DIETARY FAT INTAKE DURING PRE- AND POST-WEANING TIME PERIOD AND ITS ASSOCIATION WITH THE ONSET OF CARDIOVASCULAR DISEASE IN THE OFFSPRING

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ABSTRACT

The 'fetal origins' of cardiovascular disease (CVD) hypothesis proposes that maternal nutritional environment during pregnancy can play an important role in determining the cardiovascular health of an individual in adult life. A typical Western diet is rich in dietary fats, a fact that has been linked to the increased prevalence of CVD. In addition to the quantity of fat, the quality of fat is also known to affect the development of CVD. Whilst an increased consumption of saturated fatty acids (SFA) has been associated with higher incidence of CVD, a diet rich in polyunsaturated fatty acids (PUFA) has been suggested to lower the risk of developing CVD. Considering that nutrition patterns are shifting towards a higher-fat Western diet globally, it is of interest to understand the role of a high-fat maternal diet in the fetal origins of CVD. The current thesis was designed to understand the role of the quantity, as well as the quality, of maternal dietary fat intake during pregnancy, in the fetal origins of CVD in the adult offspring. In addition, the role of interaction between the pre- and post-natal dietary fat intake on the offspring health was assessed. Early programming experiments were conducted using C57Bl/6 mice, which have been extensively used as an animal model to investigate the dietary fat-mediated regulation of lipid metabolism. Lipid metabolism and aortic vascular function were chosen as the study outcomes to estimate the risk of developing CVD in the offspring. Results indicated that a high-fat maternal diet rich in SFA (lard) was associated with a reduced expression of hepatic low-density lipoprotein (LDL)-receptor and a higher concentration of LDL-cholesterol in the offspring. On the other hand, a high-fat maternal diet rich *n-6* PUFA (safflower oil) was associated with higher mRNA expression of hepatic lecithin: cholesterol acyltransferase and higher concentration of high-density

lipoprotein-cholesterol in the offspring. The high-fat maternal diet, irrespective of the type of fat, however was associated with reduced aortic contractile reactivity towards KCl, phenylephrine and thromboxane mimetic U44619, in the female offspring. In addition, significant interaction of pre- and post-weaning diets was reported for various outcomes studied in the offspring, suggesting the importance of both prenatal and postnatal environments in regulating the offspring cardiovascular health.

In a separate study, the effects of *n-3* PUFA-rich flax oil feeding were assessed on various parameters associated with metabolic syndrome, using the SHR/NDmcr-cp rat model. Flax oil feeding was associated with significantly lower hepatic triglycerides and cholesterol concentrations in the obese rats. In addition, flax oil feeding was associated with lower plasma insulin concentrations and oxidative stress in the obese rats. An up regulation in the hepatic expression of peroxisome-proliferator activated receptor-γ (PPAR-γ) was found to be negatively correlated with the hepatic TG and cholesterol concentrations in the obese rats, thus pointing towards the activation of PPAR-γ dependent pathways behind the hepatic lipid-lowering effects of flax oil supplementation.

Taken together, the results presented in the current thesis support the role for the quantity and the quality, of dietary fats consumed during pre- and post-weaning time periods, on the development of key parameters associated with the onset of CVD.

CO-AUTHORSHIP STATEMENT

For the work presented in chapter-3, published in *American Journal of Physiology, Regulatory, Integrative and Comparative Physiology (2009) 296: R1029-40*, I, Kanta Chechi, was involved with the design of the study, conducting the experiments, analyzing the data and preparation of the manuscript.

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AA	Arachidonic acid
ABCA-1	ATP-binding cassette transporter protein-1
ALA	α-linolenic acid
ATP-III	Adult Treatment Panel III
Apo	Apolipoprotein
ACh	Aetylcholine
ANOVA	Analysis of variance
BMI	Body mass index
CETP	Cholesteryl ester transfer protein
CYP-71	Cholesterol 7 alpha-hydroxylase
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DOHaD	Developmental origins of health and disease
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
EDHF	Endothelial derived hyperpolarizing factor
ER	Endoplasmic reticulum
EDTA	Ethylene diamine tetra acetic acid
FFA	Free fatty acids
GTT	Glucose tolerance test
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HDL	High-density lipoprotein
GLC	Gas -liquid chromatography
IDL	Intermediate-density lipoproteins
KCL	Potassium chloride
LA	Linoleic acid
LDL	Low-density lipoprotein

LCAT	Lecithin: cholesterol acyltransferase
LDL-r	Low-density lipoprotein receptor
LPL	Lipoprotein lipase
L-NAME	N (G)-nitro-L- arginine methyl ester
MUFA	Mono-unsaturated fatty acids
NAFLD	Non-alcoholic fatty liver disease
NO	Nitric oxide
NOS	Nitric Oxide synthase
PE	Phenylephrine
PSS	Physiological salt solution
PPAR	Peroxisome proliferators-activated receptor's PPAR
PUFA	Polyunsaturated fatty acids
PLTP	Phospholipids transfer protein
PI-3 Kinase	Phosphoinositide-3 kinase
PAR	Predictive adaptive response
RXR	Retinoid X receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rats
SFA	Saturated fatty acids
SR-B1	Scavenger -receptor B1
SNP	Sodium nitroprusside
SE	Standard error of mean
SD	Standard deviation
SREBP	Sterol-regulatory element binding protein
TZD	Thiazolidinedione
TG	Triglycerides
TX-A_2	Thromoboxane A_2

Type 2 DM	Type 2 diabetes mellitus
VSMC	Vascular smooth muscle cells
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very-low density lipoprotein
WHO	World health organization

LIST OF APPENDICES

CHAPTER ONE

Introduction and Overview

1.1 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is a complex and multifaceted disease that currently represents the major cause of deaths worldwide. The World Health Organization (WHO) attributed 30% of all global deaths (*i.e.* 15.3 million) as well as 10.3% of the total disability adjusted life years (DALYs) lost in 1998 to CVD (WHO, 1999). The Heart and Stroke Foundation of Canada reported a similar mortality rate in 2005, where 31% of deaths occurred due to CVD (Statistics Canada, 2009). The global burden of CVD affects all sections of society. Cardiovascular deaths in 1998 contributed to 34% of global mortality in women and 28.2% of all deaths in men (WHO, 1999). Once considered to be the 'disease of affluence' CVD is now shown to affect both developed and developing nations (Reddy and Yusuf, 1998). According to the Global Burden of Disease Study, it has been projected that a 55% rise would occur in DALY loss attributable to CVD between 1990 and 2020 in developing countries (Murray and Lopez, 1996). In addition to the increasing incidence of CVD, the early age at which it manifests would also contribute to the burden of CVD. It is projected that 6.4 million deaths would occur due to CVD in the age group of 30–69 years in developing countries by 2020 (Murray and Lopez, 1996). This rapid rate of change, together with the increasing burden of disease, is creating a major public health threat, which demands immediate and effective action.

