. (ii) the thrombus can break away into the blood stream creating an arterial blockage “myocardial thrombosis” which induces ischemia and acute myocardial infarction and sudden death (Lusis, 2000, Badimon et al., 2012, Petersen, 2007, Badimon and Vilahur, 2014).

1.3 LIPIDS AND LIPOPROTEIN METABOLISM

Dietary fats, such as triglycerides (TAGs), fatty acids (FAs), cholesterol (Chol) and its esters (CE), and small amounts of phospholipids (PPLs), play pivotal roles in energy homeostasis and

cellular physiology (Gurr, 2016, Frayn, 2010, Anderson, 2003). Eaten in a balanced diet they provide an energy-dense nutrient (37 kJ/g) containing over twice as much energy as other macronutrients such as carbohydrates (16 kJ/g) and proteins (17 kJ/g) (BNF, 2018). Lipids also provide vital elements for many cellular functions, including, intermediates of lipid biosynthesis (e.g. phosphotidate) (Frayn, 2010), precursors of steroid hormones, key elements of membrane fluidity and cell signalling (Goldstein and Brown, 2015, Bloch, 1965, Anderson, 2003) and regulate gene transcription pathways (Goldstein and Brown, 2015, Salter and Tarling, 2007). However, it has been found that consumption of dietary fats can compromise metabolic pathways, increasing supply of circulating lipids and can lead to associated diseases such as dyslipidaemia (Kingsbury and Bondy, 2003), metabolic syndrome, diabetes and atherosclerosis (Wilson, 2013, Keys et al., 1986). The following paragraphs give an overview of lipoproteins and FA metabolism.

Frayn (2010) outlines the three pathways for lipid and lipoprotein metabolism. The first is the exogenous pathway which allows for ingested dietary fats such TAG and cholesterol to enter the bloodstream and to be transported to the liver and tissues through the body's lymphatic system. Secondly, the endogenous pathway allows for the packaging of TAG and cholesterol with protein particles (lipoproteins) in the liver for transport to the cells and tissues. Finally, the third pathway focuses on the reverse cholesterol transport system, which returns cholesterol from cells to the liver for excretion in the bile.

1.3.1. Lipoproteins and Apolipoproteins

Lipoprotein (LP) is the name given to a combination of lipid and protein assembled in the body to transport hydrophobic lipids in the aqueous environment of the bloodstream and lymphatic system (Frayn, 2010). To aid this function apolipoproteins (ApoLP) bind with specific lipids and act as ligands for uptake into cells and tissues (see table 1.2) (Kingsbury and Bondy, 2003).

Table 1.2 Lipoproteins and Apolipoproteins

Lipid/Lipoprotein	Apolipoprotein	Impact on atherosclerosis risk	Function
Chylomicrons	Apo AI, AII, AIV ApoB48 (Intestinal) ApoCI, CII, CIII, ApoE	↑	Dietary TAGs. In intestine ApoCII activates LPL to hydrolyse TAG-rich chylomicrons into FFA + remnants. FAs transported to liver, muscle and adipose.
Very Low Density (VLDL)	ApoCI, CII, CIII, ApoB100 (Liver) ApoE	↑ApoB100 ligand LDLr	Cholesterol and CE. Liver synthesis of VLDL from FFA (Chylomicrons). CII activates LPL giving rise to LDL particles and FAs for adipose and muscle
Intermediate (IDL)	ApoB100, ApoC ApoE	↑ApoB100 ligand LDLr	VLDL remnant is "IDL". Hepatic lipase converts IDL to LDL or taken up by liver LDLr
Low Density (LDL)	ApoB100 (Liver)	↑ApoB100 ligand LDLr	Cholesterol and CE. Created from hydrolysed VLDL see above, delivered to liver and tissues via uptake by LDLr. Oxidized LDL particles taken up by scavenger receptors in atherosclerosis.
High Density (HDL)	Apo AI, AII, AIV ApoCI, CII, CIII, ApoE	↓ApoAI Liver receptor BI	CE and PPL. Attracts cholesterol from cells to HDL particle through SRBI by CETP or action of cholesterol esterification through activation of lecithin:acetyltransferase for transport to liver. Liver, Intestine

Source: (Kingsbury and Bondy, 2003, Rader et al., 2009, Rye et al., 2009, Schaefer, 2002)

1.3.2. Lipid Metabolism: Exogenous Pathway

The exogenous pathway allows for dietary fats including TAG and cholesterol absorption into the blood stream to the liver and tissues via the lymphatic system (Kingsbury and Bondy, 2003). TAGs are emulsified via the action of bile acids and salts and pancreatic lipases to form mixed micelles, which are then absorbed by enterocytes of the small intestine by passive or facilitated diffusion (see Table 1.3 for transport proteins) (Frayn, 2010). Some are reesterified to form new TAGs which are incorporated with other molecules such as cholesterol, cholesterol esters, proteins (e.g. ApoB48) and phospholipids to form large lipoprotein particles known as chylomicrons. Chylomicrons then enter the blood stream via the lymphatic system and transport TAGs from the intestines to the liver and adipose tissue (Frayn, 2010).

Final transport of TAGs and cholesterol to tissue receptors are dependent upon the apolipoproteins acquired e.g. ApoCII a key activator for Lipoprotein Lipase (LPL) (see Figure 1.2) (Khetarpal and Rader, 2015). The chylomicron becomes a substrate for capillaries expressing enzymes such as LPL which hydrolyses TAG to release free fatty acids (FFA) (Kingsbury and Bondy, 2003). Remaining particles are transferred to other LPs such as HDL or form chylomicron remnants with associated ApoLPs (see Table 1.2) (Kingsbury and Bondy, 2003, Rye et al., 2009). These LPs become ligands for receptors e.g. hepatic LDL receptor (LDLr) or VLDL receptor (Frayn, 2010, Khetarpal and Rader, 2015). FA regulated Transcription Factor Hepatocyte Nuclear Factor 4 (HNF) regulate chylomicrons and cholesterol metabolism e.g. via ApoLPs AII, AIV, CII and CIII (Salter and Tarling, 2007, Sladek et al 1990) see Table 1.2 and Figure 1.2a.

Table 1.3 Fatty Acid and Cholesterol Transport Proteins

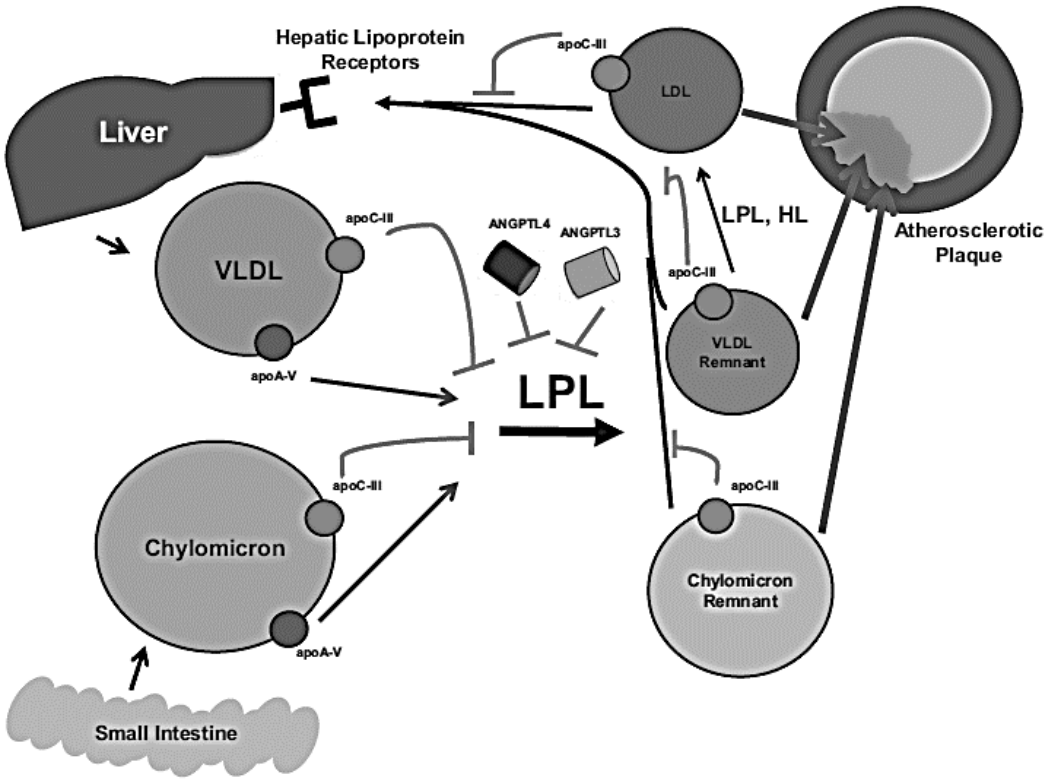
Transport Protein	**Tissue**
Fatty Acid Translocase (CD36)	Adipose tissue, small intestine, myocardium, skeletal muscle.
Fatty Acid transport Protein (FATP, isomers 1-6)	FATP1: Adipose, small intestine, skeletal muscle, brain FATP1, FATP5 Liver
Fatty Acid Binding Protein (plasma membrane) FABPM	Specific to plasma membranes.
Acetyl-CoA synthase	Transport of FAs into cells and esterification of FAs with CoA
ATP Binding Cassette (ABC) ABC-A1 ABC-G1 ABC-G5 ABC-G8	Cholesterol transport Enterocytes, ABC-A1 & ABC-G1 involved in reverse cholesterol transport - cholesterol from cells to HDL particles
Niemann-Pick C1 Like Protein (NPC1LP)	Cholesterol transport
Cholesterol Ester Transport Protein (CETP)	Involved in the reverse cholesterol transport system

Source: Frayn (2010), Khetarpal and Radar (2015), Rye et al.,(2009), Vitali et al (2017)

1.3.3. Lipid Metabolism: Endogenous Pathway

The endogenous pathway involves liver synthesis and secretion of lipoproteins (Kingsbury and Bondy, 2003). Liver TAG and cholesterol esters are packaged in the liver with apolipoproteins (ApoB100, ApoE and ApoC groups, see table 1.2) and are secreted into the bloodstream as VLDL particles. The VLDL act as a substrate to LPL within capillaries and deliver TAG from liver to tissues e.g. energy for muscle cells or storage by adipocytes (Khetarpal and Radar 2015). Redundant VLDL particles such as phospholipids, cholesterol esters or TAGs can be transferred to other lipoproteins such as HDL through the action of transport proteins e.g. Cholesterol Ester Transport Protein (CETP) or Phospholipid Transfer Protein (PPLTP) (Rader et al., 2009). The VLDL remnants and other redundant particles from circulating lipoproteins aggregate and increase the amounts of apolipoprotein (e.g. ApoB, ApoE and ApoC) in the particle which then become ligands for receptors in tissues e.g. hepatic LDL receptor, LDLr or B/E Receptor (see Table 1.2 and Figure 1.2) (Frayn, 2010, Kingsbury and Bondy, 2003). Remaining circulating particles shrink through action of lipoprotein lipase until surface components are removed and the remnant forms an Intermediate Density Lipoprotein (IDL) consisting of a core of cholesteryl ester, ApoB100, free cholesterol and phospholipids (Kingsbury and Bondy, 2003, Frayn, 2010). Cholesterol is delivered to cells through the self-limiting LDLr uptake of LDL particles and through moderation by FA regulated SCAP-SREBP2 system (Brown and Goldstein, 2009a) – see paragraphs 1.4-1.4.3 for further details.

Figure 1.2 Lipoprotein Metabolism and Atherosclerosis



Source: Khetarpal and Radar (2015)

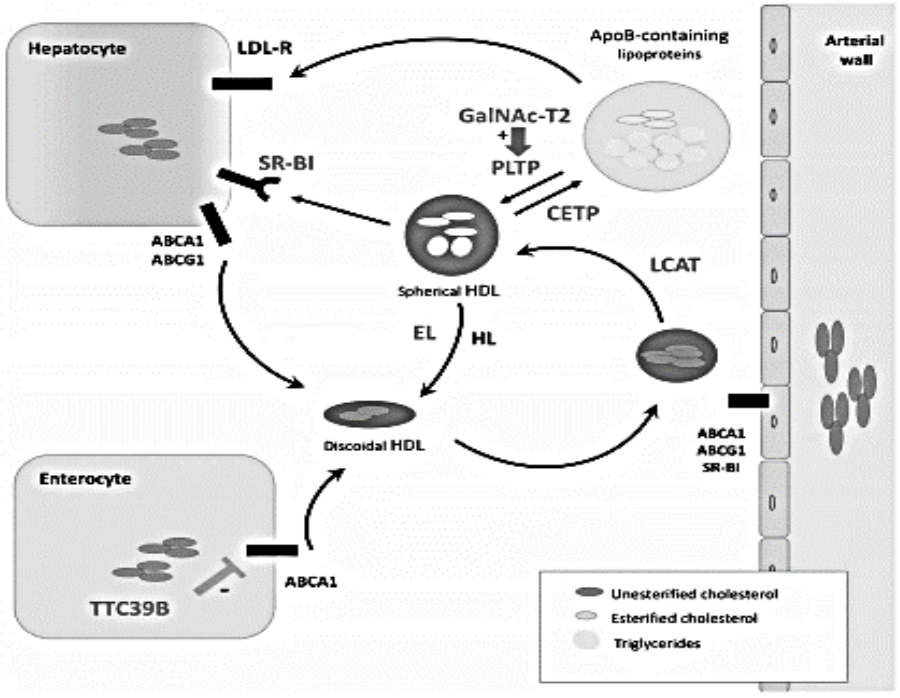
Diagram shows lipoprotein metabolism and link to atherosclerosis Key: HL: Hepatic Lipase, LPL: Lipoprotein Lipase, ANGPTL4, ANGPTL3 – secreted protein inhibitors of Lipoprotein Lipase

1.3.4. Lipid Metabolism: Reverse Cholesterol Pathway (HDL)

HDL is often referred to as "good cholesterol" due to its inherent function in humans to "collect" cholesterol from tissues and transport it to the liver for excretion via the bile – see Figure 1.3 (Frayn, 2010, Rader et al., 2009). HDL plays a key role in the transfer of cholesteryl esters, TAGs, and phospholipids between lipoproteins (Kingsbury and Bondy 2003, Rye et al., 2009). Pre β-HDL particles consist of liver and intestinal secreted Apolipoprotein AI (ApoAI) and phospholipids (PPL) to form a discoidal HDL (Frayn 2010). It accepts cholesterol and PPL from cells via action of PPL Transfer Protein (PPLTP) or ATP Binding Cassette (ABCA1) to form mature, spherical HDL_2 (larger particles) or HDL_3 (smaller particles) (Kingsbury and Bondy,

2003, Rye et al., 2009). ApoAI and Lecithin-cholesterol acyltransferase (LCAT) are essential HDL cofactors as LCAT esterifies cholesterol in cell membranes and transfers the CEs to HDL_2 via cell membrane ATP Binding Cassette proteins (ABC – see table 1.3 and Figure 1.2) to create mature, spherical particles (Kingsbury and Bondy 2003, Rye et al., 2009). These larger particles deposit cholesterol through direct interaction with scavenger receptors at hepatocytes for onward excretion in bile – recreating lipid-poor ApoAI particles for regenerative cholesterol transport (Frayn, 2010). Control of HDL cholesterol synthesis and feedback pathways are to some extent controlled by FA gene regulated transcription factors, e.g. hepatic LXR-RXR control ABCA1 involved in reverse cholesterol transport system and ApoE, LPL and CETP function (Salter and Tarling, 2007) (see paragraphs 1.4-1.4.3).

Figure 1.3 HDL Metabolism



Key: EL Endothelial Lipase HL Hepatic Lipase PLTP Phospholipid Transport Protein
LCAT: Lecithin-Cholesterol Acyltransferase CETP: Cholesterol Ester Transport Protein.
ABC ATP Binding Cassette Protein SR Scavenger Receptor
TTC39B gene promotes ubiquitination and degradation of LX Receptor reducing ABCA1 expression
GALNT2 gene encodes enzyme GaINAC-T2 and glycosylation of target proteins such as PLPT that increases PPL transfer and therefore increases HDL.

Source: Vitali et al (2017)

1.4 CHOLESTEROL-ATHEROSCLEROSIS HYPOTHESIS

There has been over 100 years' research into the impact of dietary fats on serum cholesterol (and their associated lipoproteins) and the development of atherosclerosis in both animal models and human dietary intervention studies (see section 1.5). The following paragraphs give an overview of cholesterol synthesis pathways, metabolism and its FA controlled mechanisms and association with atherosclerosis development.

Anitschow and Chalatow published their paper in German in 1913 which was translated into English in 1983 (Pelias, 1983). The translated publication describes the results of their experiments feeding cholesterol rich diets to rabbits, guinea pigs and rats and that rabbits were the most successful animal model for this type of research. They identified that egg yolk and brain fed to their experimental rabbits were high in what they described at the time as a protein "cholesterin" and that it was "absorbed through the gut lumen into the blood and can be detected there". They concluded that cholesterin had a harmful effect on many organs, including permeating the liver, adrenal glands, renal cortex, and thickening of aorta intima which was penetrated with fat like substances or "lipoids" (Anitschow and Chalatow, 1913, in Pelias, 1983). Schoenheimer and Breusch (1933) demonstrated mice could synthesise cholesterol when fed a cholesterol-free diet and conversely when mice were fed a cholesterol rich diet the cholesterol contribution from the mice was negated. This study has been noted as the first to evidence an "end-product feedback system of a biosynthetic pathway" (Goldstein and Brown, 2015). Additionally, it wasn't until 1940s that a methodology was developed to identify the classes of lipoproteins within human plasma samples through the use of ultracentrifugation and electrophoresis techniques (Gofman et al., 1949). These techniques first elucidated to the role of cholesterol Low Density Lipoprotein (LDL) and its Apolipoprotein B in the development of atherosclerosis and correlation with CHD mortality risk (Gofman et al., 1949, Gofman et al., 2007, Goldstein and Brown, 2015).

1.4.1. Cholesterol Synthesis and LDL Receptor

Dyslipidaemia is identified as an abnormal plasma lipid profile, characterised by elevated circulating levels of cholesterol or TAGs (Kingsbury and Bondy, 2003). One form of dyslipidaemia is what is now known to be a genetic disorder of lipoprotein metabolism known as Familial Hypercholesterolemia (FH). FH is characterised by elevated plasma LDL in the absence of dietary cholesterol or FA consumption and an increased risk of early CHD mortality (De Castro-Orós et al., 2010). Michael Brown and Joseph Goldstein (1974, 1977) identified the LDL receptor and its major role in cholesterol metabolism and FH. The LDLr is a transmembrane receptor, having 839 amino acids, with an external seven repeat cysteine-rich NH_2 terminal and cytoplasmic 50 amino acid COOH terminal (Goldstein and Brown, 2015). The LDLr binds LDL cholesterol with high affinity to its Apolipoprotein B and LDL enters the cell via endocytosis (Goldstein and Brown, 2015, Kingsbury and Bondy, 2003). Goldstein and Brown (1977) found that cells could regulate uptake of plasma LDL cholesterol through activation of HMG CoA reductase and associated production and localisation of LDL receptors (LDLr) at the cell surface. Excess cholesterol entering the cell reduces HMG CoA activity and suppresses de novo cholesterol synthesis, but in turn activates Acyl-CoA cholesterol transferase (ACAT) which attaches a LCFA to cholesterol to synthesise storable CEs (Goldstein and Brown, 2015, Goldstein and Brown, 1977). When cell levels of cholesterol are low, de novo cholesterol synthesis is possible from Acetyl CoA through a 20+ step enzymatic pathway (Sato, 2010). Goldstein and Brown's (1974) study into FH reported that HMG Co-A reductase activity was equated with lipoprotein binding: (i) the amount of bound LDL, (ii) VLDL as a competitor for LDL binding and acted as a HMG-CoA reductase repressor, (iii) HDL does not suppress HMG Co-A activity. They concluded that FH was caused by a genetic mutation of the LDLr causing defective binding of LDL. However, since that initial discovery over 1200 allelic mutations of the LDLr that either partially or fully destroy its function have been identified (Goldstein and Brown, 2015). Additionally it has been found that the LDLr is not alone in being causative of FH disorder, for example genetic mutation of its ligands ApoB or ApoE e.g. Apo E3 Leiden amongst others are also responsible (De Castro-Orós et al., 2010).

1.4.2. Health Initiatives for Cholesterol

The discovery of HMG CoA reductase in cholesterol metabolism led the way for the identification of a competitive inhibitor of HMG CoA reductase "compactin" (Endo et al., 1976). The subsequent discovery of a similar fungal metabolite "mevinolin" (Alberts et al., 1980) led to the development of commercial cholesterol lowering drugs commonly known as "Statins". These drugs are proven to reduce cholesterol levels and associated CHD mortality (Goldstein and Brown, 2015, Endo, 2010, Tarantino et al., 2017). Tarantino et al, (2017) noted that statins have also been reported to inhibit hepatic VLDL production due to TAG and cholesterol lowering effect of the drug potentially through other FA targeted genes e.g. PPARs. Identification of other FA activated TFs (see paragraph 1.4.5) have also been exploited for therapeutic means. For example, PPAR agonists such as fibrate are used to treat dyslipidaemia increasing LPL synthesis thus lowering TAG, LDL, hepatic VLDL and raising HDL and reducing atherosclerosis and CHD mortality (Tarantino et al., 2017, Ahmed et al., 2007).

1.4.3. Fatty Acid Activation of Gene Expression in Cholesterol and Atherosclerosis Pathways

It also became apparent through cholesterol synthesis studies in animal tissues (Dietschy et al., 1993, Schoenheimer and Breusch, 1933) that the liver was a key site for total cholesterol synthesis and regulation (Brown and Goldstein, 2009b). However, it wasn't until the 1990's onwards that key roles in activating specific transcription factors involved in the gene expression of cholesterol synthesis and atherosclerosis pathways were identified e.g. LDLr (Wang et al., 1993, Wang et al., 1994) scavenger receptors (Libby, 2002) and cholesterol synthesis enzymes (Goldstein et al., 2006). Additionally, it is recognised there are 4 families of transcription factors that are activated by FAs (Salter and Tarling, 2007) which are involved in cholesterol metabolism (Sato 2010, Brown and Goldstein 2009) and in inflammation and atherosclerosis pathways (Libby 2002, Ahmed et al 2007): (i) Sterol regulatory binding proteins (SREBPs), (ii) Peroxisome proliferator-activated receptors (PPARs), (iii) Liver X

Receptors (LXR) and, (iv) Hepatocyte nuclear factors (HNF)– see table 1.4 for isoform/sub-type information and roles (Salter and Tarling, 2007).

1.4.3.1 Sterol Regulatory Element Binding Protein (SREBP)

During the 1990's a group of membrane bound transcription factors – SREBP1a, SREBP1c and SREBP2 were characterised and light shed on the tightly regulated by a negative feedback mechanism for cell cholesterol synthesis (Wang et al., 1993, Wang et al., 1994, Sato, 2010). SREBP are nuclear receptors (Salter and Tarling 2007). They contain a basic helix-loop-helix leucine Zipper motif and are synthesised within the cell's endoplasmic reticulum (ER) (Sato 2010). Each SREBP subtype has been found to have its own role in lipid metabolism (see Table 1.4). However, the regulation and activation of SREBPs involve a complex proteolytic pathway, and endoplasmic reticulum (ER) proteins: SREBP cleavage activating protein (SCAP) and HMG Co-A reductase (Sato, 2010). In the presence of cell sterols SCAP or HMG CoA reductase undergo conformational changes and bind cholesterol. This in turn binds to two insulin-induced genes (Insig1 and Insig2) which are fundamental in the SCAP/SREBP complex being anchored in the ER (Brown and Goldstein, 2009b). This step inhibits cleavage of the membrane bound SREBP1 thereby downregulating gene expression of LDLr and HMG CoA synthase (Sato, 2010). Conversely if cell sterol levels are depleted, SREBP-SCAP moves to the Golgi via binding of SCAP-CO PII coated vesicles where site-1 (S1P) and site 2 (S2P) proteases cut the SREBP releasing it from the ER membrane and translocates to the nucleus to regulate transcription (Sato, 2010, Salter and Tarling, 2007).

Table 1.4 Fatty Acid Regulation of Transcription Factors

TF Family	TF isoforms/sub-type	Tissue	Gene regulation	Examples of Ligand Activators	Source
SREBP/SCAP	SREBPIa, SREBP1c,	Expressed in most tissues but mainly in liver and adrenal gland	FA and TAG metabolism	Sterols	Sato (2010), Wang (1993), Wang (1994) Goldstein et al 2006
	SREBP2	Ubiquitous expression	Cholesterol Metabolism LDL receptor	Sterols	Brown and Goldstein (2009)
PPAR/RXR	PPARα	Expressed in Liver, kidney, intestine	FA metabolism	SFA, MUFA and PUFAs	Salter and Tarling (2007)
	PPARβ	Expressed in small intestine, colon, heart, adipose and brain.			Salter and Tarling (2007) Tarantino et al (2015)
	PPARγ (subform γ1, γ2, γ3)	Ubiquitous expression Expressed in Adipose Macrophage	Adipocyte differentiation scavenger receptors	INFγ, TNFα LDL Cholesterol CLA isomers	Salter and Tarling (2007) Libby (2002) Viladomiu et al (2016)
LXR/RXR	LXRα	Liver, intestine, kidney, adipose, macrophage	Cholesterol metabolism	LCFA, Oxysterols:	Lee and Tontonoz (2015) Salter and Tarling (2007)
	LXRβ	Ubiquitous expression	ApoE, Lipogenesis, carbohydrate metabolism	22Rhydroxycholesterol 24,25epoxycholesterol	
HNF-4α	HNF1, HNF2, HNF3 HNF4a, HNF4γ	Liver, Intestine, Kidney and Pancreas	Lipoprotein metabolism	LCFA saturated Co-Acyl	Sladek et al (1990) Salter and Tarling (2007)

1.4.3.2 Peroxisome Proliferator-Activated Receptors (PPARs)

PPARs are steroid hormone nuclear TFs with ligand binding domain and a response element in the promotor region (Ahmed et al., 2007). Ligands include SFA, MUFAs, PUFAs and their derivatives eicosanoids (Salter and Tarling 2007). It has been identified that RTFAs – in particular Conjugated Linoleic Acid (CLA) isomers are potent activators of PPARγ (Viladomiu et al., 2016). On ligand binding PPARs change conformation and form a heterodimer complex with nuclear ligand activated receptor Retinoid X Receptor (Ahmed et al 2007, Salter and Tarling 2007). Accessory molecules are recruited (e.g. CREB-binding proteins, PPARγ co-activator = PGC-1) to create a PPAR/RXR/Accessory molecule complex which can then be phosphorylated (Ahmed et al, 2007). Ahmed et al., (2007) also noted that PPARs can also repress gene transcription through interaction with other TFs including NF-kB and signal transducer and activator of transcription proteins (STATs).

As outlined in paragraph 1.4.2 PPARs are targeted for therapeutic treatment with fibrate to treat dyslipidaemias or thiazolidinediones that treat diabetes (Ahmed et al, 2007, Tarantino et al 2017). Of particular note is the role of PPARγ and its ligands e.g. oxidised FAs, TNFα and IFNγ in regulating expression of macrophage scavenger receptors. As outlined in paragraph 1.2.1 these receptors bind modified LP ligands and CEs internalising them, creating a lipid laden “foam” cells that are the characteristic of atherosclerosis and cause of thrombosis, strokes, ischemia, and myocardial infarction (Libby 2002).

1.4.3.3 Liver X Receptors (LXRs)

LXRs are ligand activated nuclear TFs that also respond to cell cholesterol levels (Salter and Tarling, 2007). As PPARs above they form heterodimers with RXR to regulate transcription of genes through direct binding of DNA. Co-repressor proteins bind the LXR/RXR complex to silence transcription. Oxidised cholesterol derivatives e.g. oxysterols (see Table 1.3) act as

ligands, causing a conformational change and activation of gene transcription (Lee and Tontonoz, 2015). LXRs are found to have anti-inflammatory function through action of LXR/Co repressor proteins silencing inflammatory genes (Lee and Tontonoz, 2015). They are also found to control adenosine triphosphate binding cassette protein (ABCA1) involved in reverse cholesterol transport system and ApoE, Lipoprotein Lipase and CETP function (see paragraph 1.3.4) (Salter and Tarling, 2007) supporting the reported increase in HDL production and anti-inflammatory effects of LXRs described by Lee and Tontonoz (2015).

1.4.3.4 Hepatocyte Nuclear Factor-4α

Although there are several isoforms and sub-types of HNF (HNF1, HNF3 and HNF4α HNF4γ). HNF4α has been identified through its amino acid sequence that it is a ligand-dependent TF of the steroid/thyroid hormone receptors, that also play a role in differentiation and development (Sladek et al., 1990). Originally, HNFs were thought to be liver specific, however they have now been found in several tissues including kidneys, intestine and pancreas (Drewes et al., 1996). Its activation is through binding with high affinity to LCFA saturated Acyl CoA whilst unsaturated Acyl suppresses its activity (Salter and Tarling, 2007). In particular this TF regulates constituents of chylomicrons and cholesterol metabolism including lipoproteins AII, AIV, CII and CIII (Salter and Tarling, 2007, Sladek et al 1990) see Table 1.2 and Figure 1.2.

Considering the information outlined in sections 1.2, 1.3. and 1.4, it can begin to be understood that the FA pathways in the aetiology of CHD is complex and intrinsically linked. Not only does the human body rely upon nutrients supplied for survival, energy, and function, but responds to nutrient supply, or lack thereof, in FA and cholesterol metabolism, the inflammation response and associated FA controlled gene transcription expression. In the following paragraphs the impact of different FAs: SFA, PUFAs and TFAs will be discussed to try and identify the body's response to these FAs in terms of lipid profiles and atherosclerosis.

1.5 THE IMPACT OF DIFFERENT FATTY ACIDS ON SERUM CHOLESTEROL AND ATHEROSCLEROSIS

As outlined briefly in Section 1.4, animal models of cholesterol metabolism (Rader et al., 2009, Schoenheimer and Breusch, 1933, Spady and Dietschy, 1983, Goldstein and Brown, 2015) and atherosclerosis (Pelias, 1983, Zadelaar et al., 2007, Getz and Reardon, 2012) (discussed further in Section 1.7) and human epidemiological studies - see below, (Keys et al., 1965b, Keys et al., 1965a, Manttari et al., 1987, Mahmood et al., 2014) - have informed knowledge of how dietary FAs impact differentially on serum cholesterol levels and therefore atherosclerosis and CHD risk.

1.5.1. Saturated Fatty Acids

Many SFA are found in animal products such as meat and dairy but also extracted from plants e.g. palm oil. A fatty acid that contains no double bonds within the hydrocarbon chain confers a straight configuration. This fatty acid is denoted as a "saturated" fatty acid and confers a solid texture at room temperature.(Hernandez, 2013) . Animal products are also recognised as being high in cholesterol (Mensink et al 2003). Of the SFAs consumed by participants in human dietary intervention studies, many studies identified that the majority of SFA came from dairy products such as cheese, butter and milk (Kinsell., et al, 1952, Keys et al., 1965b; Keys et al., 1986, Mattinen, et al 1992, Mensink and 2003). Mensink and Katan (1992) excluded stearic acid (C18:0) from their analyses as they considered it to have hypocholesterolaemic impact on serum LDL compared to Myristic c acid (C14:0) and Palmitic acid (C16:0). Skeafe et al (2004) corroborated this by reporting that saturated fats such as Myristic acid (C14:0), Palmitic Acid (C16:0) and Lauric Acid (C12:0) are the main hypercholesterolemia inducing SFA within milk fat, with Stearic Acid (C18:0) having a lesser effect, along with Caprylic Acid (C8:0) and Capric Acid (C10:0).

1.5.2. Unsaturated Fatty Acids (UFA)

Unsaturated FAs have one (monounsaturated fatty acid "MUFA) or more than one (polyunsaturated fatty acid "PUFA") double bond within the hydrocarbon chain. The double bond binds two of the carbons together C=C. Where the bonds are positioned within the chain and on the chain lead to the formation of positional and geometric isomers. *Cis* unsaturated fatty acids have their bonds positioned on the same side of the chain causing a twist in the molecule which confers a bent configuration. *Trans* unsaturated fatty acids double bonds are positioned on opposite sides of the hydrocarbon chain and confer a straight configuration with an appearance similar to that of a SFA (Gurr, 2016). *Cis* and *trans* PUFAs can have two or more double bonds based along the chain and noted as n-3 for position the third carbon from the methyl end and so on (Gurr, 2016). The more double bonds, the greater instability of the molecule which lowers the melting point compared to saturated fats and the fatty acids become liquid (e.g. oils) at room temperature (Gurr, 2016, Hernandez, 2013). Unless otherwise stated, all MUFAs and PUFAs in this thesis are in the cis configuration.

The majority of MUFAs and PUFAs are extracted from plants (Hernandez, 2013). For example, olive oil is rich in MUFA Oleic acid (C18:1) and Rapeseed Oil is rich in PUFA Linoleic (18:2 n-6) and MUFA Oleic Acid. Olive oil in particular is linked to a "Mediterranean style" diet with beneficial impact on CHD (see paragraphs below) (Keys et al., 1986). These oils have been observed as having a beneficial impact on LDL and HDL cholesterol levels and improving the LDL/HDL ratio (Bos et al., 2010, Mensink et al., 2003) and atherosclerosis (Tonge, 2011). Additionally, essential fatty acids, Linoleic Acid (LA) (C18:2 n-6), and α-Linoleic Acid (ALA) (C18:3 n-3) are unable to be synthesised endogenously and must be provided in the diet (Mazzocchi et al 2018). LA n-6 PUFAs are prevalent in corn, soybean, safflower, and sunflower oils used worldwide (Innis 2007). However, ALA n-3 PUFAs are limited in soybean and canola oil (Innis, 2007) but can be found in "oily" fish products such as salmon and mackerel (Tonge, 2011). LA is elongated and desaturated to form Arachidonic Acid (AA, C20:4), whilst ALA is elongated and desaturated to eicosapentaenoic acid (EPA C20:5) and docosahexaenoic acid

(DHA C22:6) (Mazzocchi et al., 2018) – see Figure 1.4 Metabolism of LA to AA is important as AAs are an essential component of cellular membranes. Whilst EPA and DHA are vital to neurological, visual and brain function. Eicosanoids (see Figure 1.4) play vital roles in maternal physiology and fetal development – modulating reproductive, pulmonary, cardiovascular, and inflammatory pathways including prostaglandins, thromboxanes and leukotrienes (Innis, 2007, Mazzocchi et al 2018).

Figure 1.4 Conversation of Essential Fatty Acids to Eicosanoids and Docosanoids

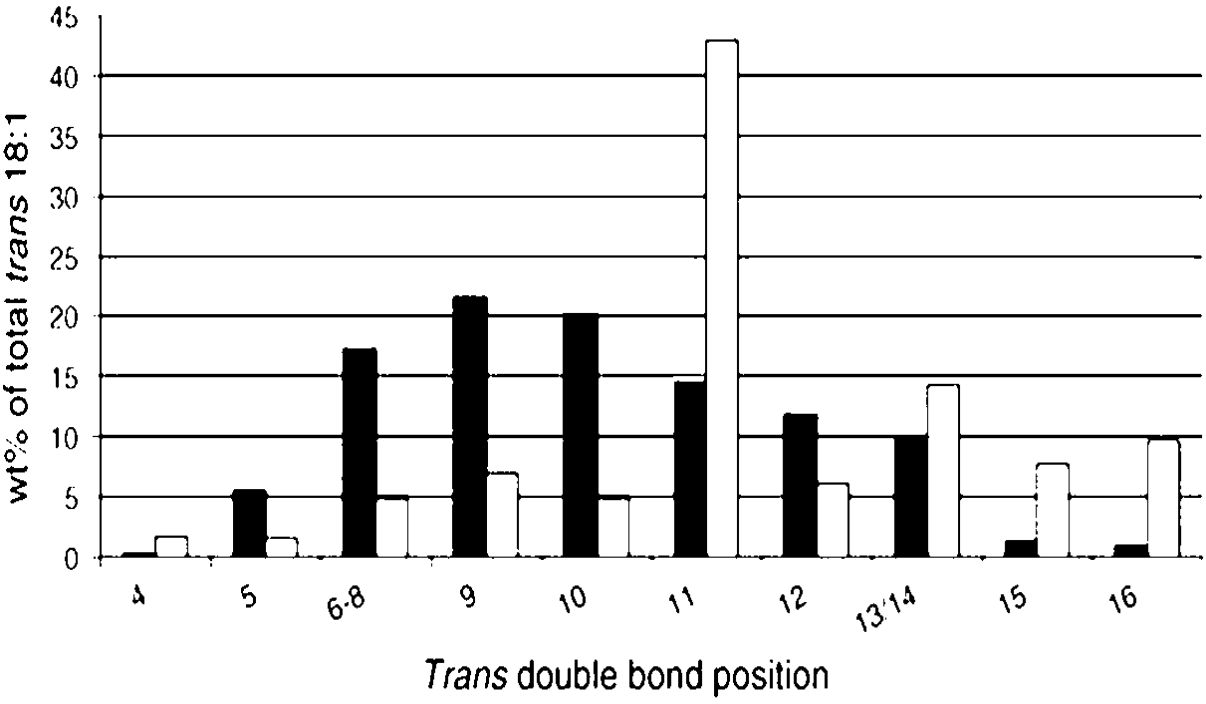
C18:2 n-6	**LA**		**C18:3 n-3**	**ALA**
↓		*Δ6-desaturase*	↓	
C18:3 n-6			C18:4 n-3	
↓		*Elongase*	↓	
C20:3 n-6			C20:4 n-3	
↓		*Δ5-desaturase*	↓	
C20:4 n-6	**AA**	EICOSANOIDS	**C20:5 n-3**	**EPA**
↓		*Elongase*	↓	
C22:4 n-6			C22:5 n-3	
↓		*Elongase*	↓	
C24:4 n-6			C24:5 n-3	
↓		*Δ6-desaturase*	↓	
C24:5 n-6			C24:6 n-3	
↓		*β-Oxidase*	↓	
C22:5 n-6		DOCOSANOIDS	**C22:6 n-3**	**DHA**

Source Mazzocchi et al 2018.

1.5.3. Trans Fatty Acids (TFA)

There are two main sources of dietary TFA – these are industrially manufactured TFA and those produced by ruminant animals e.g. cows, goats. Although both TFAs have the same C18:1 *trans* isomers present, their distribution is different - see Figure 1.5. The following paragraphs gives a brief overview of both types of TFA.

Figure 1.5 PHVO and Ruminant *trans* isomers



Source Lock et al 2005 ■ PHVO (Industrially produced) □Ruminant TFA

1.5.3.1 Partially Hydrogenated Vegetable Oil TFA (PHVO or P)

Typically, PHVO are manufactured from vegetable, fish, or animal oils to stabilise them for use in products such as margarines. *Cis* unsaturated fatty acids are saturated via a hydrogenation process where the fats and oils are mixed together with hydrogen under high pressure and in presence of a metal catalyst e.g. nickel or palladium (Hernandez, 2013, Clark,

2009). As hydrogenation increases, content of PUFAs decrease and MUFAs and TFAs increase (Figure 1.5) (Valenzuela and Morgado, 1999)

Figure 1.6 Hydrogenation of PHVO

Hydrogenation

$$CH_2{=}CH_2 + H_2 \rightarrow CH_3{-}CH_3$$

Source: Hernandez, (2013)

Three changes can occur during the hydrogenation process: (i) A double bond is changed to a single bond and two PUFAs are converted to MUFA, or MUFA converted to SFA. (ii) The double bond position moves within the chain.(iii) The double bond changes to/from *cis* and *trans* (Valenzuela and Morgado, 1999).

The USA FDA designate that an industrial hydrogenated fat is one which is solid at room temperature with between 15-25% *trans* fatty acids (TFA). *Trans* Fatty Acids due to their unsaturated and straight conformation are representative of naturally occurring saturated fats with comparative detrimental health benefits including cardiovascular diseases (Mozaffarian et al., 2006). Many countries, including the USA and UK have now taken steps towards reducing the content of *trans* fats in food products, however they are still present (2-4% daily total energy intake) in many processed and fried foods e.g. fries, snacks e.g. crisps, margarines and bakery items (Teegala et al., 2009, Mozaffarian, 2006). The majority of PHVO used in these products are *trans* fats with C18:1 configuration with a Gaussian distribution across C18:1 isomers of Elaidic Acid *trans*-9, -10, -11, -12 -see Figure 1.5. (Chardigny et al., 2008, Tyburcz et al., 2009).

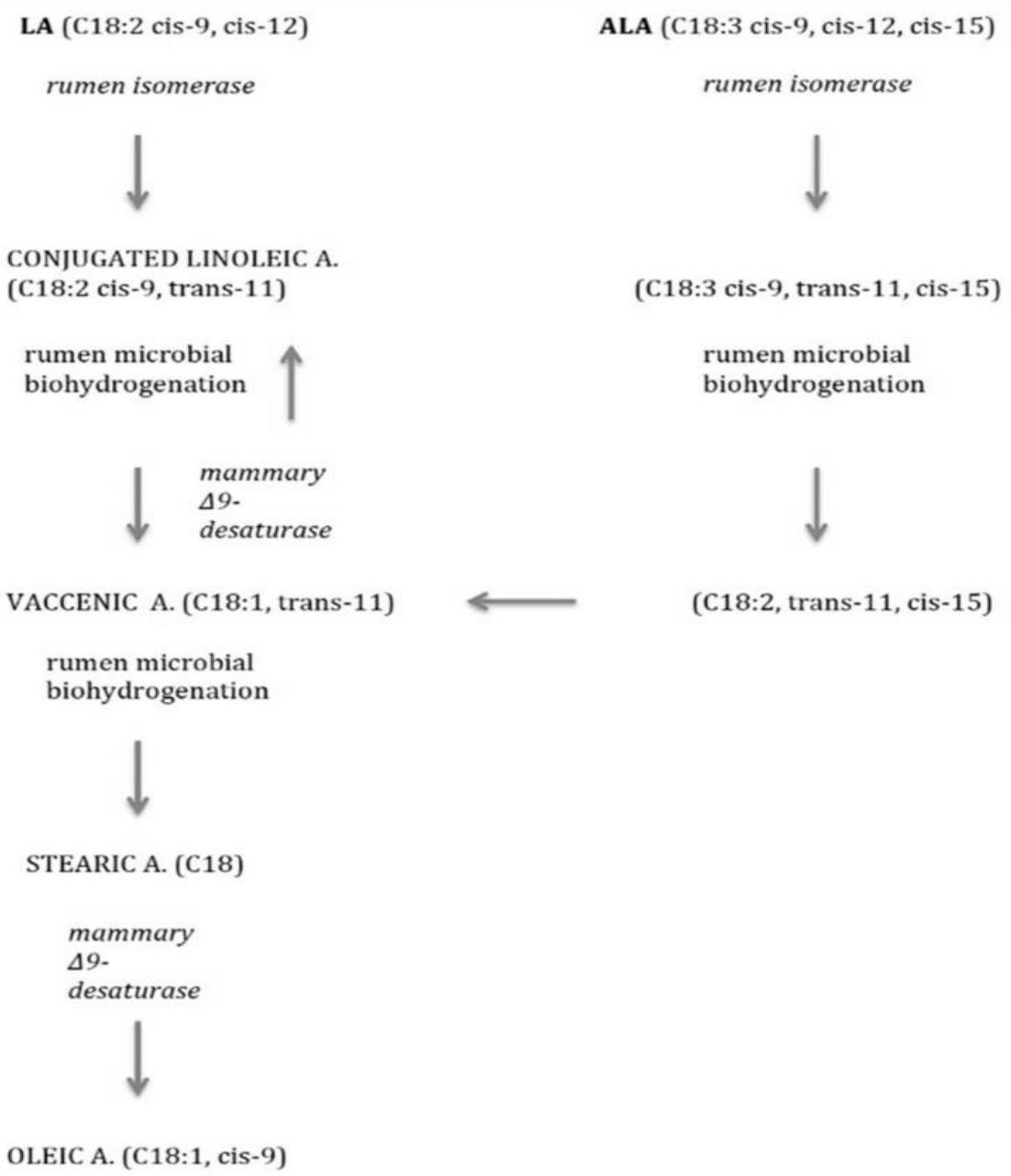
1.5.3.2 Ruminant TFA (RTFA or R)

MUFAs and PUFAs found in plant materials are processed by biohydrogenation of rumen bacteria which synthesise 'natural' *trans* fatty acids which are found in body fat, meat, milk, and other dairy products. Anaerobic *Butyrivibrio fibrisolvens* bacteria within the first stomach catalyses linoleic acid to intermediates such as cis, *trans* C18:2 n-9, n-11 (Enjalbert, 2009). These are hydrogenated further to create the main *trans* fatty acid isomer, Vaccenic Acid (VA) (*trans* C18:1 n-11) - Vaccenic Acid is a precursor for synthesis of CLA, cis-9, *trans*-11 C18:2 (Rumenic Acid – RA) and Stearic Acid (C18:0) see Figure 1.6 and Figure 1.7 Although quantity of TFA is dependent upon diet, these two TFA isomers generally represent between 1-9% of total TFAs (Chardigny et al., 2008). RTFAs are eaten in lower quantities than PHVO (2-3% daily total energy intake) at approximately 0.5% daily total energy intake (Lock et al., 2005, Mozaffarian et al., 2006, Valenzuela and Morgado, 1999).

1.5.3.3 Conjugated Linoleic Acids (CLA)

CLAs, although TFA isomers are considered to have anticarcinogenic and antiatherogenic effects in animal models (Tyburcz et al., 2009). However, many CLAs are derived from ruminant products including milk fat and meat (see Figure 1.7). They can also be synthesised from *trans* isomers of oleic acid by humans (Mazzocchi et al., 2018). CLAs are a family of positional and geometric conjugated dienoic isomers of linoleic acids e.g. cis-9, *trans*-11 CLA and *trans*-10, cis-12 CLA. They have a conformation of alternating single and double bonds which confer both *trans* and cis fatty acid configuration (Ophardt, 2003). Although CLAs are considered *trans* fats their polyunsaturated nature confers lower melting point properties and are found to have possible health benefits (see paragraph 1.5.6.2).

Figure 1.7 Ruminant Conversion of Linoleic Acid to CLA, VA and Stearic Acid



Source Mazzocchi et al 2018.

1.5.4. Impact of MUFA/PUFAs and SFAs on Cholesterol-Atherosclerosis

Since the early 1950s there has been great interest in the impact of dietary fats on cholesterol levels and mortality from atherosclerosis and CHD events. Kinsell et al., (1952), reported a difference in total cholesterol serum for hospitalised human subjects fed "dairy-derived fats" and egg yolk, when compared to vegetable diets. Epidemiological studies of ethnic sub-groups evidenced that populations such as Eskimos and Japanese farmers who consumed high levels of fish and n-3 PUFAs had lower CHD risk and mortality compared to people of the same ethnicity consuming high SFA western diets (Keys et al., 1958). Dietary intervention studies by Keys et al (1965a) confirmed this view and concluded that a high cholesterol diet (250-350mg cholesterol/1000 calories) increased serum cholesterol levels and swopping to a cholesterol-free diet reversed this outcome. Key's et al., (1986) 15 year follow up of the Seven Countries Study (SCS), initially carried out between 1958-1964, confirmed more geographic dietary anomalies and CHD mortality risk. Keys et al, (1986) reported that Northern European cohorts e.g. Finland and Northern American cohorts had a significantly higher CHD mortality rate that was strongly correlated with consuming more SFAs in diets. Equally, Southern European cohorts had lower CHD mortality that were strongly correlated consuming more MUFAs or PUFAs e.g. olive oil as Keys termed a "Mediterranean diet" as the main dietary fats – and finally the Japanese with the lowest CHD mortality rate and a mainly low-fat fish diet (n-3 PUFAs).