Besides the social burden, there are data available to indicate the economic burden of CVD. According to the American Heart Association and the American Stroke Association (2006), total direct and indirect costs of CVD and stroke were estimated to

be $403.1 billion for the year 2006. At the same time, European Union reported an annual cost of €169 billion (Leal *et al.*, 2006), while the Heart and Stroke Foundation of Canada reported an annual expenditure of $22.2 billion to be associated with the incidence of CVD (Canadian Heart Health Strategy-Action Plan Steering Committee, 2009). The overall social and economic burden of CVD points toward the need for action to strengthen the preventive measures to counter the spread of CVD epidemic, which is now being widely recognized by many countries. The World Health Assembly adopted a resolution that urged Member States to collaborate with WHO to develop "...a global strategy on diet, physical activity and health for the prevention and control of non-communicable diseases, based on evidence and best practices, with special emphasis on an integrated approach..." (WHO, 2002).

A number of chronic conditions such as dyslipidemia, visceral obesity, hypertension, glucose intolerance and type 2 Diabetes Mellitus (DM) are recognized as independent risk factors for the development of CVD. The co-existence of three or more of these conditions can also be identified as 'metabolic syndrome', which can double the risk of subsequent development of CVD and premature death (Bricker and Greydanus, 2008). The diagnostic criteria for metabolic syndrome by WHO (Alberti and Zimmet, 1998) and National Cholesterol Education Program- Adult Treatment Panel III (NCEP-ATPIII) (NCEP- Expert Panel 2001) (Grundy *et al.*, 2005) is given in Table 1.1.

Diet and nutrition have long been identified to be the key players in the development of CVD and its risk factors. It is apparent at the global level that 'nutrition

Table 1.1 Diagnostic criteria for metabolic syndrome according to the WHO and ATP III

Component	WHO crtieria (Insulin resistance* plus two of the rest)	ATP III criteria (Three of the following)
Abdominal/central obesity	Waist to hip ratio: > 0.90 (men), > 0.85 (women), or BMI > 30 kg/m^2	Waist circumference: >102 cm in men, > 88 cm in women
Hypertriglyceridemia	>= 150 mg/dL (>= 1.7 mM)	>= 150 mg/dL
Low HDL cholesterol	< 35 mg/dL (< 0.9 mM) for men, < 39 mg/dL (<1.0 mM) for women	< 40 mg/dL (<1.036 mM) for men, <50 mg/dL (<1.295 mM) for women
High blood pressure	>= 140/90 mm Hg or documented use of antihypertensive therapy	>= 130/85 mm Hg or documented use of antihypertensive therapy
High fasting glucose	Impaired glucose tolerance, impaired fasting glucose, insulin resistance, or diabetes	>= 110 mg/dL (>= 6.1 mM)
Microalbuminuria	Urinary albumin to creatinine ratio: 30 mg/g, or albumin excretion rate: 20 mcg/min	

(Alberti and Zimmet, 1998; Grundy *et al.*, 2005)

WHO = World Health Organization; ATP = Adult Treatment Panel; BMI = body mass index; HDL = high-density lipoprotein.

*Insulin resistance is identified by type 2 DM or impaired fasting glucose.

transition' has swept the entire world since the second half of the twentieth century inducing major modifications in diet, which has paralleled the rise in the incidence of CVD. The dietary changes that characterize the 'nutrition transition' include both quantitative and qualitative changes in the diet. The adverse dietary changes include shifts in the structure of the diet towards a higher energy-density diet with a greater role for fat and added sugars in foods, greater saturated fat (SFA) intake, reduced intakes of complex carbohydrates and dietary fiber, and reduced fruit and vegetable intakes (Drewnowski and Popkin, 1997). These dietary changes are compounded by lifestyle changes that reflect reduced physical activity at work and during leisure time.

While it is apparent that nutrition during adult life is important, there is increasing evidence that CVD risks begin in fetal life and continue into old age (Barker, 1995; 1997a; 1997b, 2004a; 2004b). Adult CVD has therefore been identified to reflect cumulative differential lifetime exposures to damaging physical and social environments. For these reasons, the Joint WHO/Food and Agriculture Organization Expert Consultation committee adopted a life-course approach to capture the cumulative risk as well as many opportunities for dietary intervention. While accepting the imperceptible progression from one life stage to the next, five stages were identified for convenience, which were recognized as 1). Fetal development and the maternal environment, 2) Infancy 3), Childhood and adolescence 4). Adulthood, and 5), Aging and older people. Nutritional and lifestyle interventions at each stage of this life-course approach were recognized as an important strategy to control the global epidemic of CVD by the Expert Consultation Committee (WHO, 2003).

In recent years, a lot of interest has been generated in understanding the impact of the maternal nutritional environment during gestation on the overall health of the adult individual. The concept that "*in utero*" nutrition can be critical in determining the cardiovascular health of an individual in later life was originally known as Barker's hypothesis. This concept has since been variably called 'fetal programming', 'fetal origins' and 'developmental origins of health and disease' (DOHaD) hypothesis.

1.2 FETAL ORIGINS HYPOTHESIS

The 'fetal origins' hypothesis underscores the importance of the fetal nutritional environment in the onset of adult CVD. According to this hypothesis adverse maternal nutrition during gestation can create a 'nutrionally challenged' environment' for the developing fetus. The fetus then responds by 'programming' its own growth in a way that could increase its risk of developing CVD in later life (Barker, 1995). Barker and colleagues provided the initial evidence supporting the fetal origins hypothesis, which was obtained from a number of epidemiological studies. These studies related adult disorders such as hypertension (Barker *et al.*, 1990), insulin resistance (Phillips *et al.*, 1994), vascular dysfunction (Martyn *et al.*, 1995), obesity (Yajnik, 2000) and dyslipidaemia (Barker *et al.*, 1993) to adverse intrauterine conditions that caused disproportionate fetal growth or 'low birth weight'. The proposal that 'low birth weight' could have been the direct result of maternal under-nutrition during pregnancy was later

qualified by the studies conducted on the survivors of Dutch Hunger Winter (Ravelli *et al.*, 1976; Ravelli *et al.*, 1998; Roseboom *et al.*, 2000). Exposure to the Dutch famine, where average daily caloric intake was limited to 1680–3360 kJ during early gestation, was associated with adulthood hypertension, whereas the exposure to famine during late gestation was associated with increased obesity and glucose intolerance in the individuals (Ravelli *et al.*, 1976; Ravelli *et al.*, 1998). These observations further suggested that the 'programming' effects of adverse maternal nutrition were dependent upon the 'timing of the insult', and on the tissues and systems undergoing critical periods of development at that time. A range of studies performed on populations suffering from 'poor nutrition' from South Africa (Levitt *et al.*, 1999), the Caribbean (Thame *et al.*, 2000), India (Fall *et al.*, 1998) and Australia (Hoy *et al.*, 1999) further confirmed that under-nutrition during pregnancy was strongly associated with a higher risk of developing CVD in adult humans. However, the strength of the relationship between low birth weight and adult CVD has been challenged. Huxley and coworkers have demonstrated through meta-analyses that the effect size for associations between birth weight and vascular disease indicators actually decreases as cohort size increases (Huxley *et al.*, 2002). Furthermore, a study performed on the individuals conceived during the siege of Leningrad found no relationship between intrauterine malnutrition and glucose intolerance, dyslipidemia, hypertension, or cardiovascular disease in adulthood (Stanner *et al.*, 1997). These observations were later explained on the basis of the thrifty phenotype hypothesis.