Research studies were also carried out to ascertain the cholesterol synthesis pathway (Bloch, 1965) and into a condition where hypercholesterolemia and early mortality from CHDs was present in families (Familial Hypercholesterolemia) – see paragraph 1.4.1. The FH studies added fuel to the cholesterol-atherosclerosis fire and linked dietary FA therapy of lowering plasma total cholesterol by swopping SFA with vegetable oils (Powell and Vacca, 1961). These dietary intervention and epidemiological studies influenced the development of the concept that dietary fats differentially elevated plasma total cholesterol and that elevated cholesterol (hypercholesterolemia) levels positively correlated with increased risk of CHD mortality.

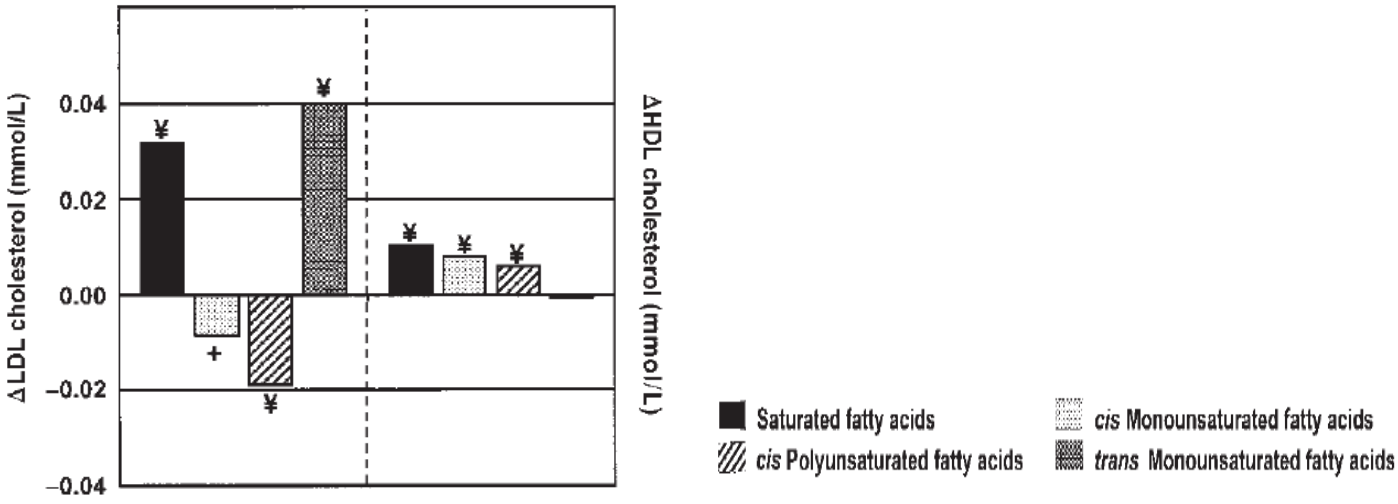
In the USA large CHD cohort "Framingham Study" was also in progress to identify causes of CHD mortality. The Framingham Study is a multi-generation, familial study that commenced in 1948 and continues to this day (Mahmood et al., 2014). These studies identified that not only elevated plasma total cholesterol was a strong correlation but other risk factors were involved in CHD aetiology e.g. sex, age, hypertension, diabetes, smoking, and left ventricular hypertrophy (Dawber et al., 1959, Kannel et al., 1961). In particular, Kannel et al (1961) confirmed the relationship of hypertension and hypercholesteremia correlated with increased CHD risk- 2.6-fold in men and 6-fold in women. The study noted that whilst blood cholesterol in men >245mg per 100ml (13.5mmol/L) were 3-fold more likely to develop CHD women were only slightly affected by this factor - an indication that differences between sexes and CHD risk factors were present.

It wasn't until the 1960s and 1970s that analytical techniques were developed to identify classes of lipids e.g. triglycerides, lipoproteins, and apolipoproteins (see Table 1.2). These analytical techniques were developed and standardised alongside another large USA dietary intervention study into CHD prevention strategies – US Lipid Research Clinics Program (LRC) (Fredrickson et al., 1967, Myers et al., 1989, NHLBI, 2013). These analytical techniques were also applied to the Framingham samples (Gordon et al., 1977, Wilson, 2013) and other LRC samples (Jacobs et al., 1990) that ascertained that plasma HDL cholesterol were strongly inversely correlated to CHD risk and mortality.

A human dietary intervention study whose objective was to reduce CHD risk through increasing plasma HDL and lowering LDL cholesterol in dyslipidaemic men commenced in the 1980s in Helsinki, Finland. (Manttari et al., 1987). The Helsinki study evidenced that their highest CHD risk group had plasma TAG >2.3 mmol/L and a ratio LDL/HDL cholesterol >5mmol/L. Their study confirmed that the most prognostic value for CHD risk was LDL/HDL ratio in conjunction with TAG (Manninen et al., 1992). Further meta-analyses of dietary manipulation of serum cholesterol studies were undertaken by Mensink and Katan (1992) and

Mensink et al., (2003). Their analyses confirmed the differential impact of FAs on serum LDL and HDL (see Figure 1.8). They estimated that MUFA and PUFAs had a positive impact by reducing LDL and increasing HDL – in a beneficial cholesterol profile for CHD risk. However, they found that SFA increased HDL more than the MUFA/PUFAs – even though LDL and its associated CHD risk were also increased. Finally, they identified that PHVO would decrease HDL but also increase LDL – shown in the ratios of total cholesterol/HDL cholesterol below – the higher the ratio the greater the CHD risk. A meta-analysis (Mozaffarian et al., 2010) corroborated the view that replacing SFA with PUFAs may reduce CHD events. Finally, a recent review of previous studies was conducted by Clifton and Keogh (2017) concluded that reducing SFA reduced CHD risk and that replacing SFA with MUFA/PUFAs in the diet further reduced CHD risk.

Figure 1.8 The effect of SFA, PUFA, MUFA and TFA on Serum Total Cholesterol-HDL Ratio and Total Cholesterol-LDL Ratio



Source: Mensink et al., (2003) Δ Predicted change in the ratio of total cholesterol/HDL ratio and total cholesterol/LDL ratio when 1% energy carbohydrates replaced by FA. *P* < 0.05; **+***P* < 0.01 ¥*P* < 0.001.

Studies in animal models have also reported similar outcomes with low fat diets compared to high-fat high cholesterol diets in being causative of atherosclerosis. The ApoE*3 Leiden (AEL) female mouse model is proven to develop atherosclerosis in response to a high fat high

cholesterol diet (Vanvlijmen et al., 1994, Tonge, 2011, Hofker et al., 1994). Using the AEL mouse model of atherosclerosis studies have consistently shown that raising or decreasing dietary SFA and cholesterol impacts on atherosclerosis lesion area: Groot et al (1996) reported that a high cholesterol diet increased lesion area whilst a study by Yates et al.,(2009) confirmed that a low fat, low cholesterol diet reduced atherosclerotic lesions. Further studies by Tonge (2011) confirmed that atherosclerosis was greater in animals fed SFA beef compared to groups fed a SFA beef diet that had been enriched with MUFA-rich Rapeseed Oil. These studies corroborate the epidemiological findings and show that atherosclerosis is correlated to dietary fatty acids.

1.5.5. Impact of FAs on Serum TAG and CHD Risk

It has been identified in several studies that hypertriglyceridemia was indicative of an elevated CHD risk – but only when taken into consideration with LDL cholesterol and other CHD risk factors e.g. diabetes, smoking (Gotto, 1992, Cambien et al., 1986, Gordon et al., 1977). These studies concluded that due to TAG interaction with both VLDL and HDL metabolism (see Table 1.2, Figure 1.2 and Figure 1.3) and HDL's strong inverse relationship with CHD risk - TAG as an individual biological marker of CHD risk was unreliable (Cambien et al., 1986, Austin, 1989, Manninen et al., 1992). These studies and meta-analyses have confirmed that MUFA/PUFAs can moderately reduce serum TAG compared to SFA (Mensink and Katan 1992, Mensink et al., 2003). Manninen (1992) concluded that the Helsinki study outcomes reflected that of animal studies, and that TAGs and HDL differentially impacted on CHD development, with TAGs being involved in thrombosis of compromised arteries (see Figure 1.2).

1.5.6. The Impact of PHVO and RTFA on Cholesterol-Atherosclerosis

Several dietary intervention studies in the 1990s reported on the impact of plasma lipids and associated cholesterol ratios in the identification of CHD risk (Mensink and Katan, 1990, Lichtenstein, 1998, Almendingen et al., 1995). Whilst Mensink and Katan 1990 confirmed previous views that consuming Oleic acid was better for cholesterol levels compared to SFA, and that PHVO diets had the worst atherogenic profile as they raised LDL and reduced HDL (see Figure 1.8). In the other two studies (Lichtenstein, 1998, Almendingen et al., 1995) it was confirmed that MUFA/PUFA soybean oils had the lowest HDL/LDL ratio, with PHVO being the highest. Further studies by Han et al., (2002) and Baer et al., (2004) confirmed the hypercholesterolemic impact of PHVO on plasma lipids in estimating CHD risk. However, they also identified that inflammatory cytokines involved in atherogenesis were raised in the PHVO groups e.g. IL-6 and TNFα (Han et al 2002), elevated CRP and E-selectins (Baer et al 2004) - see paragraph 1.2 for role in inflammatory atherogenesis pathway. The "Nurse Health Study" epidemiological review also confirmed these outcomes: that PHVO had a greater atherogenic potential compared to other FAs not only due to their adverse impact on cholesterol profiles, but also that they instigated inflammatory pathways (Chiuve et al., 2009, Lopez-Garcia et al., 2005). These views have been reconfirmed in a meta-analysis by Mozzafarian et al., (2010) who calculated that if TFA were replaced by other FAs such as animal fats or vegetable oils a 50% or 65-85% reduction respectively would occur in CHD risk. Similar results have also been reported in animal models fed TFAs (Bassett et al., 2009, Koppe et al., 2009).

Studies into the impact of dairy products including enrichment with VA and CLAs on cholesterol and atherosclerosis have been the focus of investigation for over two decades. Pfeuffer and Schrezenmeir (2006) cautioned that extracting the correct information from TFA dietary intervention and epidemiological studies was problematic due to participants often consuming high SFA dairy products e.g. butter, cheese, milk (as in the aforementioned PHVO and SFA clinical studies). It was also recognised that ruminant products also contained C18:1 TFA isomers that although similar to PHVO had different distributions, with the predominant TFA in dairy being Vaccenic Acid (VA) and including potentially beneficial CLAs (Huth, 2007)

see Figure 1.5. Huth (2007) reviewed the first five RTFA dietary intervention studies that took place between 1993-2006 that compared the impact of PHVO and RTFAs. Huth (2007) concluded that all 5 clinical studies (including data from the Nurses' Health Study and Framingham studies) showed greater risk from PHVO and especially from PHVO margarines. In comparison RTFA consumed in butter and milk fats showed little impact on serum cholesterol levels and associated CHD risk. Further dietary intervention studies followed to compare PHVO with RTFAs.

Chardigny et al (2008) noted a lack of effect in lipid profiles for subjects eating PHVO diets compared to RTFA-rich diets. In particular, women showed a significant increase in total serum cholesterol, TAG concentration and an increased concentration of large particle LDL. However, they confirmed that PHVO participants had reduced serum HDL indicating a higher CHD risk profile. This study concluded that PHVO and RTFA have differential effects on serum lipid profiles in both genders, and thus both diets confer differential CHD risk to males and females. Motard-Belanger et al (2008) remarked upon the fact that two further studies had shown RTFA had a similar negative effect on CHD risk as PHVO. Their study showed that daily high intake of RTFA (consisting of. 4 x 50g cheese 33% fat; 2 x 250ml milk 3.25% fat, 175g yogurt 3.25% fat, 8 x 5ml tsp butter) increased serum total cholesterol and LDL, and reduced HDL, consequently increasing CHD risk. However, this was not the case with a moderate RTFA diet. Their study confirmed PHVO had a greater deleterious effect on serum cholesterol profiles suggesting that PHVOs and RTFA diets do have differential effects, and that the effects of RTFAs are dependent upon quantity in the diet. Although differences in impact of PHVO RTFA were observed in the above studies, German et al's (2009) review concluded that there was no clear evidence to suggest that *trans* fats from ruminant meat and dairy products increase the risk of CHD. The view that PHVO and RTFA have different impacts on CHD risk and may be dependent upon quantities consumed was upheld by Bensden et al., (2011). However, a recent quantitative review of 30+ previous studies by Brouwer et al (2013) into the CHD risk of RTFA in comparison to PHVO calculated that RTFA, CLAs and PHVO were "gram for gram" similar. They concluded that the studies reviewed in the impact of RTFA and CLAs

risk factors would be the same as PHVO - increasing LDL, decreasing HDL, and increasing the LDL/HDL ratio and thus high CHD risk.

However, although human dietary intervention and epidemiological studies have observed an impact of RTFA - perhaps dependent upon quantities consumed (Chardigny, et al 2008, Motard and Belanger, 2008, Brouwer et al 2013) it has also been noted that it is often difficult to discern the impact of FAs due to the complexity of the FA nutrients eaten in the trials and confounding factors such as obesity (Pfeuffer and Schrenzenmeir 2006). Therefore, a succession of studies has begun to identify the different effects of separate TFA isomers on lipid profiles, inflammation and atherogenesis pathways (see paragraph 1.5.6.1). Additionally, from the 1980s onwards *trans* isomers CLAs present in RTFA have been reported to have beneficial health effects, perhaps due to the fact that RTFA VA is a precursor for endogenous CLA synthesis e.g. c9, *t*11-(RA) and CLA *t*10,c12 (Pfeuffer and Schrezenmeir, 2006).

1.5.6.1 Impact of C18:1 *trans* isomers on Cholesterol -Atherosclerosis

Further to the dietary intervention and some epidemiological studies, different impact of TFA isomers on cholesterol and atherosclerosis have been evidenced in animal models. It has been shown that VA or PHVO enriched butter diets differentially impacted on serum cholesterol compared to control standard butter diets in Hamsters (Lock et al 2005, Tyburcz et al., 2009, Kraft et al., 2011). Lock et al (2005) reported that VA fed animals had a lower VLDL and IDL/HDL ratio compared to both standard and PHVO diets, however HDL was also reduced. They confirmed that the PHVO diet animals had a lower VLDL and IDL/HDL ratio compared to standard butter. Tyburcz (2009) reported an improved serum lipid profile in hamsters that had consumed diets enriched with either EA or VA showing that EA and VA may have differential impacts on cholesterol metabolism. A study in guinea pigs, although failing to show a relationship between PHVO or RTFAs and atherosclerosis, confirmed that diets rich in ruminant milk fat which contained Vaccenic Acid had increased small HDL (Rice et al., 2010).

Kraft et al., (2011) further reported that PHVO diets split between the *trans* isomers *t4-t10* see Figure 1.5 (Sunflower PHVO) had a higher risk lipid profile compared to *t11-t16* see Figure 1.5 (Safflower PHVO) with increased total cholesterol, IDL and VLDL and reduced HDL and also differential effects on cholesterol regulating SREBPs. These studies have identified that not all TFAs are equal in their impact on lipid metabolism and consequent impact on CHD risk. However, a recent clinical dietary intervention trial that supplemented diets with VA, had a less positive outcome. Gebauer et al., (2015) replaced dietary stearic acid with CLA *c*9,*t*11, VA or PHVO. Their study reported that CLA *c*9,*t*11 did not affect cholesterol lipoprotein profiles but had lowered serum TAG. However, they found that both VA and PHVO groups had increased total cholesterol, LDL cholesterol and ratio of total cholesterol/HDL compared to the control group. However, they also found that VA had increased HDL and atherogenic markers for LDL including ApoLPs ApoA1, ApoB and LP(a). Their study concluded that VA, but not CLA should be listed under TFAs for nutritional information. In this respect the study and the views of Brouwer et al., (2013) have labelled VA as a high-risk CHD TFA. However, the undeniable link with endogenous VA-RA CLA synthesis cannot be ignored. Indeed, since the 1980's CLAs have been identified to have beneficial health effects in animal models including reduction of hypertension and diabetes, anticarcinogenic, antitumorigenic effects and reduction of age-related bone loss (Pariza et al., 2001, Rahman et al., 2007).

1.5.6.2 Impact of CLAs on Cholesterol-Atherosclerosis

VA and CLAs have been reported to have hypolipidemic, anti-atherogenic and anti-inflammatory properties. Several animal studies in rats have identified that VA and CLA *c*9,*t*11 have had a hypolipidemic impact on serum and hepatic lipids and indicated they may be useful in the treatment of non-alcoholic fatty acid liver disease (NAFLD) and metabolic syndrome (Jacome-Sosa et al., 2014, Jacome-Sosa et al., 2010, Wang et al., 2009). However, this research is also applicable to reducing CHD risk by improving cholesterol profiles. Further animal studies in New Zealand white rabbits have reported that CLA supplementation inhibited and reduced atherosclerosis lesions (Kritchevsky et al., 2004, Kritchevsky et al.,

2000). Basset et al., (2010) has also reported that LDLr$^{-/-}$ mice fed VA and cholesterol rich butter showed an athero-protective impact on lesions compared to EA and other cholesterol fed animals. In cellular studies, it was identified that CLA *t*10*,c12* controlled expression of the CD36 receptor in macrophages, regulated TAG and had a lipid reduction mechanism (Stachowska et al., 2010). A further study by Li et al (2018) identified the impact of endogenous VA conversion to CLA *c*9,*t*11 and that of CLA *t*10*c*9 on inflammatory markers in human umbilical endothelial cells (HUVEC). They found that VA was responsible for down regulating Toll-like receptor 4 (TLR4) which is responsible for the regulation of inflammatory mediators e.g. IL6, and adhesion molecules ICAM and VCAM. In contrast, Leptin inhibited VA conversion and upregulated TLR4 in a pro-inflammatory response. Of particular note is the fact that VA and CLAs appear to regulate lipoprotein and inflammatory pathways through SREBPS, PPARs, and other gene expression pathways (Viladomiu et al., 2016, Kraft et al., 2011) and may be recognised formally as being ligands for FA gene regulation. However, although cellular, animal and human studies are beginning to understand the pathways potentially FA regulated by VA and CLAs, the negative outcome in human dietary studies when combined with other nutrients is 'food for thought' and requires further investigation (Fuke and Nornberg, 2017).

1.5.7. Other Macronutrients and their Impact on Serum Cholesterol

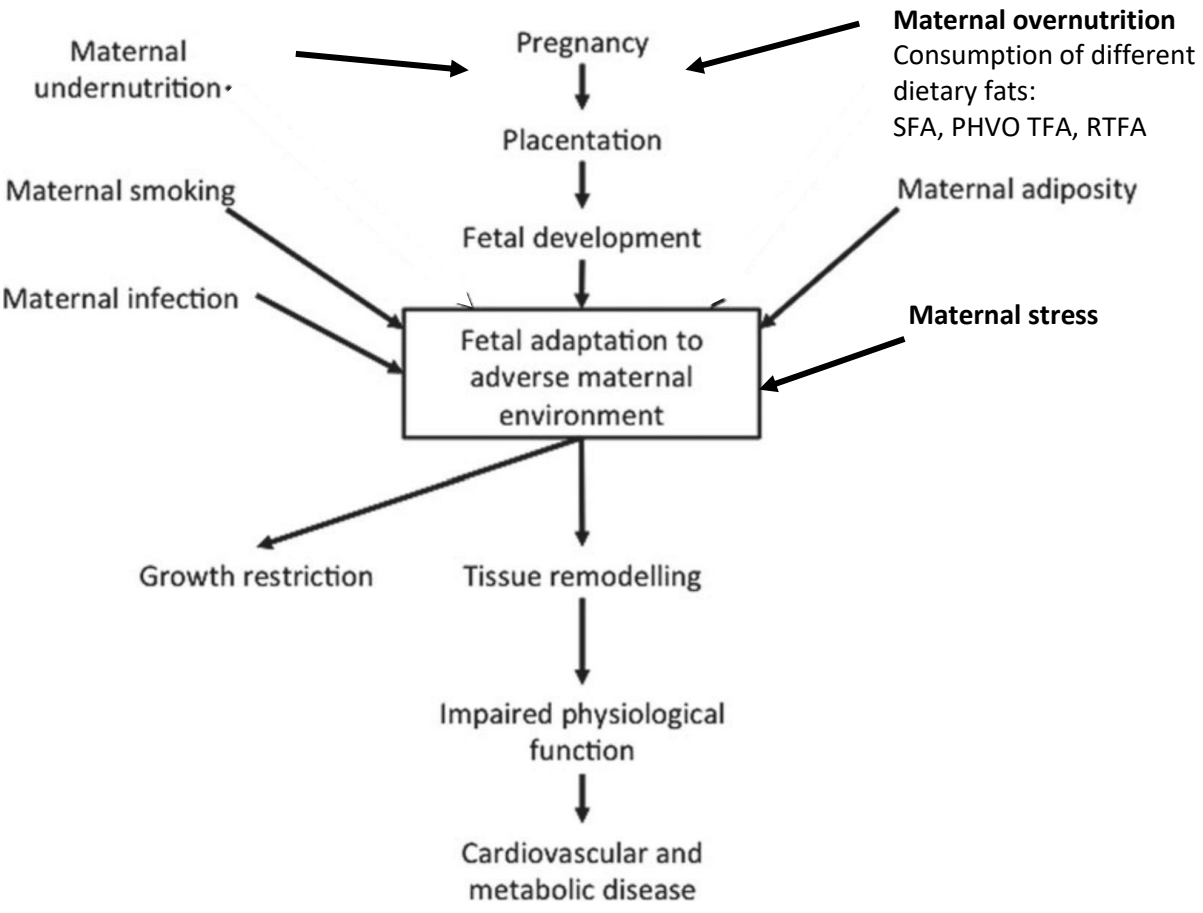
There has been considerable interest in exchanging SFAs for other macronutrients e.g. carbohydrates instead of MUFA/PUFAs. Keys et al (1965a) reported that the impact on total cholesterol appeared similar when exchanging SFA with carbohydrates. Meta analyses by Mensink and Katan of 27 trials (1992) and 60 trials (2003) respectively found that exchanging SFA for carbohydrates had little impact on LDL cholesterol. This view was upheld in a review of meta-analyses and outcomes by Clifton and Keogh (2017). Difficulties in measuring SFA in dairy products and their use in dietary intervention trials has come in part from the identification of RTFA in ruminant meat and dairy products (Huth, 2007, Campbell, 2017). Campbell (2017) also commented that animal meat or dairy proteins could be causal in elevated cholesterol metabolism. Until the roles of individual nutrients and their complex

interactions can be fully mapped, then the role of FAs in metabolism and atherogenic pathways cannot be fully comprehended.

1.6 FETAL PROGRAMMING AND DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE (DOHAD)

"Fetal programming hypothesis" or "Barker Hypothesis" was named after the first researcher to observe the associations between low birth weight and anthropometry and increased risk of mortality from CVDs in later life – see paragraph 1.6.1 (Langley-Evans, 2001). The term "programming" and DOHaD theory now encompass the understanding that a maternal insult or environmental stimulus, such as undernutrition, overnutrition, smoking or stress (see Figure 1.9), causes perturbations in the fetal environment and can permanently affect the ontogenesis of the organism, resulting in changes to the organism's metabolism and physiology and giving rise to increased susceptibility to non-communicable diseases in adulthood (Langley-Evans, 2013, 2001). The following paragraphs give an overview of the fetal and developmental programming hypothesis in humans and animal models, with focus on the impact of maternal consumption of different isomers of *trans* fats (Figure 1.5) on the susceptibility of offspring to developing atherosclerosis.

Figure 1.9 Fetal and Developmental Programming of Disease



Source: Adapted from Langley-Evans (2013)

1.6.1. Origins of Fetal Programming Hypothesis – Undernutrition in Human Retrospective Studies

A study of over 15,000 men and women born in Hertfordshire between 1911-1930 identified that those men who had the lowest body weights at birth and 1 year of age, were found to have the highest death rates form ischaemic CHD in later life (Barker et al., 1989). This study gave an indication that early childhood nutrition as well as the *in-utero* environment played a crucial role in non-communicable disease outcomes. Two further follow up studies by Barker et al., (Barker et al., 1990, Barker et al., 1993) gave more detailed birth and anthropometric data including head circumference and length, from which ponderal indices

were derived as a measure of thinness (birthweight/length^3). They found that babies born with a high placental weight/birthweight ratio or low ponderal index of thinness at birth were inversely correlated with health problems in later life including elevated blood pressure, impaired glucose tolerance, and elevated plasma fibrinogen which was considered a marker for CHD risk. Other studies in USA and Finland concurred with these outcomes. In the US Nurses' Health Study, Curhan et al (1996a) reported on two cohorts totalling over 170,000 women that lower birthweights <5-5.5lb had a higher risk of hypertension. Their analysis of over 22,000 men in this study (Curhan et al., 1996b) also had similar results to the women with hypertension and BMI in adulthood being dependent upon birthweight. However, they also reported an increased risk for Type II Diabetes in men for low birthweights <5-5.5lb. Eriksson et al (1999) confirmed that their study of 3600 men from Helsinki reflected that of Barker et al's studies (Barker et al 1989, 1993b) that CHD mortality was inversely correlated with low birth weight and thinness at birth. However, they highlighted although those men who had died of CHDs had a low birthweight, they also had increased their bodyweight to have a higher than average BMI by 7-15 years of age termed "catch up growth". They hypothesised that a poor prenatal diet was causative of fetal growth retardation during vital development periods, followed by a post-natal nutrient-rich diet that may increase childhood obesity and CHD risk through programming/tissue remodelling pathways.

One of DOHaD's premises is that "fetal adaptations to scarcity become maladaptive only when individuals are later exposed to an environment of plenty" (Schulz, 2010). This DOHaD premise was met by Eriksson et al's (1999, 2001b) analyses of the Helsinki data. However, it was also reflected in another pivotal retrospective study of maternal undernutrition and fetal programming that occurred between 1944-45 "The Dutch Famine" (Schulz, 2010) and is also recognised as the "Hunger Winter" (Lumey et al., 2007). This key study identified that the timing of the nutrient insult during the early, mid, or late gestational development period compared to post-natal nutrient availability may instigate differential effects on fetal programming of adulthood diseases (Schulz, 2010). Maternal food intake was reduced from an average of 1600 calories/day in December 1943 to under 1000 calories/day by November

1944, with the most dramatic decrease to 500 calories/day recorded in April 1945 (Lumey et al, 2007). Food availability remained low until May/June 1945 with an increase to over 2000 calories/day. This meant that nutrient availability at the time of conception, first, second and third trimesters differed throughout the pregnancy. It was found that babies born during mid-late gestational famine mothers were lighter, thinner, and shorter with smaller heads and were found to have increased hypertension in later life, compared to those offspring not exposed to the famine. Exposure to famine in early gestation had the greatest impact, and predisposed offspring in adulthood (50-59y age) to the prevalence of several poor health outcomes including: (i) higher risk of CHD, indicated by prevalence of angina pectoris (Roseboom et al., 2000b); (ii) reduced glucose intolerance (de Rooij et al., 2006b, Ravelli et al., 1998) potentially via impaired insulin-secretion pathways (de Rooij et al., 2006a) and consequent Type II Diabetes (Roseboom et al., 2011); (iii) atherogenic lipid profiles (Roseboom et al., 2000a), (iv) elevated fibrinogen, and (v) higher BMI (Roseboom et al., 2001). Finally, early and mid-gestational famine offspring had increased prevalence of obstructive airway disease (Lopuhaa et al., 2000) and impaired renal function (Painter et al., 2005).

1.6.2. Fetal Programming by Undernutrition in Animal Models

Maternal undernutrition has also been shown in several animal models, with outcomes reflecting those of epidemiological and retrospective studies. Food restriction during pregnancy was found to programme hypertension in offspring of rats (Woodall et al., 1996), guinea pigs (Kind et al., 2002) and sheep (Hawkins et al., 2000). Other effects of food restriction in the guinea pig have include (i) an elevated cholesterol metabolism in both male and female offspring (Kind et al., 1999), and hyperinsulinemia in adult male offspring (Kind et al., 2003). Fetal programming due to specific nutrient restriction e.g. maternal low protein (MLP) has also been identified in rat studies ((1994) Langley-Evans (2000)). It was found that offspring of mothers fed low protein diet during gestation, exhibited elevated hypertension compared to the chow control group in later life. Langley Evans (2000) concluded that it was the balance of nutrients that defined the outcomes observed and were crucial to maternal undernutrition in pregnancy. Fetal programming of atherosclerosis through MLP has also

been evidenced in AEL mice (Yates et al., 2009). At 15 weeks age, the AEL offspring of were found to have greater lesions, elevated plasma cholesterol and plasma TAG compared to the AEL control group. It is evident from the above animal studies in several rodent species and sheep that diet restriction and MLP diets can not only program CHD risk factors in offspring e.g. hypertension, altered cholesterol metabolism, but can also manifest itself in atherosclerosis progression.

1.6.3. Fetal Programming of CHDs by Overnutrition

In comparison to the glut of retrospective data and animal studies of maternal undernutrition that have laid the foundations for developmental programming of NCDs, there is less *historical* data to draw on for overnutrition. However, maternal obesity and associated energy-dense nutrient intake are cause for concern in fetal programming outcomes for CHDs in future populations (SACN, 2011, SACN, 2018). It is acknowledged that many energy dense foods e.g. ruminant meats, bakery products, biscuits, butter, milk, margarines and shortenings whilst containing SFAs also contain between 3-5% TFAs (PHVO and RTFAs) (Innis, 2006). Energy imbalance through overnutrition manifests itself in maternal obesity. However, although many human studies report on "obese" maternal profiles in pregnancy and lactation and that of associated metabolic problems e.g. hyperlipidaemia, they do not address specific macronutrient intakes and their impact in causing NCDs. In large cohort studies it has been observed that being overweight or obese pre-pregnancy has less influence on fetal outcomes compared to maternal gestational weight gain (Gaillard et al., 2013, Flick et al., 2010). Therefore, the impact of energy dense foods such as fats, and their impact on maternal physiology is of key importance in considering fetal programming outcomes. Maternal obesity is often a risk factor for preeclampsia (Stewart et al 2007). Preeclampsia is a condition which usually occurs mid-late gestation and can have severe consequences for mother and fetus including proteinuria, de novo maternal hypertension, maternal organ dysfunction (liver and renal) and placental dysfunction causing placental hypertrophy and fetal growth restriction (Bokslag et al., 2016). Obesity in pregnancy monitoring studies have also identified maternal

factors that may influence programming and fetal outcomes, including maternal, reduced vascular function (Meyer et al. 2013), elevated inflammatory markers (Ramsay et al., 2002, Stewart et al., 2007), dyslipidaemia including elevated fasting TAG (Ramsay et al 2002, Meyer et al 2013) and elevated small LDL (Meyer et al., 2013). Gademan et al (2014) also reported that maternal FFA levels were corelated to the child's fat percentage at birth and increased BMI. Stewart et al., (2007) emphasised that obese pregnant women already have elevated plasma lipid profile and biological markers in early pregnancy compared to average weight pregnant women. These levels increase throughout the mid-late gestation period, then reduce post-partum to the elevated starting value but do not fall below that of their average weight counterparts. Taking these risk factors in mind, it is not surprising that the observed outcomes of obese maternal studies include a significant increase in number of pre-term babies, caesarean deliveries, and either lower birth weight babies or macrosomia babies (Gailard et al, 2013, Flick et al, 2010).

1.6.4. Fetal Programming by Overnutrition in Animal Models

Many animal studies look at high SFA diets and their impact on developmental programming. It has been demonstrated in several animal models of obesity that high SFA, 'junk food' diets during pregnancy not only alter the maternal metabolism but programme NCDs and obesity in offspring. Fetal programming of Non-Alcoholic Fatty Liver Disease (NAFLD), CVD risks including insulin-resistance, hyperlipidaemia, hypertension, vascular and endothelial dysfunction and obesity have been observed when subjected to high SFA or 'Junk Food' diets *in utero*, in rats (Alfaradhi et al., 2014, Bayol et al., 2008), C57 mice (Gregorio et al., 2010, Oben et al., 2010) and non-human primates - Japanese Macaques (McCurdy et al., 2009, Fan et al., 2013, Sullivan et al., 2014, Li et al., 2019). Studies in smaller animal models often omit maternal profiles except for the factors of 'obesity' and maternal 'dietary SFAs'. Programming outcomes by maternal overnutrition include offspring with elevated lipid metabolism and associated vascular dysfunction in rats (Khan et al., 2005, Koukkou et al., 1998, Ghosh et al., 2001); impaired endothelial function in $LDLr^{-/-}$ mice (Langenveld et al., 2008); and hypertension and obesity in C57 mice (Liang et al., 2009) .

1.6.5. Fetal Programming of Atherosclerosis

As outlined above maternal overnutrition and obesity cause maternal metabolic dysfunction e.g. elevated maternal lipid and lipoprotein metabolism and inflammatory markers during pregnancy. Although these metabolic parameters in themselves are high CHD risk factors for the mother, they also appear to programme atherosclerosis in future generations. Whilst it is thought that human fetal cholesterol metabolism is mainly endogenous and that little is transferred to the offspring via the placenta (Herrera and Ortega-Senovilla, 2014, Mennitti et al., 2015), it was identified that temporary maternal hypercholesterolemia or mothers with Familial Hypercholesterolemia (see paragraph 1.4.1) were linked to early development of atherosclerotic lesions in their fetuses and children in the "Fate of Early Lesions in Children" (FELIC) study (Napoli et al., 1997, Napoli et al 1999). Napoli's et al studies (1997, 1999) reported that although hypercholesterolemic mothers increased prevalence of atherosclerotic lesions in their fetuses there were no changes observed in their offspring's lipid metabolism. These researchers went onto reproduce programming of atherosclerosis in two animal models: (i) New Zealand white rabbit (Post-natal diet (Napoli et al., 2000), and (ii) $LDLr^{-/-}$ knockout mice (Napoli et al., 2002). Their results mirrored those of the human studies, increasing atherosclerosis in the offspring, but not impacting on offspring lipid metabolism profiles, bodyweights or other parameters measured which were the same as control animals. These outcomes were again shown in offspring born to wild type or $ApoE^{-/-}$ knockout murine mothers (Palinski and Napoli, 2002).

In two further studies in the $ApoE^{-/-}$ knockout mouse model of atherosclerosis, it was observed that atherosclerosis was programmed in the absence of maternal hypercholesterolemia (Madsen et al., 2003), and presence of maternal hypercholesterolemia (Goharkhay et al., 2007). In support of Palinksi and Napoli's findings, Goharkhay et al (2007) found that maternal hypercholesterolemia *in utero* environment had programmed atherosclerosis in offspring. Finally, a recent publication has also confirmed that maternal

high SFA diet during pregnancy, caused maternal hypercholesterolemia (C57 maternal mouse) and programmed increased atherosclerosis in AEL offspring (Tarling et al., 2016).

1.6.6. Impact of TFAs on Fetal Programming

There is evidence to support that TFAs are passed to the developing fetus via the placenta or to the neonate via milk during lactation (paragraph 1.6.9)., and that the exposure timing may have an effect upon the programming of CHDs and atherosclerosis (Larque et al., 2001, Albuquerque et al., 2006, Hornstra et al., 2006). In the following paragraphs, impact of TFAs *in utero* will be discussed and thereafter a review of the impact of FAs in lactation and early post-natal life.

Many of the human TFA studies outlined below focus on the impact that of all C18:1 *trans* isomers or that of PHVO C18:1 *trans* 9 EA, and very rarely identify other *trans* isomers in RTFAs such as VA and CLAs (see figure 1.5 for distribution of C18:1 *trans* isomers). Human pregnancy and neonate studies particularly focus on the impact of TFAs on the developing fetus due to the interaction of TFAs in reducing availability of EFAs (Innis., 2006, Desci and Boehm., 2013). Innis (2006) noted that whilst n-6 PUFAs and AA are more prevalent and readily consumed in products such as olive oils, maternal intake of n-3 PUFAs are generally low. It is noted that maternal EFA requirements increase up to 4.5% total calories during pregnancy and 7% during lactation (Arbex et al., 2015). EFAs Linoleic Acid (LA) (C18:2 n-6) is desaturated and elongated to Arachidonic Acid (C20:4 n6). Whilst α-Linoleic Acid (ALA) (C18:3 n-3) is desaturated and elongated to eicosapentaenoic acid (EPA C20:5) and onwards to docosahexaenoic acid (DHA C22:6 n3) (see Paragraph 1.5 and Figure 1.4) (Innis., 2006, Desci and Boehm., 2013, Mazzocchi et al., 2018). Studies in human fibroblasts have observed that desaturase enzymes in the EFA conversion pathways are potently inhibited by PHVO Elaidic Acid(EA) and to a lesser extent by Vaccenic Acid (VA) (Rosenthal and Whitehurst, 1983). During pregnancy there is an increase in demand for maternal EFAs for the developing fetus (Holman et al., 1991) with the brain and nervous system being rich in n-3 PUFAs (Arbex et al 2015). N-6 AAs are an essential component of cellular membranes e.g. PPL and influence membrane fluidity throughout the

body (Holman et al 1991) and act as eicosanoid precursor for cellular division and signalling pathways (Innis, 2006). Whilst n-3 EPA and DHA are vital for healthy neurological, visual and brain function. EFAs therefore play key roles in maternal physiology and fetal development (Innis., 2007, Arbex et al, 2015, Mazzocchi et al., 2018).

Several studies throughout the 1990s and 2000s analysed C18:1 *cis* and *trans* isomers in cord plasma or plasma samples from mother or newborn infant (Koletzko and Muller, 1990, Elias and Innis, 2001, Hornstra et al., 2006, Houwelingen and Hornstra, 1994, van Houwelingen et al., 1996). It was noted that as TFAs are unable to be synthesised by humans, any TFAs present were the result of maternal transfer to the developing fetus/new-born infant (Hornstra et al 2006). Hornstra, et al (2006) in reviewing two cohorts in the Netherlands reported weak correlations between TFAs and birth anthropometry. The majority of the studies above reviewed monoenoic "trans" isomers, e.g. EA, and recorded an inverse relationship of maternal and fetal TFAs with reduced birth anthropometry. However, Desci and Boehm (2013) caution against the results of reduced birth anthropometry due to considerable confounding factors and weak associations when taking these factors into consideration. There are a limited number of studies which identified RTFAs, it was observed that maternal/fetal plasma lipids were positively associated for CLAs and inversely correlated with birth anthropometry (Elias and Innis., 2001) and EFA LC PUFAs (Enke et al., 2011). Enke et al., (2011) also reported that this was not the case for VA *trans* 11. This outcome would appear supportive of the cellular studies that found no effect of VA on EFAs, potentially due to its desaturase that is not involved in the EFA synthesis pathway (Rosenthal and Whitehurst, 1983, Rosenthal and Doloresco, 1984). Additionally, a meta-analysis of European women concluded that EA were the predominant TFAs present when measured during pregnancy and these TFA were inversely correlated with LC PUFAs. There is evidence to suggest that TFAs inhibit EFA LA and ALA synthesis to LC PUFAs, reducing availability of EFAs to the developing fetus and mother thus compromising fetal and neonate development – and potentially with birth anthropometry (Hornstra et al., 2006, Innis 2007, Desci and Boehm, 2013).

Studies have observed that children with low n-3 PUFAs present with visual impairments and psychological disorders e.g. attention deficit/hyperactivity disorder (Arbex et al., 2015). Fetal programming studies of maternal TFA during pregnancy and lactation in rats, found that those offspring exposed to TFA *in utero* had reduced brain n-3 EPA and remodelled hypothalamic mechanisms that increased appetite in later life compared to control animals (Albuquerque et al., 2006). Desci and Boehm (2013) aptly concluded that the studies' they had reviewed brought into question the nutritional viability of consuming TFAs during pregnancy. However, although there is weak evidence to support CLA are similar to PHVO EA in their EFA interaction, VA was reported not to inhibit desaturase activity to the same extent as EA in cellular studies nor impact on fetal and neonate EFAs, which questions whether all TFAs should be treated equally.

1.6.7. Impact of TFAs on Fetal Programming in Animal Models

Although there are many PHVO programming studies in animal models, the impact of PHVO is not ascertained whether the programming outcomes are *in utero* or early life nutrition in lactation that is causal. Several studies have noted that maternal dietary TFAs were incorporated into tissues of their offspring, in piglets (Pettersen and Opstvedt, 1992) and rats. (Komatsuzaki et al. (2013). An impact of programming on rat offspring exposed to PHVO *in utero* were found to have reduced brain n-3 EPA and remodelled hypothalamic mechanisms that increased appetite in later life compared to control animals (Albuquerque et al., 2006). This outcome was reproduced to some extent by Pimentel et al., (2012) in rat offspring exposed to PHVO during pregnancy and lactation. At d90 PHVO offspring had elevated hypothalamic inflammatory cytokines e.g. TNFα, IL-6, IL1-β compared to chow offspring and showed impaired satiety behaviour. Offspring of animal models of maternal obesity that were fed high fat/SFA diets (paragraph 1.6.4) similarly provided evidence that offspring displayed hyperphagia (Bayol et al., 2007, Sullivan et al., 2014, Thompson et al., 2017). In one study it was identified that maternal hyperlipemia could be causative of hypothalamic orexigenic neurogenesis in the developing fetus (Chang et al., 2008). Bouret (2010) explained that orexigenic hypothalamic development is early in gestation and sensitive to maternal

hormone e.g. leptin, insulin and associated nutrient supply. However, the PHVO animal studies reported above have either not recorded maternal lipid profiles nor confirmed if hyperlipidaemia was not present.

1.6.8. Mechanisms of Fetal Programming

There is extensive evidence in both human cohort and retrospective studies and animal experiments (see above) that maternal nutrition can detrimentally impact upon an individual's health in later life – see Figure 1.9 (Langley-Evans, 2015). The following paragraphs give a brief overview of the programming mechanisms that are under investigation.

1.6.8.1 The Placenta

The development of the placenta and nutrient supply to the fetus during its development is complex. In humans, the placenta progressively increases in size throughout pregnancy, with fetal/placental weight ratio increasing 40-fold from week 6 to term (Myatt, 2006). In normal pregnancy maternal plasma nutrients and hormones tightly regulate placental transporters and signalling pathways thereby influencing placental and fetal growth rates. Disruption of placental development pathways (for example gestational diabetes, maternal protein restriction or preeclampsia) can cause intrauterine growth restriction, small for gestational age or large for gestational age babies.(Jansson and Powell, 2006, Delhaes et al., 2018). Myatt (2006) notes that the timing of the nutritional or environmental insult is central to placental function. Perturbations during angiogenesis will have different effects compared to perturbations during trophoblast growth and differentiation. For example, maternal anaemia increases placental angiogenesis in the first trimester (Kadyrov et al., 1998). Retrospective studies of maternal nutrient and iron deficiency during early gestation were shown to cause placental hypertrophy and retarded fetal growth, with consequent hypertension in later life (Barker et al., 1990, Lumey, 1998). In contrast, pre-eclampsia at +20 weeks gestation, is representative of failure of placentation, reduced angiogenesis and consequent placental

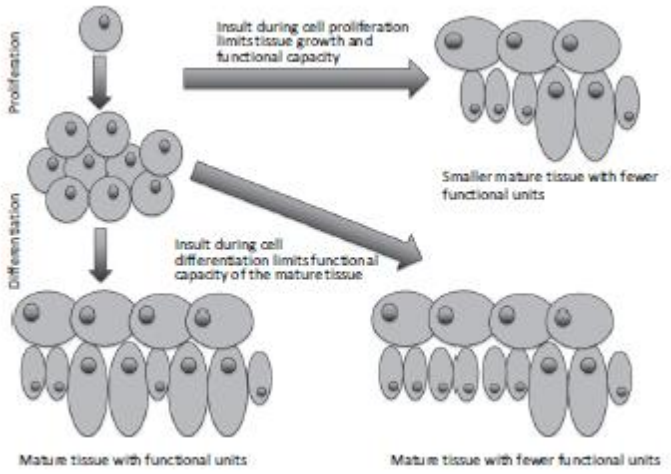
hypoperfusion also resulting in retarded fetal growth with consequent CHD risk in later life (Poston, 2006, Bokslag et al., 2016). It is noted that mothers who are obese or consume high SFA diets during pregnancy have additional factors that may contribute to placental dysfunction, including elevated inflammatory markers, adipokines and insulin resistance.

However, programming pathways in humans are not yet fully understood with the majority of placenta samples only available post-partum and not during gestation (Delhaes et al., 2018). Jansson and Powell (2006) concluded the placental programming pathway is multi-faceted. It not only depends upon maternal nutritional status but also maternal hormones (e.g. cortisol), angiogenesis, oxidative/nitrative stress, transporter expression regulation and epigenetic modification of placental genes.

1.6.8.2 Tissue Remodelling

Intrauterine growth restriction caused by maternal stress, undernutrition or overnutrition results in retarded growth of organs including the liver, spleen, thymus and kidneys (Boito et al., 2002, Greenwood and Bell, 2003). In mammalian cells the development pathway is controlled through proliferation, differentiation, replication, repair, renewal, and apoptosis, mediated with extrinsic signalling molecules, such as cytokines and growth factors. The latter mechanisms combine to create a diverse myriad of tissues and thus develop into a complex adult, multi-cellular organism (Alberts et al., 2016). The timing of the proliferative phase is key to specific organogenesis, for example skeletal muscle, heart and liver is early in development, with the kidney occurring later (Langley-Evans, 2009, Brameld et al., 2003, Si-Tayeb et al., 2010). Dependent on timing of the maternal nutritional or environmental insult it is proposed that cellular proliferation, but not differentiation, would be affected generating organs that were smaller with less cells, but with normal cellular function. However, impediments during the differentiation of tissue cells would produce organs of normal size but with altered cell type and potentially fewer functional units (Figure 1.10) (Langley-Evans, 2009).

Figure 1.10 Tissue Remodelling



Source: Langley Evans (2015)

As an example, it has been shown that an MLP diet during gestation in rats (Langley-Evans et al., 1999) and sheep (Lloyd et al., 2012) produce offspring with kidneys of similar size to controls, but with reduced nephron count. Human studies have also evidenced that low birth weight and hypertension are linked with a reduced renal volume (that is synonymous with nephron complement) (Zandi-Nejad et al., 2006, Keller et al., 2003). MLP and nephrogenesis studies in rats have specifically indicated that perturbations in nuclear cytoskeleton remodelling and cell cycle regulation that increase cellular apoptosis play a key role during embryonic development (Swali et al., 2011, Lloyd et al., 2012, Welham et al., 2002).

1.6.8.3 Genetics and Epigenetics

Epigenetics is described as “a link between genotype and phenotype” (Goldberg et al., 2007). Epigenetic factors control expression, or silencing, of genes through modifications to chromatin and its associated histone proteins during the cell cycle but does not alter the underlying base-pairing chromosome DNA sequence. It is considered that epigenetics play a key role in orchestrating an organism’s hierarchical development system, from totipotent germ line cells to finite germ layer somatic cells. These heritable modifications in turn confer

control over cell fate through signalling for gene expression or gene silencing mechanisms (Alberts et al., 2016). During the cell cycle, nuclear complexes alter DNA-histone bonds and cause transient changes in chromatin conformation. Changes include translocation of histone dimers, known as sliding, unwrapping of DNA from histone octamers and DNA loop formation (Cheung et al., 2000). DNA methylation or demethylation further exerts control of the chromatin state, in association with post-translational modifications. Histones are susceptible to a myriad of N-terminal tail post-translational covalent modifications (e.g. acetylation, deacetylation, methylation, phosphorylation), which occur transiently during replication and transcription processes. All these modifications confer initiation or repressive regulation of gene expression and can act as recognition sites in the recruitment of enzymes, protein regulatory complexes, cytokines or transcription factors (Kouzarides, 2007). The DNA and core histone modifications ascribe an inheritable genetic and underlying epigenetic histone code that has become central to understanding developmental processes, and the direction of cell fate for both normal and pathological development (Jenuwein and Allis, 2001). For example, in rats, maternal low protein has been found to decrease methylation of hepatic PPARα and glucocorticoid genes of weaned offspring (Lillycrop et al., 2005). The study noted methylation of gene promoters provide stable mechanisms for altered gene expression and can be linked with tissue remodelling and risk of disease in humans. Additionally, it has been shown that maternal hypercholesterolemia during pregnancy moderately altered fetal arterial expression of 135 genes which could play a role in atherogenesis over the course of their offspring's lifespan (Palinski and Napoli, 2002, Napoli et al., 2002). These studies proposed that maternal hypercholesterolemia caused increased lipid peroxidation and reactive oxygen species during early fetal development potentially affecting signalling pathways for genes e.g. TNF, PPARγ involved in cell recruitment, growth and differentiation, thus imprinting susceptibility to atherosclerosis during fetal development.

1.6.9. Developmental Programming of NCDs by Early Life Nutrition

Developmental programming is suggestive that the greater the disparity in *in utero* nutrition and birth outcomes compared with their post-natal environment the greater the influence on

disease outcomes in later life (Koletzko et al., 2012). For example, the impact of undernutrition *in utero,* low birth weight and catch up growth in childhood as described by Eriksson et al, leading to obesity or hypertension in later life (Eriksson et al., 2001a, Eriksson et al., 2001b, Eriksson et al., 1999). Or, through maternal obesity with offspring having a propensity for obesity in later life (Laitinen et al., 2001, Steur et al., 2011, Gademan et al., 2014, Salsberry and Reagan, 2005, Schellong et al., 2012). Therefore, not only maternal nutrition during pregnancy but also early life nutrition are essential to future health outcomes (Salsberry and Reagan, 2005). During the first year of life infants' transition from a liquid diet of either breastmilk or formula milk to solid foods (Mennella and Tribulsi, 2012). Breastfeeding is considered the best choice of nutrition for infants in the first 6-12 months and is also considered beneficial to mothers due to decreasing breast cancer risk, reducing endometritis, post-partum weight loss and BMI reduction (WHO, 2013b, SACN, 2018). Breastfeeding studies have identified health benefits for infants including improved cognitive abilities (Evenhouse and Reilly, 2005), reduced inflammatory bowel disease (Joseph et al., 2004) reduced asthma (Gdalevich et al., 2001), and reduced obesity (Arenz et al., 2004).

1.6.10. Impact of Different Sources of FAs on the Developmental Programming of NCDs by Early Life Nutrition

It is noted that humans have an extended period of breast feeding that exposes the neonate to milk fat ingestion in early life with subsequent potential health implications (Armitage et al., 2005). Lipid content of human breastmilk is considered to have a vital role providing 45-55% of total energy provided to the infant in first 6 months (Innis, 2007, Bzikowska et al., 2018, Mazzocchi et al., 2018), with ≥95% constituted from TAG, and smaller amounts of MAG, DAG, NEFAs phospholipids and cholesteryl esters (Delplanque et al., 2015). Delplanque et al., (2015) note that in addition to the energy content dietary lipids provide EFAs, lipid soluble vitamins and substrates vital to cellular structure and function, signalling pathways and lipoprotein metabolism for infant health and development. The quantity of fat within the maternal milk does not vary with maternal daily intake (Innis., 2007, Bzikowska et al., 2018). A study reviewing European and African milk samples identified that the maternal diet

influences the content of SFA MUFAs and PUFAs in breastmilk (Koletzko et al., 1992). Bzikowska et al., 2018 confirmed that during the first month post-partum, maternal BMI is also positively correlated to breastmilk energy, fat content and dry matter content and that it is the maternal body mass that is responsible for the content of breastmilk rather than daily nutrient intake per se. Maternal physiology adjusts during late pregnancy and early post-partum, mobilising maternal adipose stores through increased lipolysis and is a source of maternal FAs (Herrera and Ortega-Senovilla, 2014). Therefore, maternal milk is reflective of maternal dietary FAs e.g. SFAs, EFAs and TFA – see table 1.6 (Larque et al., 2001, Innis 2007, Delplanque et al., 2015). Innis et al (2007) confirmed that TFAs from ruminant and PHVO origin have been found in human breastmilk up to 9% total energy TFA. Mueller et al (2010) identified in over 300 women in the Netherlands KOALA birth study that PHVO and RTFA were positively correlated with maternal milk 18:1 *trans* in dose respondent manner. The highest levels of PHVO (EA) were found in the "conventional food" intake group, in contrast to highest levels of RTFAs (VA and CLA) within the "organic origin" dairy group. Their results highlighted that PHVO were considerably higher compared to RTFAs and that PHVO consumption declined with increased dairy intake. Furthermore, measurement of TFAs in human breast milk have found wide variations within countries and between different countries, for example, the highest TFA content was in USA 2.5%-13.8%; Canada 4.6%-5.3% with the lowest TFA content 0.87%-1.55% in Germany (Desci and Boehm, 2013).

Table 1.5 Fatty Acid Composition of Human Breast Milk

FA Composition	Breakdown of FAs
TAG 95-98%	• TFAs are esterified to predominantly sn1 of TAG displacing SFA • 70% Palmitic Acid (C16:0) esterified to TAG sn-2 and absorbed as glycerol-palmitate
SFA 10-12%,	• 8-10% of SFA C6:0-10:0 • Lauric Acid (C14:0) • Palmitic Acid (C16:0) see above for TAGs • Stearic Acid (C18:0)
MUFAs	• 40% of MUFAs is Oleic Acid (OA C18:1 n-9) *NB ≥40% OA where large quantities of olive oil are consumed e.g. Mediterranean diets
PUFAs & EFAs	• PUFAs are dependent upon maternal diet • EFAs, LA and ALA must be provided in the maternal diet (7%
TFA 0.5-2.5%	• Elaidic Acid (EA C18: *trans* 9) • Vaccenic Acid (VA C18:1 *trans* 11) • CLAs (RA C18:2 *cis* 9 *trans* 11)

Source: Mazzocchi et al., (2018), Larque et al (2001)

Innis (2007) noted that a ratio of 14:1 LA/ALA is consumed by mothers providing 7% daily energy to the infant via breastmilk, with the quantity of LA ingested highly variable between 3-10%. As identified in maternal and fetal plasma and cord samples above, studies of human breast milk evidenced an inverse relationship between TFA and EFAs (Szabo et al., 2007, Innis, 2007, Decsi and Boehm, 2013), indicating that EFA nutrient supply to the infant may be compromised by PHVOs in early life. Therefore, it can also be understood that infant consumption of PHVOs via maternal milk would in turn restrict infant metabolism of any EFAs to their LC PUFAs thereby impacting two-fold on infant EFA and associated development and signalling pathways. However, it was noted that all TFAs are not equal with Desci and Boehm, (2013) reporting that 18:1 *trans* and not 16:1 *trans* were inversely correlated with EFA in milk samples. Reported programming outcomes for TFAs in connection with infant early nutrition are limited. A study noted that mothers consuming 4.5g TFA/day were over five-times more likely to have body fat ≥30% and their infants twice as likely to have bodyfat ≥24%. They concluded that their study reflected that maternal consumption of TFA and subsequent breastfeeding of their infants may influence the prevalence of adiposity observed (Anderson et al., 2010).

1.6.11. Developmental Programming by Early Life Nutrition in Animal Models

Studies of animal milk (pigs and rats) have confirmed the human breast milk results that FAs are reflective of maternal diet and are incorporated into maternal milk in a dose respondent manner (Larque et al., 2000, Pettersen and Opstvedt, 1991). Larque et al., (2000) noted that in rats PHVO was incorporated into the milk and had significantly increased n-6 LA PUFAs and reduced n-3 ALA PUFAs with a ratio of 7:1 compared to milk of chow fed dams. Osso et al., (2008) reported that in rat offspring exposed to PHVO in lactation only had impaired insulin sensitivity and cardiac insulin resistance. Whilst in C57 mice Oben et al (2010) cross-fostered obese dam SFA offspring to lean chow fed dams and vice-versa during lactation. Those offspring exposed to high SFAs *in utero* only had greater insulin resistance and metabolic NAFLD characteristics. Whereas those offspring exposed to chow *in utero* and high SFA during lactation via obese dams had elevated inflammatory markers e.g. TNFa and IL6 and elevated liver TAG. Oben et al (2010) concluded that maternal obesity programmed metabolic dysfunction in offspring and a NAFLD phenotype, however that this is also dependent on the 'critical early postnatal period' lactation diet. Rat fetal programming studies, feeding 20% SFA during lactation have further demonstrated vascular endothelial dysfunction at d15 and d60 post weaning (Ghosh et al., 2001, Koukkou et al., 1998).

In conclusion, the above studies are indicative that maternal nutrition at opposite ends of the nutrient spectrum - under or over nutrition - and that of early life nutrition can impact on the development of NCDs in later life. the degree of difference between these factors may determine disease outcome in adulthood. Gillman (2005), and Langley-Evans and McMullen (2010), address criticisms of the DOHAD hypothesis, cautioning against taking the significance of such epidemiological and animal model studies at face value. They stipulated that many studies are not consistent in that they don't account for all confounding factors, for example, social class, maternal smoking, habitually poor nutrition, poverty and geographic location, all of which have been identified as influential in fetal growth and neonate anthropometry. The SACN (2011) report on maternal, pregnancy and early nutrition also commented that

identification of maternal nutritional status is lacking in many studies and thus their impact on disease later life is more difficult to ascertain.

1.7 ANIMAL MODELS OF FETAL AND DEVELOPMENTAL PROGRAMMING

Human epidemiological studies have supported and provided evidence of fetal and developmental programming hypotheses that are prevalent in current research arenas. However, caution is noted due to conflicting results, with differing reporting variables between studies thereby reducing consistency and strength of conclusions drawn (McMullen and Mostyn, 2009). Additionally, dietary intake studies such as breastfeeding, rely on observational reports, individual self-reporting, variation in timepoints or factors measured, and confounding factors e.g. smoking and lifestyle choice (Schack-Nielsen and Michaelsen, 2007). It is also acknowledged that human studies often confer greater financial implications and time constraints with epidemiological studies not evidenced until years after completion (Breckenridge, 2013). McMullen and Mostyn (2009) confirm developmental programming animal models provide essential evidence in support of human findings providing a 'causal relationship' between maternal diet and programming NCDs. Therefore animal models of developmental programming are able to control many confounding factors and variables measured whilst being able to confirm a vast array of physiological effects of maternal nutritional insults to the developing fetus (Koletzko et al., 2012) and in early post-natal life (Plagemann et al., 2012) and their influence on NCDs in later life. Taking into account species differences where caution is warned comparisons can be drawn as to the impact of specific nutrients e.g. protein carbohydrate or fatty acids, and quantities of nutrients (over nutrition or under nutrition) fed to animals at different timepoints during neonate development (Breckenridge, 2013, Williams et al., 2014).

1.7.1. Animal models of Fetal Programming of Atherosclerosis

Russell and Proctor (2006) commented that there is a need for animal models that mimic human CVDs. However, as CVDs are multifactorial in nature no single animal model is comparative to human pathophysiology. Therefore, there are several animal models that have been used to understand cholesterol-atherosclerosis aetiology and fetal programming. One of the first animal models in the 1900s by Nikolij Anikschow that demonstrated cholesterol-atherosclerosis principle was the rabbit (Pelias, 1983, Finking and Hanke, 1997). The New Zealand White Rabbit has been used to investigate cholesterol metabolism (Getz and Reardon, 2012) and the impact of different dietary fats e.g. CLAs on the development of atherosclerosis (Kritchevsky et al., 2004, Kritchevsky et al., 2000). However, as herbivores rabbits require high levels of cholesterol to achieve CVDs (Russell and Proctor, 2006). Getz and Reardon (2012) note that the Watanabe Hereditary Hypercholesterolemic rabbit has an LDLr defect gene and exhibit Familial Hypercholesterolemia. This animal model has also been used to demonstrate the role of maternal hypercholesterolemia in the fetal programming of atherosclerosis (Napoli et al., 2000) and has been key in understanding cholesterol metabolism and development of statins (Tonge, 2011). However, concerns have been raised regarding, site and location of lesions, and microbial health of animals due to *Chlamydia pneumoniae* present in atherosclerosis lesions and respiratory pathogens e.g. “snuffles” in those rabbits fed high-cholesterol diets. (Russell and Proctor, 2006).

Larger animal models such as pigs, sheep and non-human primates have also been used to identify impact of maternal dietary intake and fetal programming of NCDs (see paragraph 1.6). However, larger animals are more expensive to house, can take longer timescales for programming outcomes to be achieved and often have heightened ethical concerns (Badimon et al., 2013). Rodents such as mice and rats have been extensively used due being litter bearing abilities and cost efficacy in comparison to larger animal models (Tannock and King, 2010). Small animal models also allow for shorter generation timings enable cross generational programming studies to be elucidated. (McMullen and Mostyn, 2009).

1.7.2. Rodent Models of Fetal Programming of Atherosclerosis

Maternal overnutrition and fetal programming studies have encompassed cafeteria feeding or high fat diets (Williams et al., 2014). These diets are representative of modern "western diets" rich in SFA, MUFAs and PUFAs and consumed by animals during peri-conception, throughout fetal development and postnatally (Armitage et al., 2005). However, it is noted rodents have an inherent energy balance and even when faced with a high-fat diet they do not normally consume more nutrients than they require (Keesey and Hirvonen, 1997). Although rats have been extensively used to demonstrate fetal programming of metabolic syndrome, rodents are naturally resistant to atherosclerosis (Russell and Proctor 2006). It is also acknowledged that rodents possess a different lipoprotein metabolism compared to humans with low pro-atherosclerosis LDL and VLDL and high anti-atherosclerosis HDL, and therefore are unsuitable to be used for atherosclerosis programming studies (van den Hoek et al., 2014, Wang and Paigen, 2005) and this can impact on programming outcomes of atherosclerosis as demonstrated by Yates et al., (2009). Finally, sexual dimorphism has been demonstrated in many animal models highlighting variable programming impacts on either male or females (Getz and Reardon, 2012). For example, studies have identified no impact of programming of male C57s or AEL transgenic mice whilst female offspring did and is indicative that gender-specific hormones influence outcomes in animal models (Yates et al., 2009, Chechi et al., 2009).

Russell and Proctor (2006) note that the C57BL/6J wild type mouse develops some atherosclerosis in response to a high SFA, cholesterol-rich diet. However, a study by Yates et al (2009) found considerably reduced lesions and cholesterol metabolism in this model with differences between both males and female offspring. To reproduce a similar atherosclerosis model to humans, gene deletion 'knockout' or transgenic mouse models of atherosclerosis have been developed. The gene knockout mice impairs cholesterol clearance from the metabolism inducing a hypercholesterolemic profile with consequent atherosclerosis progression in the aorta (Zadelaar et al., 2007). The most common models include the

apolipoprotein E-deficient mouse (ApoE$^{-/-}$) and LDL receptor deficient mouse (LDLr$^{-/-}$) (Getz and Reardon, 2012). The ApoE$^{-/-}$ model alters lipoprotein metabolism to mostly lipoprotein remnants (apoB48) however the main lipoprotein in human lipoprotein metabolism is LDL. Additionally, even in chow animals lesion development commences earlier and by 15-20 weeks age advanced lesions with fibrous plaques and thrombus rupture can occur. It is also noted that ApoE can also be expressed by adrenal cells and is involved in macrophage biology, thereby potentially confounding atherosclerosis lesion outcomes (Getz and Reardon, 2012). In comparison the LDLr$^{-/-}$ mouse are less hyperlipidaemic compared to ApoE$^{-/-}$ mice with the main lipoprotein metabolism consisting of VLDL and LDL. Hypercholesterolaemia is dependent upon dietary fat and cholesterol. However, the main drawback of this model is that in chow fed animals lesions are not formed in early life and do not develop until approximately 12 months age (Getz and Reardon, 2012, Zadelaar et. 2007). Therefore, this model is less beneficial when ascertaining fetal or early life lesions in offspring.

1.7.3. ApoE*3 Leiden Mouse Model of Fetal and Developmental Programming of Atherosclerosis

The ApoE*3 Leiden (AEL) mouse develops atherosclerosis dependent upon high SFA/Cholesterol-rich diets (Zadelaar et al, 2007, Kleeman et al 2007). AEL mice are a transgenic strain that carry a rare dominant-negative mutation of the human ApoE3 gene (Wardell et al 1989). This human mutation has been added in a construct that contains the hepatic APOC1 gene and promoter element, which regulates expression of ApoE and APOC1 genes. The normal ApoE3 gene codes for 299 amino acids, whilst the AEL has an additional 7amino acid repeat (306 amino acids). AEL confers dominant expression of Type III hyperlipoproteinemia phenotype with defective binding of the LDL receptor (Wardell et al., 1989). These defects impair clearance of chylomicron and VLDL remnants which accumulate in serum inducing hypercholesterolemia (Havekes et al., 1997). The AEL mutation construct with APOC1 may also reduce lipolysis, VLDL uptake and hypertriglyceridemia (van den Maagdenberg et al., 1993). In the AEL mouse model this gene mutation causes a more human-type lipid metabolism and mimics the susceptibly to the development of

atherosclerotic plaques in the aortic arch when fed a high SFA/cholesterol-rich diet (Zadelaar et al., 2007 Havekes et al., 1997, van Vanvlijmen et al 1996). Compared to the ApoE$^{-/-}$ and LDLr$^{-/-}$, AEL mice represent a moderate model for hyperlipidaemia and dietary fat induced atherosclerosis (Zadelaar et al., 2007, Getz and Reardon, 2012). Van vlijmen et al (1996) noted that maximal lipoprotein metabolism was during rapid growth period at 45d age. In young mice VLDL and TAG increased 50%, and VLDL-apo B secretion rates increased by 75% compared to older mice, however clearance of VLDL and ApoB were similar. Furthermore, their study found that gender influenced both hepatic VLDL production and clearance rates, with hepatic VLDL/TAG and clearance of VLDL ApoB being higher in females compared to males. Their study concluded that gender and age modulated hyperlipidaemia in the AEL mouse. Investigations into the impact of diet composition (low fat vs. high SFA/Cholesterol-rich diets) on serum lipoprotein metabolism and atherosclerosis in AEL female mice found that atherosclerosis lesions were 5-10 times larger than control mice with lesions developing near the aortic arch. Significant correlations between serum cholesterol levels and lesion area indicated that the AEL mouse model was a suitable model for dietary intervention studies of atherosclerosis (Groot et al., 1996). Since then the AEL mouse has been proven to give an insight into cholesterol metabolism and atherosclerosis with varied fatty acid results (De Roos et al., 2003, Tonge, 2011), atherosclerosis inflammatory and immune response pathways (Boesten et al., 2005, van Vlijmen et al., 2001), and atherosclerosis therapeutic treatments (Kooistra et al., 2006). Additionally, it had been evidenced, using a maternal C57BL/6J mouse that are cross bred with AEL males, that maternal low protein programmed atherosclerosis in AEL offspring (Yates et al 2009). At the commencement of the fetal and developmental programming of atherosclerosis studies carried out and thus reported on herein, it was pertinent to use the maternal C57 background, cross bred with AEL males with outcomes reported in the AEL female offspring. Since the conclusion of these studies it has also been confirmed that maternal high fat diets have also programmed atherosclerosis in AEL offspring (Tarling et al., 2016).

1.8 AIM AND HYPOTHESIS

As outlined above in the General Introduction, there are different effects of P, R and W fatty acids on maternal lipoprotein metabolism and CHD risk with consequent metabolic disease and atherosclerotic CVD outcomes for offspring. Our studies aimed to investigate the impact of two types of TFA (that associated with ruminant milk and that associated with partially hydrogenated vegetable oil) during pregnancy, pregnancy + lactation, or throughout lifespan on the risk of atherosclerosis in offspring using the AEL mouse model.

Study 1 Hypothesis: That maternal consumption of ruminant derived TFA during pregnancy and fetal development, will protect the offspring from atherosclerosis, while that associated with PHVO will have the opposite effect.

Hypothesis Study 2: It was hypothesized that maternal consumption of P diet throughout pregnancy, or pregnancy + lactation would adversely alter maternal lipoprotein metabolism and transfer TFAs to the offspring increasing susceptibility to atherosclerosis to a greater extent compared to Western diets consumed during the same development periods. It was further considered that maternal consumption of R during the same development periods would improve maternal lipoprotein metabolism compared to P and W diets and decrease susceptibility to atherosclerosis in their offspring.

Hypothesis Study 3: It was hypothesised that exposure PHVO throughout lifespan (starting at conception) would increase atherosclerosis risk in offspring to a greater extent compared to Western and RTFA diets. It was further considered that the Western diet would have a greater influence on increasing atherosclerosis risk in offspring compared to the RTFA diet.

CHAPTER 2 MATERIALS AND METHODS

2. REAGENTS

All reagents were of analytical grade and purchased from Fisher Scientific or Sigma Aldrich unless otherwise stated.

2.1 DIETS FOR ANIMAL EXPERIMENTS

A standard chow, Rat and Mouse Diet Number 1 Maintenance (RM1) Diet (Special Diet Services, Essex, UK) was used for the basic maintenance of mice whilst not on feeding trials. According to the manufacturer, the diet contained 2.7% crude oil of which the majority of the fatty acids were: oleic acid (0.77%), linoleic acid (0.69%) and palmitic acid (0.31%). The FAME composition of this diet is shown in Figure 2.2. RM1 was used as the basis for the control "Chow" (C) diet used in the feeding trials. This was fed to C57BL/6J mice (dams) throughout pregnancy and lactation and also to female ApoE*3 Leiden (AEL) offspring in post-natal diets. RM1 was used also as an element of the experimental fatty acid diets – see PHVO and RTFA and Western diets in Table 2.1 below. FAME composition of experimental diets are shown in Figure 2.1.

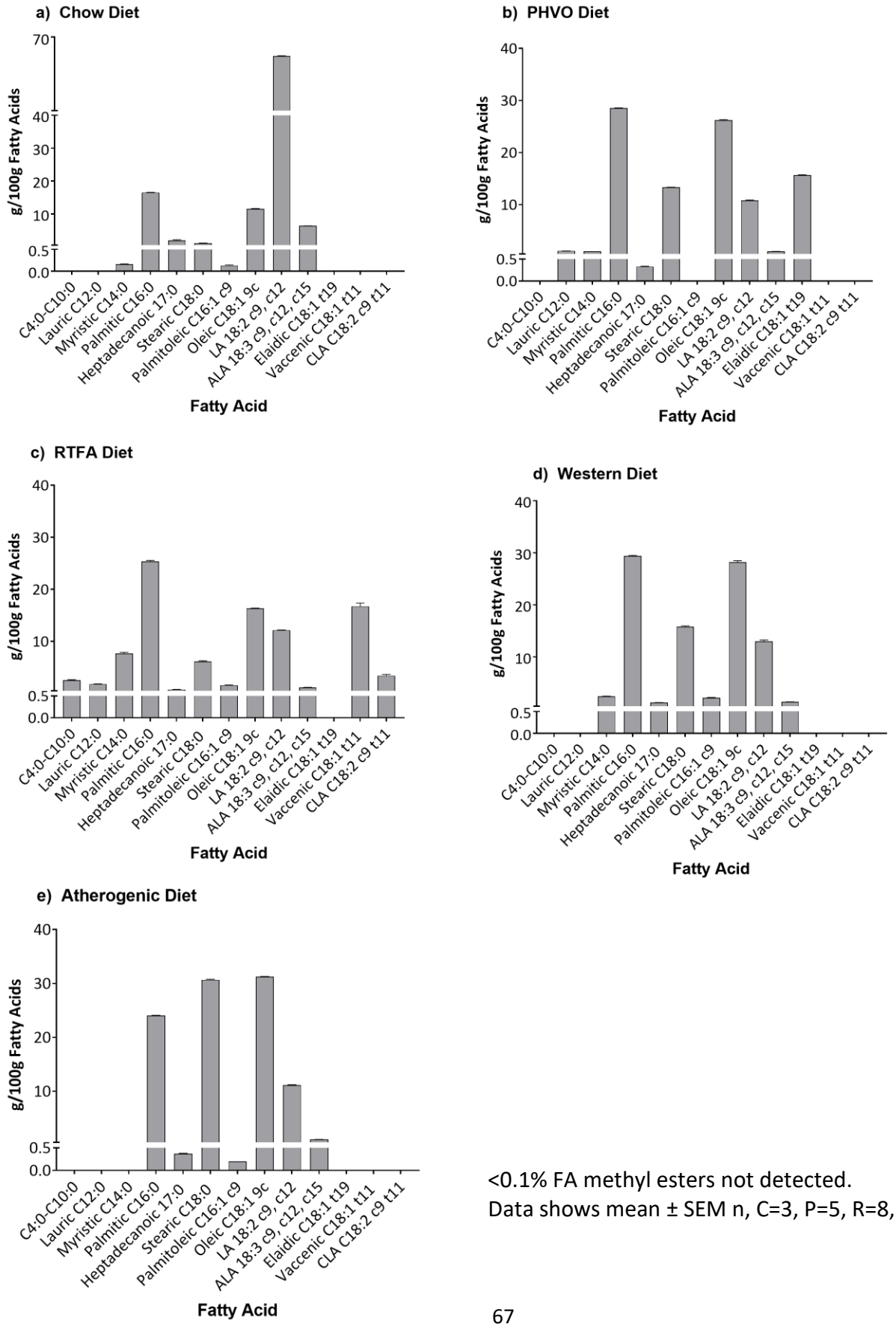
RM1 was weighed out and appropriate quantities of fats (Table 2.2) were heated to 65°C until liquid and homogenous. Fats were thoroughly mixed into the RM1 diet using an electric mixer for 15 minutes and finished by hand to ensure even fat distribution throughout. Where possible, up to 10kg of feed were made-up at one time, allocated into 1kg batches and stored at -20°C until required.

Table 2.1 Experimental Diets Fatty Acid Profile

Systematic Name	Trivial name	Shorthand	PHVO (PHVO) g/100g	Dairy TFA g/100g fat
Saturated fatty acids				
butanoic	butyric	4:0	0	3.41
hexanoic	caproic	6:0	0	1.75
octanoic	caprylic	8:0	0	0.90
decanoic	capric	10:0	0.06	1.82
dodecanoic	lauric	12:0	1.18	2.09
tetradecanoic	myristic	14:0	1.23	8.02
hexadecanoic	palmitic	16:0	31.52	22.94
octadecanoic	stearic	18:0	14.81	5.82
SUM 12+14+16			**33.93**	**33.05**
Monoenoic fatty acids				
cis-9-octadecenoic	oleic		22.76	13.37
# Other cis-9 18-1			1.69	1.99*
Monoenoic *Trans* fatty acids*				
trans 4, octadecadienoic			0	0.09
trans 5, octadecadienoic			0	0.09
trans 6/8, octadecadienoic			3.37	0.78
trans 9, octadecadienoic	**elaidic**	**18:1 ω-9 trans**	**9.83**	**0.64**
trans 10, octadecadienoic			4.42	2.42
trans 11, octadecadienoic	**vaccenic**	**18:1 ω-11 trans**	**2.32**	**13.87**
trans 12, octadecadienoic			0.80	0.78
trans 13/14, octadecadienoic			1.26	0.98
trans 15, octadecadienoic			0.50	0.29
trans 16, octadecadienoic			0.07	0.16
SUM t9+10+11			**16.57**	**16.93**
Polyunsaturated fatty acids*				
9,12-octadecadienoic	linoleic	18:2(n-6)	2.01	2.35
6,9,12-octadecatrienoic	γ-linolenic	18:3(n-6)		
9,12,15-octadecatrienoic	α-linolenic	18:3(n-3)	0.04	0.32
Polyunsaturated *trans* fatty acids				
Trans		18:2	0.87	1.99
Cis-9, *trans*-11	CLA	18:2	0	4.44

(Chardigny et al., 2008)

Figure 2.1 %FAME Experimental Diets



<0.1% FA methyl esters not detected.
Data shows mean ± SEM n, C=3, P=5, R=8, W=6, A=5.

Table 2.2 Animal Feed Experimental Diets

DIET	STUDY 1	STUDY 2 AND STUDY 3
Chow (C)	1000g RM1	1000g RM1
RTFA (R)	870g RM1. 130g Ruminant Milk Fat	850g RM1 150g Ruminant Milk fat
PHVO (P)	870g RM1 130g PHVO	850g RM1 150g PHVO
Western (W)	N/A	850g RM1. 125g Beef Tallow 15g tripalmitin 10g Corn oil
Atherogenic (A)	870g RM1 30g Cocoa Butter 2.5g Cholesterol	850g RM1. 50g Cocoa Butter 2.5g Cholesterol

2.1.1. Partially Hydrogenated Vegetable Oil *TFA* (PHVO or P)

To prepare the PHVO diet required for the studies, two different types of PHVO (35% Fuji and 65% Lipid Nutrition) which were blended with RM1.

2.1.2. Ruminant TFA (RTFA or R)

Milk fat high in ruminant *trans* fatty acids was kindly gifted by Professor Dale Bauman from a previous study at Cornell University, (Ithaca, New York, USA) where cows were fed a diet enriched with safflower oil, synthesising a milk fat predominantly rich in 18:1-*t9/11* vaccenic acid and CLA. This oil was blended with RM1 as shown in Table 2.2.

2.1.3. Western Diet (Western or W)

To produce a diet with a fatty acid profile similar to that seen in the typical human western diet, RM1 was mixed with corn oil (rich in polyunsaturated fats) and saturated fats including beef tallow and tripalmitin.

2.1.4. Atherogenic Diet (Athero or A)

In order to induce atherosclerosis in mice during the postnatal period, a high fat, high cholesterol diet was utilized. This was produced by mixing RM1 with cocoa butter (Nestlé, Vers-chez-les-Blanc, Switzerland) and cholesterol (2.5 g/kg diet).

2.2 ANIMALS

All animal experiments were approved by an independent Animal Care and Use Committee and were performed under UK Home Office licence in accordance with European Union specifications. Before the study the number of animals required was calculated using data from a previously reported study (Yates et al., 2009), with an aim to have 90% power to detect an effect of maternal diet (α=0.05, β=0.1). The nature of the study dictated that the feeding study was not performed blind, though all analyses (including atherosclerosis) was performed by animal number and without reference to the treatment group. Animals were housed in plastic cages in the University of Nottingham animal facilities under controlled conditions (20-22°C, 55% humidity, 12-hour light: dark cycle) with free access to food and water at all times. Wild type, seven-week-old, C57BL/6J (C57s) female mice were obtained from Harlan UK and initially maintained on Rat and Mouse Diet Number 1 Maintenance Diet (Special Diet Services). C57 females (dams) were matched for bodyweight and mated with male heterozygous AEL mice on C57BL/6J background (sires) at six to eight weeks age. The AEL male mice were also matched for bodyweight across groups. This cross breeding produces 50% AEL heterozygous offspring and 50% C57 wild type offspring, of which 50% will be female and 50% male. The transgenic mice were bred in our facility from founder males kindly provided by Dr Louis Havekes (TNO Pharma). Heterozygous animals were used as the AEL transgene is lethal in homozygotes. For both studies, AEL female offspring were selected due to their greater diet-induced, hyperlipidaemic profile and atherosclerosis susceptibility, when compared to AEL males (Groot et al., 1996, van Vlijmen et al, 1996).

2.2.1. ANIMAL HUSBANDRY

2.2.1.1 Mating and Pregnancy

AEL sires were paired and mated with female C57 dams for two oestrous cycles (eight days). The paired animals were housed within Individual Ventilated Cages (IVC)and after eight days males were removed and euthanised in accordance with Schedule 1, Animals (Scientific Procedures) Act 1986 (ASPA) (Home_Office, 1986). Pregnant dams remained within the IVC environment until pups were weaned. The dams were checked daily for confirmation of pregnancy and their bodyweight recorded. Throughout mating and pregnancy (18 days) dams were fed the control Chow diet (C) or one of the experimental diets (P, R or W) *ad libitum*.

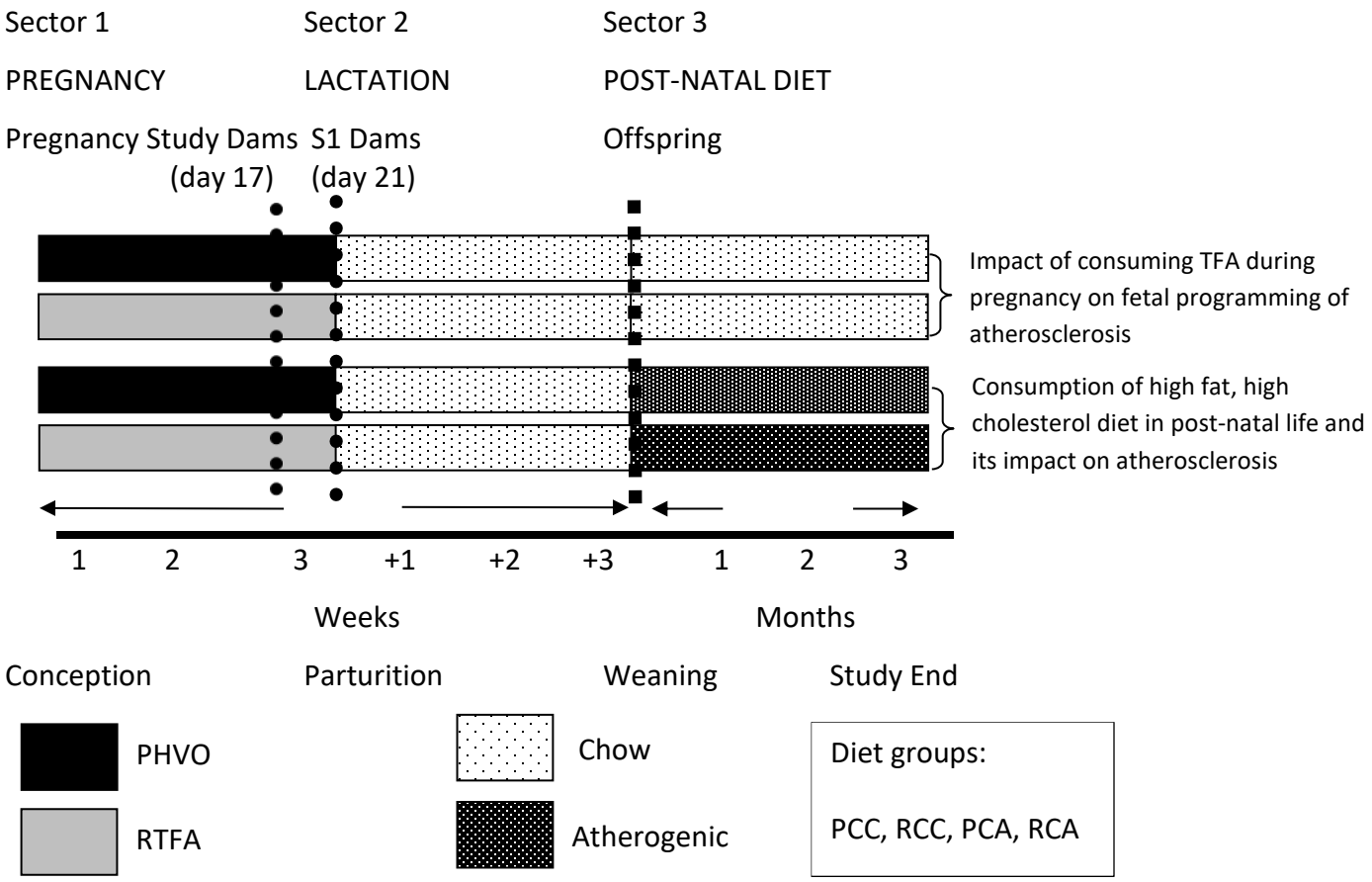
2.2.2. Parturition/Suckling

At parturition, dams continued either on a control diet or an experimental fat diet, exposing them and their suckling offspring to differing nutritional insults. Offspring were weaned onto postnatal diets at approximately 21 days of age.

2.2.3. Offspring

At 21 Days, pups were sexed and all female offspring genotyped (method 2.5). All AEL positive female offspring were weaned onto control Chow diet or experimental fat diet for a period of 84 days (12 weeks). Mice were housed singly within a controlled environment at 21°C, 55% humidity, and a 12-hour light and dark cycle. Feed was offered *ad libitum* and animals had unrestricted access to fresh water. Animals were monitored daily over the first seven days post weaning to ensure diet tolerance and health. Thereafter, all animals were weighed at weekly intervals to compare body weights between groups. See Experimental Designs for Study 1 Figure 2. and Study 2 Figure 2.3 and Study 3 Figure 2.4 for further details.

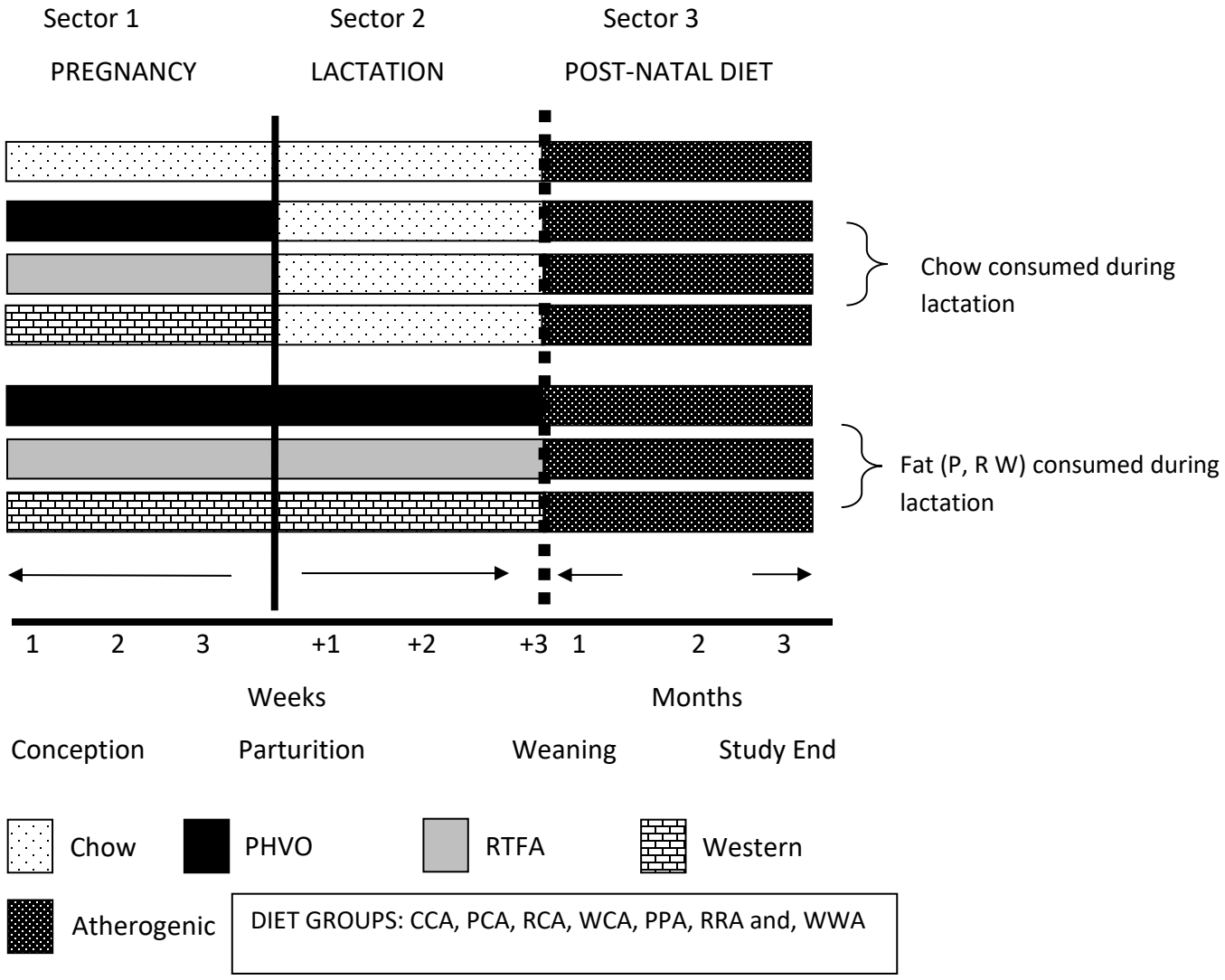
Figure 2.2 Study 1 Experimental Design: The Impact of PHVO or RTFA on the Fetal Programming of Atherosclerosis



2.2.4. Study 1 Diet Groups: The Impact of PHVO or RTFA on the Fetal Programming of Atherosclerosis

- **PCC**: Dams were fed PHVO diet during pregnancy (P) and Chow during lactation (C). Offspring were weaned onto a Chow (C) post-natal diet.
- **RCC**: Dams were fed RTFA diet during pregnancy (R) and Chow during lactation (C). Offspring were weaned onto Chow (C) post-natal diet.
- **PCA**: Dams were fed PHVO diet during pregnancy (P) and Chow during lactation (C). Offspring were weaned onto an Atherogenic (A) post-natal diet.
- **RCA**: Dams were fed RTFA diet during pregnancy (R) and Chow during lactation (C). Offspring were weaned onto an Atherogenic (A) post-natal diet.

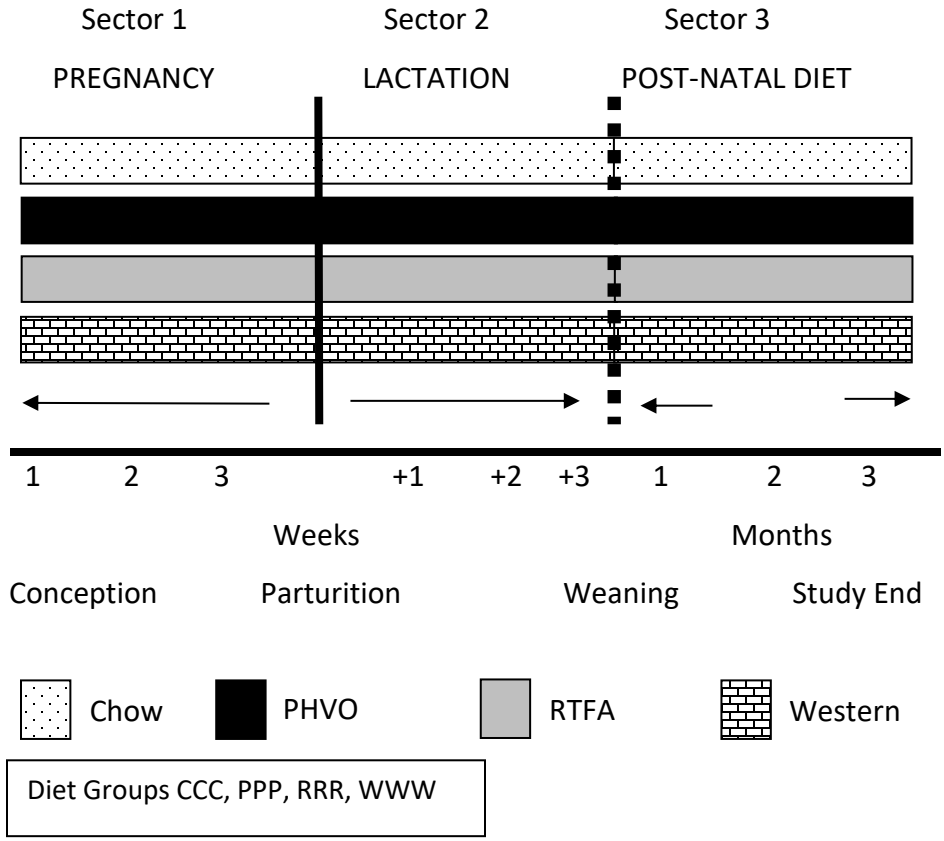
Figure 2.3 Study 2 Experimental Design: The Impact of Maternal consumption of PHVO, RTFA or Western Diet throughout Pregnancy and Lactation on the Fetal and Developmental Programming of Atherosclerosis



2.2.5. Diet Groups Study 2: The Impact of Maternal consumption of PHVO, RTFA or Western Diet throughout Pregnancy and Lactation on the Fetal Programming of Atherosclerosis

- **CCA**: Dams were fed Chow diet during pregnancy (C) and Chow during lactation (C). Offspring were weaned onto an Atherogenic (A) post-natal diet.
- **PCA**: Dams were fed PHVO diet during pregnancy (P) and Chow during lactation (C). Offspring were weaned onto an Atherogenic (A) post-natal diet.
- **RCA**: Dams were fed RTFA diet during pregnancy (R) and Chow during lactation (C). Offspring were weaned onto an Atherogenic (A) post-natal diet.
- **WCA**: Dams were fed Western diet during pregnancy (W) and lactation (W). Offspring were weaned onto an Atherogenic (A) post-natal diet.
- **PPA**: Dams were fed PHVO diet during pregnancy (P) and lactation (P). Offspring were weaned onto an Atherogenic (A) post-natal diet.
- **RRA**: Dams were fed RTFA diet during pregnancy (R) and lactation (R). Offspring were weaned onto an Atherogenic (A) post-natal diet.
- **WWA**: Dams were fed Western diet during pregnancy (W) and lactation (W). Offspring were weaned onto an Atherogenic (A) post-natal diet.

Figure 2.4 Study 3 Experimental Design: The Impact of PHVO, RTFA or Western Diet throughout Lifespan on the Fetal Programming of Atherosclerosis



2.2.6. Diet Groups Study 3: The Impact of PHVO, RTFA or Western Diet throughout Lifespan on the Fetal Programming of Atherosclerosis

- **CCC**: Dams were fed Chow diet during pregnancy (C) and lactation (C). Offspring were weaned onto a Chow (C) post-natal diet.
- **PPP**: Dams were fed PHVO diet during pregnancy (P) and lactation (P). Offspring were weaned onto a PHVO (P) post-natal diet.
- **RRR**: Dams were fed RTFA diet during pregnancy (R) and lactation (R). Offspring were weaned onto a RTFA (R) post-natal diet.
- **WWW**: Dams were fed Western diet during pregnancy (W) and lactation (W). Offspring were weaned onto a Western post-natal diet (W).

2.3 TISSUE SAMPLES

Dependent upon the study, animals were euthanised in accordance with Schedule 1, ASPA (Home_Office, 1986). Several different tissues were excised, weighed, snap frozen in liquid nitrogen, then stored at -80°C until analysis (Table 2.3).