1.2.1 Thrifty phenotype hypothesis

In an attempt to explain the association between maternal under-nutrition and adult disease, Hales and Barker proposed the thrifty phenotype hypothesis (Hales and Barker, 1992), which suggests that under a poor *'in utero'* environment, the developing fetus undergoes certain adaptive responses to maximize uptake and conservation of the available nutrients, resulting in a conservative metabolism. These adaptations enable the survival of the fetus in a potentially threatening prenatal environment. When the infant is then exposed to a nutritionally 'poor' postnatal environment, the programming of thrifty phenotype would prove advantageous, as these individuals would be biologically prepared to survive in a nutritionally 'poor' environment. The problem, however, appears when the postnatal nutritional environment is plentiful as compared to the *'in utero'* nutrition, because the individual is not biologically prepared to handle the excess of nutrients and as a result, disease manifests. Since the nutritional environment during the developmental period as well as the postnatal period was 'poor' during the siege of Leningrad, according to the thrifty phenotype hypothesis, the fetal 'predictive adaptations' might have offered a biological advantage to these individuals for better survival. As a result, no relationship was observed between the maternal under-nutrition and adult CVD during the siege of Leningrad study (Stanner *et al.*, 1997). In addition, data from other population-based studies further suggest that growth restriction *in utero* is less harmful to long-term health when nutrition in postnatal life is compromised to a similar degree (Primatesta *et al.*, 2003). Conversely, 'catch-up growth', where babies are born small but grow rapidly in the first months of life to eventually achieve normal

centiles of birth weight, has been identified as a particular risk factor for the later development of CVD (Eriksson *et al.*, 1999).

1.2.2 Predictive adaptive response hypothesis

The concept of 'thrifty phenotype' was revisited by Hanson and Gluckman when they proposed the predictive adaptive response (PAR) hypothesis in 2004 (Gluckman and Hanson, 2004). According to this hypothesis, the developing fetus can sense its prenatal environment and based on these 'environmental cues', it undergoes certain adaptive responses to survive in its 'predicted' postnatal environment. If the predictions made by the fetus are 'matched' by its postnatal environment, the individual will have a healthier life span. However, the problem appears when there is a 'mismatch' between the prenatal and the postnatal environment, as physiological adaptations made by the fetus during the developmental period are not able to meet the expectations of a different postnatal environment.

Although being conceptually similar, the distinction between the two hypotheses can be made based on the fact that the thrifty phenotype hypothesis calls for immediate adaptive changes in the developing fetus to an immediate environmental challenge (*e.g.* maternal under-nutrition) by reducing either body growth or the growth of individual organs to promote chances of its immediate survival. It then has to 'cope' throughout its life with the consequences of the irreversible components of this prenatal adaptive strategy. In contrast, PAR confers no immediate advantage but is induced to confer long-

term advantages later in life (Hanson and Gluckman, 2005). The current understanding of the thrifty phenotype or PAR hypothesis in relation to the physiological outcome in the offspring remains unclear.

1.3 MATERNAL UNDER-NUTRITION AND FETAL ORIGINS OF CVD: ANIMAL STUDIES

In addition to the epidemiological evidence, a number of animal studies have modeled poor '*in utero*' environment by employing various approaches such as 'caloric restriction' or by feeding 'low protein diets' during pregnancy to rodents, pigs and sheep models. Reduced β-cell mass and β-cell number was reported in one-day old Wistar rat offspring born to mothers that were subjected to 50% restriction of *ad-libitum* food intake during the last week of pregnancy (Garofano *et al.*, 1997). Continued restriction of the mother during suckling resulted in permanent reduction in β-cell mass and number, and impaired glucose tolerance in the rat offspring (Garofano *et al.*, 1998). Another study reported that 30% restriction of maternal *ad-libitum* food intake intake resulted in higher systolic blood pressure, higher fasting insulin concentration, hyperphagic behavior and obesity in the Wistar rat offspring (Vickers *et al.*, 2000). Recent studies in sheep have shown that maternal nutrient restriction over the period of maximal placental growth, *i.e.* between 28 and 80 days of gestation, resulted in offspring adiposity with increased mRNA abundance of uncoupling protein-2 and peroxisome proliferator activated receptor-α (PPARα), key components of fetal fat metabolism, in offspring adipose tissue

(Bispham *et al.*, 2005). In addition, nutrient restriction of ewes during late pregnancy has been shown to cause glucose intolerance in one-year old offspring, which was further associated with reduced expression of glucose transporter-4 in adipose tissue and increased adipose tissue mass (Gardner *et al.*, 2005). Offspring of twin sheep pregnancies compared to singletons, were found to exhibit hypertension and reduced glomerular filtration rates (Ross *et al.*, 2005). Furthermore, in a study comparing low and high birth weight pigs, low birth weight was shown to be associated with glucose intolerance at 12 months of age which was ascribed to reduced insulin sensitivity (Poore and Fowden, 2004).

Based on the animal studies dealing with maternal under-nutrition, a number of plausible mechanisms have been proposed to explain the phenomenon of 'nutritional-programming'.

1.3.1 Fetal tissue remodeling

Changes in the number of cells or types of cells present within a tissue could have a profound effect on organ function, and this has been proposed as one of the mechanism underlying fetal nutritional programming. All tissues are essentially derived from small populations of embryonic progenitor cells that differentiate into more specialized cells as the organs mature during development. Thus, it is plausible that nutritional imbalances during development affect the tissue/organ differentiation in a way that results in long-term consequences. Maternal low-protein diet during pregnancy has been shown to result

in 30% reduction in nephron number in the kidney of adult rat offspring (Langley-Evans *et al.,* 2003). Reduced renal mass has been proposed to result in glomerular hyperfiltration leading to hypertension in remnant nephrons with subsequent glomerular injury, proteinuria and systemic hypertension (Brenner *et al.,* 1996).

Besides affecting organ function, tissue remodeling may affect the profile of genes expressed within a tissue, may alter cell-cell signaling pathways, modify hormone production and may also affect the capacity of cells to respond to various hormone signals. Altered insulin production and glucose homeostasis has been reported as a result of reduced number of islets in the pancreas of the Wistar rat offspring born to mothers fed low-protein diet during pregnancy (Dahri *et al.*, 1990). Moreover, changes in the neuronal density and volume of key hypothalamic centers have also been reported in the Wistar rat offspring born to mothers fed low-protein diet during pregnancy. These changes have been shown to cause a shift in neuropeptides, which are known to affect the feeding behavior in the offspring, thereby affecting the development of obesity (Plagemann *et al.*, 2000)

1.3.2 Fetal glucocorticoid exposure

Glucocorticoids are steroid hormones that can freely cross the placenta. They are known to act as powerful modulators of gene expression and hence play an important role during fetal development. In most species, there is a massive gradient of glucocorticoids across the placenta with maternal concentrations being 100-1000 times higher than those in the

fetal circulation (Edwards *et al.*, 1993). It is believed that the placental enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2 acts as gatekeeper and protects the developing fetal tissue from overexposure to maternal glucocorticoids by converting the active corticosteroids into inactive forms (Seckl and Brown, 1994). Since glucocorticoids have potent effects on tissue development, overexposure to maternal glucocorticoids has been proposed as yet another potential mechanism of nutritional programming (Benediktsson *et al.*, 1993; Bertram *et al.*, 2001). A down-regulation in the mRNA expression of placental 11β-HSD2 has been reported in the Wistar rat offspring of mothers fed low-protein diet during pregnancy, which was further associated with high systolic blood pressure (SBP) in these offspring at 4-, 8- and 12 weeks of age (Bertram *et al.*, 2001).

1.3.3 Epigenetic mechanisms

Epigenetics describes genomic markings (chemical modifications) which are heritable from one cell generation to the next but do not involve changes in the primary DNA sequence (Bernstein *et al.*, 2007). The best-known epigenetic marks are (i) methylation of cytosines in CpG dinucleotides and (ii) post-translational modifications of histones. Since epigenetic marks can be modified by environmental factors, the epigenome is a means to connect environmental exposure with gene expression and cell/tissue function.

Both DNA methylation and histone acetylation patterns have been known to play an important role in fetal development through their influence on fetal gene expression,

thereby making them potential candidates as the mediators of fetal physiological programming (Mathers and McKay, 2009). The overall level and pattern of DNA methylation in the developing embryo or fetus is governed by flux through the methionine-homocysteine pathway, suggesting that it is sensitive to the supply of nutritional factors such as amino acids, folic acid and vitamins (Young *et al.*, 2004). Changes in the DNA methylation pattern of hepatic PPAR-α and glucocorticoid receptor genes have been reported in the offspring of Wistar rats fed a protein- restricted diet (*i.e.* 9%) during pregnancy (Lillycrop *et al.*, 2005). Hypomethylation of these genes, which was associated with their increased expression in the offspring liver, was found to be reversed when the mothers were fed a protein-restricted diet supplemented with folic acid (Lillycrop *et al.*, 2005).

Despite the variation in the timing and extent of the 'programming stimulus' as well as the species and strain differences, the 'caloric-restriction' and 'low-protein maternal diet' studies have proved invaluable in establishing the role of maternal under-nutrition as well as the underlying molecular mechanisms in the fetal origins of adult disease. However, these studies have limited relevance to Western populations, where the abundance of 'energy dense' nutrition has been implicated as one of the major causes behind the onset of CVD (Cordain *et al.*, 2005; Lichtenstein AH *et al.*, 1998). As described earlier, dietary patterns are changing across the world. Most countries in Asia, Latin America, North Africa, the Middle East and the urban areas of sub-Saharan Africa have experienced a shift in the overall structure of their dietary patterns over the last few decades. With an increased consumption of fat and sugar combined with a fall in total

cereal and fiber intake, an inexorable shift towards the higher-fat Western diet has occurred, which is reflected in a large proportion of the population consuming over 30% of energy from fat (Popkin, 1999; 2001; Popkin and Gordon-Larsen, 2004). Inevitably, these changes in the everyday dietary pattern may be associated with an increased consumption of dietary fat during pregnancy.

1.4 DIETARY FATS AND FETAL ORIGINS HYPOTHESIS

High-fat diets are generally not related to fetal growth retardation. However considering their abundance in the 'everyday diet' worldwide, as well as their association with the onset of CVD, it is paramount to question their role in the fetal origins of adult CVD. To my knowledge, none of the epidemiological studies have investigated the role of dietary fat consumption during pregnancy on outcomes of CVD in the offspring. Some clinical studies, however, have investigated the role of maternal dietary excess during pregnancy and its impact on cardiovascular function in the offspring. In a post-mortem study of the Italian population, Napoli *et al.* reported that fetuses and children born to hypercholesterolemic mothers exhibited enhanced aortic fatty streak formation, compared to the offspring from normocholesterolemic mothers (Napoli *et al.*, 1997). A number of studies conducted on Pima Indians have further suggested that maternal diabetes during pregnancy may likely program a predisposition to adult insulin resistance in their children (Dabelea *et al.*, 2000; Dabelea and Pettitt, 2001; Schaefer-Graf *et al.*, 2005). Moreover, maternal hyperglycemia has been shown to cause fetal hyperinsulinemia and

fat deposition (Dorner, 1994; Hillier *et al.*, 2007), whereas substantial evidence suggests that offspring of obese women are at a greater risk of developing metabolic disorders (Boney *et al.*, 2005; Schaefer-Graf *et al.*, 2005). Lack of epidemiological data concerning the maternal dietary fat consumption during pregnancy and its impact on the burden of CVD points towards a need to conduct more of such studies in the future, especially considering that dietary fat consumption during pregnancy is on the rise.

1.4.1 Dietary fat consumption and fetal origins of CVD: Animal Studies

Despite the lack of epidemiological data, a number of animal studies have modeled an energy-rich '*in utero*' environment where excess calories are obtained from dietary fats. These studies have provided the current understanding of the dietary fat-mediated programming of adult CVD and related conditions such as dyslipidemia, endothelial dysfunction, hypertension, obesity, insulin resistance and type 2 DM.

1.4.1.1 Dietary fats and fetal origins of dyslipidemia

Dyslipidemia is a term reflecting abnormal lipid levels that are known to increase the risk of developing CVD in humans. The guidelines for the clinical diagnosis of dyslipidemia, based on NCEP-ATPIII are given in Table 1.2. Targeting dyslipidemia has been considered as an important part of the preventive strategy against CVD (Thompson, 2004). Use of pharmacological means to improve blood lipid levels has proved beneficial

in the reduction of CVD risk (Heart Protection Study Collaborative Group, 2002; Cannon *et al.*, 2004). In the West of Scotland Heart Study, use of pravastatin resulted in 20% reduction in plasma cholesterol levels, which was associated with a 30% reduction in mortality and morbidity due to CVD (Shepherd *et al.*, 1995). The recent 'Targets of New Therapy' trial exhibited an additional ~35% decrease in the level of low-density lipoprotein (LDL)-cholesterol, and a proportionate reduction in cardiac event rate in a cohort of CVD patients with multiple risk factors, when treated aggressively with atorvastatin (80 mg/day) as opposed to a moderate treatment with 10 mg/day (Waters *et al.*, 2004). However, besides the pharmacological means, nutritional factors, including dietary fats, are well known to modulate the outcome of dyslipidemia.

Increased intake of SFA has been shown to increase plasma triglycerides (TG), total-cholesterol, LDL-cholesterol, and to reduce the levels of high-density lipoprotein (HDL)-cholesterol, all of which are independently known to increase the risk of developing CVD (reviewed in Mensink, 1993 and Hu *et al.*, 2001). Omega-3 (***n-3***) polyunsaturated fatty acids (PUFA) intake is known to reduce plasma TG levels effectively (reviewed in Russo, 2009). However, data concerning the effects of ***n-3*** PUFA consumption on plasma LDL-cholesterol remain controversial. Fish oil supplementation containing 6 g/day of ***n-3*** fatty acids eicosapentaenoic acid (EPA, 20:5 ***n-3***) and docosahexaenoic acid (DHA, 22:6 ***n-3***) has been reported to increase LDL-cholesterol (Connor *et al.*, 1993), whereas large amounts of fish oil could lower LDL-cholesterol levels (Harris *et al.*, 1983). Both fish oil and the replacement of SFA in the diet have been shown to lower plasma LDL-cholesterol levels (reviewed in Harris, 1997). Opposite

to *n-3* PUFA, higher omega-6 (*n-6*) PUFA intake, especially diets rich in linoleic acid (LA, 18:2 *n-6*), has been associated with a reduction in serum cholesterol and TG levels, thus promoting LA as one of the most effective fatty acids in the prevention of dyslipidemia (reviewed in Hayes, 2000 and Mensink *et al.*, 2003).