Table 2.3 Tissue Samples

Breed	Study/Chapter	Tissue Sample	Collection Time point
C57 Females (dams)	Pregnancy Study ASLG01/09/Mat CHAPTER 4	Blood, Liver, Perirenal adipose tissue and gonadal adipose tissue, placentas, 50% whole fetus, 50% fetal liver.	Day 17 pregnancy.
C57 Females (dams)	Study 2 and Study 3 ASLG01/11 CHAPTER 5	Blood, Brain, mammary, glands, Liver, Heart, Perirenal adipose tissue and gonadal adipose tissue.	After 21 days lactation period at weaning of pups
AEL Females	Study 1 ASLG01/09	Blood, Heart (mounted), Aortic Arch, Thoracic Aorta, Liver, Perirenal adipose tissue and gonadal adipose tissue.	After 84 days on a post-natal diet (15 weeks of age)
AEL Females	Study 2 and Study 3 ASLG01/11	Blood, Brain, Heart (mounted), Aortic Arch, Thoracic Aorta, Liver, Perirenal adipose tissue and gonadal adipose tissue.	After 84 days on a post-natal diet (15 weeks of age)

2.4 SERUM COLLECTION

Immediately after confirmation of death by cervical dislocation, up to 1ml blood was extracted by cardiac puncture, collected into clotting serum gel tubes (Sarstedt), and placed on ice. Blood serum tubes were centrifuged at 4°C for 12 minutes at 16,000g. Serum was kept on ice, and dependent upon quantity, split into several 50µl aliquots per animal and stored at -80°C until required for analysis.

2.5 GENOTYPING

All female pups were sampled between 18-21 days age to detect the AEL genotype. For initial genotyping ear tissue was collected from pups by BRU staff in line with ASPA regulations (Home_Office, 1986). Reaffirmation of genotype at the end of the study was undertaken utilising 10mg frozen crushed liver tissue. Extract-N-Amp^{TM} Tissue PCR Kit (Sigma Aldrich, Catalogue Number XNAT2) was used to extract and amplify genomic DNA from tissue samples.

2.5.1. DNA Extractions

Each tissue sample was placed into individual, labelled 1.5ml eppendorf tube and 10µl of Extract-N-Amp^{TM} Extraction Buffer added. Each tissue sample was mechanically disrupted using a sterilised pestle and incubated at room temperature for ten minutes. Samples were incubated at 95°C for three minutes, and then 100µl Extract-N-Amp^{TM} Neutralisation Buffer added, vortexing each sample to mix well. Neutralised extracted samples were stored at 4°C until PCR was carried out.

2.5.2. PCR Amplification

Reagents were added (Table 2.4) to create a “master mix” and 16µl placed into thin walled PCR plates, to which 4µl tissue extract sample was added giving a total reaction volume of 20µl per sample. Each sample was gently mixed by pipette to ensure reaction homogeneity. AEL 20bp

primers were previously designed (Yates et al., 2009) and ordered from Eurofins MWG Operon, the sequences are set out below.

AEL Primers

AEL Forward Mus: Sequence (5' -> 3'): GCC CCG GCC TGG TAC ACT GC

AEL Reverse Mus: Sequence (5' -> 3'): GGC ACG GCT GTC CAA GGA GC

Table 2 4 PCR Extract-N-Amp Solution

Reagent	Volume (µl)
Extract-N-Amp^{TM} PCR reaction mix	10
Forward AEL Primer	0.8
Reverse AEL Primer	0.8
PCR grade water	4.4
Sample	4
Total Volume	20

Three controls were used: (i) positive control - 4µl of confirmed AEL tissue extract; negative controls (ii) 4µl of confirmed C57BLJ6 tissue extract and (iii) 4µl PCR grade water. A PCR thermal cycler (PTC200, MJ Research) was used with cycling parameters optimised for the AEL genotype (Table 2.5).

Table 2.5 PCR Thermocycler Parameters for the AEL Genotype

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	10 minutes.	1
Denaturation	95°C	30 seconds	
Annealing	68°C	30 seconds	32
Extension	72°C	30 seconds	
Final Extension	72°C	7 minutes	1
Hold	4°C	Indefinitely	1

2.5.3. Gel Electrophoresis

A 1.5% (w/v) agarose gel (analytical grade agarose, Melford Laboratories Ltd) was prepared with 1x TAE buffer. The agarose solution was placed in a conical beaker and heated until fully dissolved and transparent, then cooled under cold running water. The agarose gel was poured into a gel former (Bio Rad) with combs and left to set for one hour at room temperature and either utilised immediately or stored overnight at 4°C. Combs were removed from the set gel, and the gel and gel former submerged in an electrophoresis tank containing 1x TAE buffer. 5µl of Type II loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 15% ficoll (type 400) was added to each PCR reaction and pipetted up and down to mix. 12µl of every sample was loaded into consecutive wells of the submerged agarose gel. 10µl of five parts 100bp DNA ladder (Promega) and one-part loading buffer was used to identify genomic bands and loaded into one well per gel. The gel was run at 80 volts for 40-50 minutes until the ladder and samples had run an appropriate distance. The gel was removed from the running tank and stained in an Ethidium Bromide solution (0.5µg/ml) for 30 minutes and imaged using a UV platform and camera. Images were captured using GelDoc 2000 software (Bio Rad) and the image printed out for genotype results analysis.

2.6 SERUM ANALYSES

Serum total cholesterol, Triglyceride or glucose content were determined using either colorimetric assays (methods 2.6.1 and 2.6.2) or using a RX Imola Randox Chemistry Analyser (method 2.6.3).

2.6.1. Colorimetric Assays –Serum Total Cholesterol and Triglyceride

Quantitative analysis of serum total cholesterol and Triglyceride were carried out using Infinity™ reagents. Dependent upon the assay, respective cholesterol or TAG standards were made up using distilled water. 10µl of each standard were pipetted in duplicate on a 96 well microassay plate with 10µl of distilled water used as the blank. For each sample, a total volume of 10µlwas diluted as per protocol (Table 2.6) and placed in duplicate onto the microassay plate. 200µl of

appropriate reagent (cholesterol or Triglyceride) was added to each well and samples incubated at 37°C for 15 minutes. Results were read on a plate reader (BioRad 680XR) using Microplate Manager software and a dual wavelength reading: measurement filter 550nm and reference filter 650nm.

Table 2.6 Infinity™Assay Dilutions

	Cholesterol	Triglyceride
Sample dilution with distilled water	Neat or 1:2	1:4

2.6.2. Colorimetric Assay – Serum Glucose

A set of seven standards were made up from a 20mM glucose/distilled water solution.1.5ml eppendorf tubes were labelled with sample and standard identification numbers and with total sample volume of 10µl (1:2 dilution distilled water)for samples and standards were pipetted into their corresponding tubes**.**500µl of Glucose Oxidase reagent (Sigma Aldrich) was added to each tube and briefly vortexed to mix. The sample assay was incubated at room temperature for 20 minutes then 200µl of each sample and standard set out a 96 well microassay plate in duplicate, using distilled water as the blank. Results were read on a plate reader (Bio Rad 680XR) using Microplate Manager software and a dual wavelength reading: measurement filter 550nm and reference filter 650nm.

2.6.3. Serum Lipid Analysis (Randox)

A Randox Imola RX -series Chemistry Analyser (Randox Laboratories Ltd, Crumlin, UK) was utilised and training provided by Dr N. Kendall, University of Nottingham School of Veterinary Medicine and Science. Plasma HDL/LDL analyses were carried out using direct clearance methodology (Randox, 2011a, Randox, 2011b). Assays were carried out on plasma Total Cholesterol by CHOD-PAP method (Randox, 2011c) and plasma Triglycerides by GO-PAP method (Randox, 2011d). Reagents were purchased from Randox Lt, Crumlin, UK. Randox calibrants, lipid and sera control reagents were prepared to manufacturer's instructions and 1ml of each set up in vials on the inner carousel of the Randox Imola analyser with distilled water as a blank.

The minimum amount of serum required for these analyses was 100µl. Where less that 100µl serum was available samples were diluted with distilled water and dilutions recorded for final calculation. Standards and samples were placed in 500µl Eppendorf tubes and floated in LP4 tubes which were placed in consecutive order onto the outer carousel of the analyser. The Randox Imola program for each reagent progressed through the sample set twice providing duplicate sampling.

2.7 FATTY ACID ANALYSIS

2.7.1. Direct Fatty Acid Methyl Ester (FAME) Synthesis

Direct FAME synthesi*s* (O'Fallon et al., 2007)of diets and their constituents was performed before analysis by gas chromatography (GC). 1g of feed or 0.1g dietary fat were placed into a glass methylation tube.0.7ml 10M potassium hydroxide in water was added followed by 5.3ml methanol. Samples were incubated in a water bath at 55°C for one hour 30 minutes and vortexed for five seconds every 20 minutes. Samples were then cooled below room temperature in cold tap water bath. Once cooled 0.58ml of 12M sulphur acid in water was added and carefully mixed by inversion. Samples were returned to the water bath and incubated at 55°C for a further 1.5 hours and vortexed for five seconds every 20 minutes. As before samples were cooled in a cold tap water bath before 3ml of hexane was added and vortexed for 30 seconds. Samples were placed in a centrifuge at 500g for five minutes. The top hexane layer was transferred to a glass LP4 tube and 1ml of this was placed into a GC vial (VWR International Ltd) and capped. Samples were stored at -20°C until analysis.

2.7.2. Gas Chromatography of Fatty Acid Methyl Esters (FAME)

Analysis of FAME samples were carried out using a Perkin Elmer Clarus 500 Gas Chromatograph with flame ionisation detector (FID) running TotalChrom software. The GC was fitted with a 100m CP-Sil 88 column (Varian, Walton on Thames, Surrey). The FAME samples in GC vials (method 2.7.1) were loaded onto the automatic sampler carousel with a C4-C24 standard (Supelco 189-19 AMP) for identification of known fatty acid components. The gas chromatograph specification and

programme are shown in Table 2.7. Chromatographs of each sample were obtained and the exported into Excel format for analysis. The results reported each sample FAME as a percentage allowing for comparison of samples.

Table 2.7 FAME Analysis Gas Chromatography Specifications and Programme

- Injector temperature: 255°C
- Detector temperature: 255°C
- Detector range: 1
- Carrier gas: Hydrogen
- Inlet pressure: 41.5 psi
- Split ratio: 1:20
- Airflow rate: 450ml/min
- Hydrogen: 45ml/min
- Injection speed and volume: Fast speed, 1.0µl
- Pre-injection sample washes: 2
- Post-injection solvent washes: 2

Programme:

1. Initial temperature: 45°C, hold for 4 minutes
2. Ramp 1: 13°C/minute to 175°C, hold for 27 minutes
3. Ramp 2: 4°C/min to 215°C, hold for 35 minutes

Total run time: 86 minutes per sample

2.7.3. Fatty Acid Analysis of Perirenal Adipose Tissue

Perirenal tissue samples were exported to Dr A. L. Lock, Department of Animal Science, Michigan State University, for analysis of *trans* fatty acids by gas chromatography. The results are shown in their respective chapters.

2.7.4. Lipid Extraction from Liver Tissue

300mg of crushed liver tissue was placed into LP4 tubes and homogenised in 1.6ml sodium sulphate (1g/15ml distilled water). The homogenate was decanted into a 15ml falcon tube containing 5.4ml of three-parts hexane to two-parts isopropanol. Another 2ml of sodium sulphate was added and samples vortexed for 30 seconds to mix well. Samples were centrifuged at 1200g for 15 minutes at room temperature. The top layer of solvent containing the lipid extraction was transferred to a new LP4 tube and dried down under nitrogen. Lipid extracts were re-suspended in 1ml hexane, capped, and stored at -20°C until use.

2.7.5. Liver Cholesterol and Triglyceride Assays

For each sample 100µl of hexane lipid extract (method 2.7.4) was transferred into a corresponding 500µl eppendorf tube. The lipid extract was dried down under nitrogen and re-suspended in 100µl isopropanol. For the assays, a total sample volume of 10µl was used (Triglyceride extracts were diluted 1 in 8 in isopropanol). 10µl standards, samples and blank were pipetted in duplicate onto a 96 well microassay plate. 200µl of appropriate reagent (Infinity^{TM}Cholesterol or Triglyceride) was added to each well and samples incubated at 37°C for 15 minutes. Results were read on a plate reader (Bio Rad 680XR) using Microplate Manager software and a dual wavelength reading: measurement filter 550nm and reference filter 650nm.

2.8 HISTOLOGICAL ANALYSIS OF THE AORTA AND ATHEROSCLEROSIS QUANTIFICATION

The heart and aorta dissection techniques and methods used for histological analysis of the aorta and atherosclerosis quantification are included in Chapter 3, Paragraph 3.1.

2.9 STATISTICAL ANALYSIS

Results are shown as mean data ± Standard Error of the Mean (SEM). Statistical analyses were carried out using SPSS 25 and GenStat software. Data was assessed for normality using Shapiro-Wilk test or Levene's test of homogeneity. Non-normally distributed data were transformed by square root "a" or log10 "c" before parametric statistical analyses were performed by the appropriate statistical test e.g. Student T-Test, repeated measures ANOVA, and ANOVA with Bonferroni post-hoc tests. Non-parametric data were analysed by Independent sample Mann-Whitney U "b" or Kruskal-Wallis H "d" statistical tests. Significance $P<0.05$.

CHAPTER 3 METHOD DEVELOPMENT FOR ATHEROSCLEROSIS LESION QUANTIFICATION IN APOE 3*LEIDEN MICE

3. INTRODUCTION

Over the last 20 years many authors (Qiao et al (1994), Groot et al (1996), Gijbels et al (1999), Van Vlijmen et al (2001), Kooistra et al (2006), Yates et al (2009)) have quoted Paigen et al (1987) for their heart and aortic valve dissection techniques or quantification methodology. As these techniques and methods are referred to frequently, they begin to be adopted and interpreted for standard murine atherosclerosis analyses and to some extent were utilised for atherosclerosis quantification of the studies herein. However, analysis of quantification methodologies used has not been reviewed.

Paigen et al's (1987) studies considered 3 different quantification methods: (a) all sections, with lesion size visually estimated using a microscope grid eyepiece, (b) all sections, ORO stained cross-sections with photomicrographs taken and computer graphics software quantification, and (c) selecting sections with the largest lesions across all samples. They found that the latter method had greater statistical power compared to the other two due to utilising five independent, equidistant sections at 80µm intervals. They also found utilising method (a) and (c) together to be the most efficient for quantification as no photomicrographs were required.

Although the studies outlined above and shown in Table 3.1 have sectioned the same region of aorta and aortic valves, they identified their own preference in terms of the section thickness (5µm-10µm), distance (e.g. alternate 10µm, 40µm, and three 120µm), and number of sections quantified (n=3, 4, 5, 10, 15). Due to the variation in these methods and a different choice for this study (7µm sections at a distance of 14µm), it was determined to fully evaluate the atherosclerosis results for Study 1 to ensure they were (i) representative of the whole and (ii) no important data was omitted. It was also an opportunity to identify patterns of atherosclerosis expression throughout the aortic valve region.

Table 3.1 Comparison of Atherosclerosis Quantification Techniques[1]

	Publication/Author	Sections	Quantification
1.	Paigen et al(1987)	10µm alternate cross sections.	All sections: grid microscope measurement. photomicrograph and computer software. 5 alternate 10µsections at 80µm intervals
2.	Qiao et al (1994)	10µm alternate cross sections	Analysis 20-30 sections, alternate sections at 10µm intervals
3.	Groot et al (1996)	10µm alternate cross sections	Up to 40 sections imaged, all analysed. 10 alternate sections at 10µm intervals.
4.	Gijbels et al (1999)	7 µm cross sections	3 sections at 120µm intervals
5.	Van Vlijmen et al (2001)	5 µm cross sections	4 sections of aortic valve area at 40µm intervals
6.	Kooistra et al(2006)	5 µm cross sections	4 sections aortic valve area at 30 µm intervals.
7.	Yates et al(2009)	10µm alternate cross sections	15 sections aortic valve area, alternate sections at 10µm intervals.

[1] The data shown in the table above refers to cross sectional analyses in the aortic valve region only and does not detract from additional morphometric and categorical analyses carried out by the authors.

3.1 MATERIALS AND METHODS

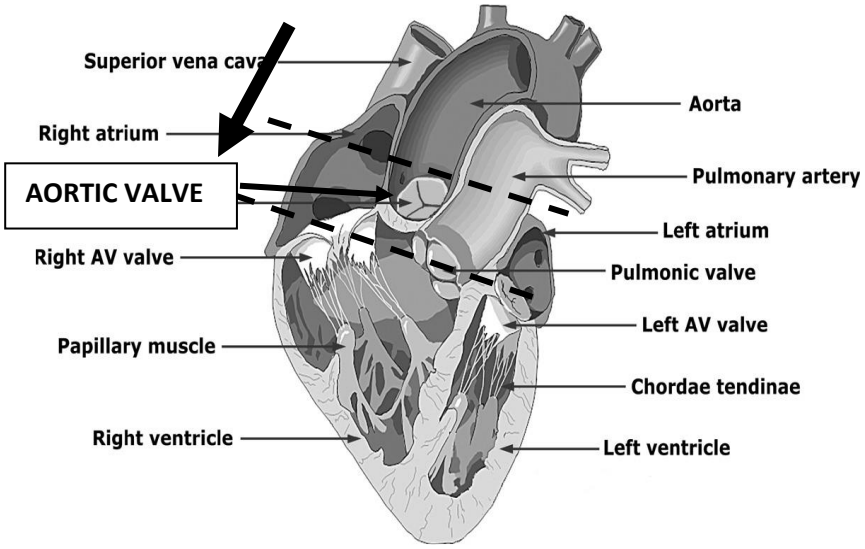
3.1.1. Mice and Diets

Diets and mice are previously described in Chapter 2 Methods, Paragraphs 2.1 and 2.2, respectively. C57BLJ6 female mice were cross bred with AEL males. During gestation (21 days) the pregnant dams were fed either a PHVO (P) or RTFA (R) diet thereby exposing the fetus to two types of TFA during development *in utero.* On the birth of the pups, dams were transferred onto chow diet (C) which was consumed by the dams throughout suckling. At 21 days of age, the AEL female pups were weaned onto a post-natal diet of either Chow (C) or cholesterol-rich Atherogenic (A) diet, resulting in four diet groups: (i) PCC, (ii) PCA, (iii) RCC, and (iv) RCA. AEL offspring remained on their post-natal diets for 12 weeks before being culled and tissues excised at 15 weeks of age.

3.1.2. Heart Dissection and Microtomy

The heart, aortic arch and descending aorta were excised at culling. The heart was dissected out, weighed, and flushed with histological OCT mounting media (Raymond A Lamb). As described by Paigen *et al* (1987) see Figure 3.1, the heart was sectioned perpendicular to the axis of the aorta. The sectioned hearts were mounted on a histology cork (Raymond A Lamb) with OCT, snap-frozen in liquid nitrogen and stored at -80°C until analysis (Figure 3.2).

Figure 3.1 Orientation of Heart and Aortic Valves for Dissection and Microtomy

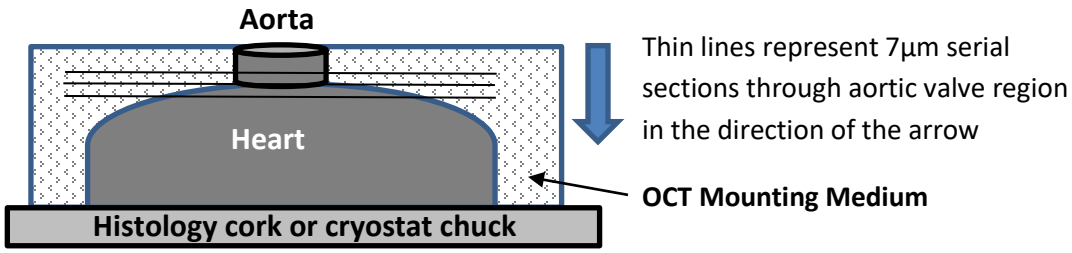


Source: Image adapted from Donnelly (2008)

3.1.3. Histological Analysis of Atherosclerosis within the Aorta

Each heart was remounted onto a cryostat chuck using OCT and brought to temperature (-20°C) one hour prior to sectioning (Figure 3.2). Serial cross-sections of the aorta (7µm thick) were taken using a cryostat (Bright Instruments, Huntingdon, Cambs, UK). The sections were placed onto charged slides (VWR International) from the appearance of the aortic valve leaflets, throughout the valves up to the heart atrium.

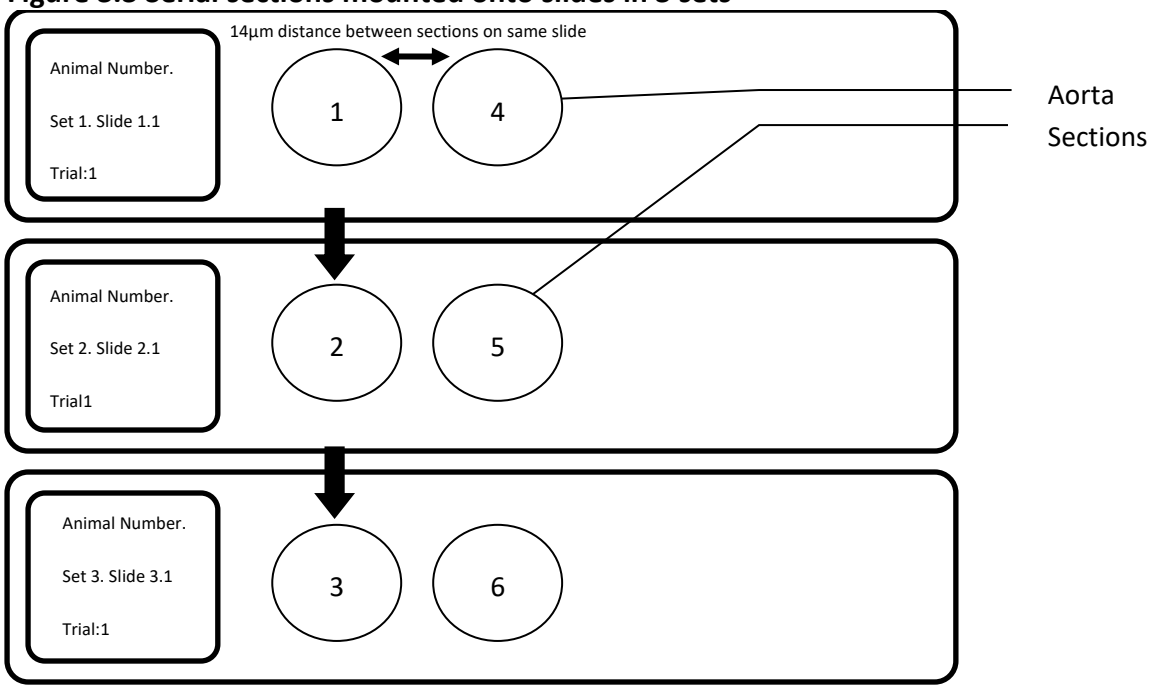
Figure 3.2 Diagram of Mounted Heart for Microtomy



Source: Adapted from Tonge (2011)

A series of three slides were used for each aorta. The first three sections were placed one on each of the three slides and this process repeated (Figure 3.3). This meant that the sections on each slide were 14µm apart.

Figure 3.3 Serial sections mounted onto slides in 3 sets



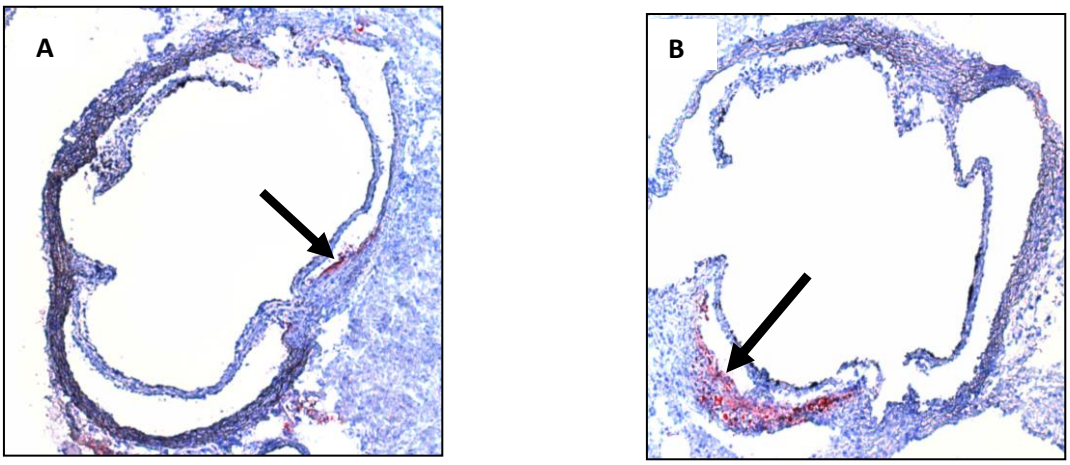
3.1.4. Oil Red O Staining

An Oil Red O (ORO) stock solution (0.5%w/v Oil Red O in isopropanol) was heated at 60°C for two hours, allowed to cool, then stored in the dark at room temperature until use. A working solution was made up of two parts ORO stock solution and three parts of 1% dextrin. This was mixed for an hour at room temperature and filtered before use. The slides were placed in staining racks and immersed in isopropanol for 15 seconds and ORO for 20 minutes, followed by rinsing in isopropanol for 15 seconds. Sections were counterstained in Mayers Haematoxylin (Raymond A Lamb) for five minutes, rinsed in tap water and blued in Scots tap water (distilled water containing 2% w/v sodium bicarbonate and 0.35% w/v magnesium sulphate). Finally, the slides were rinsed in tap water to remove any residue. An aqueous glycerol mounting medium was used (50% v/v glycerol and 50% dH_2O v/v) and the edges of the cover slips were sealed with clear lacquer.

3.1.5. Imaging and Quantification

All ORO stained sections were imaged with a light microscope (Leica DM5000B) using Leica Application software version 2.1 at 10x magnification. All sections were imaged, and ORO stained plaques quantified using Image-Pro® Plus 5.1.2 software. Analyses were performed blind without knowledge of diet group. Lesions were quantified by number of red pixels (Oil Red O lipid staining) present and converted to lesion area (μm^2).

Figure 3.4: Photomicrographs of Aorta Sections stained with Oil Red O



Images show aortic valve leaflets. Arrows indicate atherosclerotic lesions. Image (A) Shows small lesions in post-natal chow fed animals. Image (B) shows large lipid filled atherosclerotic lesion in animals fed a post-natal high fat/high cholesterol "atherogenic" diet.

3.2 RESULTS - ANALYSIS AND QUANTIFICATION OF ATHEROSCLEROSIS

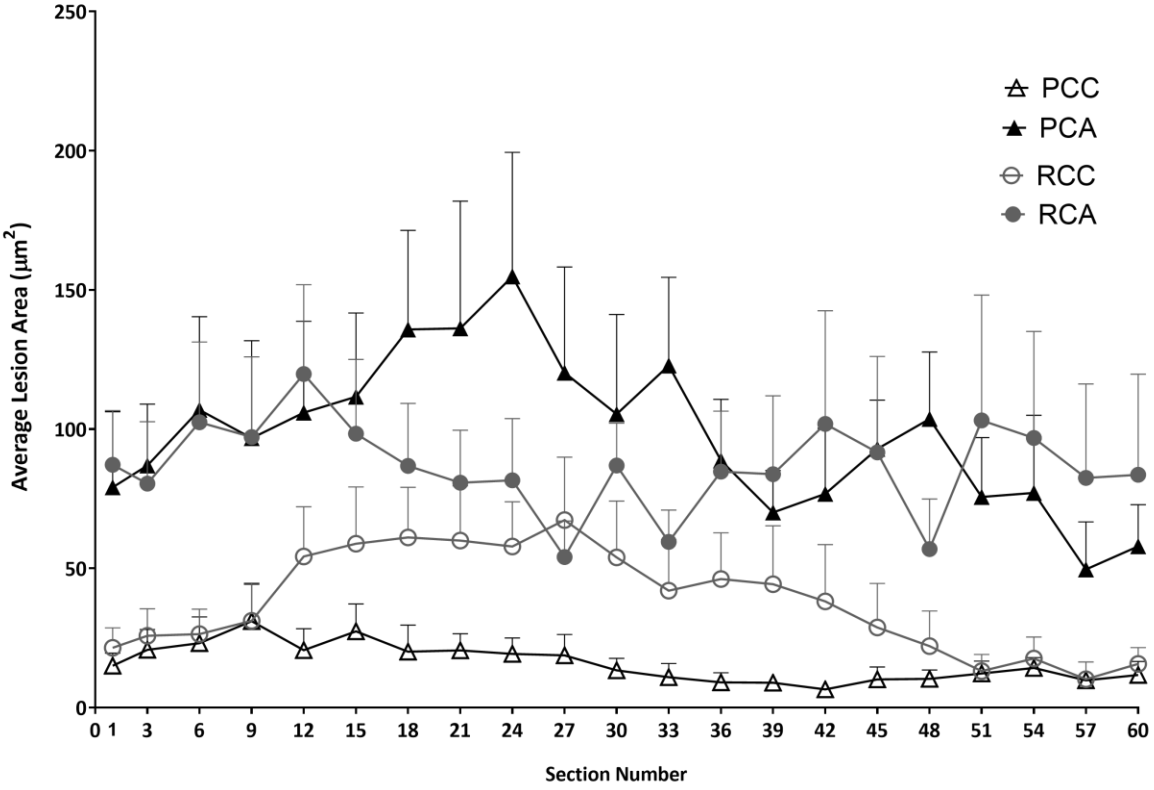
As previously described (paragraph 3.1.1) 7µm sections were analysed at a distance of 14µm between sections from the appearance of the aortic valves from aortic arch towards the heart. For Study 1, 21 specimens across four diet groups were quantified.

3.2.1. Pattern of Atherosclerosis Expression throughout Aortic Valves of ApoE*3 Leiden Mice

Sections were aligned to where three aortic valves were clearly observed (Figure 3.4). The average lesion area (μm^2) was calculated for every section and plotted to identify the pattern of atherosclerosis expression for each diet group (Figure 3.5). The pattern of lesion expression showed distinct differences between offspring diet groups. The offspring Atherogenic diet groups (A: PCA and RCA) were defined by a higher and protracted peak compared to chow groups (C: PCC and RCC) giving an immediate visual impact of the atherogenic diet on severity and prevalence of atherosclerosis. A second difference observed was the lack of lesions in the chow groups at the

beginning and end of the valves compared to atherogenic diet groups. It was also observed that PCC groups had lower levels of atherosclerosis compared to RCC throughout the sections 12-48.

Figure 3.5 Pattern of Atherosclerosis Lesions in the Aortic Valves of ApoE*3 Leiden Mice



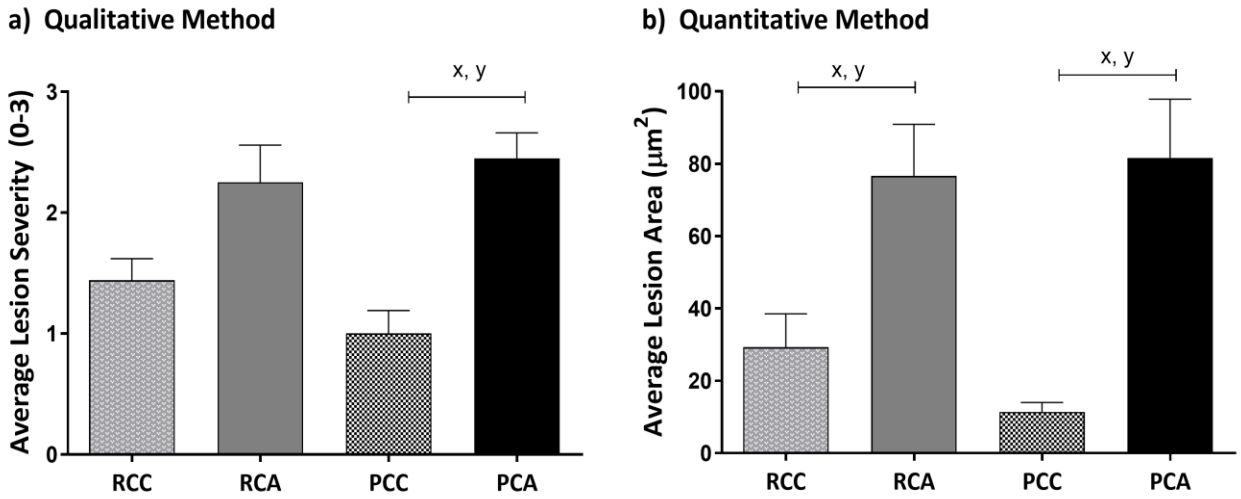
Mean data ±SEM shown for Maternal Pregnancy and lactation diet and Offspring post-natal diet respectively (PCC=8, RCC=9; PCA=11, RCA=8).

3.2.2. Comparison of Qualitative and Quantitative Analysis of All Sections

Atherosclerosis was quantified by two methods: (i) qualitative: visually scoring photomicrographs specimens dependent on atherosclerotic lesion severity: 0=none; 1=mild; 2=moderate; 3=severe. All sections assessed by eye and each animal allocated a final score between 0-3; (ii) quantitative image analysis of ORO lesion stained areas (method 3.1.5). Both methods analysed atherosclerosis present in all sections and produced similar overall statistical outcomes (see Results Table 3.2). However, from Figure 3.6 it was apparent qualitative analysis overestimated the severity of lesions in the chow groups. This lack of sensitivity was picked up by quantitative

analyses and Bonferroni post-hoc analysis clearly indicating that quantitative analyses had greater statistical power.

Figure 3.6 Results of Qualitative and Quantitative Analyses of Atherosclerosis



Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation, and post-natal diets respectively (PCC=8, RCC=9; PCA=11, RCA=8). Data analysed by two-way ANOVA with Pregnancy diet and post-natal diet as factors and Bonferroni post-hoc test. Pairs of unlike letters (x,y) significantly different P<0.001.

3.2.3. Quantitative Analysis of Subsets of Atherosclerosis Lesions

In order to check the viability of analysing a smaller subsets of sections as described by Paigen et al (1987) and other authors (see Table 3.1), n=3, n=4, n=5, n=10 and n=15 sections were analysed from Section 1 where the aortic valves were clearly present towards the heart and compared to all sections analysed (Table 3.2).

The results for all sections (N=21, S1-60, Figure 3.5) were comparative with smaller subsets of sections quantitative analyses and the qualitative analysis (Table 3.2). All the results were consistent in showing that there was no effect of maternal diet (P or R) on atherosclerosis and confirmed that there was a significant difference in atherosclerosis between offspring post-natal (A or C) diet groups (P<0.05). However, the Bonferroni post-hoc test gave slightly different outcomes. Quantification of all sections and N=5 sections at 84μm intervals both identified a significant difference between PCC vs PCA and that of RCC vs RCA. However these effects were

not observed in any other quantification of subsets of sections, with three out of five analyses not detecting a difference in PCC vs. PCA (N=5 at 84µm distance, N=10 at 14µm distance, and N=4at 42µm distance, Table 3.2).

Repeated measures ANOVA with maternal diet, offspring diet and section number as factors, gave another perspective on the atherosclerosis quantified (Table 3.2) There was a strong effect of section number observed for all sections across the aortic valve towards the heart ($P<0.001$). There was trend for section number shown for N=4 sections at 28µm distance, showing that as sections progressed towards the heart atherosclerosis increased ($P=0.051$, Figure 3.5).

Quantification of sections N=10 sections at 14µm distance also reflected the pattern of atherosclerosis expression with a strong effect of section number observed ($P<0.05$). However, this analysis also identified that those offspring exposed to P diet during pregnancy decreased, compared to R offspring whose atherosclerosis increased over the same distance (Section x Maternal Diet: $P<0.01$, see Figure 4.9). There was no interaction of section x offspring diet, showing consistency in the amount of atherosclerosis across these sections in C and A groups, respectively.

The remainder of the analyses of smaller subsets of sections of atherosclerosis did not show any effect of section number or section x diet interaction.

Table 3.2 Results of the Analysis of Different Quantities of Aortic valve cross-sections

			Offspring Post-natal Diet			REPEATED MEASURES ANOVA (*P*)			
Table 3.1 Author	**Number of Sections analysed**	**Maternal diet**	**C**	**A**	**Total**	**Section (S)**	**Maternal Diet (M)**	**Offspring Diet (O)**	**Interaction M x O**
Paigen 1987	**Qualitative scoring 0-3**	P	^{x}1.00 ±0.19	^{y}2.45 ±0.21	1.84 ±0.22	N/A	0.381	<0.001	0.1
	per aorta (all sections)	R	1.44 ±0.18	2.25 ±0.31	1.82 ±0.20		SxM: N/A	SxO: N/A	SxMxO: N/A
		Total	^{x}1.24 ±0.14	^{y}2.37 ±0.14					
Paigen 1987	**All Sections [a]**	**P**	^{x}15.75 ± 3.7	^{y}87.73 ±17.9	22.84 ±2.5		0.509	<0.001	0.600
Qiao, 1994	**N=21 at 14µm interval**	**R**	^{x}38.51 ±11.5	^{y}96.94 ±20.2	30.86 ±6.8	<0.001	SxM:0.039	SxO:0.992	SxMxO:0.988
Groot 1996		**Total**	^{x}27.80 ±6.8	^{y}91.61±12.8					
Paigen 1987	**Sections 1,15,30,45,60 [a]**	**P**	^{x} 15.24 ±3.16	^{y}90.51 ±19.8	58.82 ±14.3		0.977	<0.001	0.764
	N=5 at 84µm interval	**R**	^{x}35.86 ±10.9	^{y}91.64 ±18.8	62.12 ±12.4	0.095	SxM:0.864	SxO:0.916	SxMxO:0.651
		Total	^{x}26.11 ±6.4	^{y}90.98 ±16.2					
Groot 1996	**Sections 1,3,6,9,12**	**P**	22.19 ± 7.1	88.13 ±17.9	60.41 ±13.1		0.383	0.002	0.759
Yates 2009	**15,18,21,24,27 [a]**	**R**	46.75 ±12.8	111.53 ±30.5	77.20 ±17.3	0.049	SxM:<0.01	SxO:0.877	SxMxO:0.963
	N=10 at 14µm interval	**Total**	^{x}35.15 ±7.9	^{y}98.10±16.2					
Gijbels 1999	**Sections 1, 24, 51 [a]**	**P**	^{x}16.71 ± 4.8	^{y}77.60 ±15.6	51.96 ±11.3		0.913	<0.001	0.764
	N=3 at 126µm interval	**R**	35.71 ±11.0	91.10 ±22.2	61.81 ±13.5	0.200	SxM:0.241	SxO:0.952	SxMxO:0.923
		Total	^{x}26.8 ±6.6	^{y}83.38 ±12.5					
Van Vlijmen	**Sections 1, 9, 18, 27 [a]**	**P**	21.25 ± 6.9	80.77 ±15.9	55.71 ±11.7		0.352	0.002	0.834
2001	**N=4 at 42µm interval**	**R**	45.28 ±13.3	108.91 ±29.3	75.23 ±16.9	0.173	SxM:0.011	SxO:0.641	SxMxO:0.955
		Total	^{x}34.10 ±8.1	^{y}92.62 ±15.3					
Kooistra	**Sections 1, 6, 12, 18 [a]**	**P**	^{x}19.39 ±6.7	^{y}92.07 ±21.5	61.47 ±15.1		0.606	<0.001	0.591
2006	**N=4 at 28µm interval**	**R**	40.80 ±11.3	18.42 ±29.9	72.62 ±17.0	0.051	SxM:0.101	SxO:0.932	SxMxO:0.777
		Total	^{x}30.72 ±7.1	^{y}99.10 ±17.3					

Mean data ±SEM shown for Maternal Pregnancy and lactation diet and post-natal offspring diet respectively (PCC=8, RCC=9; PCA=11, RCA=8). Data transformed to square root "a" and analysed by three-way repeated measures (for section number) ANOVA, with maternal diet and offspring post-natal diet as factors, blocking for animal and litter from which the samples were derived. Pairs of unlike letters (x,y) are significantly different P<0.05.

3.3 DISCUSSION

For this study, we assessed the impact of maternal consumption of two different *trans* fat diets (P and R) during pregnancy on the susceptibility of AEL offspring to atherosclerosis in adult life. Quantification of atherosclerosis in the aortic valve lesions was after 12 weeks on post-natal diets, over a distance of 294µm in the aortic valve region. Due to the variation in the reported number of sections quantified over different distances (Table 3.1) and different approaches to quantification used (e.g. qualitative vs. quantitative) it was necessary to assess whether these methods and quantification of smaller subsets of sections were comparative to whole aortic valve analyses.

It was found that the qualitative scoring of lesions was much quicker and simpler compared to the computer aided quantitative analysis. However, using this approach it was not possible to accurately plot the expression of atherosclerosis throughout the aortic valve region. Paigen *et al* (1987) used a microscope grid eyepiece that they concurred improved lesion assessment accuracy; however, it would also increase the time and effort spent analysing atherosclerosis sections. Additionally, if these sections were needed for reanalysis, there is the drawback that tissue integrity may deteriorate over time and introduce variability. In our study, photomicrograph and computer software quantification produced greater statistical power and facilitated comprehensive data analyses. It also provided an image of the lesions which can be accessed long after the stained sections may have lost their integrity or been archived. This method was worthwhile as it provided accurate, rigorous data which was able to be plotted and assessed visually and statistically. However, this method was labour intensive and time consuming. Our results showed that utilising qualitative scoring (with or without grid eyepiece) would be sufficient for a general overview of atherosclerosis. However, caution is advised in using this solely to quantify atherosclerosis as statistical sensitivity was lower compared to quantitative methods and outcomes could be overlooked.

Overall, our results showed that all sections quantified gave the same results (e.g. maternal diet: P vs R; offspring diet: C vs A) as the smaller subsets of sections analysed. This robust approach

supports the results by other authors who analysed all sections and the reduced number of sections (Paigen et al., 1987, Qiao et al., 1994, Groot et al., 1996). The consistent outcome across these analyses conferred confidence in the results presented and that quantification of a lower number of sections would be appropriate. Although N=5 sections (as per Paigen et al 1987 methodology) gave the same outcome as N=21 sections in our study, caution is warranted as it does not give the investigator a full picture of expression of atherosclerosis as it progresses throughout the valves. Furthermore, closer analysis of the smaller number of sections using Bonferroni post -hoc tests, indicated a lack of sensitivity with two out of five analyses (N=10 at 14µm distance, and N=4at 42µm distance, Table 3.2) not indicating a difference between PCC and PCA diet groups. This suggests that a smaller number of sections may not give a full representation of the results, particularly if later sections leading towards the heart are omitted.

Analysis of subsets of atherosclerosis by repeated measures ANOVA with section number as a factor, gave a good indication of the magnitude of atherosclerosis as it progressed through the aortic valve towards the heart, with a strong effect of section number observed in N=4 sections at 28µm distance (P=0.051, Figure 3.5). Equally quantification N=10 sections at 14µm distance displayed a strong effect of section number ($P<0.05$), with section x maternal diet interaction ($P<0.01$). Plotting the maternal diet (see figure 4.9), identified that as the sections progressed towards the heart offspring exposed to P maternal diet started to decrease in atherosclerosis and R maternal increase in atherosclerosis. None of the other subsets of sections showed any effect of section number indicating homogeneity of atherosclerosis between diet groups across the sections quantified.

For our study, we considered that the most robust approach was to quantify all sections (N=21), in conjunction with N=10 sections due to the interaction of the section number with diet over this region. The latter analysis of 10 sections did not detract from the overall results, but added another facet by which to interpret the data and describe the pattern of atherosclerosis as it advanced through the valve sections towards the heart, which is often excluded from the results.

3.4 CONCLUSION

For our study, the most robust analysis was N=21 sections and N=10 sections from the aortic valve (Sections 1-27, Figure. 3.5). From the smaller number of sections we analysed (N=3, N=4, N=5, N=10, N=15) we concluded it was inappropriate to use these as a single measurement of atherosclerosis as it could generate incomplete or skewed results. Caution is warranted due to differential atherosclerosis expression throughout the aortic valve region. Therefore, we concluded that the most rigorous and prudent analysis would be for atherosclerosis to be quantified throughout the whole of the aortic valve region using accurate quantification methodology, such as computer aided analysis. Thereafter, a smaller number of sections could be utilised to describe the pattern of expression through the aortic valve region. Combining both analyses, adds weight and credence to the reported results and ensures that key statistical outcomes are not omitted.

CHAPTER 4: THE IMPACT OF A PHVO OR RTFA DIET DURING PREGNANCY ON THE DEVELOPMENT OF ATHEROSCLEROSIS IN OFFSPRING (APOE*3 LEIDEN MOUSE)

4. INTRODUCTION

As outlined in the General Introduction epidemiological studies and animal experiments have shown that poor maternal diet during pregnancy can cause increased susceptibility to a range of chronic diseases in the offspring, including hypertension, type 2 diabetes and atherosclerotic CVD (Langley-Evans, 2015, Symonds et al., 2013, Hanson and Gluckman, 2014, Mone et al., 2004).

More recently, it has become apparent that maternal overnutrition, and consumption of energy dense foods rich in SFA and TFA, can manifest in maternal adiposity. In human pregnancy monitoring studies obese mothers are found to have elevated inflammatory markers (Ramsay et al., 2002, Stewart et al., 2007), reduced vascular function (Meyer et al., 2013) and dyslipidaemia (Meyer et al., 2013, Ramsay et al., 2002). Observed outcomes of obese mothers include, pre-term babies, lower birth weight babies and macrosomia babies that have greater susceptibility to obesity and CVDs in childhood and later life (Gademan et al., 2014, Flick et al., 2010, Gaillard et al., 2013). In post-mortem studies of pre-term fetuses. It was found that those offspring of hypercholesterolemic mothers had atherosclerotic lesions within their aortas (Napoli et al., 1999). In animal experiments, it has also been shown that maternal hypercholesterolemia induces atherosclerosis in offspring of rabbits (Napoli et al., 2000), LDLr-knockout mice (Napoli et al., 2002) and ApoE-knockout mice (Goharkhay et al., 2007).

Dietary TFA, particularly those in PHVO rich in *trans* C18:1 isomers, adversely affects lipoprotein concentrations (Mensink et al., 2003) and increase risk of developing atherosclerotic CVD (Mozaffarian and Clarke, 2009). Ruminant-derived meat and dairy products represent another source of dietary TFA. However, while PHVO contains a wide range of isomers, the specific

composition of which depends on the parent oil, ruminant-derived products contain predominantly VA (C18:1 *t11*), which is produced by the bacterial population of the rumen (Figure 1.5). This is particularly significant as a proportion of dietary VA is converted to CLA isomer C18:2*c*9*t*11, through the action of stearoyl CoA desaturase in the tissues of animals, including humans (Turpeinen et al., 2002, Kuhnt et al., 2006). It has also been demonstrated that there are potential beneficial effects of butter enriched in VA and CLA C18:2*c*9*t*11 on lipoprotein profiles (Lock et al., 2005).

In humans and animals, TFA in the maternal diet can be transferred across the placenta into the circulation and tissues of the developing offspring with possible metabolic consequences (Innis, 2006). A human cohort study suggested that maternal TFA consumption, during the second trimester of pregnancy, was positively associated with fetal growth rates (Cohen et al., 2011), although studies in mice exposed to TFA-enriched milk fat show retarded growth rates (Kavanagh et al., 2010).