Similar to the adult life, fetal exposure to high-fat diets during intrauterine and neonatal life has been shown to induce dyslipidemia during adulthood. A lard-rich diet fed to the mothers during pregnancy and suckling was associated with reduced HDL-cholesterol, and raised plasma TG levels, in 160-day-old and 1-year-old male and female offspring of Sprague-Dawley rats (Ghosh *et al.*, 2001; Khan *et al.*, 2003). Another study involving diet-induced hypercholesterolemic pregnant rabbits, however reported normal plasma cholesterol levels, despite the presence of aortic lesions, in the offspring (Palinski *et al.*, 2001). In contrast, a continuous exposure to *n-3* PUFA-rich diet during pre- and post-weaning time periods was associated with lower plasma cholesterol levels in the 2-month-old and 3-month-old macrosomic male offspring of diabetic Wistar rats (Yessoufou *et al.*, 2006). In addition, a recent study reported lower body weight, lower SBP, lower LDL- and higher HDL-cholesterol concentrations in the offspring of hypercholesterolemic C57 mice mothers, treated with pravastatin during late pregnancy, compared to the offspring obtained from hypercholesterolemic mothers (Elahi *et al.*,

Table 1.2 Diagnostic criteria of dyslipidemia by ATPIII

Component	WHO criteria (Insulin resistance* plus two of the following)	ATP III criteria (Three of the following)
Abdominal/central obesity	Waist to hip ratio: > 0.90 (men), > 0.85 (women), or BMI > 30 kg/m^2	Waist circumference: >102 cm in men, > 88 cm in women
Hypertriglyceridemia	>= 150 mg/dL (>= 1.7 mM)	>= 150 mg/dL
Low HDL cholesterol	< 35 mg/dL (< 0.9 mM) for men, < 39 mg/dL (<1.0 mM) for women	< 40 mg/dL (<1.036 mM) for men, <50 mg/dL (<1.295 mM) for women
High blood pressure	>= 140/90 mm Hg or documented use of antihypertensive therapy	>= 130/85 mm Hg or documented use of antihypertensive therapy
High fasting glucose	Impaired glucose tolerance, impaired fasting glucose, insulin resistance, or diabetes	>= 110 mg/dL (>= 6.1 mM)
Microalbuminuria	Urinary albumin to creatinine ratio: 30 mg/g, or albumin excretion rate: 20 mcg/min	

(Expert Panel, 2001) (Thompson, 2004)

ATP III = Adult Treatment Panel III, LDL = Low-density lipoprotein, HDL = high-density lipoprotein, TG= triglycerides

2008). This study indicates that overt dyslipidemia during gestation has the potential to program the onset of adult CVD in the offspring, which can be prevented using the nutritional and drug-based interventions.

Overall, the available data indicate that maternal intake of dietary SFA during gestation and lactation may promote, whereas maternal intake of dietary *n-3* PUFA may prevent, the outcome of dyslipidemia in the offspring. None of the studies have however, looked at the effects of a maternal diet rich in *n-6* PUFA on the regulation of lipid metabolism in the offspring. Considering that LA is very effective in lowering plasma lipid levels, it would be relevant to investigate the effects of a maternal diet rich in *n-6* PUFA on the outcome of dylipidemia in the offspring.

1.4.1.2 Dietary fats and fetal origins of endothelial dysfunction

Vascular endothelium plays a central role in maintaining cardiovascular homeostasis in the body. Through the synthesis and release of vasoactive agents such as nitric oxide (NO), endothelial derived hyperpolarizing factor (EDHF), endothelin-1 and thromboxane A_2, the endothelium is known to regulate the tone of underlying vascular smooth muscle cells (VSMC) thereby contributing to the regulation of blood flow (Forgione *et al.*, 2000). In addition, through the expression of various adhesion molecules such as vascular cell adhesion molecule-1 and P-selectin, endothelial cells are capable of controlling leukocyte and platelet adhesion to the vessel wall that plays an important role in atherosclerotic plaque formation. As a result, endothelial cell dysfunction has been

implicated in a number of conditions including essential hypertension (Perticone *et al.*, 2001), insulin resistance, type 2 DM (Hsueh and Quiñones, 2003) and in the etiology of atherosclerosis (Bonetti *et al.*, 2003). Its assessment in coronary arteries in man is now recognized as a predictive index of CVD (Halcox *et al.*, 2002).

A number of epidemiological studies have related SFA consumption with endothelial dysfunction (de Roos *et al.*, 2001; Fuentes *et al.*, 2001; Keogh *et al.*, 2005). It has been proposed that the SFA-induced increase in LDL-cholesterol may lead to endothelial dysfunction, as both *in vitro* and *in vivo* studies have shown that endothelial function may be abnormal within a few hours of exposure to increased levels of LDL-cholesterol (Jayakody *et al.*, 1987; Cohen *et al.*, 1988; Tamai *et al.*, 1997). In addition, low HDL-cholesterol concentration has been associated with a reduction in flow-mediated dilatation in healthy young men (Toikka *et al.*, 1999). Although LA consumption has been reported to have beneficial effects on plasma LDL- cholesterol levels, it has been associated with endothelial cell dysfunction (Turpeinen *et al.*, 1999). LA is proposed to enhance oxidative modification of LDL-cholesterol (Reaven *et al.*, 1991; Abbey *et al.*, 1993). In addition, LA-mediated increase in oxidative stress of endothelial cells may lead to the activation of certain stress-responsive transcription factors, inflammatory cytokine production, and the expression of adhesion molecules (Ascherio *et al.*, 1996). An increased consumption of *n*-6 PUFA is also known to result in the accumulation of arachidonic acid (AA, 20:4 *n-6*), which can increase the production of prothrombotic eicosanoids and thromboxane A_2, known to promote platelet aggregation and vasoconstriction (Renaud, 1990). In contrast to LA, α-linolenic acid

(ALA, 18:3 *n-3*) is known to improve endothelial dysfunction (Ros *et al.*, 2004; Zhao *et al.*, 2004). ALA is the precursor for EPA and DHA, which have also been shown to increase endothelium-dependent relaxation *in vitro* (Okuda *et al.*, 1997; Omura *et al.*, 2001). Moreover, *n*-3 PUFA have also been shown to mediate anti-inflammatory effects by decreasing the expression of adhesion molecules and cytokines, thus affecting leucocyte–endothelium interactions (reviewed in Das, 2000). EPA and DHA are also known to compete with arachidonic acid (AA, 20:4 *n-6*) for the use of cyclooxygenases, enzymes involved in the production of eicosanoids. The eicosanoids derived from *n-3* PUFA are known to be anti-thrombotic and anti-inflammatory in nature such as prostacyclins, which are known to promote vasodilation and reduce platelet aggregation, thereby also reducing plaque formation (reviewed in Kang and Leaf, 1996).

To my knowledge, epidemiological studies have not identified the role of maternal dietary fat consumption during pregnancy in the etiology of adult vascular dysfunction. However, several animal studies have suggested that endothelial dysfunction can be programmed '*in utero*' by high-fat maternal diet. Reduced endothelium-dependent dilation has been reported in small mesenteric vessels of the adult offspring born to Sprague-Dawley rats fed a lard-rich diet during pregnancy (Ghosh *et al.*, 2001; Khan *et al.*, 2003). Another study reported endothelial dysfunction in the adult offspring of Sprague-Dawley rats exposed to a lard-rich diet during lactation (Khan *et al.*, 2005). Besides endothelial dysfunction, prenatal exposure to a high-fat diet rich in SFA was shown to reduce aortic distensibility, which was further associated with reduced

endothelial cell volume and VSMC number in the aortas of Sprague-Dawley rat offspring as compared to the controls (Armitage *et al.*, 2005).

Available data thus far indicates that SFA consumption by mothers can induce endothelial dysfunction in the offspring. Considering that *n-3* PUFA has been attributed beneficial effects, while *n-6* PUFA intake has been associated with deleterious effects on endothelial function, it would be important to delineate the effects of maternal diets rich in *n-3* and *n-6* PUFA consumed during pregnancy, on the outcome of endothelial function in the offspring.

1.4.1.3 Dietary fats and fetal origins of hypertension

Hypertension is a major risk factor for the development of CVD. An age-specific increase of 20 mmHg in SBP and/or 10 mmHg in diastolic blood pressure (DBP) above the population average more than doubles the risk of CVD (Lewington *et al.*, 2002). According to the International Society of Hypertension guidelines for the management of hypertension, a SBP of 140 mmHg or greater and a DBP of 90 mmHg or greater is identified as hypertension in humans (Whitworth *et al.*, 2003). The degree of hypertension is subsequently divided into three grades. Grade I hypertension is defined as SBP of 140-159 mmHg, or DBP of 90-99 mmHg; Grade II is a SBP of 160-179 mmHg or a DBP of 100-109 mmHg; Grade III is a SBP of greater than 180 mmHg or DBP of greater than 110 mmHg. Risk is then stratified based on other cardiovascular risk factors, target organ damage, and associated clinical conditions (Whitworth *et al.*, 2003).

Increased consumption of dietary SFA has been reported to be associated with a progressive increase in SBP in both human and animal studies (Zheng *et al.*, 1999; Tamaya-Mori *et al.*, 2002), whereas the data regarding *n-6* PUFA consumption on the onset of hypertension are rather inconsistent (reviewed in Pietinen, 1994). An inverse relationship between LA intake and blood pressure has been reported previously (Miura *et al.*, 2008). Moreover, LA intake was associated with reduced SBP and lowered prevalence of blood pressure in white men (Djoussé *et al.*, 2005). However, a number of other studies have demonstrated no relationship between dietary *n-6* PUFA intake and blood pressure (Berry and Hirsch, 1986; Brussaard *et al.*, 1981; Marget *et al.*, 1984). EPA and DHA consumption, on the other hand, have been reported to lower blood pressure in both human (Morris, 1994) and rat studies (Frenoux *et al.*, 2001; Rousseau *et al.*, 2003). DHA supplementation of spontaneously hypertensive rats (SHR) for 6 weeks resulted in SBP measurements of 34 mmHg less than the normotensive Wistar Kyoto rats (Frenoux *et al.*, 2001). Similarly EPA and DHA supplementation of a rat model of hyperinsulinemia resulted in delayed onset of hypertension (Rousseau *et al.*, 2003). A number of meta-analyses have also reported beneficial effects for *n-3* PUFA supplementation in lowering blood pressure of patients with untreated hypertension (Appel *et al.*, 1993; Morris *et al.*, 1993; Morris, 1994).

The role of maternal dietary fat consumption during pregnancy on the onset of hypertension in adult offspring has been addressed in animal studies. A diet rich in SFA fed to the mothers during gestation and lactation was associated with higher SBP in 7-week-old rat offspring (Langley Evans *et al.*, 1996). Similarly, feeding animal lard to the

mothers during gestation and lactation was associated with higher SBP and DBP in 6- and 12-month-old female offspring of Sprague-Dawley rats, but not in the male offspring, which instead were reported to have lower heart rate (Khan *et al.*, 2003). Another study reported higher SBP in the adult offspring exposed to a diet rich in SFA during suckling alone, indicating the importance of nutrition both during gestation and lactation on the programming of hypertension in the adult offspring (Khan *et al.*, 2005). As opposed to SFA, maternal diet rich in *n-6* PUFA has been associated with lower blood pressure in the Sprague-Dawley rat offspring (Jensen *et al.*, 2004). Furthermore, a maternal diet rich in *n*-6 PUFA fed during gestation and lactation was associated with higher renal Na^+, K^+-ATPase activity in the offspring compared to a maternal diet rich in SFA, which may play a role in the regulation of blood pressure in these offspring (Armitage *et al.*, 2005; Armitage *et al.*, 2008a). Moreover, *n-3* PUFA deficiency during perinatal time period has been associated with higher mean arterial pressure in offspring of Sprague-Dawley rats (Weisinger *et al.*, 2001; Armitage *et al.*, 2003).

Overall, the available data from animal studies indicate that dietary SFA intake by mothers during pregnancy may increase, while both *n-6* and *n-3* PUFA intake may reduce the risk of developing hypertension by the offspring. More studies should be conducted in this area to confirm the role played by various dietary fats in the fetal programming of hypertension.

1.4.1.4 Dietary fats and fetal origins of obesity

Obesity is a highly complex disease defined as the accumulation of excess fat caused by an imbalance between energy intake and energy expenditure. Clinical determination of obesity is made based on the body mass index (BMI) and in terms of fat distribution *via* the waist to hip ratio. According to the WHO, a BMI of 30 kg/m^2 or greater is identified as obese. BMI is a measure of total body fat, while waist circumference (> 102 cm in men and > 88 cm in women) and waist to hip ratio (the circumference of the waist divided by that of the hips; > 0.9 for men and > 0.85 for women) are both used as measures of central obesity. Compared to the BMI, however, central obesity is considered to be a stronger predictor of the onset of CVD (Yusuf *et al.*, 2004). Obesity is fundamentally a disorder of energy balance in which energy intake exceeds energy expenditure, thus pathways involved in the regulation of appetite and food preferences play an important role in the development of obesity. It is well established that the control of appetite is mediated by a collection of hypothalamic neuropeptides that are principally expressed in the arcuate nucleus (ARC) of the hypothalamus. The neurons that express these neuropeptides have extensive projections into other hypothalamic nuclei including the dorsomedial hypothalamus (DMH), lateral hypothalamus (LH) and paraventricular nucleus (PVN), where further appetite regulating neurons are recruited. Neuropeptide Y (NPY) and agouti-related protein (AgRP) are among the orexigenic peptides that are known to increase food intake, while pro-opiomelanocortin (POMC)-derived α-melanocyte-stimulating hormone (MCH) and cocain-amphetamine regulated transcript (CART) are well-known anorexigenic peptides (reviewed in Kalra *et al.*, 1999).