4.1 AIM AND HYPOTHESIS

In this study, the aim was to investigate the impact of two types of TFA (that associated with ruminant milk and that associated with partially hydrogenated vegetable oil) during pregnancy on the development of atherosclerosis in the offspring using the AEL mouse model (see Methods, Figure 2.3 for experimental design).

Hypothesis: That maternal consumption of ruminant derived TFA during pregnancy and fetal development, will protect the offspring from atherosclerosis, while that associated with PHVO will have the opposite effect.

4.2 DIETS

In order to examine the study's hypothesis, chow diets were supplemented with 13% TFA, equivalent to 4% daily energy in mice. Dietary fats were similar to those initially used by

Chardigny *et al* (2008) in the human TRANSFACT study (see Methods Chapter 2,Table 2.1). Although there are some specific differences including CLAs within the RTFA diet, where possible the distribution and sum of other fatty acids SFA, MUFAs and PUFAs were equivalent in the dietary groups. Fatty acid composition of experimental diets (% FAME) are shown in Chapter 2, Methods, Figure 2.2. See Methods paragraph 2.2.4 for full descriptive of diet groups.

4.3 MICE

Seven-week-old wild type female (n=10 per diet group) C57BL/6J mice (dams) were mated with randomly selected AEL males (sires) (approx. eight weeks age) over a two oestrous cycle period of eight days. Groups were matched for variations in bodyweight. During mating the experimental Ruminant TFA (R), or PHVO TFA (P), diets were fed to both sires and dams. On the eighth day the sires were removed, and dams remained on the allocated experimental TFA diets throughout pregnancy (see Figure 2.2 Sector 1"Pregnancy").

4.3.1. Pregnancy Study (Dams/Fetus): Wild type C57BL/6J mice

On day 17 of pregnancy dams were sacrificed with maternal and fetal tissues harvested (see Methods paragraph 2.3 and results paragraph 4.4).

4.3.2. Study 1 Dams: Wild type C57BL/6J mice

On birth of pups (see Figure 2.2, Sector 2 "Lactation") dams were transferred onto a chow diet (C), thereby exposing their offspring to a chow diet during suckling for a period of 21 days giving two dam diet groups: (i) RTFA/Chow (RC); and (ii) PHVO/Chow (PC). At 21 days age, pups were sexed, genotyped (see Methods paragraph 2.5) and weaned onto a postnatal diet. Dams were culled in accordance with Schedule 1 procedures, ASPA (Home_Office, 1986), no maternal tissues were harvested.

4.3.3. Study 1 Offspring: ApoE*3 Leiden Mice

Female AEL pups were weaned onto a post-natal diet (see Figure 2.2, Sector 3 “Post-natal”) of either standard chow “C” or a high fat, high cholesterol Atherogenic diet “A” (see Methods Table 2.3) giving rise to four offspring diet groups:

- PHVO groups: PCC and PCA.
- RTFA groups: RCC and RCA.

Offspring remained on post-natal diets for 12 weeks (84 days). After 12 weeks on experimental diets, at the age of 15 weeks, mice were culled in accordance with Schedule 1 procedures, ASPA and tissues harvested (Home_Office, 1986) (see Methods paragraph 2.3 and offspring results paragraph 4.5).

4.4 RESULTS - THE IMPACT OF CONSUMING A PHVO OR RTFA DIET DURING PREGNANCY ON MATERNAL PHYSIOLOGY (C57 MOUSE)

4.4.1. The Impact of consuming a P or R Diet during Pregnancy on Maternal Body Weight

From conception to study end point, it was observed that pregnancy weight gain trajectory and overall weight gain was similar between both diet groups. These effects were corroborated through similar average feed intake and energy efficiency see Table 4.1

Table 4.1 The Impact of consuming a P or R Diet consumed during Pregnancy on Maternal Body Weight

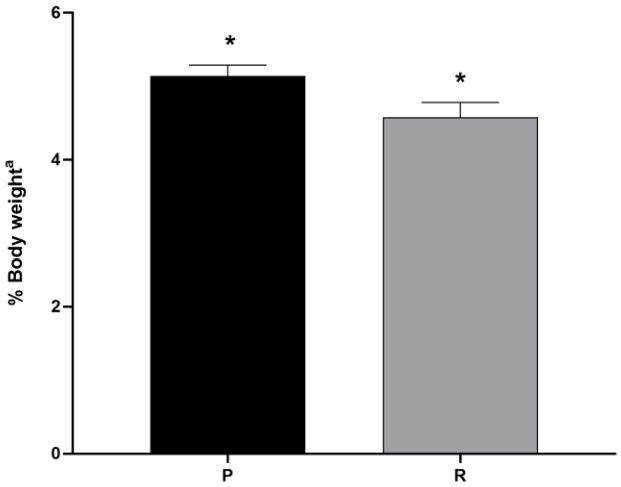
	P	R	T-TEST P Value
Start weight (g)	16.58 ±0.23	17.02 ±0.25	0.664
Bodyweight at day 17 pregnancy (g)	28.73 ±0.53	29.96 ±0.77	0.210
Pregnancy weight gain Day 17 (g)	12.15 ±0.61	12.94 ±0.58	0.377
Average daily feed intake (g)	2.20 ±0.10	1.98 ±0.11	0.182
Feed Efficiency (g weight / g feed intake)	7.62 ±0.34	7.74 ±0.37	0.771

Data are shown as mean ±SEM for n observations per group, showing pregnancy diet (P=6, R=5). Significance P<0.05.

4.4.2. The Impact of consuming a P or R Diet during Pregnancy on Maternal Body Composition and Organ Weight

Organ and tissue weights are expressed as a percentage of body weight (%BW). At day 17 gestation animals that had consumed P had heavier livers compared to those animals that had consumed R during pregnancy (P<0.05, Figure 4.1).

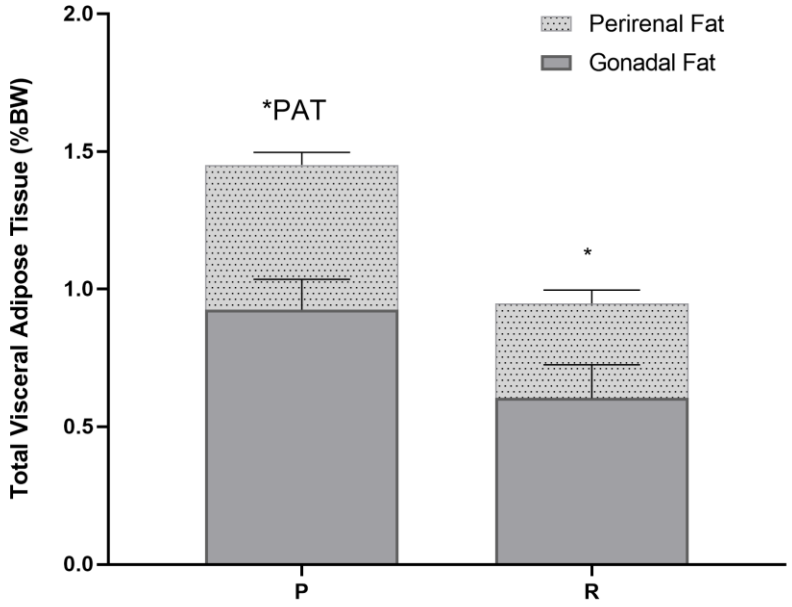
Figure 4.1 The Impact consuming a P or R Diet during Pregnancy on Maternal Liver Weight



Data are shown as mean ±SEM for n observations per group, showing pregnancy diet (P=6, R=5). Nonparametric data transformed square root "a" and analysed by T-Test. Significance, *P<0.05.

Animals that had consumed P during pregnancy had larger total visceral adipose depots compared to animals that had consumed R ($P<0.05$, Figure 4.2). Break down of visceral adipose results showed that P animals had larger perirenal adipose depots ($P<0.05$) but not gonadal adipose tissue ($p=0.082$).

Figure 4.2 The Impact of consuming a P or R Diet during Pregnancy on Maternal Visceral Adipose Tissue



Data are shown as mean ±SEM for n observations per group, showing pregnancy diet (P=6, R=5) and analysed by T-Test. For PAT (Perirenal Adipose Tissue) Significance, *P<0.05.

4.4.3. The Impact of consuming a P or R Diet during Pregnancy on Fetal and Placenta Development

There were no differences observed between the two diet groups at day 17 gestation for maternal bodyweight, placental weight or fetus body weight (see Table 4.2 below). Dams consuming R diet during pregnancy had more fetuses present compared to dams consuming P (P=0.001). This difference was observed in the number of fetuses within the left uterine horn (P<0.05).

Table 4.2 The Impact of consuming a P or R Diet during Pregnancy on Fetal and Placenta Development

	P	R	T-TEST P
Body weight d17 pregnancy	28.73 ±0.53	29.96 ±0.77	0.210
Average number of fetuses per pregnancy	**6.17 ±0.40**	**8.60 ±0.24**	**0.001**
# fetus left uterine horn	**1.67 ±0.42**	**4.00 ±0.82**	**0.023**
# fetus right uterine horn	4.50 ± 0.43	4.75 ±0.85	0.779
Total fetus absorptions	0.83 ± 0.31	0.25 ±.025	0.214
Placenta % BW	0.39 ± 0.13	0.38 ±0.12	0.922
Fetus weight (g)	0.55 ±0.02	0.51 ±0.02	0.313
Fetal/placenta ratio	4.70 ±0.20	4.9 ±0.18	0.778

Data are shown as mean ±SEM for n observations per group, showing pregnancy diet (P=6, R=5). Fetuses and placentas (P=37, R=43). Significance P<0.05.

4.4.4. The Impact of consuming a P or R Diet during Pregnancy on Maternal Serum Lipids and Serum Glucose

Serum Cholesterol and Serum TAG measurements were performed by Colorimetric Assays using Infinity reagents (Method 2.6.1 and Table 2.7). Serum total cholesterol and TAG were found to be greater in animals consuming P compared to R ($P<0.05$).

Serum glucose was measured by Colorimetric Assay using glucose oxidase reagent (Method 2.6.2) and were found to be similar between both diet groups (Table 4.3).

Table 4.3 The Impact of consuming a P or R Diet During Pregnancy on Maternal Serum Lipids and Serum Glucose

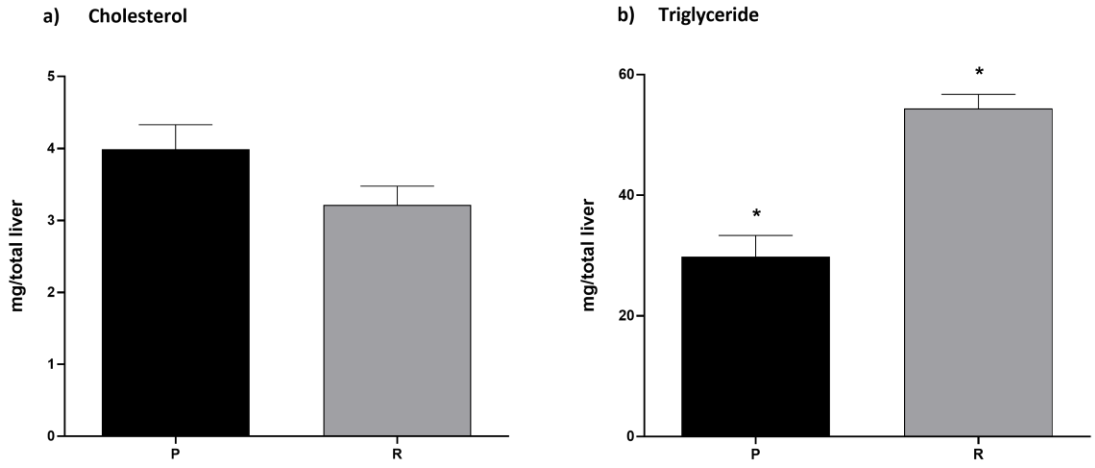
	P	R	T-TEST P
Total Cholesterol (mmol/L)	**3.08 ±0.16**	**2.29 ±0.22**	**0.044**
TAG (mmol/L)	**2.62 ±0.24**	**1.63 ±0.28**	**0.029**
Glucose (mmol/L)	12.42 ±1.17	11.72 ±1.46	0.718

Data are shown as mean ±SEM for n observations per group, showing pregnancy diet (P=6, R=5). Significance $P<0.05$.

4.4.5. The Impact of consuming a P or R Diet during Pregnancy on Maternal Liver Lipids

Lipids were extracted from livers (Method 2.7.4) and TAG and cholesterol assays performed (Method 2.7.5). Liver lipids are expressed as mg/total liver. At day 17 pregnancy there were no differences in liver cholesterol (Figure 4.3a). Liver TAG (Figure 4.3b) were higher in animals fed R compared to P ($P<0.05$).

Figure 4.3 The Impact of consuming a P or R Diet during Pregnancy on Maternal Liver Lipids

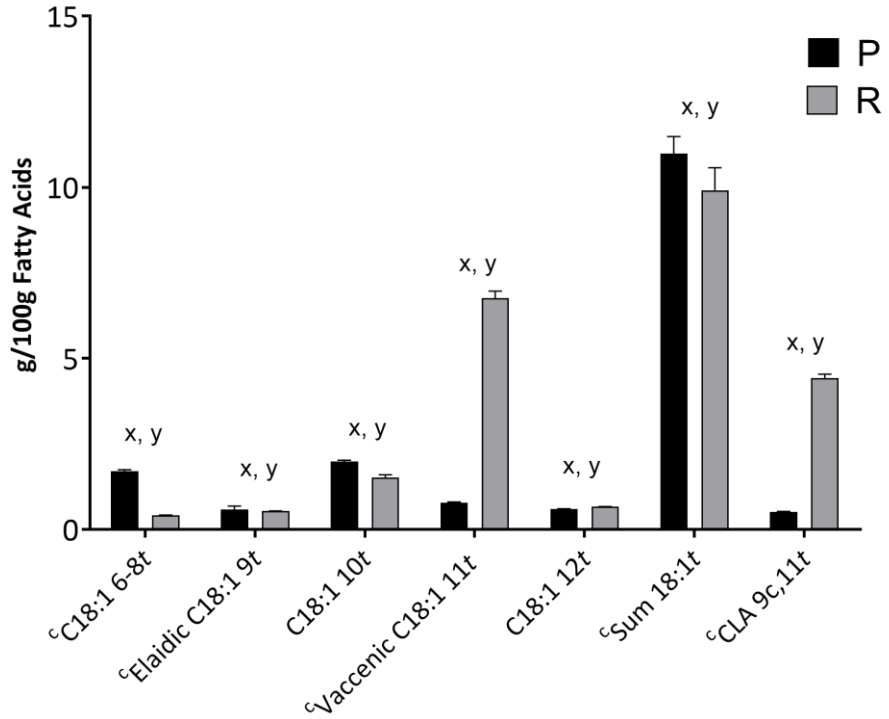


Data are shown as mean ±SEM for n observations per group, showing pregnancy diet (P=6, R=5) and analysed by T-Test. Significance *P<0.05.

4.4.6. The Impact of consuming a P or R Diet during Pregnancy on the Fatty Acid Composition of Maternal Perirenal Adipose Tissue (%FAME)

FAME FAs were extracted from perirenal adipose tissue and measured (Method 2.7.1., A. Lock, University of Michigan). The distribution of *trans* fatty acids in the perirenal adipose tissue is shown in Figure 4.4. As expected, the TFA isomers in the maternal perirenal adipose tissue reflected that of the diets consumed (Table 2.1 and Figure 2.1). The results showed that animals that had consumed R during pregnancy had greater quantities of Vaccenic Acid (C18:1 *t11*) and CLA (9*c*11*t*) in their perirenal adipose tissue compared to P animals. Those animals that had consumed P during pregnancy had a wider distribution of *trans* isomers across C18:1 6-12*t* isomers, and total C18:1 *trans* compared to R animals.

Figure 4.4 C18:1 *trans* Fatty Acid Composition of Maternal Perirenal Adipose Tissue



Data are shown as mean ±SEM for n observations per group, showing pregnancy diet (P=6, R=5). Nonparametric data transformed to Log10 "c" and analysed by T-Test. For each *trans* FA isomer, bars with unlike letters (x,y) are significantly different P<0.05.

4.5 RESULTS - THE IMPACT OF MATERNAL CONSUMPTION OF A PHVO OR RTFA DIET DURING PREGNANCY ON ATHEROSCLEROSIS DEVELOPMENT IN OFFSPRING (AEL MOUSE)

For a full description of offspring diet groups, see paragraph 2.2.4.

4.5.1. The Impact of Maternal Consumption of P or R Diet during Pregnancy on the Body Weight of Offspring

AEL female offspring were weaned at approximately three weeks of age and weighed daily over the first seven days. Thereafter animals were weighed weekly for a period of 11 weeks. There were no effects of the maternal diet (P or R) or offspring post-natal diet (C or A) observed on offspring body weight on Day 0 weaning nor after 84 days on post-natal diets, and consequently overall weight gain was similar (Table 4.4). Analysis of weight gain trajectory from Day0 to Day84 by repeated measures ANOVA confirmed that there was no difference in growth patterns between the diet groups.

Table 4.4 The Impact of Maternal Consumption of P or R Diet during Pregnancy on the Body Weight of Offspring

		Offspring Post-natal Diet			TWO-WAY ANOVA P		
	Maternal diet	C	A	Total	Maternal Diet	Offspring Diet	Maternal x offspring
Weaning body weight Day 0 (g)	**P**	7.74 ±0.22	7.77 ±0.32	7.76 ±0.20	0.340	0.775	0.861
	R	7.88 ±0.35	8.32 ±0.32	7.95 ±0.23			
	Total	7.81 ±0.21	7.88 ±0.24				
Body weight Day 84 (g)	**P**	21.61 ±0.51	20.91 ±0.41	21.21 ±0.35	0.325	0.366	0.517
	R	21.68 ±0.33	21.65 ±0.35	21.71 ±0.24			
	Total	21.69 ±0.32	21.22 ±0.30				
Mean weight gain days 0-84 (g)	**P**	13.88 ±0.57	13.14 ±0.46	13.45 ±0.22	0.589	0.284	0.609
	R	13.89 ±0.41	13.63 ±0.34	13.77 ±0.26			
	Total	13.88 ±0.17	13.34 ±0.30				

Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation, and post-natal diets respectively (PCC=8 RCC=9; PCA=8, RCA=11). Data was analysed by two-way ANOVA with Bonferroni post hoc test. Significance $P<0.05$.

4.5.2. The Impact of Maternal Consumption of P or R Diet during Pregnancy on the Body Composition and Organ Weights of Offspring

Organ and tissue weights are expressed as a percentage of body weight (%BW). There were no effects of the maternal diets) or offspring post-natal diets (C or A) observed on liver size (see Table 4.6).

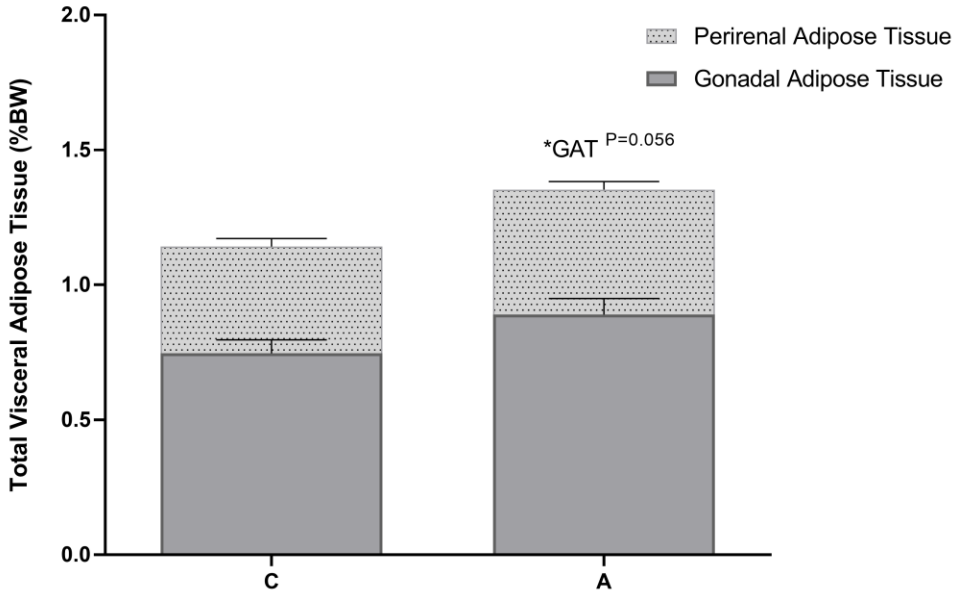
Table 4.5 The Impact of Maternal Consumption of P or R Diet during Pregnancy on the Body Composition and Organ Weights of Offspring

		Offspring Post-natal Diet			TWO-WAY ANOVA P		
	Maternal diet	C	A	Total	Maternal Diet	Offspring Diet	Maternal x offspring
Liver (%BW)	**P**	5.08 ±0.18	5.34 ±0.17	5.23 ±0.12			
	R	5.06 ±0.16	5.23 ±0.15	5.14 ±0.11	0.720	0.200	0.777
	Total	5.07 ±0.05	5.30 ±0.11				
Perirenal Adipose (%BW)	**P**	0.35 ±0.04	0.44 ±0.04	0.40 ±0.03	0.112	0.092	0.880
	R	0.43 ±0.04	0.50 ±0.05	0.46 ±0.03			
	Total	0.40 ±00.03	0.46 ±0.0.03				
Gonadal Adipose (%BW)	**P**	0.68 ±0.08	0.86 ±0.08	0.80 ±0.06	0.153	0.056	0.758
	R	0.81 ±0.06	0.93 ±0.08	0.86 ±0.05			
	Total	0.74 ±0.05	0.89 ±0.06				

Data are shown as mean ±SEM for n observations per group showing pregnancy, lactation, and post-natal diets respectively (PCC=8 RCC=9; PCA=8, RCA=11). Data was analysed by two-way ANOVA with Bonferroni post hoc test. Significance $P<0.05$.

There were no effects of the maternal P or R diet observed on visceral adipose depots. However, it was found that there was a trend for offspring that had consumed a post-natal A diet to have greater gonadal adipose depots compared to C (P=0.056, Table 4.5 and Figure 4.5).

Figure 4.5 The Impact of a Post-natal Atherogenic Diet on Visceral Adipose Tissue in offspring



Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation, and post-natal diets respectively (Chow: PCC=8 RCC=9; Atherogenic: PCA=8, RCA=11). Data was analysed by two-way ANOVA with Bonferroni post hoc test. GAT: Gonadal Adipose Tissue. Significance P<0.05.

4.5.3. The Impact of Maternal Consumption of P or R Diet during Pregnancy on the Serum Glucose and Serum Lipids of Offspring

Serum glucose was measured by Colorimetric Assay using glucose oxidase reagent (Method 2.6.2). There were no effects of the maternal pregnancy diet (P or R) or offspring post-natal diet (C or A) observed on serum glucose levels (Table 4.6).

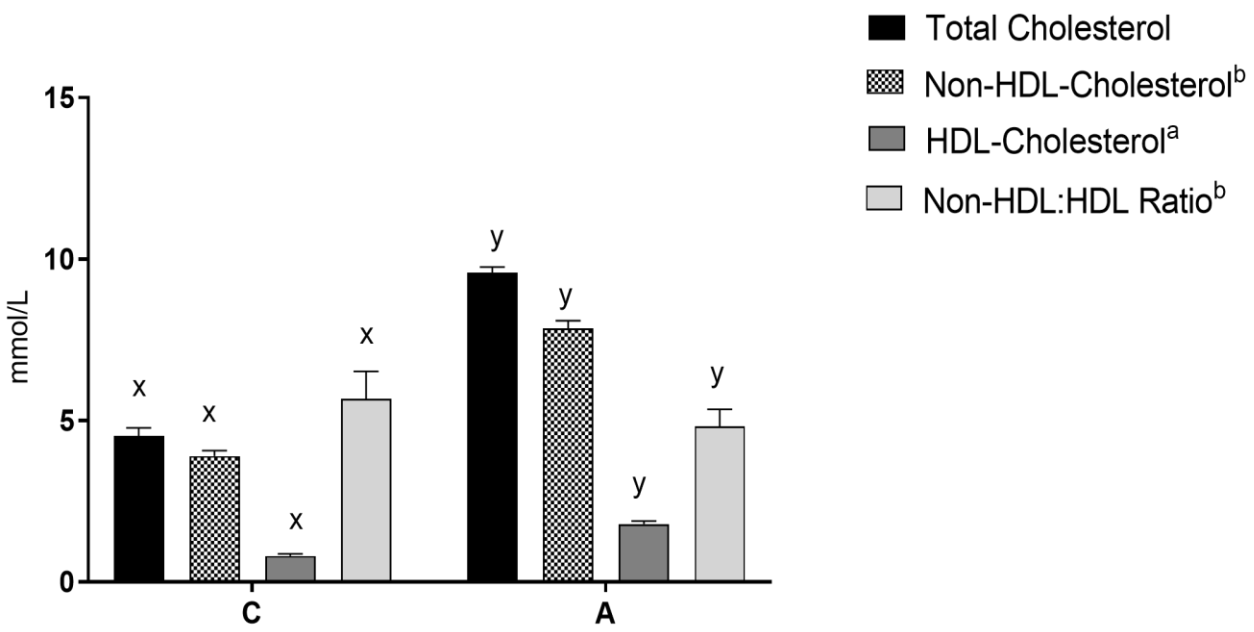
Serum Cholesterol and serum TAG measurements were performed in duplicate with a Randox Imola RX chemistry analyser via a direct clearance method (HDL, LDL) or colorimetric assay (Total Chol, TAG) (Method 2.6.3). There were no effects of maternal P or R diet observed on serum total cholesterol, HDL-cholesterol, non-HDL-cholesterol, or serum TAG. Total cholesterol and non-HDL cholesterol were higher in offspring post-natal A diet groups compared to C ($P<0.05$, Figure 4.6). The A diet caused an increase in HDL-cholesterol, and as a result the non-HDL:HDL ratio was reduced in these diet groups when compared to chow ($P<0.05$). Serum TAG was lower in the A diet groups compared to C ($P<0.05$, Table 4.6).

Table 4.6 The Impact of Maternal Consumption of P or R Diet during Pregnancy on Serum Glucose and Serum TAG of Offspring

		Offspring Post-natal Diet			TWO-WAY ANOVA P		
	Maternal diet	C	A	Total	Maternal Diet	Offspring Diet	Maternal x offspring
Glucose (mmol/L)	**P**	14.32 ±1.02	14.57 ±0.81	14.47±0.62			
	R	14.04 ±0.91	14.83 ±1.40	14.43±0.81	0.989	0.620	0.797
	Total	14.18 ±0.66	14.68 ±.029				
TAG (mmol/L)	**P**	2.63 ±0.25	1.43 ±0.09	1.94 ±0.18			
	R	2.94 ±0.28	1.59 ±0.12	2.30 ±0.23	0.244	0.0001	0.709
	Total	^{x}2.80 ±0.25	^{y}1.50 ±0.07				

Data are shown as mean ±SEM for n observations per group (PCC=8, RCC=9; PCA=11, RCA=8) and analysed by two-way ANOVA. Unlike letters (x,y) are significantly different $P<0.05$.

Figure 4.6 The Impact of a Post-natal Atherogenic Diet on Serum Cholesterol in Offspring

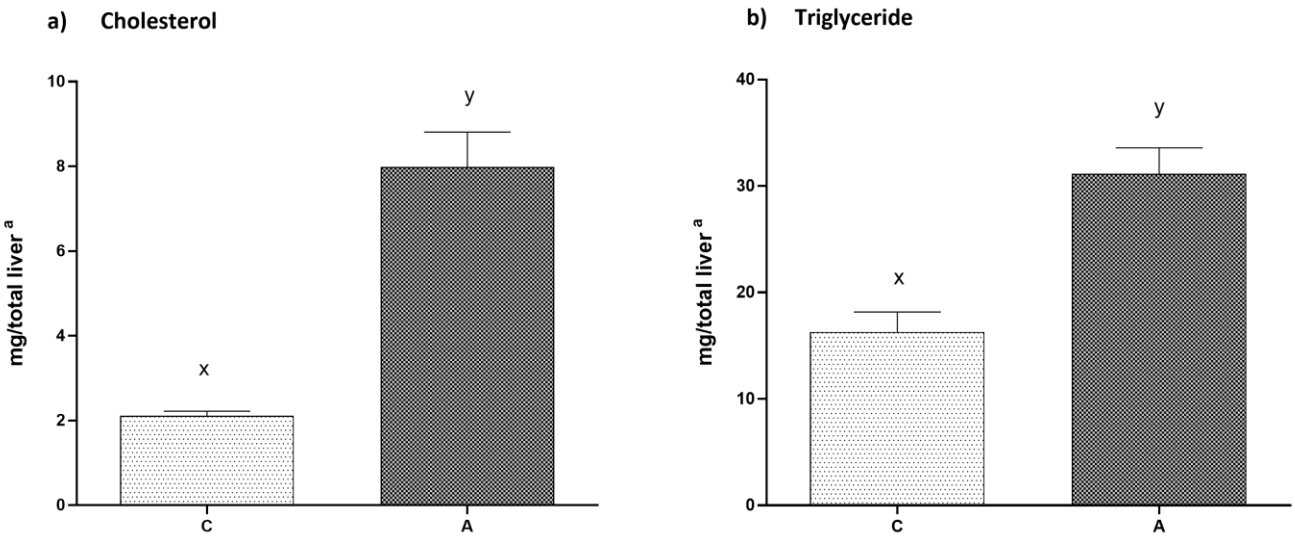


Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation, and post-natal diets respectively (Chow: PCC=8 RCC=9; Atherogenic: PCA=8, RCA=11). Data transformed to log 10 "a" and analysed by two-way ANOVA with Bonferroni post hoc test. Nonparametric data were analysed by Independent sample Mann Whitney "b". For each corresponding cholesterol column (C vs A), unlike letters (x,y) are significantly different $P<0.05$.

4.5.4. The Impact of Maternal Consumption of P or R Diet during Pregnancy on Liver Lipids of offspring

Lipids were extracted from livers (Method 2.7.4) and TAG and cholesterol assays performed (Method 2.7.5). Liver lipids are expressed as mg/total liver. There was no effect of maternal P or R diet on offspring liver cholesterol or TAG. The post-natal A diet group showed a three-fold difference in hepatic cholesterol (Figure 4.7a) compared to C and two-fold higher hepatic TAG P<0.001 (Figure 4.7b).

Figure 4.7 The Impact of a Post-natal Atherogenic Diet on Liver Lipids in Offspring



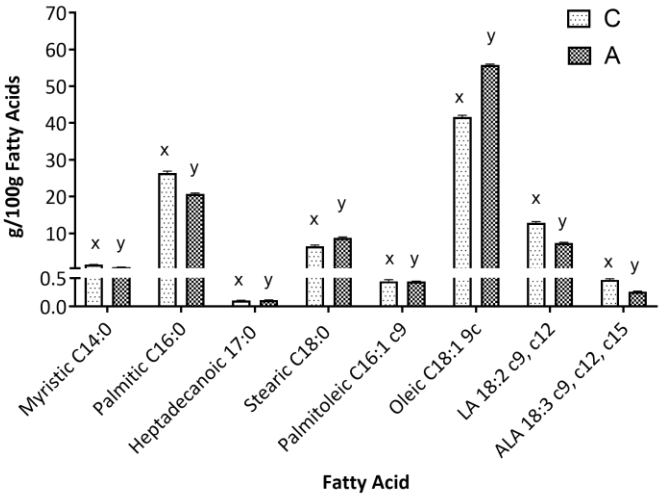
Data are shown as mean ±SEM for n observations per group showing pregnancy, lactation, and post-natal diets respectively (Chow diet groups: PCC=8 RCC=9; Atherogenic diet groups: PCA=8, RCA=11). Nonparametric data was transformed to log 10 "a" and analysed by two-way ANOVA with Bonferroni post hoc test. For each liver lipid unlike letters (x,y) are significantly different P<0.001.

4.5.5. The Impact of Maternal Consumption of P or R Diet during Pregnancy on the Fatty Acid Composition of Perirenal Adipose Tissue (%FAME)

FAME were extracted from perirenal adipose tissue and measured (Method 2.7.1., A. Lock, University of Michigan). There was no effect of maternal diet (P or R) with no TFAs being found in the adipose tissue. The pattern of FA distribution in the perirenal adipose of offspring (Figure 4.8a) is similar to that contained within the post-natal diets (Figure 4.8b). However, LA C18:2 *c*9, *c*12 that is greater in post-natal C offspring (P<0.05) compared to A offspring, whilst C16:0 Palmitic acid and Stearic Acid was predominant in the adipose of post-natal A offspring (P<0.05) compared to C offspring.

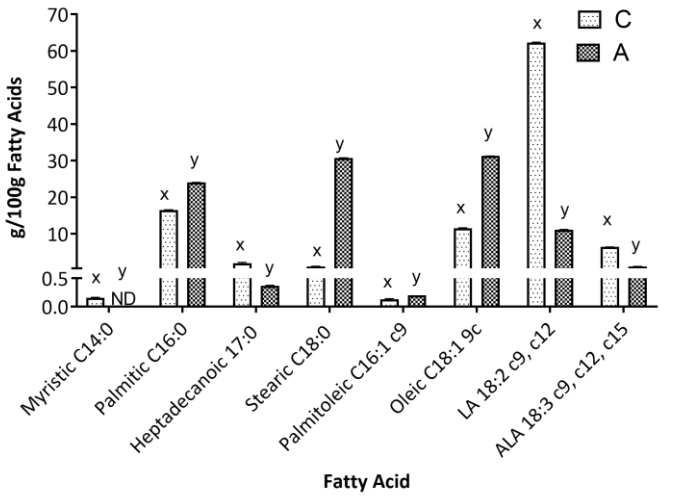
Figure 4.8 The Distribution of Fatty Acids in Post-natal Chow and Atherogenic Perirenal Adipose Tissue of Offspring (%FAME)

a) Perirenal Adipose Tissue (FAME)



<0.1% FAME Not Detected (ND). Data are shown as mean ±SEM for n observations per diet sample (C=3, A= 5). Data analysed by T-Test. For each FA paired column unlike letters (x,y) are significantly different P<0.05.

b) Post-natal Atherogenic and Chow diets (FAME)



<0.1% FAME Not Detected (ND). Data are shown as mean ±SEM for n observations diet group (C=10, A= 10). Data analysed by T-Test. For each FA paired column unlike letters (x,y) are significantly different P<0.05.

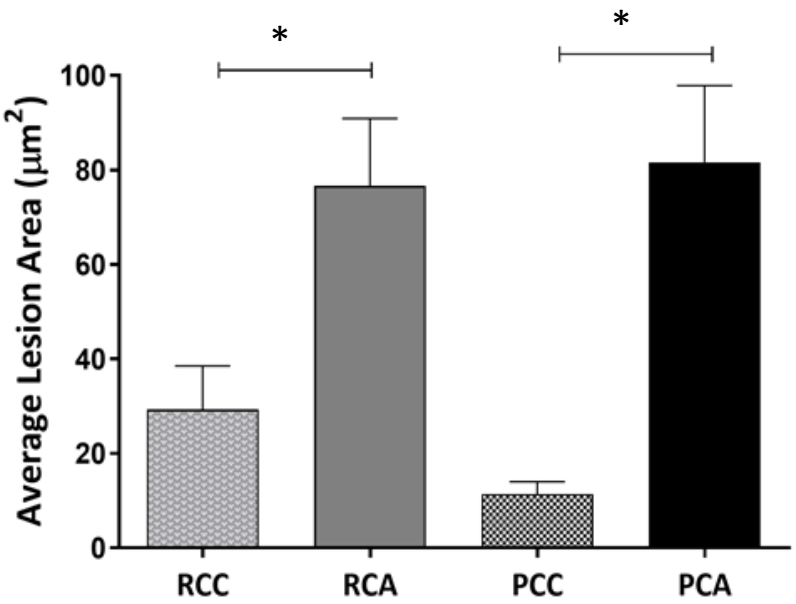
4.5.6. The Impact of Maternal Consumption of P or R Diet during Pregnancy on the Development of Atherosclerosis in Offspring

As previously described 7µm sections were captured onto slides and the lipid stained with Oil Red O (Method 3.1). These were analysed at a distance of 14µm between sections from the appearance of the aortic valves from aortic arch towards the heart, and then imaged and quantified (Method 3.2). All analyses were performed blind without knowledge of diet group. Mean atherosclerotic area (μm^2) was calculated for each section and plotted to show pattern of atherosclerosis expression (Figure 3.5). N=10 equidistant sections from each diet group from the three valves joining (Section 1) were analysed for the results.

The effect of maternal diet during pregnancy and the effect of offspring's post-natal diets on the development of atherosclerosis was statistically analysed in two different ways. Initially the data was analysed by two-way ANOVA. The mean results of the 10 sections showed there was no overall effect of maternal pregnancy diet (P=0.383) nor maternal x post-natal offspring diet interaction (P=0.759, Figure 4.9) on offspring's atherosclerosis. However, the results confirmed that there was a strong effect of post-natal atherogenic diet, with three-fold greater atherosclerosis compared to offspring consuming chow in post-natal life (P<0.01).

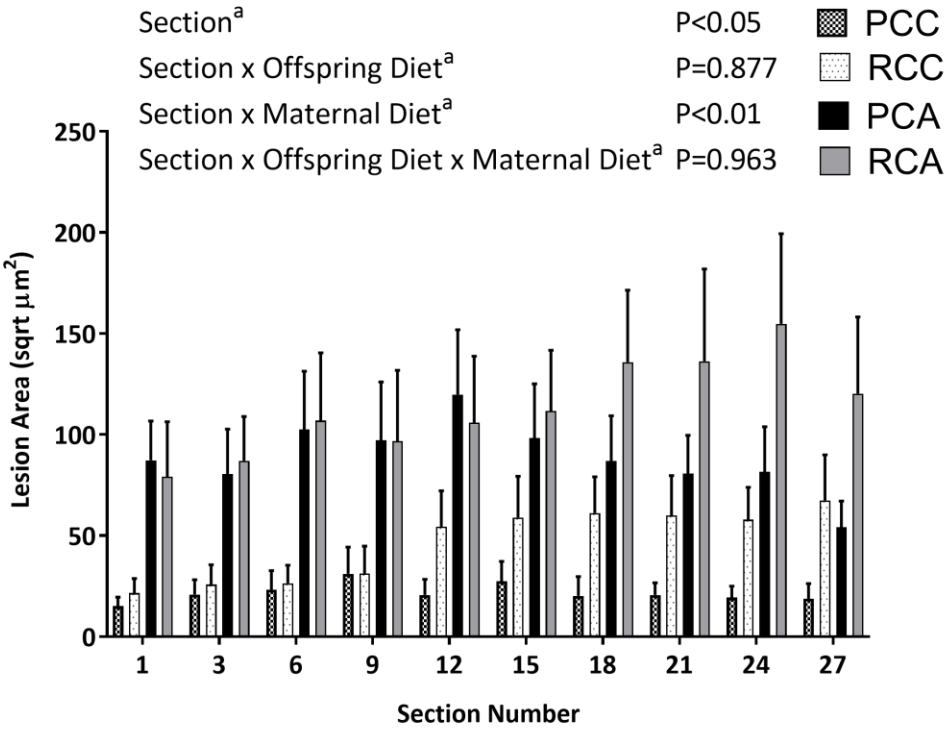
Secondly, the data was analysed by repeated measures (for section number) ANOVA blocking for animal and litter from which offspring were derived. As can be seen in Figure 4.10 there was an effect of section number (P<0.05) with atherosclerosis of R offspring increasing progressively in sections closest to the heart, and atherosclerosis of P offspring decreasing over the same area. This fluctuation in atherosclerosis across sections was reflected by the maternal diet x section interaction (P<0.01). There was no interaction of offspring diet x section number (P=0.877) indicating a consistent difference in atherosclerosis across all sections between post-natal Atherogenic and Chow diet groups.

Figure 4.9 The Impact of Maternal Consumption of P or R Diet during Pregnancy on the Mean Atherosclerosis Lesion Area in Offspring



Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation, and post-natal diets respectively (PCC=8, RCC=9; PCA=11, RCA=8). Data analysed by two-way ANOVA with Pregnancy diet and post-natal diet as factors and Bonferroni post-hoc test. Significance *P<0.01.

Figure 4.10 The Impact of Maternal Consumption of P or R Diet during Pregnancy on the Pattern of Atherosclerosis Expression in Offspring



Data are shown as mean ±SEM for n observations per diet group, showing pregnancy diet groups (P: PCC=8, PCA=11, R: RCC=9, RCA=8). Data transformed to square root "a" and analysed by repeated measures (for section number) ANOVA, maternal pregnancy diet and offspring post-natal diet were factors, with blocking for each individual animal and litter from which the animals were derived. P values for the effect of diet, section, and interaction between the two are presented. Significance P<0.05.

4.6 DISCUSSION

The hypothesis for this study was that fetal exposure to PHVO or RTFA in the maternal diet would have a different impact on maternal physiology and cause a change in the susceptibility to development of atherosclerosis in offspring due to the differing TFA isomer profiles. Study 1 found that there were differences in the pattern of atherosclerosis across the section of aorta analysed between R and P maternal diet groups. It also found that atherogenic post-natal diets increased atherosclerosis three-fold compared to chow post-natal diets. Paragraph 4.6.1 discusses Study 1 and the effects of maternal and post-natal diets on atherosclerosis expression in offspring. Thereafter, Paragraph 4.6.2 discusses the Pregnancy Study that showed that dietary P and R TFAs differentially altered maternal physiology during fetal development.

4.6.1. Study 1: The Impact of a P or R Diet during Pregnancy on the Development of Atherosclerosis in AEL Offspring

There was no effect of the maternal diet on offspring's organ weight, tissue weight, serum glucose or serum lipids. The results indicated differences in atherosclerosis offspring of P and R- fed mothers along the length of aorta studied resulting in a significant maternal diet x section interaction observed $P<0.01$. Atherosclerosis in P offspring reduced whilst atherosclerosis of R offspring increased as sections 21-27 progressed towards the heart. As outlined in Chapter 3, caution is warranted in ascribing biological significance to these results due to differential atherosclerosis expression throughout the aortic valve region. Further, robust atherosclerosis analyses utilised in this study (see Chapter 3) indicated that this was a variation in atherosclerosis expression over a short number of sections, and not a significant effect of maternal diet on offspring atherosclerosis expression.

However, there were significant effects observed of the post-natal atherogenic diet compared to post-natal chow diet on offspring's lipid metabolism and atherosclerosis.

Meta-analyses of FAs and their impact on cholesterol metabolism have shown that high SFA-cholesterol rich diets increase lipid metabolism and CHD risk in humans (Figure 1.8, Mensink et al., 2003). It is recognised that replacing SFAs and cholesterol-rich diets with MUFA/PUFA (low fat) diets, have a positive impact on cholesterol metabolism, reducing LDL and increasing HDL, thus lowering CHD risk (Clifton and Keogh, 2017, Mozaffarian et al., 2010). It has consistently been shown in several AEL atherosclerosis studies that a post-natal SFA/cholesterol-rich diet has increased atherosclerotic lesions compared to low fat chow animals (Groot et al., 1996, van Vlijmen et al., 1996, Tonge, 2011, Tarling et al., 2016) with Groot et al., (1996) reporting a 5-fold increase in atherosclerotic lesion area in 6 month old AEL mice. The results of this study were obtained in female AEL mice at 15 weeks of age, after 12 weeks on their respective atherogenic and chow post-natal diets. Considering the cholesterol-atherosclerosis theory explored in paragraph 1.4, it was found that offspring consuming the atherogenic-diet had a two-fold increase in total cholesterol compared to chow-fed offspring (Figure 4.7, $P<0.05$). This diet induced serum cholesterol metabolism influenced the progression of atherosclerosis with the results showing that the lesion area was three times greater compared to offspring consuming a chow post-natal diet (Figure 4.9 $P<0.001$). The results of this study supported the AEL studies that female AEL offspring had greater atherogenic-diet induced atherosclerotic lesions compared to chow-fed animals.

4.6.2. Pregnancy Study: The Impact of a P or R Diet during Pregnancy on Maternal and Fetal Physiology

As already discussed, TFAs in the maternal diet are able to passed to the developing offspring across the placenta (Innis, 2006). This study showed that the TFA derived from PHVO and RTFA accumulated in the adipose tissue of dams. R-fed dams accumulated a disproportionately greater amount of CLA C18:2*c*9*t*11 in their adipose tissues predominantly through the action of Stearoyl-CoA desaturase on the C18:1*t*11 in the dietary fat. It is therefore appropriate to assume that the developing fetuses were exposed to different TFA isomers *in utero* dependent on the experimental diet fed.

It has been reported that PHVO increases serum cholesterol and other risk factors for atherosclerosis, e.g. serum TAG (Mensink et al., 2003, Mozaffarian and Clarke, 2009). It has also been evidenced in human (Ramsay et al., 2002, Stewart et al., 2007) and animal studies (McCurdy et al., 2009, Oben et al., 2010) that maternal dyslipidaemia can lead to an increase in inflammatory markers such as TNFa and IL6 (Innis, 2007) and reduced vascularity function in mothers (Meyer et al., 2013, Ramsay et al., 2002, Stewart et al., 2013). Furthermore, it has been shown that maternal hypercholesterolemia can cause onset of atherosclerosis in offspring in humans (Napoli et al., 1999) and animal studies (Napoli et al., 2000, Napoli et al., 2002, Goharkhay et al., 2007). In the AEL mouse it has been shown that a maternal high SFA/cholesterol-rich diet doubled maternal serum cholesterol levels through the course of the pregnancy and caused changes in the offspring's lipid metabolism and atherosclerosis burden (Tarling et al., 2016). In this study the P-diet increased maternal serum total cholesterol concentrations by 25% ($P<0.05$) and serum TAG by 37% ($P<0.05$) compared to R-fed animals. There was no control chow group to which to compare the R-fed animals, therefore it cannot be ascertained if the R diet had a hyperlipidaemic effect. However, the serum lipid results of the P-fed animals supports the outcomes of previous studies that PHVO increased serum cholesterol and TAG. It is recognised that these factors are not only important to maternal health but can also provide a nutritional insult during fetal development that could contribute to the aetiology of diseases such as atherosclerosis.

At day 17 gestation, there were no differences observed in maternal body weight of dams consuming P or R diets. This outcome was supported by finding no difference in average daily feed intake or feed efficiency measurements. The weights of fetuses and placentas and the fetal:placental ratio were similar between the two diet groups, indicating that the placenta was matching that of fetal growth and no indication of placental hypertrophy that could contribute to impaired fetal development (Bokslag et al., 2016, Langley-Evans, 2001). However, there were significant differences found in the number of fetuses present, with those animals consuming the P diet during pregnancy having significantly less fetuses compared to R-fed dams ($P<0.01$), with the difference observed in the left uterine horn compared to right uterine horn ($P<0.05$). It was previously reported that mice have a reduction in local reproductive hormones and reduced vein

vascularity that influenced uterine transmigration of up to 40% of fetuses (Forbes and Taku, 1975). The results of this study that P-fed dams displayed dyslipidaemia that could increase inflammatory markers and reduce vascular function may be pertinent. However, other studies have reported that mice have a bias for fetus survival in the right uterine horn (Wiebold and Becker, 1987), and that the right ovary produce a greater number of ova compared to the left (Brown, 2007). Similar to the result observed in P dams in this study, Wiebold and Becker (1987) noted that the right uterine horn has twice the survival rate compared to the left uterine horn. However, as there were no differences observed in the number of fetuses or placentas in the right uterine horn between R or P-fed dams, the outcome of this result is not conclusive. To confirm these findings further experimental work would be required to compare hormone levels, inflammatory markers, vascular properties and TFA isomers present within fetal and placental tissues from both left and right uterine horns, respectively.

Although final bodyweight was similar between the two diet groups, P-fed dams had a lower number of fetuses which would point a lower final bodyweight being observed in P-fed dams. To account for this disparity, it was found that dams fed the P-diet had greater perirenal adipose depots ($P<0.05$), and heavier livers ($P<0.05$) compared to R dams. However, the adiposity in P dams was very modest (combined increased weight of both depots equals about 1.5% of total body weight) and more detailed experiments of body composition would be required to confirm the tissue differences observed in P-fed animals.

The pregnancy study above highlighted that different C18:1 *trans* isomers were present in the maternal adipose and that the P and R diets had a differential impact on maternal lipid profiles. Therefore, it was appropriate to assume that a similar maternal physiology would be present for the Study 1 dams which gave rise to the AEL offspring.

4.6.3. Study Limitations

Although the pregnancy study gave an indication of maternal physiology during pregnancy, the maternal profile for Study 1 offspring cannot be categorically ascertained. Rodents are known to adjust their food intake when fed hyperenergetic diets (Sampey et al., 2011) and as C57 mice are prone to stress-related loss of pregnancy, feed intake data during pregnancy and lactation was not collected and other macronutrient e.g. protein intake during pregnancy could not be ascertained. Additionally, the Study 1 dams nursed their offspring whilst consuming a chow lactation diet for 3 weeks prior to the weaning of pups onto their post-natal diets, and therefore no maternal tissues were harvested during their pregnancies.

It is acknowledged that AEL mice produce very little atherosclerosis on chow post-natal diets (Groot et al., 1996., Yates et al., 2009., Tonge, 2011). It has been reported in other mouse models such as the LDLr knockout mouse, that on a chow diet they require up to 12 months to develop atherosclerotic lesions (Getz and Reardon, 2012). Therefore, it can be suggested that as the AEL offspring in this study were euthanised after 12 weeks on post-natal diets, only a quarter through the reported 12 month timescale, that atherosclerosis development in the chow group could have progressed later in a time respondent manner. Finally, as there was no control group to give a base line for the impact of maternal diet, it is difficult to conclude whether exposure to TFAs *in utero* had a better or worse profile compared to offspring that had not. However, it was interesting to note that AEL offspring of both P and R mothers consuming post-natal chow or atherogenic diets were similar in thereby conferring consistency in reported outcomes.

4.7 CONCLUSION

In conclusion, the main outcomes for this study is that in the maternal C57 mouse, consumption of PHVO during pregnancy caused significant changes to maternal perirenal adipose tissue weight, liver weight, increased serum lipid metabolism and decreased fecundity compared to dams consuming RTFA. These results suggest that the maternal PHVO diet conferred a nutritional insult to the mother and an environmental stimulus for the developing fetus and has the potential to contribute to fetal programming pathways. Feeding P and R diets to mothers during pregnancy caused some variability in offspring atherosclerosis across several sections of aortic valve leading towards the heart. However, robust analyses (see Chapter 3) confirmed that neither TFA maternal diet changed overall atherosclerosis expression in offspring. Feeding an atherogenic diet in post-natal life clearly increased the extent of aortic atherosclerosis compared to chow fed offspring.

CHAPTER 5: STUDY 2 - THE IMPACT OF MATERNAL CONSUMTION OF PHVO, RTFA OR WESTERN DIET DURING PREGNANCY AND LACTATION ON THE DEVELOPMENT OF ATHEROSCLEROSIS IN THE APOE*3 LEIDEN MOUSE

5. INTRODUCTION

As discussed in the General Introduction and Chapter 4 introduction, maternal overnutrition, particularly energy dense high-fat diets, rich in SFA often result in maternal adiposity and can also programme metabolic diseases in the offspring (Flick et al., 2010, Dong et al., 2013). Risk of CVD and premature death in the offspring of obese women has been demonstrated (Reynolds et al., 2013). In animal studies, maternal obesity is associated with increased adiposity, impaired glucose homeostasis, impaired endothelial function and hypertension in the offspring (Samuelsson et al., 2008, Li et al., 2019, McCurdy et al., 2009, Sullivan et al., 2014).

The impact of SFAs and PHVO on increasing lipoprotein metabolism and CHD risk are established (Mensink et al., 2003, Mozaffarian et al., 2009) with the impact of ruminant derived TFA on serum lipoproteins being equivocal in human dietary studies (Chardigny et al., 2008, German et al., 2009, Bendsen et al., 2011). However, RTFA are purported to have potential health benefits with VA being converted to CLA C18:2*c*9*t*11 by the action of Stearoyl Co-A desaturase in tissues of animals and humans (Kuhnt et al., 2006, Turpeinen et al., 2002). It has been shown in animals that butter enriched with CLA C18:2*c*9*t*11 had beneficial effects on lipoprotein profiles (Lock et al., 2005). .

Maternal hypercholesterolemia is associated with increased susceptibility to atherosclerosis in both animal models (Napoli et al., 2002, Napoli et al., 2000) and humans (Napoli et al., 1999). In the AEL mouse feeding a diet enriched in animal fat (beef tallow) and cholesterol (similar to the diet fed in this study) raised maternal plasma cholesterol and increased the development of atherosclerosis in offspring, independently of changes in plasma cholesterol or TAG (Tarling et al., 2016).

In humans and animals, maternal intake of TFA is transferred across the placenta and into the circulation and tissues of the developing offspring. (Innis, 2007). SFA and TFAs are incorporated into maternal adipose stores during pregnancy. These fat stores are mobilised during late pregnancy during maximal fetal growth phase and early post-partum periods (Herrera and Ortega-Senovilla, 2014). Studies of human breast milk confirmed that maternal body mass is positively correlated with breast milk TFAs in a dose respondent manner and are representative of the mother's diet (Mueller et al., 2010, Innis, 2007). Studies in humans show that maternal TFAs impair EFA availability in fetal and neonate tissues causing cognitive difficulties and physiological disorders in offspring (Arbex et al., 2015), and propensity for obesity in childhood (Anderson et al., 2010). In animal studies, offspring of obese mothers that continued to suckle through early postnatal development were found to have metabolic disorders such as insulin resistance and vascular endothelial dysfunction (Ghosh et al., 2001, Koukkou et al., 1998, Fan et al., 2013). Oben et al., (2010) concluded that in C57 mice the "critical early postnatal period" lactation diet was pivotal in development of metabolic disease in offspring.

5.1 AIM AND HYPOTHESIS

In this Study we included both sources of TFA (P and R) and also included a "Western" (W) diet group, rich in beef tallow SFAs for comparison. This study aimed to consider the impact of the different maternal fat diets (P, R, W) during different developmental periods: pregnancy or pregnancy and lactation on the susceptibility of offspring to development of atherosclerosis.

Considering the different effects of P, R and SFA/Cholesterol 'Western' diets on lipoprotein metabolism and CHD risk, it was hypothesized that maternal consumption of P diets throughout pregnancy and lactation would alter maternal metabolism and cause development atherosclerosis in their offspring to a greater extent compared to Western diets consumed during the same development periods. It was further considered that maternal consumption of RTFA during the same development periods would decrease susceptibility to atherosclerosis in their offspring compared to PHVO and Western diets.

5.2 METHOD

In order to examine the study's hypothesis, chow diets were supplemented with 15% TFA, equivalent to 5% daily energy in mice. Dietary fats were similar to those initially used by Chardigny et al (2008) in the human TRANSFACT study (see Methods Chapter 2, Table 2.1). Fatty acid composition of experimental diets (% FAME) are shown in Chapter 2, Methods, Figure 2.2. See Methods paragraph 2.2.5 for full descriptive of diet groups.

Seven-week-old wild type female (n=10 per diet group) C57BL/6J mice (dams) were mated with AEL males (sires, approx eight weeks age) over a two oestrous cycle period (eight days). Diet groups were matched for variations in bodyweight. During mating 25% of the mice were fed Chow "C", with the remaining 75% mice being fed one of three experimental fat diets: P, R, W (Chapter 2 Methods, Figure 2.3 Sector 1- Pregnancy). On the eighth day the sires were removed, and dams remained on their allocated diets throughout pregnancy.

On the birth of the pups (Chapter 2, Methods, Figure 2.3, Sector 2, Lactation) Chow (C) dams remained on a chow diet (C) throughout lactation giving rise to a control Chow group for pregnancy and lactation (CC). The animals consuming fat (P, R, W) in pregnancy either remained on their allocated fat diet giving rise to three fat groups throughout lactation (PP, RR, WW) or were transferred onto a chow diet (C) which gave rise to fat during pregnancy and chow during lactation diet groups (PC, RC, WC). On weaning of pups, dams were sacrificed, and tissues harvested (Chapter 2 Methods, paragraph 2.3).

<u>Maternal Diet Groups (C57 mice)</u>

- Chow group: CC
- RTFA groups: "RR" "RC"';
- PHVO groups: "PP" "PC";
- Western groups: "WW" "WC".

At 21 days of age, pups were sexed and genotyped. Female AEL pups were weaned onto a post-natal atherogenic diet "A" (Figure 2.3 Sector 3, Post-natal). This gave rise to a Chow control group (CCA); three fat in pregnancy and chow lactation groups (PCA, RCA, WCA) and three fat in pregnancy and lactation diet groups (PPA, RRA, WWA). Offspring remained on post-natal diets for 12 weeks (84 days), after which, at the age of 15 weeks, mice were culled by Schedule 1 procedure in accordance with ASPA and tissues harvested (Chapter 2, Methods, paragraph 2.3).

<u>Offspring Diet groups (AEL Mice</u>)

- Chow: "CCA" (control);
- PHVO: "PCA" and "PPA";
- RTFA: "RCA" and RRA";
- Western: "WCA" and "WWA".

5.3 MATERNAL RESULTS –THE IMPACT OF CONSUMPTION OF PHVO, RTFA OR WESTERN DIET DURING PREGNANCY AND LACTATION ON MATERNAL BODY COMPOSITION AND TISSUES

5.3.1. The Impact of Maternal consumption of PHVO, RTFA OR Western diet on Pregnancy Weight Gain

The following results reflect data for all dam weight measurements during pregnancy. Each dietary group was matched for bodyweight at day 0 mating (g). There were no differences observed in body weight between dams fed Chow (C) throughout pregnancy compared to any of the fat diets (P, R or W) (Table 5.1).

Table 5.1 The Impact of consumption of PHVO, RTFA OR Western diet during Pregnancy on Maternal Body Weight

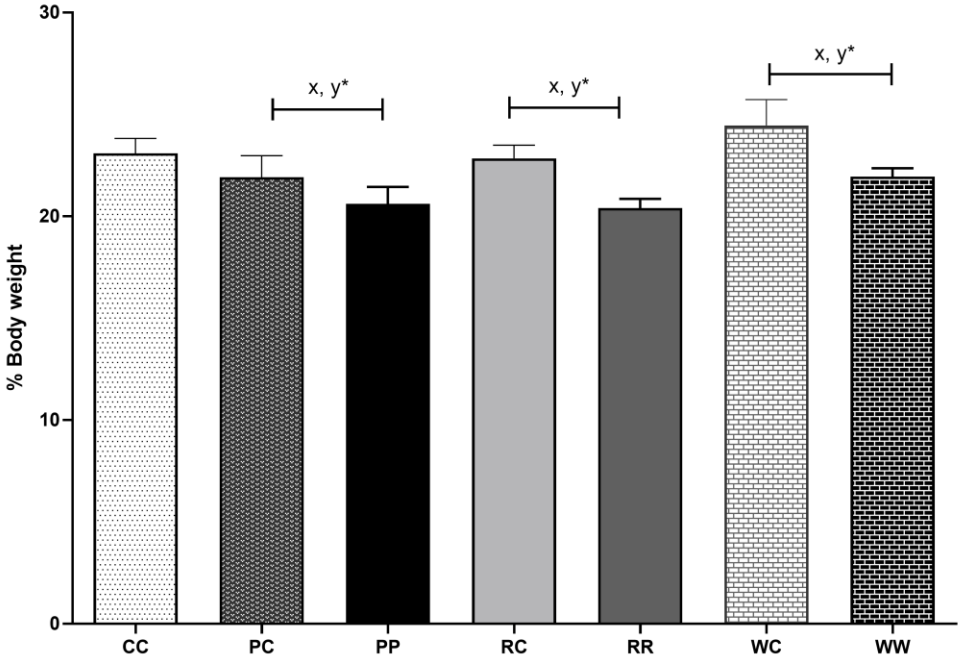
	Pregnancy Diet	Body Weight (g)	Kruskal-Wallis (*P*)
Start Body weight (g)[d]	C	16.98 ±0.43	
	P	16.00 ±0.31	0.294[d]
	R	16.03 ±0.22	
	W	16.69 ±0.39	
Day 18 Pregnancy Body weight (g)[d]	C	33.06 ±0.84	
	P	31.85 ±0.56	0.088[d]
	R	30.65 ±0.56	
	W	32.23 ±0.90	
Pregnancy weight gain (g)[d]	C	16.08 ±0.65	
	P	15.35 ±0.45	0.262[d]
	R	14.62 ±0.44	
	W	15.54 ±0.83	

Data are shown as mean ±SEM for n observations per group, showing pregnancy diet (C=13, P=18, R=20, W=18). Non-parametric data were analysed by Independent sample Kruskal-Wallis "d". Significance $P<0.05$.

5.3.2. The Impact of Maternal consumption of PHVO, RTFA OR Western diet during Lactation on Post-weaning Maternal Bodyweight

After 3 weeks suckling pups, it was found that those dams that had continued on the fat diet during lactation (PP, RR, WW) had reduced maternal body weight by 11% ($P<0.001$) independently of the nature of the test fat (Figure 5.1). Due to C57 maternal sensitivity to disruption with potential resultant pregnancy absorption, feed intake was not measured and therefore feed efficiency calculations are not presented.

Figure 5.1 The Impact of Consumption of a P, R or W Diet during Lactation on Post-Weaning Maternal Body Weight



Data are shown as mean ±SEM for n observations per group showing pregnancy and lactation diet respectively (CC=13, PC=7, PP=11, RC=6, RR=14, WC=6, WW=12). Data were analysed by two-way ANOVA and Bonferroni post-hoc tests. For each parameter bars with unlike letters (x, y) are significantly different from each other and from Chow (*). Significance $P<0.05$.

5.3.3. The Impact of Maternal consumption of P, R or W diet during Pregnancy and Lactation on Maternal Body Weight, Organs and Tissues

Liver weights were also lower in dam fed fat during pregnancy and lactation (PP, RR, WW) compared to those dams consuming fat in pregnancy alone (PC, RC, WC) independently of test fat ($P<0.001$) (Table 5.2).

There was no difference in visceral adipose tissue between any of diets consumed during pregnancy and lactation (Table 5.2).

Table 5.2 The Impact of consumption of P, R or W diet during Pregnancy and Lactation on Maternal Tissue

	Pregnancy Diet	Lactation Diet C		Lactation Diet P, R, W		TOTAL		TWO-WAY ANOVA (*P*)		
		Mean	SEM	Mean	SEM	Mean	SEM	Pregnancy	Lactation	Preg*Lact
Liver (%BW)	**C**	7.57	0.33							
	P	7.71	0.32	7.07	0.14	7.31	0.16			
	R	8.43	0.29	7.35	0.34	7.67	0.27	0.232	<0.001	0.506
	W	7.62	0.26	6.32	0.16	6.75	0.20			
	Total	7.91	0.18	6.93	0.16					
Perirenal Adipose (%BW)	**C**	0.28	0.03							
	P	0.43	0.11	0.38	0.09	0.40	0.07			
	R	0.49	0.11	0.49	0.07	0.49	0.17	0.355	0.750	0.965
	W	0.38	0.09	0.59	0.16	0.52	0.11			
	Total	0.43	0.06	0.49	0.06					
Gonadal Adipose (%BW)	**C**	1.03	0.09							
	P	1.14	0.23	1.57	0.27	1.17	0.14			
	R	1.42	0.29	1.64	0.22	1.58	0.17	0.122	0.381	0.839
	W	1.19	0.19	1.54	0.20	1.42	0.15			
	Total	1.24	0.13	1.47	0.12					

Data are shown as mean ±SEM for n observations per group showing pregnancy and lactation diet respectively (CC=13, PC=7, RC=6, WC=6, PP=11, RR=14, WW=12). Data were analysed by two-way ANOVA and Bonferroni post-hoc tests. Significance P<0.05.

[ł]Post hoc test not significant (P v W *P*=0.066/R v W *P*=0.061).

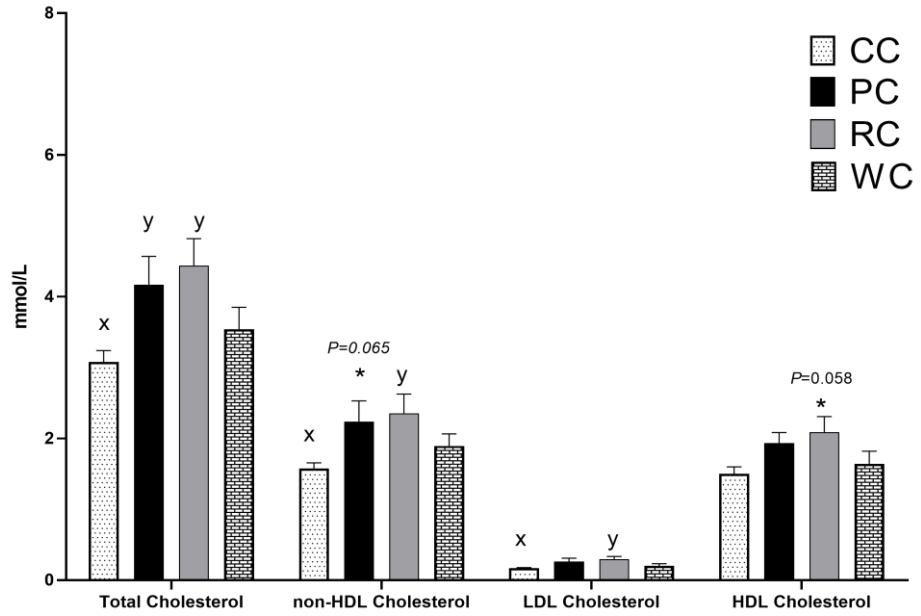
5.3.4. The Impact of consumption of P, R or W Diet during Pregnancy and Lactation on Maternal Serum Lipids and Serum Glucose

Serum Cholesterol and serum TAG measurements were performed in duplicate with a Randox Imola RX chemistry analyser via a direct clearance method (HDL, LDL) or colorimetric assay (Total Chol, TAG) (Method 2.6.3). Maternal serum total-, non-HDL-, LDL- and HDL-cholesterol were all increased in animals fed either the P or R (but not W) diets compared to those fed C during pregnancy (Figure 5.2a). Continuing the fat diet (P, R or W) during lactation further increased total-, non-HDL- and HDL-cholesterol independently of the type of test fat. Overall, the highest maternal cholesterol levels were seen in dams fed the R diet throughout pregnancy and lactation (RR), which was 88% higher than that seen in animals fed chow throughout these periods (CC) (Figure 5.2b). However, as LDL, non-HDL- and HDL-cholesterol increased proportionately, there was no difference in the ratios (Table 5.3a). There was a trend (P=0.06) for those animals consuming fat in pregnancy to have a higher LDL:HDL ratio compared to C, although this was not significantly different.

There were no differences observed between maternal pregnancy and lactation diets on serum TAG (Table 5.3a).

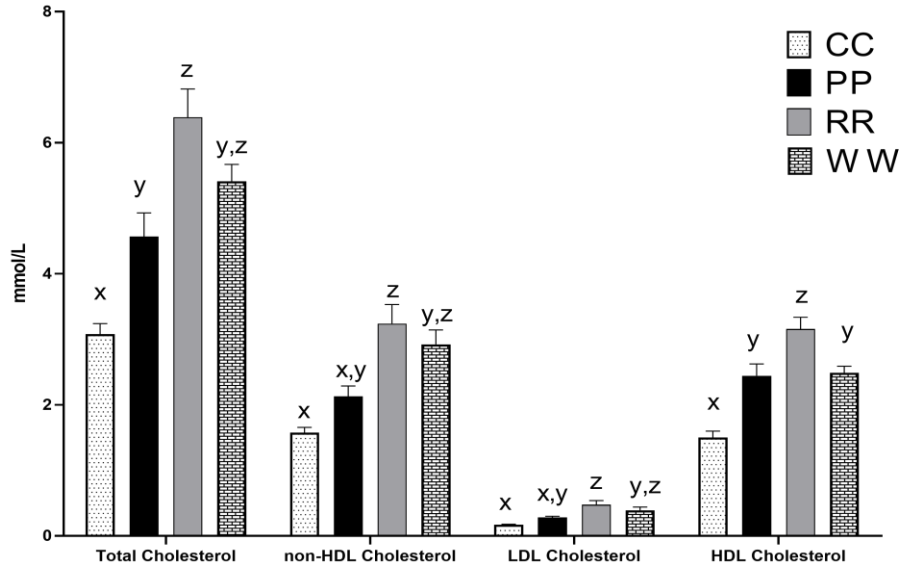
Serum glucose was measured by Colorimetric Assay using glucose oxidase reagent (Method 2.6.2). There were no differences observed between maternal pregnancy and lactation diets on serum glucose (Table 5.3b).

Figure 5.2a The Impact of a P, R or W Diet during Pregnancy on Maternal Serum Cholesterol (Post-weaning)



Data are shown as mean ±SEM for n observations per group showing pregnancy and lactation diet respectively (CC=13, PC=7, RC=6, WC=6). Data were analysed by two-way ANOVA and Bonferroni post-hoc tests. For each parameter bars with unlike letters (x, y) are significantly different from each other. Significance P<0.05. and "*"Post-hoc test not significantly different from Chow.

Figure 5.2b The Impact of a P, R or W Diet throughout Pregnancy and Lactation on Maternal Serum Cholesterol (Post-weaning)



Data are shown as mean ±SEM for n observations per group showing pregnancy and lactation diet respectively (CC=13, PP=11, RR=14,WW=12). Data were analysed by two-way ANOVA and Bonferroni post-hoc tests. For each parameter bars with unlike letters (x, y, z) are significantly different from each other. Significance P<0.05.

Table 5.3a The Impact of consumption of P, R or W Diet during Pregnancy and Lactation on Maternal Serum Lipids

		Lactation Diet								
		C		P, R, W		TOTAL		TWO-WAY ANOVA (*P*)		
	Pregnancy Diet	Mean	SEM	Mean	SEM	Mean	SEM	Pregnancy	Lactation	Preg* Lact
Total TAG (mmol/L)	C	1.01	0.10							
	P	1.45	0.21	1.12	0.12	1.24	0.11			
	R	1.44	0.20	1.37	0.18	1.39	0.13	0.220	0.392	0.572
	W	1.09	0.14	1.13	0.09	1.12	0.07			
	Total	1.33	0.11	1.22	0.08					
Non-HDL:HDL ratio	C	1.08	0.07							
	P	1.17	0.12	0.93	0.11	1.12	0.08			
	R	1.17	0.15	1.03	0.06	1.07	0.06	0.364	0.151	0.573
	W	1.19	0.12	1.19	0.09	1.19	0.07			
	Total	1.17	0.07	1.06	0.05					
LDL:HDL ratio	C	0.11	0.07							
	P	0.13	0.01	0.13	0.01	0.13	0.01			
	R	0.14	0.01	0.15	0.01	0.14	0.01	0.06	0.368	0.459
	W	0.12	0.01	0.16	0.01	0.14	0.01			
	Total	0.13	0.01	0.14	0.01					

Data are shown as mean ±SEM for n observations per group showing pregnancy and lactation diet respectively (CC=13, PC=7, RC=6, WC=6, PP=11, RR=14, WW=12). Data were analysed by two-way ANOVA with Bonferroni post-hoc tests. Significance P<0.05

Table 5.3b The Impact of consumption of a P, R or W Diet during Pregnancy and Lactation on Maternal Serum Glucose

		Lactation Diet								
		C		P, R, W		TOTAL		TWO-WAY ANOVA (*P*)		
	Pregnancy Diet	Mean	SEM	Mean	SEM	Mean	SEM	Pregnancy	Lactation	Preg* Lact
Serum Glucose (mmol/L)	C	9.86	0.93							
	P	9.55	1.41	8.59	0.49	8.94	0.59			
	R	10.19	1.39	8.14	0.50	8.75	0.57	0.673	0.252	0.304
	W	8.51	1.40	9.11	0.45	8.91	0.53			
	Total	9.42	0.78	8.59	0.28					

Data are shown as mean ±SEM for n observations per group showing pregnancy and lactation diet respectively (CC=13, PC=7, RC=6, WC=6, PP=11, RR=14, WW=12). Data were analysed by two-way ANOVA with Bonferroni post-hoc tests. Significance P<0.05

5.3.5. The Impact of consumption of P, R or W Diet during Pregnancy and Lactation on Maternal Liver Lipids

Lipids were extracted from livers (Method 2.7.4) and TAG and cholesterol assays performed (Method 2.7.5). Liver lipids are expressed as mg/total liver. There were no differences observed in liver cholesterol. Consumption of P diet during pregnancy and lactation increased liver TAG by 35% compared to P in pregnancy alone. Consuming P or R throughout pregnancy and lactation increased liver TAG by 50% compared to Chow (Table 5.4, $P<0.05$). There was no effect of W diet during pregnancy and lactation on liver TAG.

Table 5.4 The Impact of consumption of P, R or W Diet during Pregnancy and Lactation on Maternal Liver Lipids

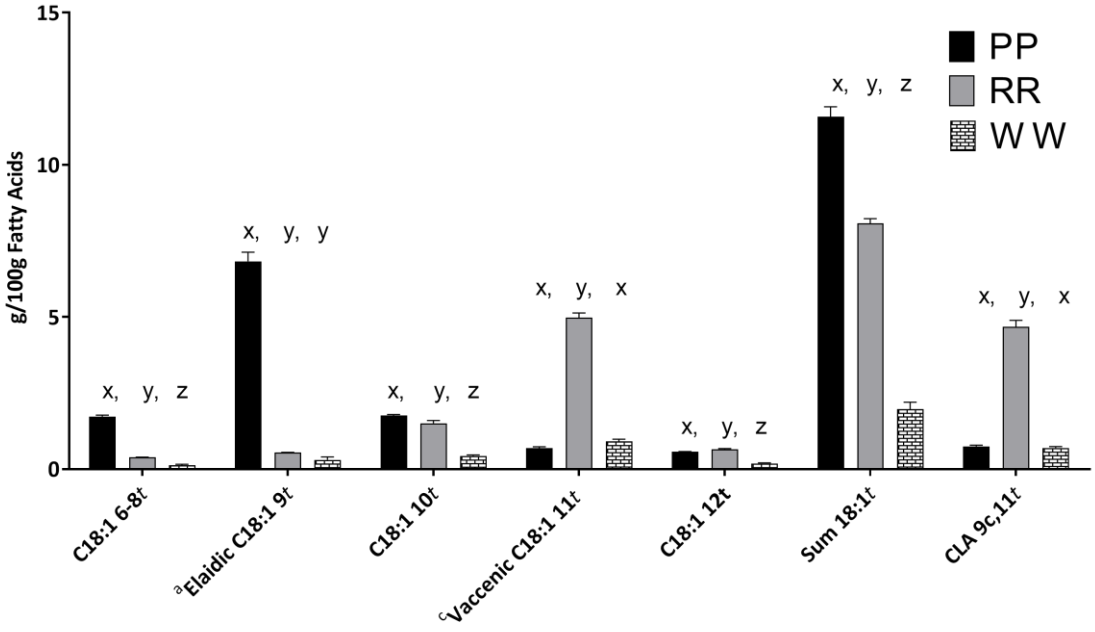
		Lactation Diet				TOTAL		TWO-WAY ANOVA (*P*)		
		C		P, R, W						
	Pregnancy Diet	Mean	SEM	Mean	SEM	Mean	SEM	Pregnancy	Lactation	Preg* Lact
Liver Cholesterol	C	6.22	1.24							
(mg/total liver)	P	4.35	0.32	5.40	0.39	5.02	0.29	0.415[d]	0.629[b]	N/A
	R	4.97	0.50	5.25	0.34	5.17	0.34			
	W	5.83	0.90	3.90	0.41	4.54	0.41			
	Total	5.01	0.36	4.87	0.25					
Liver TAG (mg/total liver)	C	[*]22.56	4.22							
	P	[X]28.73	7.08	[y*]56.74	6.10	44.66	5.38	0.696[a]	0.003[a]	0.122[a]
	R	27.91	3.32	[*]51.58	6.72	43.69	5.38			
	W	33.18	4.44	33.98	3.35	33.95	2.61			
	Total	29.87	3.02	46.62	3.47					

Data are shown as mean ±SEM for n observations per group showing pregnancy and lactation diet respectively (CC=13, PC=7, RC=6, WC=6, PP=11, RR=14, WW=12).
Non-parametric data transformed to Square root "a" or analysed by Kruskal Wallis "d" and Mann Whitney "b". Different letters (x,y) are significantly different from each other or from Chow "*" P<0.05.

5.3.6. The Impact of consumption of a P, R or W Diet during Pregnancy and Lactation of the Fatty Acid composition of Maternal Perirenal Adipose Tissue

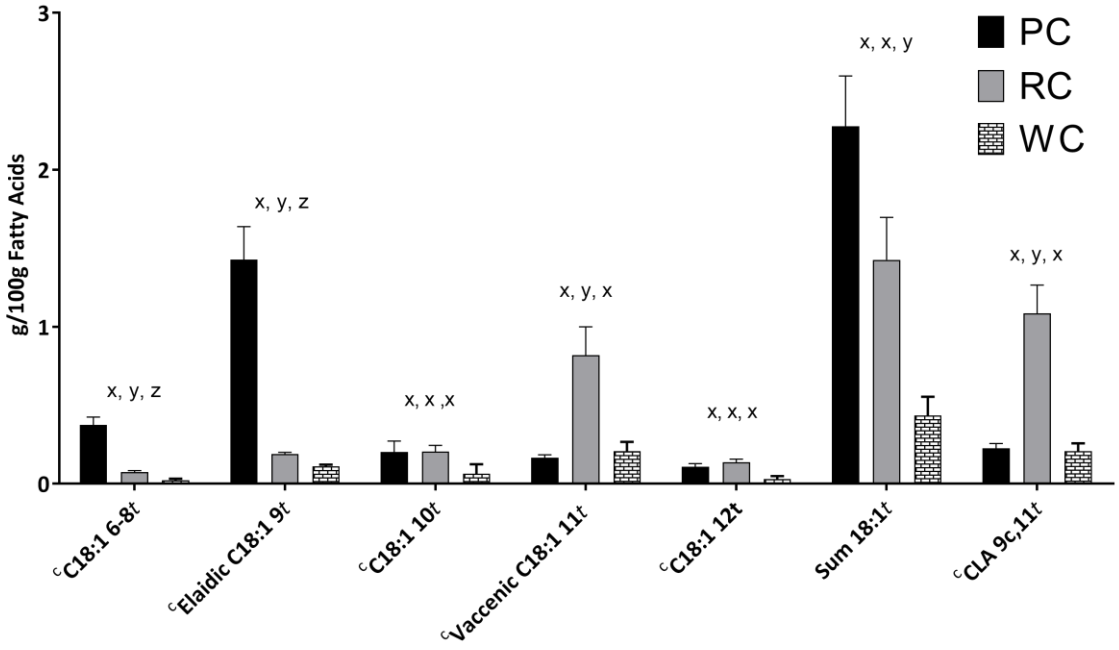
FAME were extracted from perirenal adipose tissue and measured (Method 2.7.1., A. Lock, University of Michigan). The fatty acid composition of perirenal adipose tissue in dams culled at weaning are shown in Figure 5.3a and Figure 5.3b. As expected, those mothers fed the P and R diets throughout pregnancy and lactation accumulated TFA in their adipose tissue and the *trans* isomer distribution mirrored that of the diets (Table 2.1 and Figure 2.1). As such, animals fed the P diet accumulated predominately C18:1*t*9 and those fed the R diet C18:1*t*11. However, it was notable that the R-fed animals accumulated C18:2*c*9,*t*11, in amounts that were disproportionate to the amount in the diet. As would be expected, when dams were transferred to chow during lactation, marked reductions in all TFA were observed in the adipose tissue (Figure 5.3b).

Figure 5.3a Distribution of C18:1 *Trans* FAs in Maternal Perirenal Adipose Tissue of mothers consuming P, R or W Diet during Pregnancy and Lactation (PP, RR, WW)



<0.1% FAME Not Detected (ND). Data are shown as mean ±SEM for n observations per group showing pregnancy and lactation diet respectively (CC: ND, data not shown, PP=5, RR=5, WW=5)Nonparametric data transformed to square root "a" or log10 "c" and analysed by one-way ANOVA. For each isomer bars with unlike letters (x,y,z) are significantly different P<0.05.

Figure 5.3b Distribution of C18:1 Trans FA in Maternal Perirenal Adipose Tissue of mothers P, R or W diet during Pregnancy and Chow diet in Lactation (PC, RC, WC)



<0.1% FAME Not Detected (ND). Data are shown as mean ±SEM for n observations showing pregnancy and lactation diet respectively (CC: ND, data not shown, PC=5, RC=5, WC=5,). Nonparametric data transformed to square root “a” or log10 “c” and analysed by one-way ANOVA. For each isomer bars with unlike letters (x,y,z) are significantly different P<0.05.

5.4 OFFSPRING RESULTS – THE IMPACT OF MATERNAL CONSUMPTION OF PHVO, RTFA OR WESTERN DIET DURING PREGNANCY AND LACTATION ON THE DEVELOPMENT OF ATHEROSCLEROSIS IN OFFSPRING (APOE*3 LEIDEN MOUSE)

These results assess the outcome of maternal consumption a high fat P, R, W diet throughout pregnancy and lactation (PP, RR, WW) compared to a chow lactation diet (PC, RC, WC) diet on the development of atherosclerosis in offspring. Offspring were weaned onto an atherogenic post-natal diet (PPA, RRA, WWA vs. PCA, RCA, WCA, and Control CCA).

5.4.1. The Impact of Maternal Consumption of a P, R or W Diet throughout Pregnancy and Lactation on Bodyweight of Offspring

There was no impact of maternal diet on final body weight of offspring (Table 5.5). All groups consumed an atherogenic post-natal diet and bodyweight and weight gain trajectory were similar between all diet groups.

Table 5.5 The Impact of Maternal Consumption of a P, R or W Diet throughout Pregnancy and Lactation on Bodyweight of Offspring fed an atherogenic (A) diet

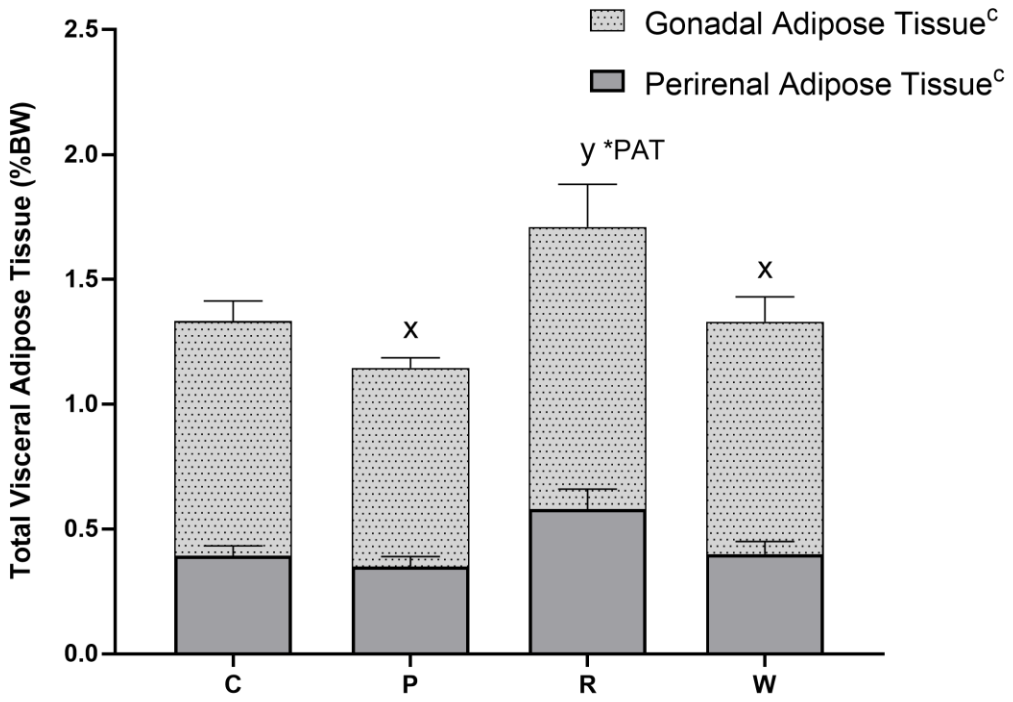
	Pregnancy Diet	Lactation Diet: C		Lactation Diet: P, R, W		TOTAL		Two Way ANOVA (*P*)		
		Mean	SEM	Mean	SEM	Mean	SEM	Pregnancy	Lactation	Preg*Lact
Day 0: weaning body weight (g)	C	7.26	0.38							
	P	7.02	0.16	5.76	0.28	6.32	0.23			
	R	6.91	0.47	6.60	0.28	6.74	0.26	0.213	0.147	0.148
	W	6.54	0.46	6.66	0.28	6.61	0.25			
	Total	6.80	0.20	6.36	0.17					
Day 84 body weight (g)	C	20.96	0.37							
	P	19.61	0.54	19.76	0.28	19.72	0.28			
	R	20.06	0.51	19.05	0.32	19.53	0.31	0.923	0.230	0.861
	W	19.83	0.35	19.29	0.46	19.54	0.30			
	Total	19.80	0.35	19.36	0.22					
Mean weight gain (g) days 0-84	C	13.70	0.37							
	P	12.59	0.56	14.04	0.25	13.93	0.33			
	R	13.15	0.58	12.46	0.32	12.79	0.33	0.185	0.915	0.761
	W	13.29	0.30	12.63	0.26	12.93	0.21			
	Total	13.04	0.02	13.00	0.20					

Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diet, respectively. (CCA=11, PCA=8, RCA =10, WCA=10, PPA=10, RRA=12, WWA=12). Data were analysed by two-way ANOVA with Bonferroni post-hoc test. Significance $P<0.05$.

5.4.2. The Impact of Maternal Consumption of P, R or W Diet throughout Pregnancy and Lactation on the Body Composition and Organ Weights of Offspring

Feeding the R diet during pregnancy increased both perirenal and gonadal tissue weights compared with both P and W diets ($P<0.05$, Figure 5.4) and increased perirenal adipose ($P<0.05$) compared to Chow diets.

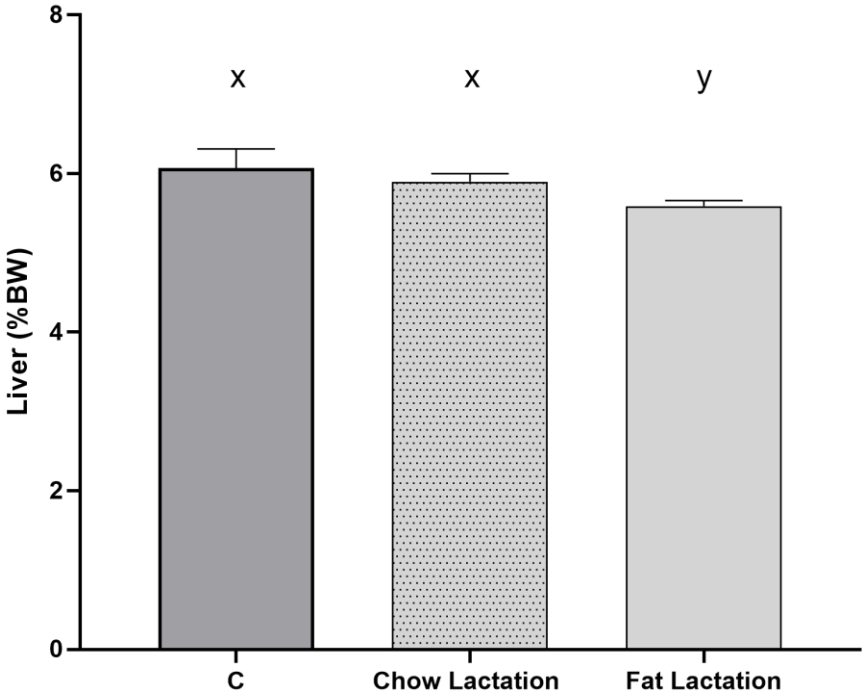
Figure 5.4 The Impact of Maternal Consumption of a RTFA Diet throughout Pregnancy and Lactation on the Adipose Tissue of Offspring fed an atherogenic (A) diet



Data are shown as mean ±SEM for n observations per group showing pregnancy, lactation and post-natal diet, respectively. (CCA=11, P:PCA=8,PPA=10, R:RCA=10, RRA=12, W:WCA=10,WWA=12) Data transformed to log10 "c" and analysed by two-way ANOVA with Bonferroni post-hoc test. Unlike letters (x,y) significantly different for both Gonadal and Perirenal Adipose Tissue. *PAT - Perirenal Adipose Tissue significantly different from Chow $P<0.05$.

Maternal consumption of fat diet during pregnancy and lactation reduced the weight of livers of offspring, independently of type of test fat compared offspring exposed to chow in lactation ($P<0.05$, Figure 5.5).

Figure 5.5 The Impact of Maternal Consumption of a P, R or W Diet throughout Pregnancy and Lactation on Liver Weight of Offspring fed an atherogenic (A) diet



Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diets, respectively. (CCA=11, Chow Lactation: PCA=8, RCA =10, WCA=10, Fat Lactation: PPA=10 RRA=12, WWA=12). Data analysed by two-way ANOVA with Bonferroni post-hoc test. Unlike letters (x,y) significantly different $P<0.05$.

5.4.3. The Impact of Maternal Consumption of a P, R or W Diet throughout Pregnancy and Lactation on Serum Lipids and Serum Glucose of Offspring

There was no impact of maternal diet on serum total cholesterol, non-HDL cholesterol or HDL cholesterol or TAG lipids (Table 5.6a) and this outcome was reflected in both the HDL/non-HDL ratio and LDL/HDL ratio (Table 5.6b).

There was no impact of maternal diet or post-natal diet on offspring serum glucose (Table 5.6c).

Table 5.6a The Impact of Maternal Consumption of a P, R or W Diet throughout Pregnancy and Lactation on Serum Lipids of Offspring fed an atherogenic (A) diet

		Lactation Diet								
		C		P, R, W		TOTAL		Two Way ANOVA (*P*)		
	Pregnancy Diet	Mean	SEM	Mean	SEM	Mean	SEM	Pregnancy	Lactation	Preg*Lact
Total TAG (mmol/L)	C	2.25	0.15							
	P	2.09	0.14	2.11	0.14	2.10	0.09	0.089	0.725	0.929
	R	2.52	0.16	2.50	0.19	2.51	0.12			
	W	2.40	0.17	2.54	0.22	2.47	0.14			
	Total	2.4	0.17	2.40	0.11					
Total Cholesterol (mmol/L)	C	9.57	0.44							
	P	[ŧ]9.04	0.58	8.68	0.32	8.84	0.30	[ŧ]0.062	0.857	0.971
	R	9.86	0.50	9.35	0.56	9.62	0.37			
	W	[ŧ]10.40	0.48	9.43	0.56	9.64	0.31			
	Total	9.60	0.30	9.15	0.25					
Non-HDL (mmol/L)	C	7.99	0.43							
	P	7.22	0.46	7.02	0.41	7.11	0.30	0.192	0.964	0.963
	R	7.75	0.44	7.91	0.95	7.84	0.53			
	W	8.34	0.68	8.41	0.68	8.38	0.47			
	Total	7.81	0.42	7.82	0.31					
Total LDL (mmol/L)	C	3.50	0.22							
	P	3.01	0.23	2.96	0.17	2.98	0.13	0.096	0.696	0.794
	R	3.19	0.23	3.02	0.18	3.11	0.15			
	W	3.47	0.20	3.33	0.16	3.40	0.13			
	Total	3.20	0.13	3.10	0.10					
Total HDL (mmol/L)	C	1.64	0.09							
	P	1.83	0.15	2.02	0.08	1.93	0.07	0.816	0.733	0.929
	R	2.10	0.11	2.00	0.11	2.05	0.08			
	W	2.07	0.10	1.87	0.10	1.96	0.07			
	Total	2.01	0.07	1.96	0.06					

Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diet, respectively. (CCA=11, PCA=8, RCA =10, WCA=10, PPA=10 RRA=12, WWA=12). Data were analysed two-way ANOVA with Bonferroni post-hoc test. Significance $P<0.05$. [ŧ]post-hoc test P vs W not significant.

Table 5.6b The Impact of Maternal Consumption of a P, R or W Diet throughout Pregnancy and Lactation on HDL-LDL and Non-HDL/HDL Ratios of Offspring fed an atherogenic (A) diet

Table 5.6 continued		Lactation Diet								
		C		P, R, W		TOTAL		Two Way ANOVA (*P*)		
	Pregnancy Diet	Mean	SEM	Mean	SEM	Mean	SEM	Pregnancy	Lactation	Preg*Lact
Non-HDL:HDL ratio	C	4.17	0.37							
	P	3.52	0.20	3.34	0.20	3.42	0.14	0.758	0.533	0.625
	R	3.74	0.23	3.02	0.22	3.40	0.18			
	W	3.45	0.16	3.40	0.28	3.43	0.16			
	Total	3.58	0.12	3.25	0.13					
LDL:HDL ratio	C	1.90	0.17							
	P	1.51	0.08	1.48	0.10	1.49	0.06	0.458	0.589	0.694
	R	1.53	0.09	1.31	0.09	1.42	0.07			
	W	1.57	0.07	1.56	0.12	1.56	0.07			
	Total	1.54	0.05	1.45	0.06					

Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diet, respectively. (CCA=11, PCA=8, RCA =10, WCA=10, PPA=10, RRA=12, WWA=12). Data were analysed two-way ANOVA with Bonferroni post-hoc test. Significance P<0.05.

Table 5.6c The Impact of Maternal Consumption of a P, R or W Diet throughout Pregnancy and Lactation on Serum Glucose of Offspring fed an atherogenic (A) diet

		Lactation Diet								
		C		P, R, W		TOTAL		Two Way ANOVA (*P*)		
	Pregnancy Diet	Mean	SEM	Mean	SEM	Mean	SEM	Pregnancy	Lactation	Preg*Lact
Serum Glucose (mmol/L)	C	8.22	0.59							
	P	7.97	0.99	9.51	0.68	8.83	0.59	0.667	0.606	0.809
	R	8.34	0.51	8.78	0.64	8.57	0.41			
	W	8.76	0.52	7.55	0.37	8.10	0.33			
	Total	8.40	0.40	8.56	0.35					

Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diet, respectively. (CCA=11, PCA=8, RCA =10, WCA=10, PPA=10, RR=12, WWA=12). Data were analysed two-way ANOVA with Bonferroni post-hoc test. Significance P<0.05.