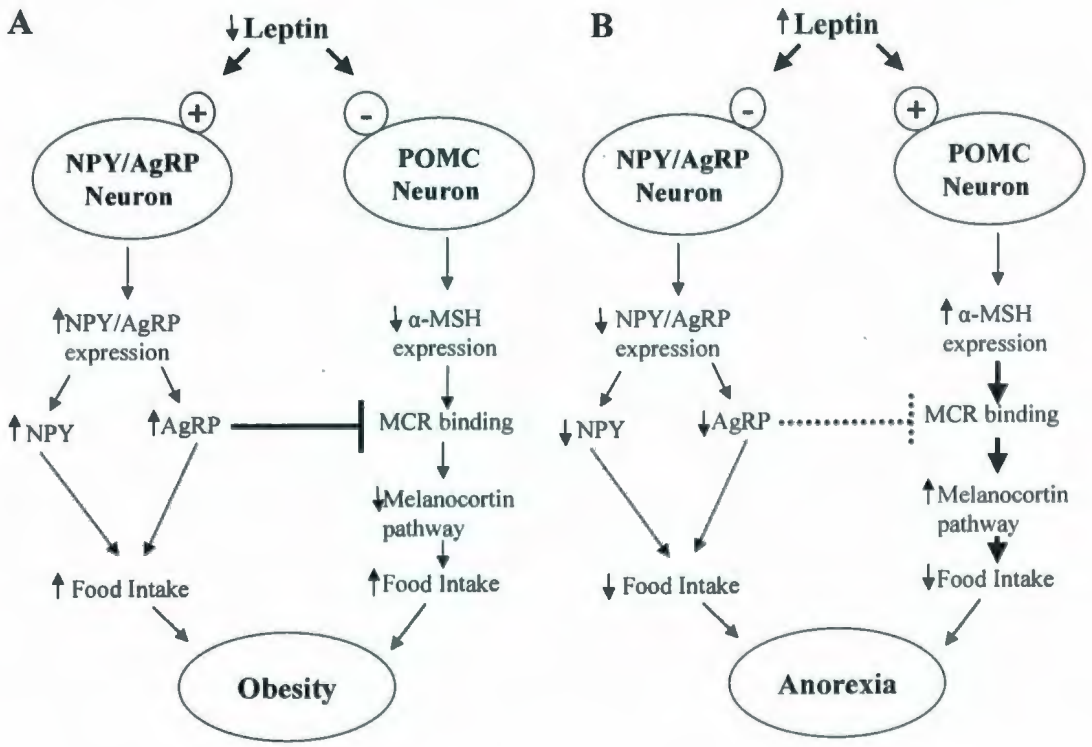

Fig. 1.1. Control of food intake under conditions of (A) low and (B) high levels of leptin

Modified from Schwartz *et al.*, 2000

NPY = neuropeptide Y, AgRP = Agouti-related protein, POMC = pro-opio melanocorticotrophin, α-MSH = α- melanocyte-stimulating hormone, MCR = melanocortin receptors

An increase in the mRNA expression of NPY has been reported in response to food restriction and fasting (Schwart *et al.,* 1998), whereas a downregulation of its mRNA expression has been reported in response to signals of increased energy stores *i.e.* leptin, glucose and insulin (Dallman *et al.,* 1995; Elias *et al.,* 1999). In contrast, high-energy exposure has been associated with an up-regulation of POMC mRNA expression (la Fleur *et al.,* 2010), while a down-regulation of its mRNA expression has been reported during periods of fasting and food restriction (Mizuno *et al.,* 1999). Leptin is well-known to regulate the expression of these neuropeptides, thus regulating the food intake in adults. The long form of leptin-receptor (OBRb) is expressed on NPY/AgRP and POMC/CART neurons within the hypothalamus, and it has been demonstrated that central (Wang *et al.,* 1997) or peripheral administration (Stephens *et al.,* 1995) of leptin decreases NPY/AgRP gene expression and increases POMC/CART mRNA expression within the hypothalamic neurons (Cheung *et al.,* 1997) (Fig.1.1).

SFA consumption has been associated with increased body weight as well as increased adiposity (reviewed in Bray and Popkin, 1998). Similarly, increased *n-6* PUFA intake has been argued to be associated with the development of obesity (reviewed in Ailhaud *et al.*, 2008). On the other hand, literature suggests a beneficial role for *n-3* PUFA supplementation in reducing obesity both in humans and animal models (Couet *et al.*, 1997; Flachs *et al.*, 2005). Dietary fat intake has also been reported to affect the expression of various neuropeptides involved in the regulation of food intake and energy expenditure. One-week feeding of high-fat diets rich in SFA were associated with lower mRNA expression of NPY and AgRP in the ARC of C57Bl/6 mice, compared to diets

rich in *n-3* and *n-6* PUFA. Moreover, continued feeding of SFA-rich diets until 7-weeks resulted in higher plasma leptin concentrations in these mice compared to the diets rich in *n-3* and *n-6* PUFA (Wang *et al.,* 2002). Another study reported hyperphagia, weight gain and a reduction in the mRNA expression of POMC in the ARC of Wistar rats fed SFA-rich diets for a period of 6-weeks, compared to the diets rich in *n-3* and *n-6* PUFA (Dredzeic *et al.,* 2007).

Animal studies have presented strong evidence for programming the hypothalamic control of food intake resulting in offspring obesity by a high-fat maternal diet. An exaggerated feeding response (maximum sensitivity to lowest dose of NPY) to injections of NPY in to the lateral brain ventricle was reported in the 6-month-old offspring of Long-Evans rats fed a diet rich in SFA during gestation and lactation. This hyperphagia however was not associated with any changes in the hypothalamic NPY concentrations (Kozak *et al.,* 2000). Another study reported reduced expression of NPY, and increased expression of POMC, in the hypothalamus of male pups at postnatal day 20, born to the Sprague-Dawley rats fed a high fat-cafeteria style diet 5 weeks before and then during gestation (Chen *et al.,* 2008). These changes were associated with reduced plasma leptin levels in one-day-old male and female pups of high-fat fed Wistar rats studied during a subsequent study (Morris and Chen, 2009). In contrast, hyerleptinemia and hypothalamic leptin resistance were reported in the 6-week-old male and female offspring of Wistar rats fed high-fat diets during gestation and lactation (Férézou-Viala *et al.,* 2007). Lack of leptin during early life in the leptin-deficient mouse has been shown to compromise the neuronal organization of hypothalamic nuclei involved in the control of

food intake, which is restored by administering leptin during the neonatal period. However, when the exogenous leptin was administered to adult *ob/ob* mice, it did not improve the neuronal projections, suggesting that leptin plays a very important role in the development of appetitive structures that occurs during critical windows of development (Bouret *et al.*, 2004).