5.4.4. The Impact of Maternal Consumption of a P, R or W Diet throughout Pregnancy and Lactation on Liver Lipids of Offspring

FAME FAs were extracted from perirenal adipose tissue and measured (Method 2.7.1., A. Lock, University of Michigan). There was no impact of maternal diet (P, R or W) throughout pregnancy and lactation on hepatic cholesterol or hepatic TAG (Table 5.7) of offspring.

Table 5.7 The Impact of Maternal Consumption of a P, R or W Diet throughout Pregnancy and Lactation on Liver Lipids of Offspring fed an atherogenic (A) diet

		Lactation Diet								
		C		P, R, W		TOTAL		Two Way ANOVA (*P*)		
	Pregnancy Diet	Mean	SEM	Mean	SEM	Mean	SEM	Pregnancy	Lactation	Preg* Lact
Liver Cholesterol	C	9.93	0.79							
(mg/total liver)	P	8.01	1.00	6.60	0.77	7.18	0.62	0.089	0.481	0.240
	R	10.88	1.16	9.02	1.12	9.91	0.81			
	W	7.32	0.79	8.74	1.17	8.13	0.75			
	Total	8.88	0.65	8.18	0.62					
Liver TAG	C	47.61	6.12							
(mg/total liver)	P	40.19	6.19	28.52	2.59	31.20	2.29	0.085	0.091	0.396
	R	51.70	6.16	35.34	4.24	43.13	4.02			
	W	38.36	4.57	34.31	3.92	36.15	2.94			
	Total	43.65	3.35	32.90	2.15					

Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diet, respectively. (CCA=11, PCA=8, RCA =10, WCA=10, PPA=10, RRA=12, WWA=12). Data was analysed by two-way ANOVA with Bonferonni post-hoc test. Significance $P<0.05$.

5.4.5. The Impact of Maternal Consumption of a P, R or W Diet throughout Pregnancy and Lactation on the Fatty Acid Composition of Perirenal Adipose Tissue of Offspring

There was no impact of maternal diet on the fatty acid composition of offspring perirenal adipose after 12 weeks on post-natal atherogenic diet, with no TFAs observed in those offspring of R and P mothers (Table 5.8).

Table 5.8 The Impact of Maternal Consumption of a P, R or W Diet throughout Pregnancy and Lactation on the Fatty Acid Composition of Perirenal Adipose Tissue of Offspring fed an atherogenic (A) diet

		Lactation Diet								
		C		P, R, W		TOTAL		Two Way ANOVA (*P*)		
	Pregnancy Diet	Mean	SEM	Mean	SEM	Mean	SEM	Pregnancy	Lactation	Pregnancy x Lactation
[1]**Σ SFA** (g/100g fat)	C	33.21	1.47							
	P	32.68	0.29	33.42	0.35	33.05	0.29	0.216	0.141	0.841
	R	32.18	0.56	32.95	0.48	32.52	0.98			
	W	33.74	0.58	34.02	0.41	33.88	0.34			
	Total	32.87	0.33	33.50	0.25					
[2]**Σ MUFA *Cis*** (g/100g fat)	C	59.44	1.44							
	P	60.01	0.29	58.61	0.26	59.31	0.30	0.078[b]	0.121[d]	N/A
	R	60.32	0.47	59.09	0.51	59.78	0.39			
	W	58.52	0.44	58.31	0.30	58.41	0.25			
	Total	59.62	0.30	58.64	0.21					
[3]**Σ PUFA *Cis*** (g/100g fat)	C	7.13	0.15							
	P	6.94	0.18	7.51	0.21	7.23	0.16	0.688	0.051	0.132
	R	7.12	0.14	7.60	0.09	7.36	0.12			
	W	7.45	0.20	7.32	0.21	7.40	0.14			
	Total	7.17	0.11	7.48	0.11					
[4]**Σ *Trans* FA**(g/100g fat)	C	ND								
	P	ND		ND	ND			N/A	N/A	N/A
	R	ND		ND	ND					
	W	ND		ND	ND					
	Total	ND		ND	ND					
[5]**Σ CLA** (g/100g fat)	C	ND								
	P	ND		ND	ND			N/A	N/A	N/A
	R	ND		ND	ND					
	W	ND		ND	ND					
	Total	ND		ND	ND					

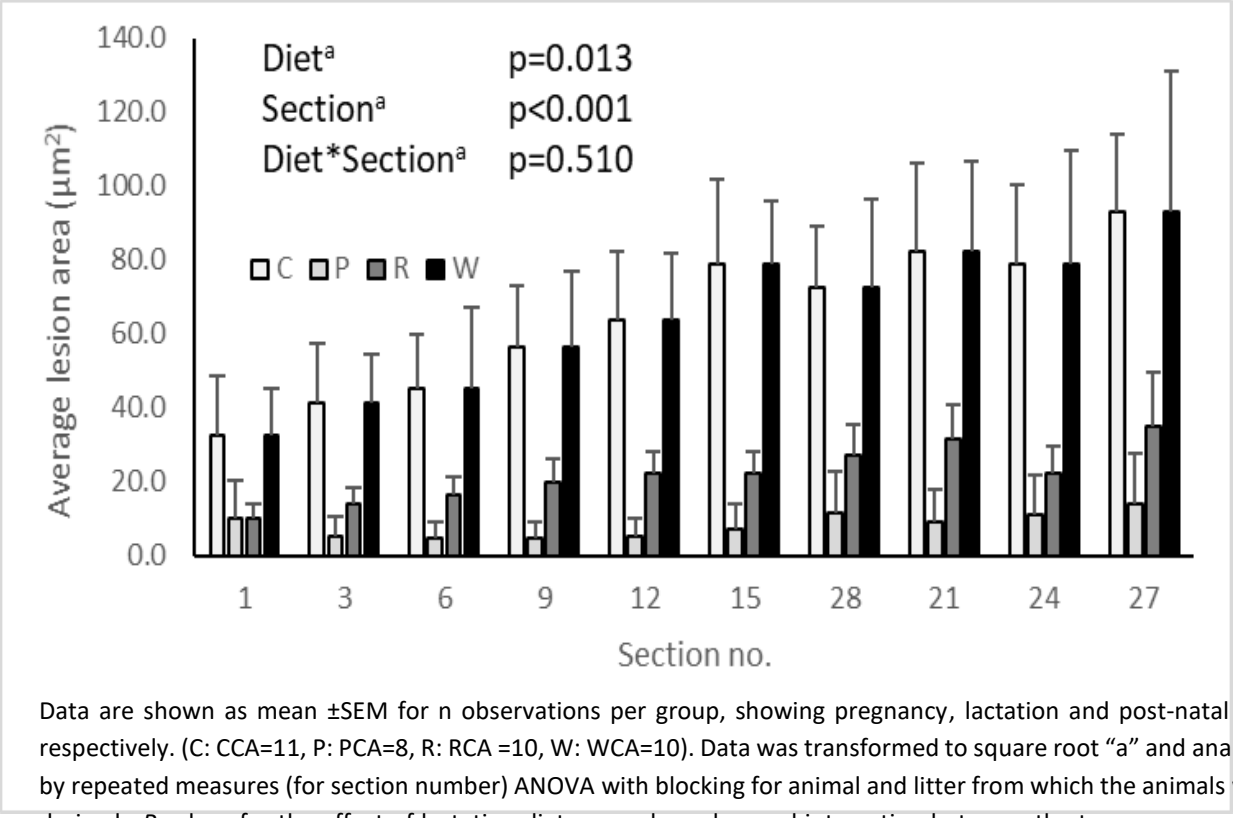
ND – Not Detected <0.1%. Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diet respectively, (CCA=5, PCA=5, RCA=5, WCA=5, PPA=5, RRA=5, WWA=5). Data analysed by two-way ANOVA with Bonferroni post-hoc test. Nonparametric data analysed by Mann Whitney "b" or Kruskal-Wallis "d". Significance P<0.05. [1]SFA sum of FAs: C10+C12:0+C14:0+C15:0+C16:0+C17:0+C18:0+C19:0+C20:0+C22:0+C23:0+C24:0 [2]MUFA *cis* sum of FAs: C14:1+C16:1,7*c*/8*c*+C16:1,9*c*+C18:1, 9*c*+C18:1,11*c*+C18:1,12*c*+C18.1,13*c*+ C18:1,14*c*/16*t*+C20:1,11*c*+C24:1,15*c*. [3]PUFA*cis* sum of FAs: C18:2 *n-6*+C18;3 *n-3*+C20:2 *n-9*+C20:3 *n-3*+C20:4 *n-6*+C22:4 *n-6*+C22:5 *n-3*+C22:6 *n-3*. [4]*Trans* sum of FAs: C18;1,6-8*t*+C18;1,9*t*+C18:1,10*t*+C18;1,11*t*+C18;1,12*t*+ [5]CLA sum of FAs: CLA 9*c*,11*t* + CLA 9*t*, 11*c*

5.4.6. The Impact of Maternal Consumption of a P, R or W Diet throughout Pregnancy and Lactation on the Development of Atherosclerosis in Offspring

As previously described 7µm sections were captured onto slides and the lipid stained with Oil Red O (Method 3.1). These were analysed at a distance of 14µm between sections from the appearance of the aortic valves from aortic arch towards the heart, and then imaged and quantified (Method 3.2). All analyses were performed blind without knowledge of diet group. Mean atherosclerotic area (μm^2) was calculated for each section and plotted to show pattern of atherosclerosis expression (data not shown). N=10 equidistant sections from each diet group from the three valves joining (Section 1) were analysed for the results.

The effect of maternal diet during pregnancy on the development of atherosclerosis was statistically analysed in 3 different ways. Initially, the impact of different pregnancy diets (C,P,R.W), followed by C diet during lactation, was analysed by repeated -measures (for section number) ANOVA, with blocking for animal and litter from which offspring were derived. As can be seen in Figure 5.6, the area of atherosclerotic lesions increased progressively in sections closest to the heart, with a strong effect of section number ($P<0.001$). There was also a significant effect of pregnancy diet ($P=0.013$), with the two TFA diets clearly resulting in lower levels of atherosclerosis compared to either the C or W diet. There was no significant interaction between pregnancy diet and section number ($P=0.510$).

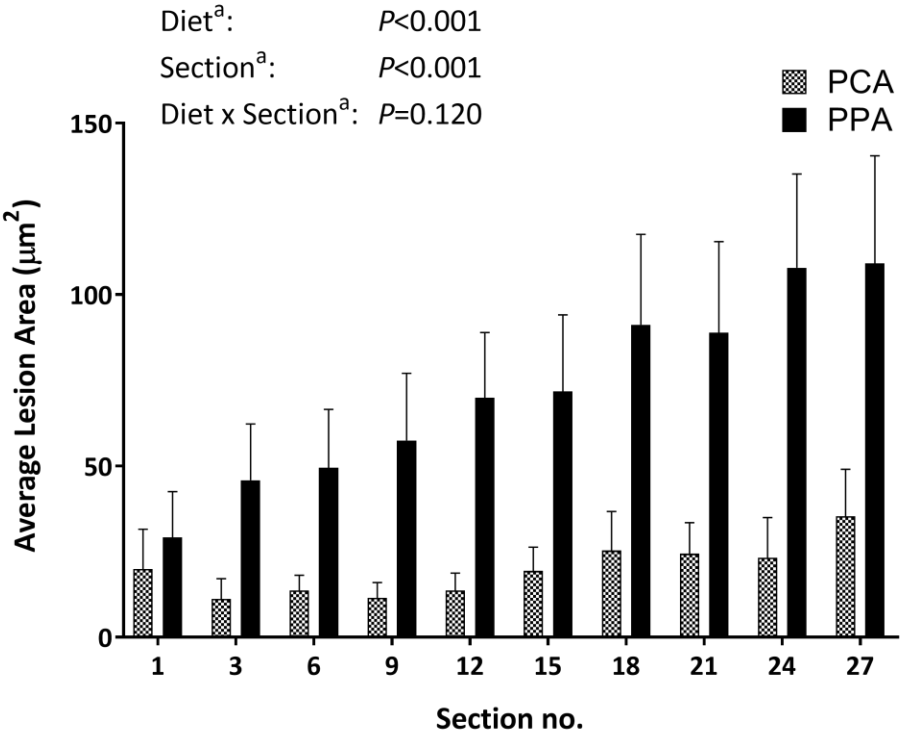
Figure 5.6 Impact of maternal pregnancy diet on development of atherosclerosis in the aorta of the offspring fed an atherogenic (A) diet



Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diet, respectively. (C: CCA=11, P: PCA=8, R: RCA =10, W: WCA=10). Data was transformed to square root "a" and analysed by repeated measures (for section number) ANOVA with blocking for animal and litter from which the animals were derived. *P* values for the effect of lactation diet, second number and interaction between the two are presented. Significance *P*<0.05.

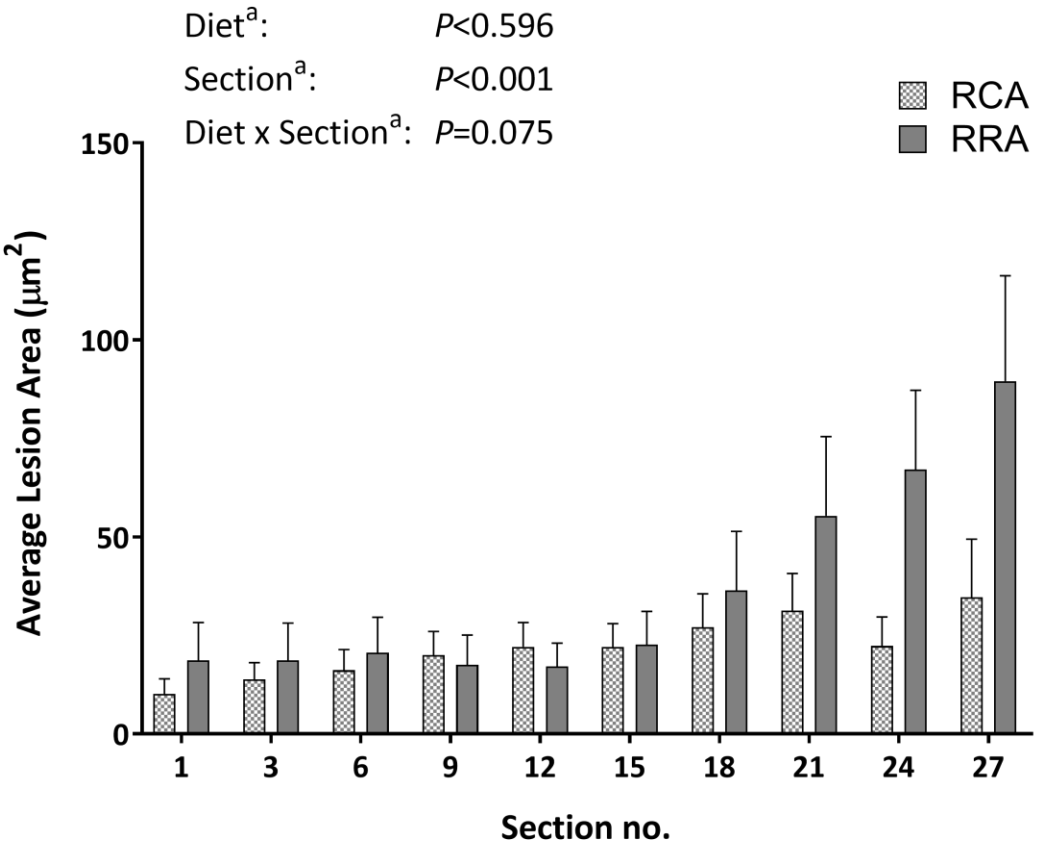
Next, the impact of each of the experimental fat diets during pregnancy and lactation was analysed separately (Figure 5.7(a-c)) by repeated measures (for section number) ANOVA. In offspring of dams fed the P-rich diets there was a strong effect of lactation diet (Figure 5.7(a), P<0.001), with those offspring from mothers fed P during lactation clearly displaying more atherosclerosis than those fed C. In offspring of mothers fed the R-diet (Figure 5.7(b), P=0.596) or W diet (Figure 5.7(c), P=0.901) there was no effect of continuing to feed the experimental fat diets during lactation.

Figure 5.7a The Impact of Maternal Consumption of P Diet throughout Pregnancy and Lactation on the Development of Atherosclerosis in Offspring fed an atherogenic (A) diet



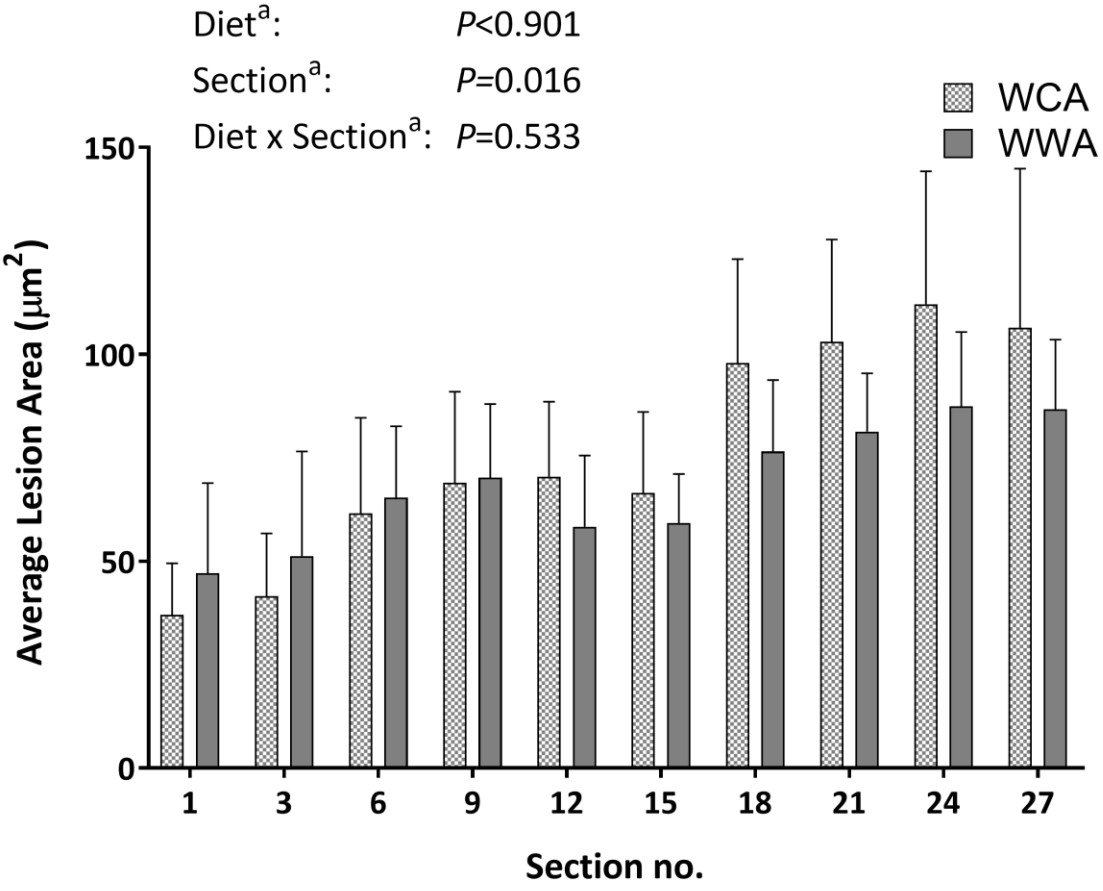
Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diet, respectively. (PCA=8, PPA=10). Data was transformed to square root "a" and analysed by repeated measures (for section number) ANOVA with blocking for animal and litter from which the animals were derived. *P* values for the effect of lactation diet, second number and interaction between the two are presented. Significance *P*<0.05.

Figure 5.7b The Impact of Maternal Consumption of R Diet throughout Pregnancy and Lactation on the Development of Atherosclerosis in Offspring fed an atherogenic (A) diet



Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diet, respectively. (RCA =10, RRA=12). Data was transformed to square root "a" and analysed by repeated measures (for section number) ANOVA with blocking for animal and litter from which the animals were derived. *P* values for the effect of lactation diet, second number and interaction between the two are presented. Significance *P*<0.05.

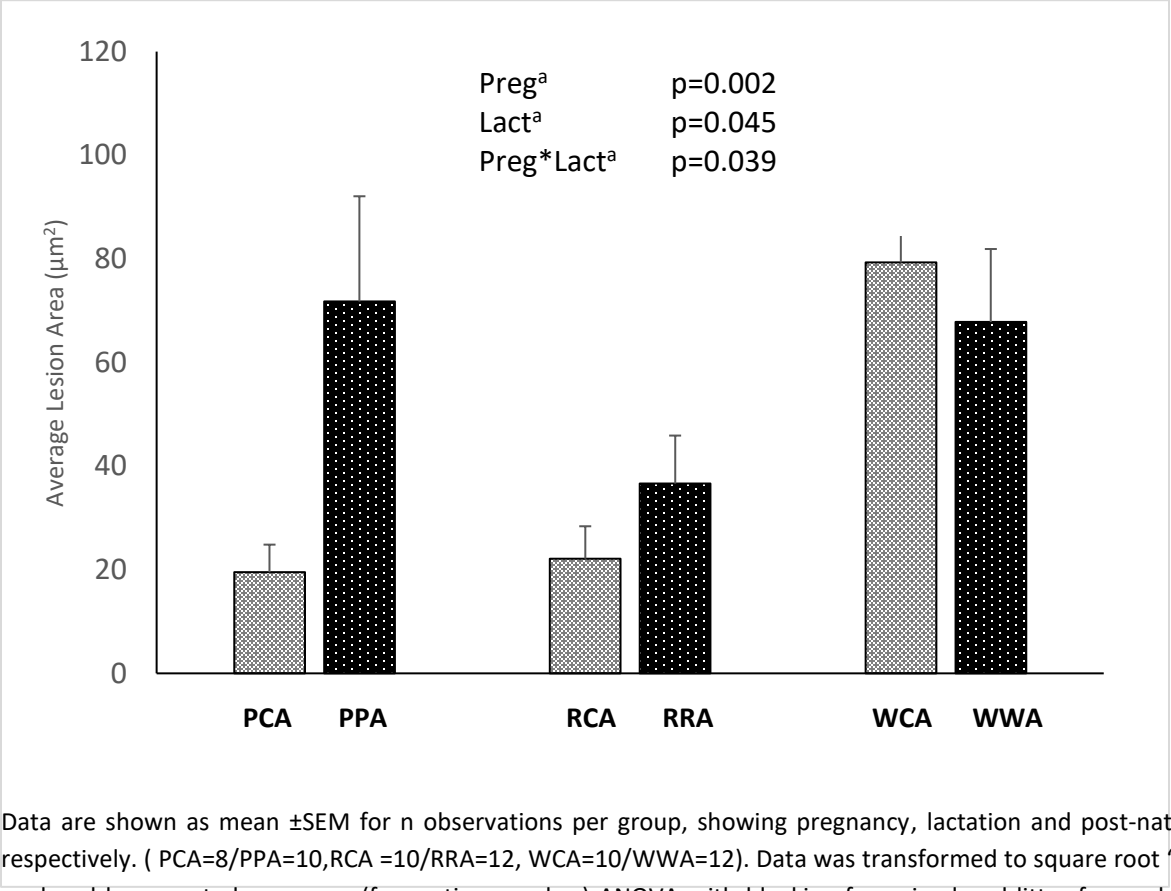
Figure 5.7c The Impact of Maternal Consumption of W Diet throughout Pregnancy and Lactation on the Development of Atherosclerosis in Offspring fed an atherogenic (A) diet



Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diet, respectively. (WCA=10, WWA=12). Data was transformed to square root "a" and analysed by repeated measures (for section number) ANOVA with blocking for animal and litter from which the animals were derived. *P* values for the effect of lactation diet, second number and interaction between the two are presented. Significance *P*<0.05.

Finally, the impact of continuing to feed the experimental fat diet during lactation, was analysed by repeated measures (for section number) ANOVA with pregnancy diet and lactation diet as factors. While section number had a clear effect ($p<0.001$) there was no interaction between section number and pregnancy ($p=0.868$), lactation diet ($p=0.371$) or both ($p=0.139$). Therefore, the data presented in Figure 5.8 represents the mean of all sections. These results showed that feeding a maternal TFA diet during pregnancy reduced postnatal atherogenic diet-induced atherosclerosis in offspring compared to offspring of mothers fed W ($P=0.002$). However, this effect was abolished when feeding of P, but not R, continued into lactation, and the nature of the lactation diet had no effect in the offspring of W-fed dams. Overall, this resulted in a significant interaction between pregnancy and lactation diets ($p=0.039$).

Figure 5.8 Impact of Maternal Pregnancy and Lactation Diet on Development of Atherosclerosis in the Aorta of Offspring fed an atherogenic (A) diet



Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diet, respectively. (PCA=8/PPA=10,RCA =10/RRA=12, WCA=10/WWA=12). Data was transformed to square root "a" and analysed by repeated measures (for section number) ANOVA with blocking for animal and litter from which the animals were derived. *P* values for the effect of lactation diet, second number and interaction between the two are presented. Significance $P<0.05$.

5.5 DISCUSSION

The original hypothesis for this study was that fetal and neonatal exposure to the two sources of TFA in the maternal diet would have differing effects on the development of atherosclerosis due to the differing TFA isomers profiles. Few studies have looked at the effects of TFA in the maternal diet on disease susceptibility in the offspring. As already discussed, such fatty acids cross the placenta and are secreted in the mother's milk proportionately to the amount in the diet (Innis, 2006). This study shows that TFA derived from PHVO and those from ruminant milk fat accumulate in the adipose tissue of dams. Those fed R showed a disproportionate accumulation of CLA C18:2*c*9*t*11, predominantly through the action of stearoyl Co-A desaturase on the 18:1*t*11 in the dietary fat. It therefore seems appropriate to assume that the developing fetuses were exposed to different TFA isomer concentrations depending upon the diet fed to the dams. As no impact of maternal diet was seen on maternal body weight, differences in susceptibility of the offspring to atherosclerosis could not be attributed to maternal adiposity.

It has been previously shown that the AEL mouse model MLP diet during pregnancy increased the susceptibility of the offspring to high dietary fat/cholesterol induced atherosclerosis (Yates et al., 2009). However, this was with increased plasma cholesterol concentrations in the offspring. In contrast, it has also been shown that a diet enriched in cholesterol and SFA (similar to the W diet used in this study but including 0.25% cholesterol) also enhanced the development of atherosclerosis but in the absence of changes in plasma cholesterol in the offspring (Tarling et al., 2016). This diet resulted in maternal plasma cholesterol concentrations more than doubling through the course of pregnancy. This supports earlier studies by Napoli et al., (1999) who showed that dramatic hypercholesterolemia induced by feeding cholesterol to pregnant ApoE knock-out mice also increased atherosclerosis in the offspring in the absence of changes to the offspring plasma cholesterol. In this study, no cholesterol was added to the maternal diet and maternal serum cholesterol was not increased in mothers fed the W diet, compared with those fed chow, and the offspring of these animals showed no increased susceptibility to

atherosclerosis. Both the P and R diets tended to modestly increase both non-HDL and HDL-cholesterol with no change in the ratio of the two.

It was noticeable that the high fat diets fed during pregnancy had an effect on food intake data for the animals (as C57 mice are prone to stress-related loss of pregnancy, this data was not collected), it is difficult to pinpoint the exact reason for this. However, given that rodents effectively defend energy intake and adjust food intake when fed hyperenergetic diets (Sampey et al., 2011), it is likely that intakes of protein and micronutrients were lower in these groups. This makes comparison of the data in this study to the other previous study of MLP in AEL mice (Yates et al., 2009) particularly pertinent. Demands for protein and micronutrients would be particularly high during lactation.

After offspring had been exposed to an atherogenic post-natal diet for 12 weeks, the impact of maternal diet on the development of atherosclerosis was assessed. The W diet showed no effect (either during pregnancy or pregnancy + lactation) compared with the chow diet (Figure 5.7c). However, both sources of TFA, when fed only during pregnancy, appeared to provide protection from postnatal atherogenic diet-induced atherosclerosis to approximately equal extents (Figures 5.6, 5.7a, 5.7b). When maternal TFA feeding was extended through lactation, much of the protective effect of P was lost and clear differences were seen in the distribution of atherosclerosis along the length of the aorta (Figure 5.7a, Figure 5.8). Unlike the results of Yates et al's (2009) study investigating MLP, these changes in susceptibility were not associated with changes in offspring plasma cholesterol. It is possible that unidentified risk factors are responsible for the observed differences and these could include the aforementioned differences in protein and micronutrient intakes that may exist between chow fed mice and mice fed diets of altered fatty acid composition. However, an alternative explanation may be differences in the development of lesions in neonatal life. This has been previously advocated to explain differences in the development of atherosclerosis in heterozygous ApoE-deficient mice born to wild type or homozygous ApoE knock-out mothers (Palinski and Napoli, 2002). While the offspring showed no difference in plasma lipids, those born of ApoE-deficient mothers exhibited a greater

susceptibility to atherosclerosis. The authors showed specific changes in gene expression in the aortas of affected animals and suggested that susceptibility to atherosclerosis may be imprinted during neonatal life. It is similarly possible that in this experiment, during fetal life alterations to the biology of the developing aorta, in response to the type of fat in the mother's diet, have resulted in the differences in susceptibility to atherosclerosis in later life. In contrast, direct exposure to P during suckling increased susceptibility to atherosclerosis. These results suggest that the impact of TFA on the early genesis of atherosclerosis are complex and that the effects *in utero* are different to the effects during suckling. The impact of TFA on atherosclerosis in adult animals are well documented. Bassett et al (2009) reported that whilst PHVO induced atherosclerosis in LDL receptor-deficient mice, ruminant TFA have an anti-atherogenic effect (Bassett et al., 2010), but this is the first report of their effects during early development. It might be speculated that the differential effects of TFA sources during pregnancy and lactation could be due to differences in the pattern of expression of pro- (and/or anti-) inflammatory factors and further work would be required to confirm this. It is of note, however, that such programming persists even when offspring have been challenged with an atherogenic diet for 12 weeks. This adds weight to the hypothesis that the TFA impact upon early lesion development during fetal and neonatal life. Further investigation will be required to examine early lesions in fetal and neonatal vessels in order to confirm this. Combining such an investigation with transcriptomic analysis of the fetal vasculature and maternal liver will contribute to a more detailed mechanistic understanding of the relationship between TFA, maternal cholesterol and early lesion development.

The only other phenotypic effect was an increase in adipose tissue weight, both perirenal and gonadal, in the offspring of mothers fed R. This study provides no obvious explanation for the 'programming' of increased adiposity associated with maternal consumption of R. It should be noted that the effect was very modest (combined increased in weight of both depots equals about 0.4% of total body weight) and that this needs to be confirmed in more detailed experiments of body composition and include measurements of food intake. Feeding high fat diets during lactation had no impact upon offspring body weight or adiposity, which is consistent

with some other, but not all, rodent studies of overfeeding in lactation (Akyol et al., 2012). However, any effect of maternal diet during suckling may have been masked by feeding of the atherogenic diet post-weaning. It has previously been shown in rats that phenotypes programmed in lactation can be modulated by post-weaning diets (Akyol et al., 2012, Gugusheff et al., 2013).

5.6 CONCLUSION

In conclusion, consumption of TFA during pregnancy appeared to protect offspring from later postnatal atherogenic diet-induced atherosclerosis, independently of the isomeric distribution of the TFA. However, if feeding is maintained during lactation, this protection may be partly lost, particularly when TFA derived from P are consumed. The mechanisms underlying these changes remain to be established, but they are not associated with changes in lipoprotein concentrations at the point when atherosclerosis was measured.

CHAPTER 6: STUDY 3 - THE IMPACT OF PHVO, RTFA OR WESTERN DIET THROUGHOUT LIFESPAN ON THE DEVELOPMENT OF ATHEROSCLEROSIS IN THE AEL MOUSE

6. INTRODUCTION

In this study we addressed the impact of exposure to the different TFA isomers (P and R) and SFAs (W) throughout the lifespan of offspring: Dams consumed the different experimental fat diets throughout pregnancy and lactation, and then offspring were weaned onto a corresponding fat diet in post-natal life. As outlined in the General Introduction, the impact of consumption of dietary SFAs and TFA on CHD Risk are established (Mensink et al., 2003, Mozaffarian and Clarke., 2009). Ruminant dairy and meat products contain *trans* VA that can be converted to CLA (C18:2*c*9*t*11) by the action of stearoyl Co-A desaturase in the tissues of animals and humans. In animals diet enriched in CLA (C18:2*c*9*t*11) have been shown to have a beneficial impact on lipoprotein metabolism (Lock et al., 2005).

Energy dense diets rich in SFA and TFA manifest in maternal obesity with dyslipidaemia and elevated inflammatory markers present (Flick et al., 2010, Dong et al., 2013). In humans, maternal obesity has recently been linked with premature death and CVD in offspring (Reynolds et al., 2013). In animal studies offspring of obese mothers that continued to suckle through early postnatal development were found to have increased adiposity and vascular endothelial dysfunction (Ghosh et al., 2001, Koukkou et al., 1998, Fan et al., 2013). Additionally, in humans (Napoli et al., 1999) and the AEL mouse it has been shown that maternal hypercholesterolemia can increase susceptibility to atherosclerosis in offspring (Tarling et al., 2016).

it is recognised that TFAs can be passed across the placenta during development and through breast milk to the neonate (Innis, 2006). In human cohorts, maternal consumption of TFAs during pregnancy have been reported to reduce EFA availability for the developing fetus and neonate, and potentially a negative impact on birth anthropometry (Hornstra et al., 2006, Innis 2007, Desci

and Boehm, 2013). Studies in children with low EFAs have been found to have visual impairments and psychological disorders (e.g. attention deficit/hyperactivity disorder) (Arbex et al., 2015). In mice, maternal consumption of TFAs during pregnancy and lactation have also been reported to retard growth of offspring (Kavanagh et al., 2010). Oben et al., (2010) observed that in obese C57 mice the "critical early postnatal period" lactation diet was pivotal in development of metabolic disease in offspring.

The results of the dams are discussed in Chapter 5. However, in summary, dams consuming the fat diets during pregnancy and lactation (PP, RR or WW) significantly increased serum total cholesterol concentration by 80% for all fat diet groups, with RR dams having 88% higher concentrations compared to C. As no cholesterol was added to the diets these results clearly indicate that the fat diets and particularly the R and P diets, increased maternal cholesterol metabolism. Another important factor was that the dams that continued to consume fat diets throughout lactation reduced their bodyweight by 11%, independently of type of test fat. It is acknowledged that mice defend energy intake when fed hyperenergetic diets (Sampey et al., 2012). However, as C57 mice are highly sensitive to stress -induced pregnancy and litter loss, maternal feed intake measurements were not obtained for these studies. It was therefore proposed that as demand for nutrients is high during lactation, protein and micronutrient intake may have been reduced in these dams.

6.1 AIM AND HYPOTHESIS

The previous two chapter were designed to specifically look at the impact of maternal consumption of trans fatty acids, during pregnancy, or pregnancy and suckling, on atherosclerotic risk in the offspring. This final study investigates the effect of exposure of the offspring throughout life (starting at conception) to different sources of trans fatty acids.

Hypothesis: It was hypothesised that exposure PHVO throughout lifespan would increase susceptibility to atherosclerosis in offspring to a greater extent compared to Western and RTFA

diets. It was further considered that the Western diet would have a greater influence on programming atherosclerosis in offspring compared RTFA diet.

6.2 METHOD

In order to examine the study's hypothesis, chow diets were supplemented with 15% TFA, equivalent to 5% daily energy in mice. Dietary fats were similar to those initially used by Chardigny et al (2008) in the human TRANSFACT study (see Methods Chapter 2, Table 2.1). Fatty acid composition of experimental diets (% FAME) are shown in Chapter 2, Methods, Figure 2.2. See Methods paragraph 2.2.6 for full descriptive of diet groups.

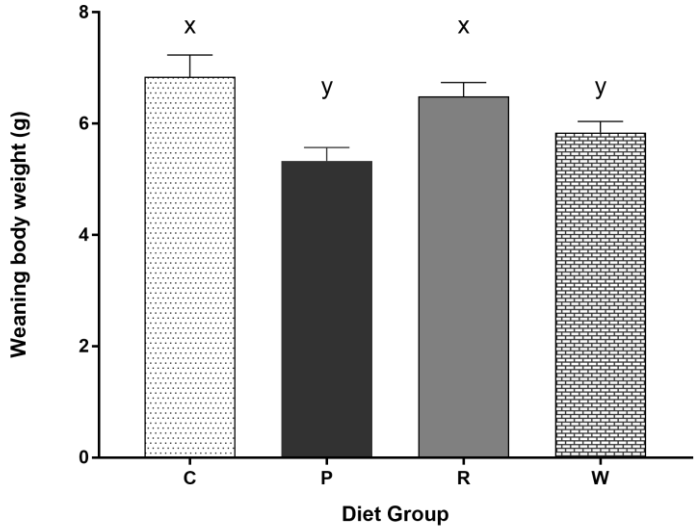
Seven-week-old wild type female (n=10 per diet group) C57BL/6J mice (dams) were mated with randomly selected AEL males (sires) (approx. eight weeks age) over a two oestrous cycle period of eight days. Groups were matched for variations in bodyweight. During mating 25% of the mice were fed Chow "C", with the remaining 75% mice being fed one of three experimental fat diets (P, R, W Figure 2.4). On the eighth day the sires were removed and dams remained on their allocated diets throughout pregnancy and lactation (see Figure 2.4 "Pregnancy" and "Lactation").

Female AEL pups were weaned onto either Chow giving rise to a control group C (CCC) or their corresponding maternal experimental fat diet giving rise to three fat diet groups P (PPP), R (RRR), and W (WWW), (Figure 2.4, Sector 3, Post-Natal). The post-natal diets (P, R, W) did not contain additional dietary cholesterol. Offspring remained on post-natal diets for 12 weeks (84 days), after which, at the age of 15 weeks, mice were culled by Schedule 1 procedure in accordance with ASPA (Home Office, 1986) and tissues harvested (Chapter 2, Methods, paragraph 2.3).

6.3 OFFSPRING RESULTS: THE IMPACT OF MATERNAL CONSUMPTION OF PHVO, RTFA OR WESTERN DIET THROUGHOUT LIFESPAN ON THE DEVELOPMENT OF ATHEROSCLEROSIS IN OFFSPRING (AEL MOUSE)

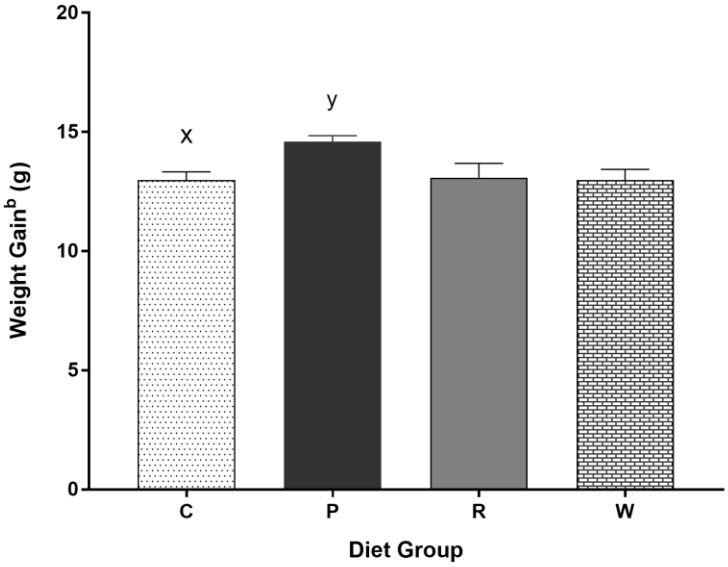
It was observed that exposure to a maternal P or W diet during pregnancy and lactation caused offspring to have significantly lower bodyweight at weaning (Day 0) compared to offspring exposed to C or R diet (Figure 6.1a $P<0.01$). However, at the end of 12 weeks on their corresponding post-natal diets, (C, P, R or W), P offspring had gained significantly more weight compared to C offspring (Figure 6.1b). However, due to the lower weaning weights of P, R and W offspring there was no difference in the final bodyweight (C: 20.6g±0.37, P: 19.9g±0.41, R: 19.7g±0.33, W: 18:2g±0.52).

Figure 6.1a The Impact of Maternal PHVO or Western Diet throughout Pregnancy and Lactation on the Weaning body weight of Offspring



Data are shown as mean ±SEM for n observations per group, showing pregnancy andlactation diets respectively: (C: CC=11, P: PP=10, R: RR=9, W: WW=10). Data analysed by ANOVA with Bonferroni post hoc test. Unlike letters (x,y) are significantly different $P<0.05$.

Figure 6.1b The Impact of a PHVO diet on Weight Gain of Offspring

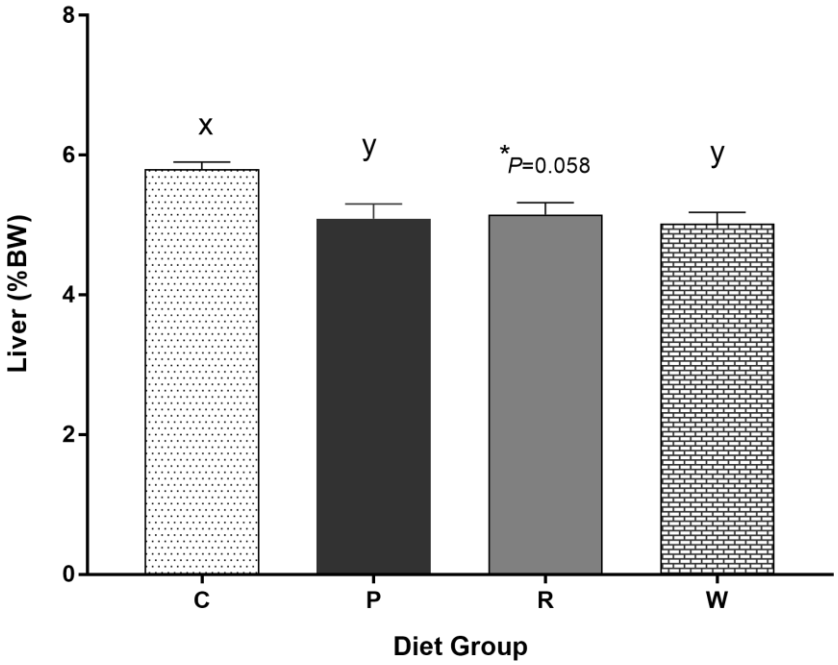


Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diets respectively: (C: CCC=11, P: PPP=10, R: RRR=9, W: WWW=10).. Nonparametric data analysed by Mann Witney (b). Unlike letters (x,y) are significantly different P<0.05. W offspring weight gain not significantly different from P: *P*=0.057.

6.3.1. The Impact of P, R or W Diet throughout Lifespan on the Body Composition and Organ Weights of Offspring at 15 weeks of age

It was found that offspring exposed to P and W fat diets, had reduced liver weight compared to C offspring (Figure 6.2). R offspring liver weight was lower *P*=0.058 compared to C-offspring.

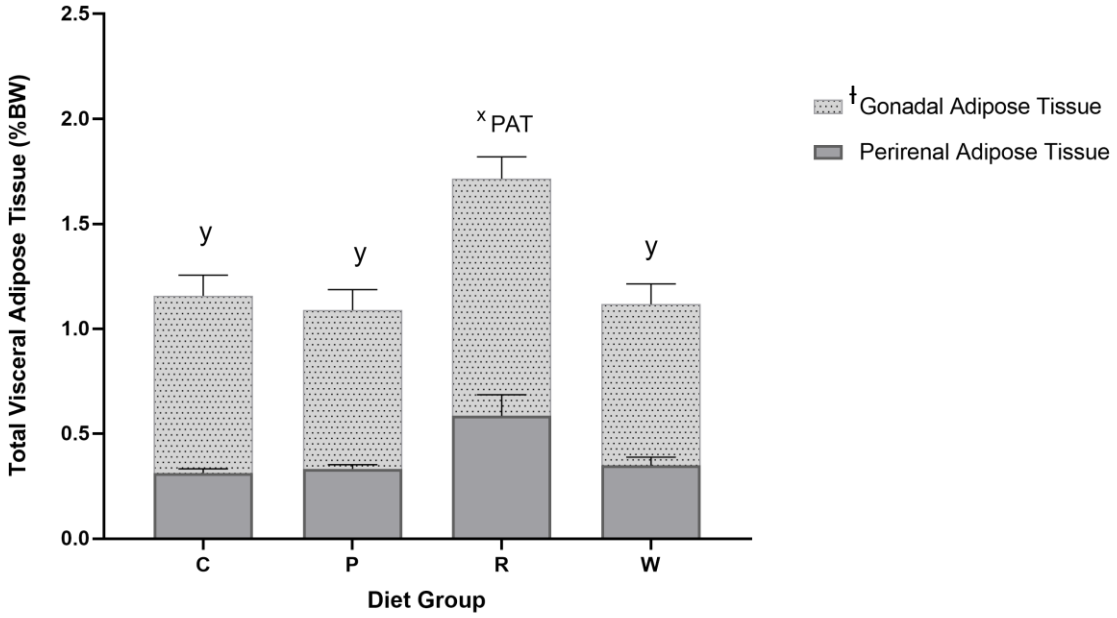
Figure 6.2 The Impact of a P, R or W diet throughout Lifespan on Liver weight of Offspring



Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diets respectively: (C: CCC=11, P: PPP=10, R: RRR=9, W: WWW=10). Data analysed by ANOVA with Bonferroni post hoc test. Unlike letters (x,y) are significantly different P<0.05. *R vs. C *P*=0.058.

Overall, it was found R offspring had significantly more total visceral adipose tissue compared to offspring C, P, and W diet groups (P<0.05). Analysis of the adipose tissue showed that these differences were predominantly found in the amount of perirenal adipose tissue (P<0.01, Figure 6.3).

Figure 6.3 The Impact of an RTFA Diet throughout Lifespan on Visceral Adipose Tissue of Offspring



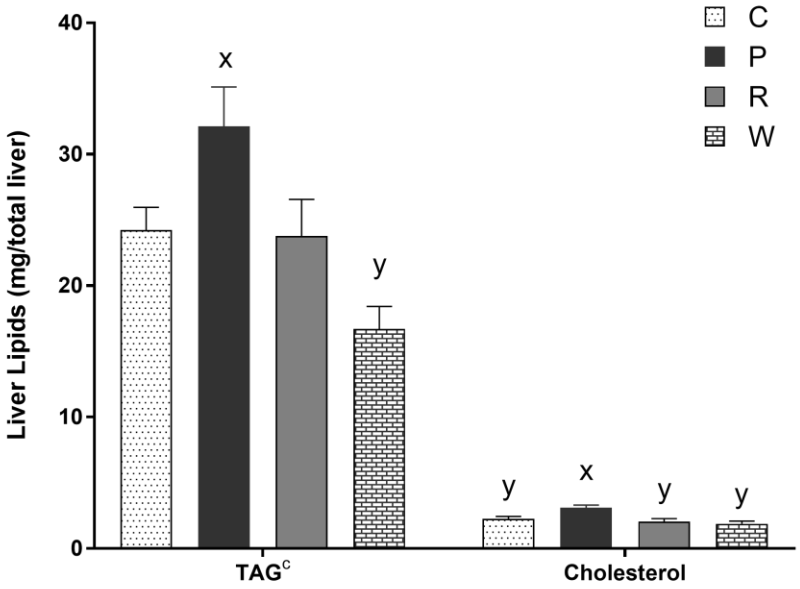
Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diets respectively: (C: CCC=11, P: PPP=10, R: RRR=9, W: WWW=10). Data analysed by ANOVA with Bonferroni post hoc test. PAT: Perirenal Adipose Tissue unlike letters (x,y) are significantly different P<0.05.

†Gonadal Adipose Tissue P=0.047. Bonferroni post hoc test not significant: R vs C Not significant, R vs P *P*=0.078, R vs W *P*=0.096

6.3.2. The Impact of a P, R or W diet throughout Lifespan on Liver Lipids of Offspring at 15 weeks of age

Offspring consuming the P post-natal diet had increased liver TAG compared to offspring consuming the W diet ($P<0.05$), but not C or R offspring (Figure 6.4, TAG). Offspring consuming the P diet also showed higher levels of liver cholesterol compared to offspring consuming the R, W or C diet ($P<0.01$, Figure 6.4 Cholesterol).

Figure 6.4 The Impact of P Diet on Liver Lipids of Offspring



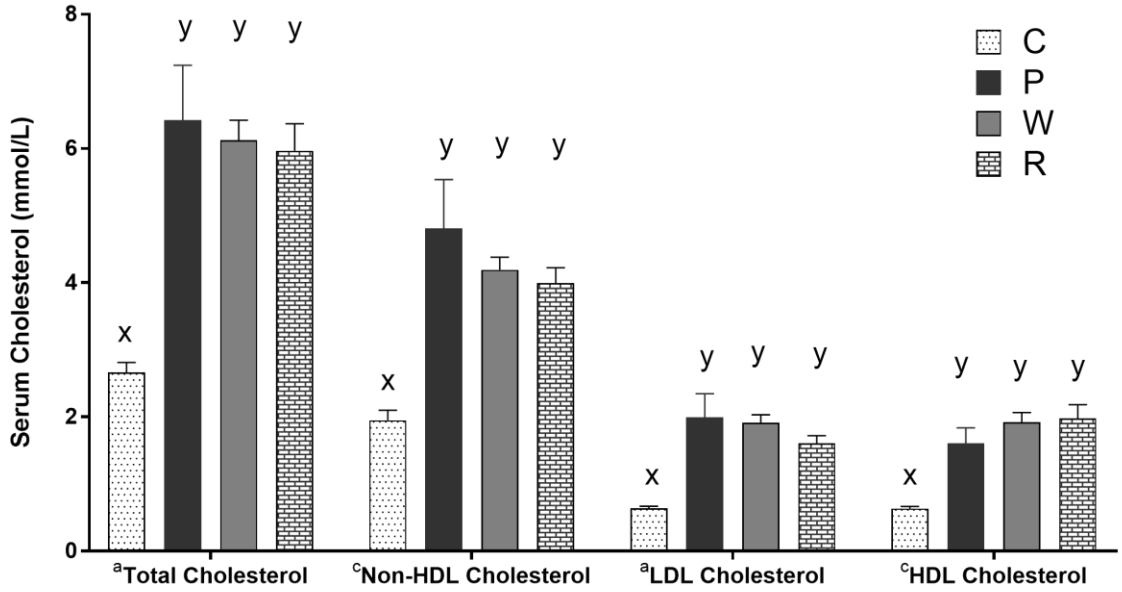
Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diets respectively: (C: CCC=11, P: PPP=10, R: RRR=9, W: WWW=10). Data transformed square root "c" and analysed by ANOVA and Bonferonni post hoc tests. For each liver lipid, unlike letters (x,y) are significantly different $P<0.05$.

6.3.3. The Impact of Different Dietary Fats throughout Lifespan on Serum Glucose and Serum Lipids of Offspring at 15 weeks of age

There were no differences observed between groups for serum glucose or serum TAG (Table 6.1).

Offspring consuming experimental fat diets had almost a two-fold increase in serum cholesterol compared to C offspring, independently of type of test fat (P<0.01, Figure 6.5). There were no differences observed in the LDL:HDL ratio (Table 6.1). However, R offspring had the highest HDL-cholesterol concentrations of all the diet groups and lowest non-HDL cholesterol of the three fat groups (see Figure 6.5). This resulted in a lower Non-HDL:HDL ratio (P=0.058, Table 6.1) compared to chow fed offspring.

Figure 6.5 The Impact of P, R or W Diet on Serum Cholesterol of Offspring



Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diets respectively: (C: CCC=11, P: PPP=10, R: RRR=9, W: WWW=10). Data transformed to log 10 "a" or Square root "c" and analysed by ANOVA with Bonferonni post-hoc test. Unlike letters (x,y) are significantly different P<0.05.

Table 6.1 The Impact of P, R or W Diet on Serum Glucose, TAG and Cholesterol ratios of Offspring

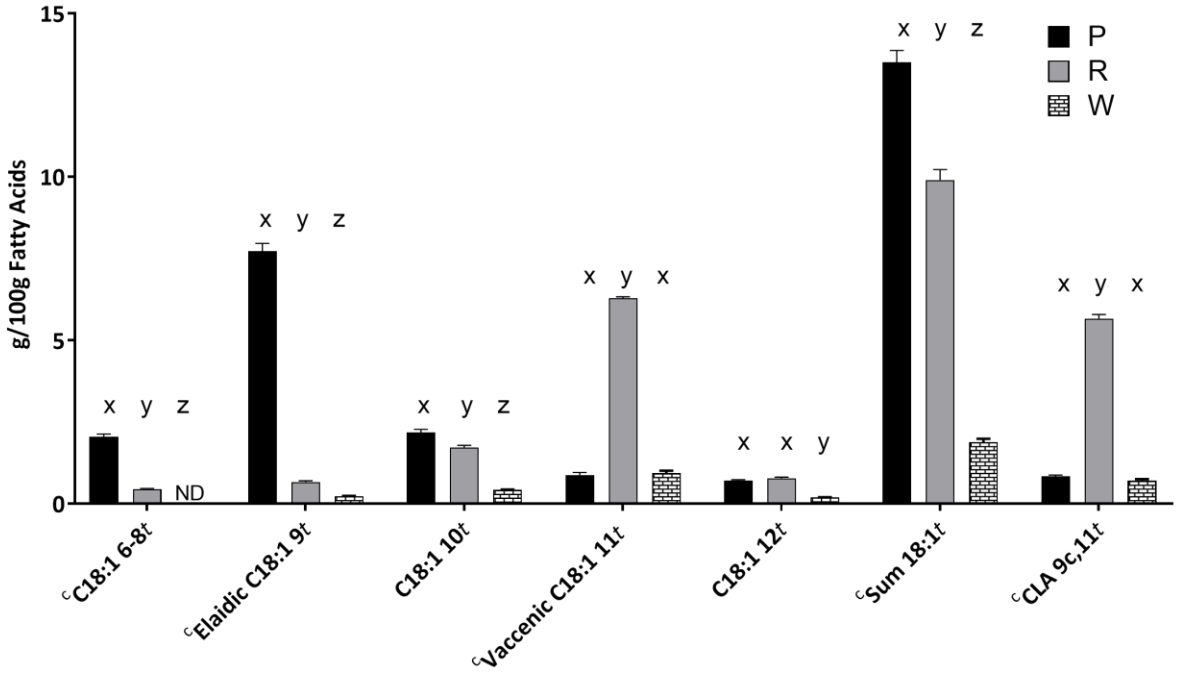
	Diet	Mean	SEM	ANOVA (*P)*
Serum Glucose (mmol/L)	C	8.69	0.34	0.508
	P	8.43	0.79	
	R	9.75	0.59	
	W	9.44	0.93	
	Total	9.19	0.45	
Total TAG (mmol/L)[a]	C	2.64	0.14	0.871[a]
	P	2.65	0.31	
	R	2.43	0.16	
	W	2.53	0.13	
	Total	2.53	0.12	
Non-HDL:HDL ratio[b,d]	C	2.81	0.26	0.058[d]
	P	2.26	0.26	
	R	1.89	0.06	
	W	2.10	0.09	
	Total	2.08	0.09	
LDL:HDL ratio	C	0.91	0.07	0.182
	P	1.05	0.14	
	R	0.78	0.03	
	W	0.96	0.06	
	Total	0.93	0.05	

Data are shown as mean ±SEM for n observations per group showing pregnancy, lactation and post-natal diets respectively: (C: CCC=11, P: PPP=10, R: RRR=9, W: WWW=10). Data transformed to log 10 “a” and analysed by ANOVA with Bonferonni post-hoc test. Nonparametric data were analysed by Independent sample Mann Whitney “b” or Kruskal-Wallis “d”.

6.3.4. The Impact of P, R and W diets on Perirenal Adipose Tissue fatty acid profile in Offspring at 15 weeks of age

FAME FAs were extracted from perirenal adipose tissue and measured (Method 2.7.1., A. Lock, University of Michigan). The distribution of *trans* fatty acids in the perirenal adipose tissue are shown in Figure 6.6. As expected, the TFA isomers in the offspring perirenal adipose tissue reflected that of the P and R diets consumed (for diets see Table 2.1 and Figure 2.1) with P offspring having greater amounts of C18:1*t*9, and R predominantly C18:*t11* and CLA ($P<0.01$, Figure 6.6). P Offspring had significantly greater total C18:1 *trans* compared to R and W offspring ($P<0.01$).

Figure 6.6 C18:1 *trans* Fatty Acid Composition of Perirenal Adipose Tissue of Offspring



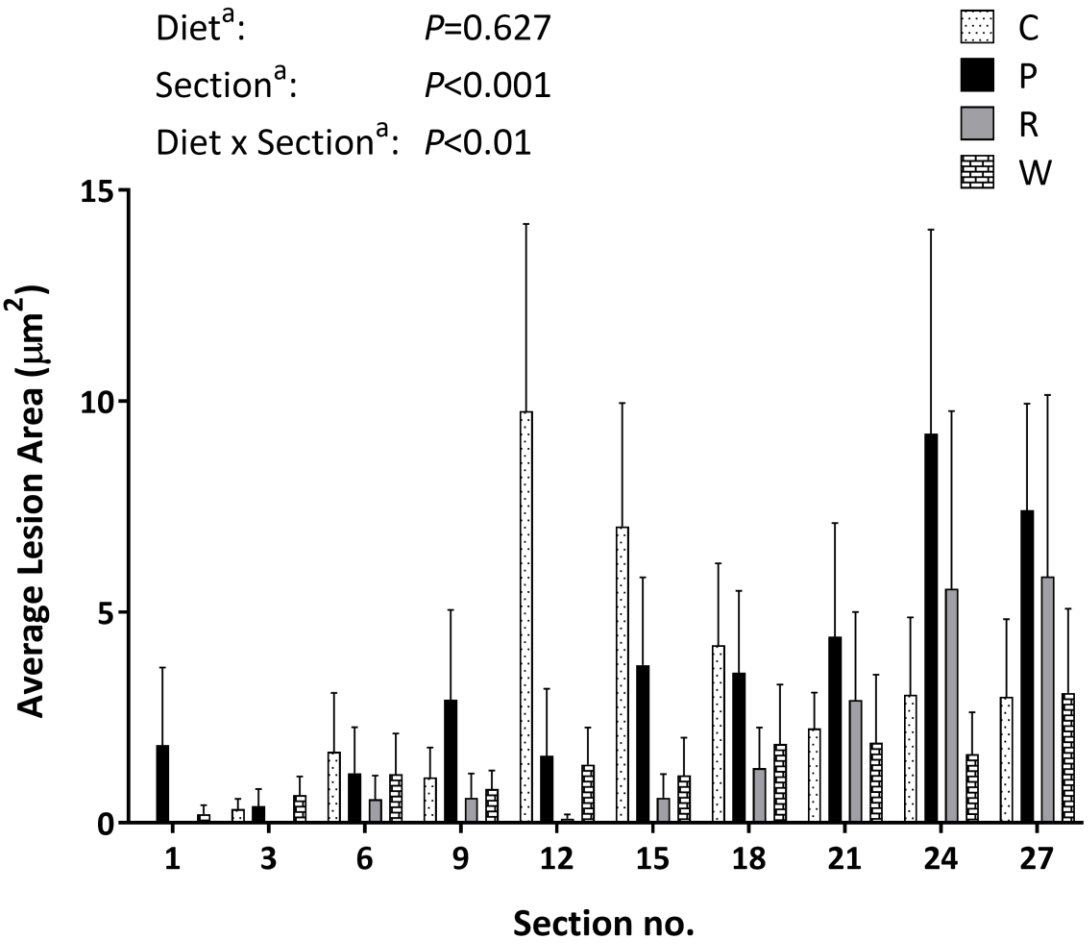
<0.1% FAME Not Detected (ND). Data are shown as mean ±SEM for n observations per group showing pregnancy, lactation and post-natal diets respectively: (C: CCC=11, ND data not shown, P: PPP=10, R: RRR=9, W: WWW=10).. Nonparametric data transformed to Log10 "c" and analysed by ANOVA with Bonferroni post-hoc test. For each isomer bars with unlike letters (x, y, z) are significantly different P<0.05.

6.3.5. The Impact of Different Dietary Fats throughout Lifespan on the Fetal Programming of Atherosclerosis in Offspring

As previously described 7µm sections were captured onto slides and the lipid stained with Oil Red O (Method 3.1). These were analysed at a distance of 14µm between sections from the appearance of the aortic valves from aortic arch towards the heart, and then imaged and quantified (Method 3.2). All analyses were performed blind without knowledge of diet group. Mean atherosclerotic area (μm^2) was calculated for each section and plotted to show pattern of atherosclerosis expression (data not shown). N=10 equidistant sections from each diet group from the three valves joining (Section 1) were analysed for the results.

The effect of exposure to a high fat diet (P, R ,W) throughout the lifespan of offspring (pregnancy, lactation and 12 weeks on post-natal diets), and consequent susceptibility to atherosclerosis was analysed by repeated measures (for section number) ANOVA with blocking for animal and litter from which offspring were derived. There was a significant interaction between section number and diet ($P<0.01$, Figure 6.7). This was primarily due to a relatively high level of atherosclerosis in earlier sections (12 and 15) of aortas from control-fed animals compared to those on high fat diets. This was confirmed when statistical analysis of high fat diets alone showed no such differences ($P=0.577$) nor diet x section interaction ($P=0.512$).

Figure 6.7 The Impact of P, R or W Diet throughout Lifespan on the Development of Atherosclerosis in Offspring



Data are shown as mean ±SEM for n observations per group, showing pregnancy, lactation and post-natal diets respectively: (C: CCC=11, P: PPP=10, R: RRR=9, W: WWW=10). Data was transformed to square root "a" and analysed by repeated measures (for section number) ANOVA with blocking for animal and litter from which the animals were derived. *P* values for the effect of lactation diet, second number and interaction between the two are presented. Significance $P<0.05$.

6.4 DISCUSSION

This study aimed to ascertain if dietary PHVO and ruminant-derived *trans* fatty acids would have different effects on maternal lipoprotein metabolism during fetal and neonate development that would cause changes in the offspring's physiology and lipid metabolism, increasing their risk to atherosclerosis development in later life. PHVO contain a wide distribution of *trans* isomers, the composition of which is dependent on the parent oil from which they are derived. PHVO adversely affect lipoprotein concentrations (Mensink et al., 2003) and increase CVD risk in humans (Mozaffarian et al., 2009). In contrast, ruminant derived TFA (RTFA) present in dairy and meat products are thought to improve lipoprotein metabolism (Mensink et al., 2003). Ruminant-derived products contain predominantly VA (C18:1 *t11*). This is particularly significant as a proportion of dietary VA is converted to CLA isomer C18:2*c*9*t*11, through the action of stearoyl CoA desaturase in the tissues of animals, including humans (Turpeinen et al., 2002, Kuhnt et al., 2006). It has also been demonstrated that there are potential beneficial effects of butter enriched in VA and CLA C18:2*c*9*t*11 on lipoprotein profiles in animals (Lock et al., 2005). In humans and animals, maternal dietary PHVO TFAs are transferred across the placenta into the circulation and tissues of developing offspring. Additionally they can be passed to the neonate in the mother's milk.

The hypothesis for this study was that fetal, neonate and post-natal exposure to two sources of TFA (P, R), and SFAs in the Western (W) diet, would have differing effects on the development of atherosclerosis. It is acknowledged that dietary TFAs can cross the placenta and are present in the mother's milk, proportional to maternal dietary intake (Innis, 2006). Both P and R-specific TFA isomers were found to be present in the maternal adipose tissue, with R dams having disproportionate amounts of CLA C18:2*c*9*t*11 through the action of stearoyl Co-A desaturase on dietary VA. In this study offspring were weaned onto their maternal corresponding fat diet (P, R W). Both P and R-specific TFA isomers were found in offspring's adipose tissue. As for the maternal analysis above, offspring consuming R showed a disproportionate accumulation of CLA.

It was therefore appropriate to conclude that offspring were exposed to these different FA isomers during fetal and neonate development and post-natal life.

In the mouse it has been shown that maternal nutrient availability during pregnancy and lactation can influence atherosclerosis development in their offspring. Yates (2009) showed that a maternal protein-deficient diet increased susceptibility to postnatal atherogenic diet-induced atherosclerosis in AEL offspring, with an associated diet-induced increase in offspring's lipoprotein metabolism. In comparison, Tarling et al., (2016) reported that a maternal SFA-cholesterol rich diet (similar to the Western diet but cholesterol was not added in our study) doubled maternal serum cholesterol concentrations during pregnancy and increased susceptibility to atherosclerosis in AEL offspring, but with no changes to offspring lipoprotein metabolism. The impact of the experimental fat diets on maternal physiology are discussed fully in Chapter 5, Paragraph 5.5. However, in summary and relevant to this study it was noted that: (i) maternal serum total cholesterol concentrations were significantly increased, independently of test fat, with R-fed dams having 88% higher concentrations compared to C. As no cholesterol was added to the experimental fat diets it was considered that different TFA isomers and SFA in W diet were pivotal to this outcome. (ii) dams consuming fat diets during suckling reduced their bodyweight by 11%, independently of type of test fat, compared to C. The difference in body weight could not be explained by tissue differences, with adipose depots and liver weights similar between all diet groups. An explanation for this could be that the dams adjusted their feed intake, as rodents defend energy intake when fed hyperenergetic diets (Sampey et al., 2012). However, this data was not collected as C57 mice are prone to stress-related pregnancy loss. Taking this into consideration, it was proposed that intake of protein and micronutrients were lower in these groups, particularly during lactation when demand for nutrients are high. It was therefore appropriate to assume that offspring were exposed to different maternal TFA isomers, hypercholesterolemia and potentially reduced protein and micronutrient availability during fetal development and suckling.

Offspring consuming fat diets in post-natal life had double the serum cholesterol concentrations compared to C offspring, independently of type of test fat. However, it was interesting to note that of the three fat diet groups, offspring consuming R had the highest HDL-cholesterol and lowest Non-HDL cholesterol concentrations, resulting in a lower Non-HDL:HDL ratio compared to C fed animals (P=0.058). The improved serum cholesterol concentrations observed in R-fed offspring is supported by other animal studies that found diets enriched with VA improved serum cholesterol concentrations for example lowering VLDL and IDL/HDL ratio (Tyburcz et al., 2009, Lock et al., 2005) or increasing small-HDL (Rice et al., 2010). In our study, R offspring also displayed significantly greater quantities of CLA and VA within the adipose tissue compared to other offspring. These study outcomes add credence to the hypothesis that endogenous VA-RA CLA synthesis could have a beneficial health impact on serum cholesterol and confer a reduced CHD-risk. Despite the two-fold difference in serum cholesterol concentrations observed in the fat groups no clear effect of diet on atherosclerosis was seen. However, a significant diet x section number interaction was observed in atherosclerosis between all diet groups (C, P, R, W $P<0.01$). This was primarily due to a relatively high level of atherosclerosis in earlier sections (12 and 15) of aortas from control-fed animals compared to those on high fat diets which was confirmed when statistical analysis of high fat diets alone (P, R, W: P=0.557) showed no differences nor diet x section interaction (P=0.512). As outlined in Chapter 3, there is variation in atherosclerosis expression throughout the aortic valve region. As rigorous analyses of atherosclerosis data did not show any further differences, there is no conclusive explanation for the anomaly observed in the two early sections of aorta of chow fed offspring. However, the lack of difference in atherosclerosis between the fat groups support a previous study in guinea pigs by Rice et al., (2010). They fed post-natal diets rich in P or R (similar to those used in this study) and although the diets increased serum cholesterol concentrations there were no differences in atherosclerosis compared to C-fed animals. It has been suggested that dietary cholesterol is key to the development of atherosclerotic lesions in guinea pigs (Kind et al, 1999). A previous study has shown that maternal Western diets (similar to the diet used in this study but containing 0.25% cholesterol) increased susceptibility to atherosclerosis development in AEL offspring (Tarling et al., 2016). The effect of the lack of cholesterol in the post-natal diets, and subsequent lack of

atherosclerosis, in this study is substantiated by comparison with Chapter 5 offspring atherosclerosis results. These offspring were likewise exposed to maternal dietary test fats during pregnancy and suckling but were weaned onto a post-natal atherogenic diet that contained SFAs and 0.25% cholesterol. These offspring had a 30% increase in serum cholesterol concentrations and a remarkable 95% increase in atherosclerosis compared to the offspring on post-natal experimental fat diets in this study. It was also interesting to note that for all offspring exposed to fat during pregnancy and lactation, although significantly different in the amount of atherosclerosis present due to the post-natal A diet, showed similar patterns of atherosclerosis as there were no differences in atherosclerosis between C, P, R, W diet groups. These results confirm that AEL mice are highly sensitive to post-natal high fat/cholesterol diet-induced atherosclerosis. Furthermore, it is noted that in some mouse models (e.g. LDL receptor deficient) mice on chow and low-fat diets, atherosclerotic lesions can take up to 12 months to develop (Getz and Reardon, 2012). Other atherosclerosis studies using the AEL mouse and feeding chow or fat diets without cholesterol confirm this outcome (Yates et al., 2009, Tonge, 2011). The results of our studies support that if a post-natal diet without cholesterol is used for atherosclerosis assessment in AEL mice, the trial should be extended beyond 15 weeks of age to allow for atherosclerosis manifestation.

Another phenotypic outcome in offspring that can be ascribed to maternal dietary factors during pregnancy and suckling, is that P offspring weighed significantly less at weaning compared to C offspring. P offspring 'caught-up' in bodyweight by day 4 post-weaning, presumably by increasing their feed intake, however feed intake data was not available to confirm this. After 12 weeks on post-natal P diet offspring had gained significantly more weight compared to C offspring. It is recognised in MLP rat studies (Langley-Evans, 2000) and human maternal undernutrition and obesity, offspring are prone to low birth weights, and rapid weight gain during early development that is termed "catch up growth" are correlated with increased prevalence of CVDs in later life (Eriksson et al., 2001a, Eriksson et al., 1999, Kajantie et al., 2005). The potential for maternal protein and micronutrient restriction during fetal and neonate development outlined above may be pertinent to this outcome. However, as there were no differences in offspring weaning

bodyweight of R and W offspring compared to C, the results indicate that this is a P diet-specific effect of exposure during pregnancy and suckling. Maternal P consumption is shown to have an inverse relationship with EFA availability and offspring birthweight in the first week of life in human cohorts (Hornstra et al., 2006, Innis, 2007), rats (Bayol et al., 2010), and C57 mice (Kavanagh et al., 2010). The pups in our study were not weighed at birth as C57 mice are prone to stress-related litter loss. However, the fact that after suckling for 3 weeks P offspring still showed retarded growth adds weight to this maternal-TFA hypothesis. Additionally, the catch-up growth identified in the first week post-weaning is synonymous with that described in the low-birth weight studies above. To identify if maternal nutrient supply was restricted during suckling (e.g. reduced FAs, protein, or EFAs) in our study, further analyses of milk samples would be required.

Offspring bodyweight at 15 weeks of age was similar between the diet groups. However, there were notable differences in tissue weights. In conjunction with the reported outcome for retarded growth early post-weaning, it was found that offspring consuming P and W diets (but not R, *P*=0.058) had smaller livers compared to chow offspring. Liver lipid analysis showed that P-offspring had significantly greater liver TAG compared to W offspring and liver cholesterol compared to P, R and W offspring. These results support research studies in human cohorts and animals, that maternal undernutrition can cause fetal growth restriction which result in growth retardation of organs such as the liver and kidneys (Greenwood and Bell, 2003, Boito et al., 2002, Langley-Evans et al., 1999). These results add weight to the argument that maternal protein and micronutrients were reduced during fetal and neonate development for all offspring in terms of liver organogenesis. However, the effect of P isomers during fetal and neonate development cannot be ignored, with retarded growth, reduced liver size and elevated liver TAG and cholesterol prevalent.

Adiposity observed in R offspring in this study was not able to be attributed to maternal diet due to offspring consuming fats throughout lifespan. However, offspring exposed to R had significantly more total adipose tissue (for both perirenal and gonadal depots) compared to C, P,

and W, offspring. However, it is noted that the effect was very modest (combined increase in both depots equals 0.4% of total body weight) and this would need to be confirmed through detailed experiments of body composition and food intake measurements. It has been shown in rats (Akyol et al., 2012, Gugusheff et al., 2013) and mice (Oben et al., 2010), that phenotypes caused by post-natal diets (from lactation onwards) can be modulated by feeding low fat, chow diets. These outcomes reflect that early life and post-natal nutrition is key.

It is acknowledged that limitations of this study include the lack of feed intake data for C57 mothers due to stress-related pregnancy and litter loss that would have indicated the reason for weight loss and nutrient availability during fetal and neonate development. Also, for the latter reason litters were not disturbed during early development and pup birth weights and daily body weights were not collected over the three weeks during suckling. Therefore, it cannot be categorically ascertained if the offspring of R and W dams had retarded growth during fetal development. In order to understand retarded growth of P and reduced liver size in fat groups was an effect of maternal nutrient deficiency or an effect of the dietary FAs, more detailed investigation into maternal body composition and composition of milk samples would be required.

In order to clarify the effect of FAs during development, investigations of offspring whole body composition, kidneys (potential for reduced nephron number), liver morphology, cellular composition, and hepatic metabolism markers (e.g. PPARα, SREBP).

6.5 CONCLUSION

In conclusion, although the diets increased maternal and offspring lipoprotein metabolism, there was no effect of maternal P, R, or W diet on the development of atherosclerosis in offspring. Offspring of P-fed dams showed phenotypic outcomes that pertain to maternal undernutrition and/or an effect of the dietary P *trans* isomers. These outcomes included significant growth retardation at weaning compared to C offspring, retarded liver growth and elevated liver TAG and Cholesterol. W offspring (but not R P=0.058) also had retarded liver growth. Offspring consuming a post-natal R diet had significantly greater adipose tissue compared to P, W and C offspring.

7. GENERAL DISCUSSION

As discussed in the introduction and Chapter 5, a maternal low protein diet increased risk of atherosclerosis in offspring when fed high fat/cholesterol post-natal diets, with associated increased lipoprotein metabolism (Yates et al., 2009). In contrast, a western maternal diet (similar to the one used in this study but with 0.25% cholesterol added) caused maternal cholesterol metabolism to increase two-fold and elevated risk of atherosclerosis in offspring in the absence of changes to cholesterol metabolism (Tarling et al 2016). PHVO are known to adversely affect serum cholesterol concentrations and increase CVD risk (Mensink et al., 2003). RTFA has been shown to have a beneficial impact on lipoprotein profiles in animals (Lock et al., 2005).

The hypothesis for this study was that fetal, neonate and post-natal exposure to two sources of TFA (P, R), and SFAs in the Western (W) diet, would have differing effects on the risk of atherosclerosis development in later life. It is acknowledged that dietary TFAs can cross the placenta and are present in the mother's milk, proportional to maternal dietary intake and have possible metabolic consequences (Innis, 2006). It is noted that the experimental fat diets (P, R W) fed to dams and offspring in this study did not contain cholesterol and therefore maternal and offspring outcomes are reflective of the FA and *trans* isomer compositions of these diets.

7.1.1. The Effect of Maternal Consumption of P and R Diets on Maternal Physiology and Lipoprotein Metabolism During Pregnancy and Lactation (C57 Mouse)

The maternal results showed that both P and R-specific TFA isomers were found to be present in their adipose tissue, with R dams having disproportionate amounts of CLA (C18:2*c*9*t*11) mainly through the action of stearoyl Co-A desaturase on dietary VA (Fig 1.5). These results confirmed that TFAs were present in the maternal metabolism and able to be passed to the offspring during pregnancy and lactation development periods. During pregnancy (at day 17 gestation) serum total cholesterol and serum TAG concentrations were increased in P-fed animals compared to R. However, in Study 2 when measurements were taken after 3 weeks of suckling, it was found that

P or R-fed animals (but not W) had similar and significant increases in total serum cholesterol compared to C-fed animals. Continuing to consume the fat diets (P, R, W) throughout lactation, caused significant increases in serum total cholesterol concentrations for all experimental fat diet groups (P, R, W) compared to C. R-fed animals had an 88% higher serum total cholesterol compared to C, and significantly greater concentrations compared to both P and W. These results indicated that P and R diets during pregnancy alone, or P, R or W diets consumed during pregnancy and lactation could instigate maternal hypercholesterolemia with the potential to confer perturbations in the fetal and neonate environment during development.

7.1.2. The Effect of Maternal Consumption of P, R, W Diets on Offspring Physiology and Atherosclerosis Development (AEL Mouse)

Study 1 confirmed previous studies that reported AEL mice are highly sensitive to post-natal A diet-induced (rich in SFA and cholesterol) atherosclerosis (Groot et al., 1996, Gijbels et al., 1999) The atherogenic diet increased serum total cholesterol concentrations by 71% and atherosclerotic area by 94% compared to C offspring ($P<0.01$, Figure 4.9 and 4.10). However, as there was no chow control group for Study 1, the effect of the maternal TFA diets on atherosclerosis risk in offspring could not be categorically confirmed. There was a maternal diet x section interaction observed with atherosclerosis increasing in R offspring and decreasing in P offspring in sections closest to the heart ($P<0.01$). However, rigorous analysis of the pattern of atherosclerosis expression (see Chapter 3) did not explain this anomaly, with mean lesion area similar between the two maternal diet groups ($P=0.383$).

Study 2 assessed the impact of P, R and W diets during pregnancy, or pregnancy and lactation on atherosclerosis risk in offspring. All offspring were weaned onto an atherogenic diet for 12 weeks. The atherogenic diet caused a uniform increase in offspring cholesterol metabolism across all diet groups with no differences observed (C, P, R and W). However, it was found that maternal TFA consumption during pregnancy appeared to protect offspring from postnatal atherogenic diet-induced atherosclerosis in later life, irrespective of isomeric distribution of the TFA ($P=0.013$, Figure 5.6). However, this effect was lost and atherosclerosis increased if the TFA diet was

continued to be fed during lactation and early development periods (Figures, 5.7a, 5.7b and Figure 5.8). Previous maternal studies in several mouse models (e.g. AEL, ApoE deficient, and LDLr deficient mouse) have shown that maternal hypercholesterolemia has induced changes in offspring susceptibility to atherosclerosis, both with (Napoli et al., 2002, Napoli et al., 1999), and without (Tarling et al., 2016), diet-induced changes to offspring lipoprotein metabolism. Napoli et al (1999, 2002) showed specific changes in aortic gene expression may be imprinted during neonatal life. Therefore, it is plausible that in response to maternal dietary TFAs during fetal organogenesis, alterations in aortic biological development could occur resulting in differences to susceptibility to atherosclerosis. It is also of note that offspring displayed this biological difference, even after being challenged by an atherogenic diet for 12 weeks (Figure 5.6 and Figure 5.8). In LDLr deficient mice it has been reported that PHVO have increased atherosclerosis (Bassett et al., 2009), whilst RTFAs have an anti-atherogenic effect (Bassett et al., 2010). However, this is the first report of both P and R TFA having athero-protective effects during early development. It is acknowledged that the effects of pro-anti-inflammatory factors in the TFA diets could have differential effects in pregnancy and lactation development periods.

7.1.3. The impact of P, R or W Throughout Lifespan on Atherosclerosis Development in Offspring (AEL Mouse)

In Study 3, offspring were weaned onto corresponding maternal diets (CCC, PPP, RRR, WWW). The fat diets increased offspring serum cholesterol concentrations by 50% compared to C offspring, with R offspring having the lowest Non-HDL:HDL ratio compared to C ($P=0.058$). There was a significant interaction between section number and diet ($P<0.01$, Figure 6.7), with control offspring having greater atherosclerosis in two early sections of aorta. However, rigorous analyses of the atherosclerosis data did not show any further differences between all diet groups with mean atherosclerosis lesion area similar between all groups ($P=0.627$). There was no conclusive explanation for the anomaly observed in the two early sections of aorta of chow fed offspring. However, it was considered that a lack of cholesterol in the post-natal fat diets contributed to the lack of atherosclerosis observed in these offspring (95% less atherosclerosis compared to offspring that had consumed an atherogenic post-natal diet: CCA, PPA, RRA, WWA

diet groups). Studies in animals and humans of fetal growth restriction and maternal undernutrition suggest that this can result in growth retardation of organs (e.g. liver and kidneys) (Greenwood and Bell, 2003, Boito et al., 2002). In this study we observed that offspring consuming fat diets (P, R and W) had significantly smaller livers compared to C offspring. An explanation for could be that the mothers of these offspring had reduced protein and micronutrient availability during fetal development. C57 mice defend energy intake when fed hyperenergetic diets (Sampey et al., 2012). It was observed that mothers consuming the fat diets throughout pregnancy and lactation had reduced their bodyweight by 11% compared to C, potentially reducing their feed intake. As C57 mice are prone to stress related pregnancy and litter loss, feed intake data was not obtained. However, this explanation is plausible and therefore it was considered that maternal protein and micronutrients would have been lower during this period when demand for nutrients is high.

It is recognised in maternal low protein rat studies (Langley-Evans, 2000) and human maternal undernutrition and obesity, offspring are prone to low birth weights, and rapid weight gain during early development that is termed “catch up growth”, and are correlated with increased prevalence of CVDs in later life (Eriksson et al., 2001a, Eriksson et al., 1999, Kajantie et al., 2005). Therefore, the maternal nutrient deficiency proposed above could also have played a role in the retarded growth of P offspring at weaning that “caught up” in body weight by day 4 post-weaning, and after 12 weeks on post-natal diets had gained significantly more weight compared to C offspring. Although all offspring were potentially exposed to maternal nutrient deficiency during development, it is acknowledged that R and W offspring did not display this effect. However, as the effect of retarded growth was still present after 3 weeks of suckling, it adds weight to the outcome that this was an effect of the maternal P-diet on offspring development.

The only other phenotypic effect was an increase in adipose tissue weight in the offspring of mothers fed R in Study 2. However, the combined increase in weight of both gonadal and perirenal adipose depots is very modest (0.4% of total body weight) compared to C, P and W offspring. Finally, adiposity observed in R offspring in Study 3 was not able to be attributed to

maternal diet due to offspring consuming fats throughout lifespan. However, as for study 2, R-fed offspring had a significant (but modest 0.4%) increase in both adipose depots compared to C, P and W offspring. In order to confirm these outcomes, feed intake analysis and more detailed experiments on body composition would be required. Feeding high fat diets during lactation had no impact upon offspring body weights or adiposity of P, W and C, which is consistent with some other, but not all, rodent studies of overfeeding in lactation (Akyol et al., 2012). However, any effect of maternal diet during suckling may have been masked by feeding of the atherogenic diet post-weaning. It has been shown in rats (Akyol et al., 2012, Gugusheff et al., 2013) and mice (Oben et al., 2010), that phenotypes caused by post-natal diets (from lactation onwards) can be modulated by feeding low fat, chow diets.

7.2 STUDY LIMITATIONS

In the pregnancy study and study 1, no chow group was included, therefore outcomes for RTFA on maternal physiology and the impact of both maternal PHVO and RTFA during pregnancy on atherosclerosis development in offspring could not be categorically ruled out.

A limitation of Study 2 and Study 3 was that due to the extensive workload and time constraints of large cohort animal studies not all diet groups were included for example, the effect of fat during lactation, (e.g. CPC, CWC, CRC); or the effect of exposure to fat during development and post-natal chow groups, (e.g. PPC, RRC, WWC).

In hindsight in Study 3, Fat throughout Lifespan groups (PPP, RRR, WWW), the addition of cholesterol to the post-natal diets may have instigated differential atherosclerosis outcomes. It has recently been shown that a maternal Western diet (similar to the one used in this study, but with added 0.25% cholesterol) influenced the development of atherosclerosis in AEL offspring (Tarling et al., 2016). In our study without cholesterol, these offspring groups (P, R W) showed remarkably little atherosclerosis at 15 weeks of age and were similar to the C offspring. It is also noted that in some mouse models (e.g. LDL receptor deficient) mice on chow and low-fat diets,

atherosclerotic lesions can take up to 12 months to develop (Getz and Reardon, 2012). Other atherosclerosis studies using the AEL mouse and feeding chow or fat diets without cholesterol confirm this outcome (Yates et al., 2009, Tonge, 2011). The results of our studies support that if a post-natal diet without cholesterol is used for atherosclerosis assessment in AEL mice, the trial should be extended beyond 15 weeks of age to allow for atherosclerosis manifestation.

As previously discussed, rodents defend energy intake when fed hyperenergetic diets (Sampey et al., 2012). Therefore, feeding high fat and TFA-rich diets during pregnancy and lactation could have resulted in the mothers reducing their feed intake. This is particularly pertinent as mothers consuming the fat diets throughout pregnancy and lactation reduced their body weight by 11% compared to C fed mothers. Therefore, it was proposed that these mothers would have reduced protein and micronutrient availability during lactation, when demand for nutrients is high. Collecting Feed intake data of mothers and offspring would have helped to identify changes in feeding patterns. Therefore, caution is advised when considering offspring phenotypic outcomes as reduced protein and micronutrient intake confer an additional perturbation to the developing neonate.

Finally, comparisons are being drawn upon the atherosclerosis presented in murine aortic arch/heart valve samples whereas in humans it would be the heart coronary arteries and associated vasculature (Zadelaar et al., 2007, Kleemann et al., 2007).. It is also acknowledged that mice and humans differ in the lipid and lipoprotein metabolism and atherosclerosis pathways. In human physiology LDL-cholesterol metabolism drives atherosclerosis progression (Wang and Paigen, 2005), whilst in AEL mice Chylomicron, IDL, VLDL and remnants are prevalent (van den Maagdenberg et al., 1993).

7.3 MAIN IMPLICATIONS AND FUTURE WORK

The UK Government's Public Health Outcomes Framework 2013-2016 set out key areas for improvement that address reducing premature mortality from CVDs through public health

improvements, including reducing the number of low birth weight term babies, implementation and support for breastfeeding initiatives, and, reducing excess body weight in adults and children through diet (DH, 2012c). Langley-Evans (2015) notes that pregnancy and infant nutrition provide a significant opportunity for improving the health of future generations, especially as parents are often more willing to make lifestyle changes during this period for the sake of their children. This makes our studies into maternal consumption of FAs during pregnancy and lactation and their impact on susceptibility to atherosclerosis development in offspring particularly relevant.

The results showed that exposure to TFA during pregnancy protected offspring from atherosclerosis, irrespective of dietary TFA isomer distribution, however this effect was lost if the TFA diets continued to be consumed during lactation. This study indicates that the effects of pro-and-anti-inflammatory factors in the TFA diets could have differential effects in pregnancy and lactation development periods. For the effect of TFAs and athero-protective outcome observed in Study 2offspring, further investigation and examination of early lesions in fetal and neonate vessels would be required. Combining this with transcriptomic analysis of fetal vasculature and maternal liver will contribute to a more detailed understanding of the relationship between TFA, maternal cholesterol and early lesion development.

Additionally, mothers consuming (P, R or W) throughout pregnancy and lactation, reduced in bodyweight (see study limitations), potentially conferring reduced protein and micronutrient availability during this period. Therefore, in order to clarify the maternal nutrient status further investigation of maternal total body composition and milk samples would be necessary. These investigations would also shed light on the impact of maternal diet on observed outcomes in Study 3 offspring: P offspring had retarded growth at weaning, and all offspring had reduced liver weights. Further studies of whole-body composition for offspring, and morphology of liver and kidney tissue could indicate whether maternal low protein/nutrient restriction was responsible tissue remodelling outcomes. Whole body composition of offspring in Study 2 and 3, would also confirm the phenotype of adiposity in offspring of R-fed dams, and those offspring consuming R in post-natal life.

7.4 CONCLUSION

In conclusion, our studies provided an insight into the impact of maternal consumption of different isomers of *trans* fatty acids on the development of atherosclerosis in offspring. Our studies consistently showed that C18:1 *trans* isomers derived from dietary PHVO (predominantly EA) and ruminant milk fat (predominantly VA and CLA) accumulated in the adipose tissue of dams. It was therefore appropriate to assume that the developing fetus and neonate were exposed to different TFA isomer concentrations depending on the diet fed to the dams. However, it was found that maternal consumption of TFA diets during pregnancy conferred an anti-atherogenic effect in offspring fed an atherogenic-diet postnatally independently of the isomeric distribution of the TFA. However, if the maternal TFA diet is maintained during lactation this protection is lost and postnatal atherogenic diet-induced atherosclerosis increases particularly when PHVO TFA are consumed. The mechanisms underlying these changes remain to be established, but they are not associated with changes in lipoprotein concentrations at the point when atherosclerosis was measured. There was an unexpected outcome of no effect of the maternal Western diet on the development of atherosclerosis in offspring. However, this may be due to the absence of added cholesterol in these diets.

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Rat and Mouse No. 1 Maintenance

Calculated Analysis

NUTRIENTS		Total	Supp (9)
Proximate Analysis			
Moisture (1)	%	10.00	
Crude Oil	%	2.71	
Crude Protein	%	14.38	
Crude Fibre	%	4.65	
Ash	%	6.00	
Nitrogen Free Extract	%	61.73	
Digestibility Co-Efficients (7)			
Digestible Crude Oil	%	2.47	
Digestible Crude Protein	%	12.92	
Carbohydrates, Fibre and Non Starch Polysaccharides (NSP)			
Total Dietary Fibre	%	17.05	
Pectin	%	1.52	
Hemicellulose	%	10.17	
Cellulose	%	4.32	
Lignin	%	1.68	
Starch	%	44.97	
Sugar	%	4.05	
Energy (5)			
Gross Energy	MJ/kg	14.74	
Digestible Energy (15)	MJ/kg	11.90	
Metabolisable Energy (15)	MJ/kg	10.74	
Atwater Fuel Energy (AFE)(8)	MJ/kg	13.75	
AFE from Oil	%	7.42	
AFE from Protein	%	17.49	
AFE from Carbohydrate	%	75.09	
Fatty Acids			
Saturated Fatty Acids			
C12:0 Lauric	%	0.02	
C14:0 Myristic	%	0.14	
C16:0 Palmitic	%	0.31	
C18:0 Stearic	%	0.04	
Monounsaturated Fatty Acids			
C14:1 Myristoleic	%	0.02	
C16:1 Palmitoleic	%	0.09	
C18:1 Oleic	%	0.77	
Polyunsaturated Fatty Acids			
C18:2(ω6) Linoleic	%	0.69	
C18:3(ω3) Linolenic	%	0.06	
C20:4(ω6) Arachidonic	%	0.13	
C22:5(ω3) Clupanodonic	%		
Amino Acids			
Arginine	%	0.91	
Lysine (6)	%	0.66	0.07
Methionine	%	0.22	0.04
Cystine	%	0.24	
Tryptophan	%	0.18	
Histidine	%	0.35	
Threonine	%	0.49	
Isoleucine	%	0.54	
Leucine	%	0.98	
Phenylalanine	%	0.66	
Valine	%	0.69	
Tyrosine	%	0.49	
Taurine	%		
Glycine	%	1.11	
Aspartic Acid	%	0.67	

NUTRIENTS		Total	Supp (9)
Glutamic Acid	%	3.17	
Proline	%	1.20	
Serine	%	0.56	
Hydroxyproline	%		
Hydroxylysine	%		
Alanine	%	0.16	
Macro Minerals			
Calcium	%	0.73	0.63
Total Phosphorus	%	0.52	0.04
Phytate Phosphorus	%	0.24	
Available Phosphorus	%	0.28	0.04
Sodium	%	0.25	0.19
Chloride	%	0.38	0.32
Potassium	%	0.67	
Magnesium	%	0.23	
Micro Minerals			
Iron	mg/kg	159.30	82.50
Copper	mg/kg	11.50	1.94
Manganese	mg/kg	72.44	19.22
Zinc	mg/kg	35.75	
Cobalt	µg/kg	634.10	550.00
Iodine	µg/kg	1202.69	1085.00
Selenium	µg/kg	298.99	100.00
Fluorine	mg/kg	10.49	
Vitamins			
β-Carotene (2)	mg/kg	0.16	
Retinol (2)	µg/kg	2566.38	2400.00
Vitamin A (2)	iu/kg	8554.27	8000.00
Cholecalciferol (3)	µg/kg	15.54	15.00
Vitamin D (3)	iu/kg	621.70	600.00
α-Tocopherol (4)	mg/kg	76.45	56.82
Vitamin E (4)	iu/kg	84.10	62.50
Vitamin B_1 (Thiamine)	mg/kg	8.58	1.96
Vitamin B_2 (Riboflavin)	mg/kg	4.33	2.94
Vitamin B_6 (Pyridoxine)	mg/kg	4.81	0.98
Vitamin B_{12} (Cyanocobalamine)	µg/kg	7.49	6.00
Vitamin C (Ascorbic Acid)	mg/kg	2.59	
Vitamin K (Menadione)	mg/kg	10.17	9.36
Folic Acid (Vitamin B_9)	mg/kg	0.79	
Nicotinic Acid (Vitamin PP) (6)	mg/kg	61.32	2.45
Pantothenic Acid (Vitamin B_{5})	mg/kg	20.17	5.80
Choline (Vitamin B_{4})	mg/kg	1080.14	366.60
Inositol	mg/kg	2369.59	
Biotin (Vitamin H) (6)	µg/kg	277.13	

Notes

1. All values are calculated using a moisture basis of 10%.
   Typical moisture levels will range between 9.5 - 11.5%.
2. a. Vitamin A includes Retinol and the Retinol equivalents of β-carotene
   b. Retinol includes the Retinol equivalents of β-Carotene.
   c. 0.48 µg Retinol = 1 µg β-carotene = 1.6 iu Vitamin A activity
   d. 1 µg Retinol = 3.33* iu Vitamin A activity
   e. 1 iu Vitamin A = 0.3 µg Retinol = 0.6 µg β-carotene
   f. The standard analysis for Vitamin A does not detect β-carotene
3. 1 µg Cholecalciferol (D_3) = 40.0 iu Vitamin D
4. 1 mg all-*rac*-α-tocopherol = 1.1 iu Vitamin E activity
   1 mg all-*rac*-α-tocopherol acetate = 1.0 iu Vitamin E activity
5. 1 MJ = 239.23 Kcalories = 239.23 Calories = 239,230 calories
6. These nutrients coming from natural raw materials such as cereals may have low availabilities due to the interactions with other compounds.
7. Based on in-vitro digestibility analysis.
8. AF Energy = Atwater Fuel Energy = ((CO%/100)*9000)+((CP%/100)*4000)+((NFE%/100)*4000)/239.23
9. Supplemented nutrients from manufactured and mined sources.
15. Calculated.