In addition to the hypothalamic changes, SFA feeding during pregnancy has been shown to increase body weight and fat composition in the adult Sprague-Dawley rat offspring (Khan *et al.*, 2003; Khan *et al.*, 2005). Moreover, a continuous exposure to animal lard during gestation and lactation was associated with increased adiposity and visceral fat depots in one-year-old offspring of Sprague-Dawley rats, even when they were fed a control diet post-weaning (Khan *et al.*, 2004). Frank obesity was observed in these offspring when they continued on animal lard post-weaning, indicating an effect of interaction between prenatal and postnatal environments (Khan *et al.*, 2004).

Although the role of high SFA consumption has been studied, to my knowledge, none of the animal studies have investigated the effects of high maternal PUFA consumption (*n-6* or *n-3*) during pregnancy on offspring obesity. Some epidemiological studies have related *n-3* PUFA intake during pregnancy (Oken *et al.*, 2004) and umbilical cord blood EPA and DHA content to reduced fetal growth (Rump *et al.*, 2001). However, whether this reduced fetal growth is associated with offspring obesity remains unknown. Considering that an obesity epidemic is sweeping the world's population, it would be important to investigate the effects of maternal diet rich in various fatty acids on the development of obesity in the offspring.

1.4.1.5 Dietary fats and fetal origins of insulin resistance and Type 2DM

Subjects with type 2 DM have two major defects: *1*) insulin resistance in skeletal muscle and liver and *2*) impaired β-cell function. Insulin resistance occurs early in the natural history of type 2 DM which is defined as the body's inability to properly respond to insulin action. This results in a concomitant increase in blood glucose concentration but is compensated by increased β-cell secretion of insulin. Insulin is involved in many metabolic pathways, thus the body's ability to promote glucose disposal, regulate lipogenesis and fatty acid oxidation also become impaired under the conditions of insulin resistance, resulting in a progressive metabolic failure. When β-cell failure ensues, hyperinsulinemia no longer can compensate for insulin resistance, and glucose homeostasis deteriorates. Initially, this manifests as impaired glucose tolerance (IGT), which eventually progresses to overt diabetes (reviewed in Defronzo, 2004). According to WHO, clinical diagnosis of diabetes can be made if the fasting plasma glucose ≥ 7.0 mM (126 mg/dL) or if the plasma glucose values are ≥ 11.1 mM (200 mg/dL), two hours after the oral dose of 75g glucose during a glucose tolerance test. Both insulin resistance and type 2 DM are very strongly correlated with the increased prevalence of CVD (WHO/International Diabetes Federation, 2006).

Dietary fat is well known to be involved, although not causally, in insulin resistance. The effects of dietary fats on insulin action are highly complex and not well understood. However, the majority of studies, both epidemiological as well as experimental, support the notion that SFA consumption worsens insulin sensitivity (reviewed in Riccardi *et al.*, 2004). Consumption of *n-6* PUFA does not entirely have a

positive effect on insulin action (reviewed in Rivellese *et al.*, 2002), whereas *n-3* PUFA, especially EPA and DHA have consistently been shown to improve insulin sensitivity in humans and in various animal models (reviewed in Olalla *et al.*, 2009).

Hyperglycemia has been reported in the offspring of Sprague-Dawley rats fed a diet rich in SFA during pregnancy (Khan *et al.*, 2003). Moreover, a continuous exposure to animal lard during gestation and lactation was associated with whole body insulin resistance and pancreatic β-cell dysfunction in the 1-year-old offspring of Sprague-Dawley rats (Taylor *et al.*, 2005). Another study comparing the role of SFA *vs. n-3* PUFA reported increased insulin secretory response to an oral glucose load in the adult offspring of rats fed a SFA-rich diet during pregnancy. On the other hand, glucose tolerance was normal in the offspring born to rats fed an *n-3* PUFA-rich diet (Siemelink *et al.*, 2002). Morphological studies on the pancreatic tissue of these offspring revealed that the SFA-rich diet inhibited islet development resulting in lower number of islet cells, whereas the *n-3* PUFA-rich diet resulted in higher number of pancreatic islet cells (Siemelink *et al.*, 2002). Future studies would be required to investigate the effects of an *n-6* PUFA-rich maternal diet consumed during pregnancy on the development of insulin resistance in the offspring.

Taken together, the available evidence indicates the importance of maternal dietary fat consumption in the 'fetal origins' of various conditions associated with the onset of metabolic syndrome and adult CVD. However, the mechanisms by which dietary fat intake results in the 'fetal programming' of adult CVD are not entirely established. Moreover, the role of interaction between the maternal dietary fat consumption and the

postnatal diets of the offspring, on the risk of developing CVD in the offspring, remains to be understood. Dietary fat intake is known to directly affect the regulation of lipid and lipoprotein metabolism, thus regulation of 'fetal lipid metabolism' by maternal dietary fat intake during pregnancy could be one plausible mechanism underlying the 'programming' of adult metabolic disorders in offspring. The different pathways involved in dietary fat-mediated regulation of lipid and lipoprotein metabolism, and their association with the onset of metabolic syndrome and CVD is described in the next section. This understanding may help in the identification of the potential players/pathways that may operate during the fetal developmental period leading to the 'programming' of adult CVD.

1.5 DIETARY FAT-MEDIATED REGULATION OF LIPID METABOLISM

1.5.1 Dietary fats and lipoprotein metabolism

Once ingested, dietary fats are released from the intestine in the form of chylomicrons into the circulation and are acted upon by lipoprotein lipase (LPL), converting them into chylomicron-remnants, which are then taken up by the liver. Fatty acids released as a result of LPL action are taken up by adipose tissue and/or muscle for utilization or storage as TG. Liver is capable of *de novo* lipogenesis, synthesis of complex lipids such as phospholipids, and oxidation of fatty acids for the production of energy. In addition,

fatty acids and cholesterol can be packaged as very-low density lipoprotein (VLDL) in the liver, which are then released into the circulation. Once acted upon by LPL, VLDL is converted to LDL, which can be cleared from the circulation with the help of LDL-receptors (LDL-r) present on the membrane of liver cells. In addition, the liver can also secrete intermediate density lipoproteins (IDL), which are also converted to LDL in the circulation. HDL, on the other hand, is involved in the reverse transport of cholesterol to the liver. HDL extracts excess cholesterol from the macrophages and peripheral tissues with the help of ATP-binding cassette transporter protein-1 (ABCA-1) and brings it back to the liver *via* scavenger receptor-B1 (SR-B1) for secretion into bile acids or for the synthesis of steroids (reviewed in Rader and Daugherty, 2008). An overview of various pathways involved in lipid and lipoprotein metabolism is given in Fig. 1.2. Deviations in these pathways may lead to various metabolic abnormalities associated with the onset of CVD.

1.5.2 Abnormal lipid metabolism and onset of CVD

Accumulation of TG and cholesterol, especially LDL-cholesterol, with a reduction in HDL-cholesterol in the blood plasma is identified as dyslipidemia, a well-known risk factor for the development of CVD. On the other hand, accumulation of TG and cholesterol in the liver causes fatty liver leading to the onset of non-alcoholic fatty liver disease (NAFLD), which can further increase the risk of developing CVD (reviewed in Kotronen *et al.*, 2007). Excess accumulation of lipids in adipose tissue causes obesity,

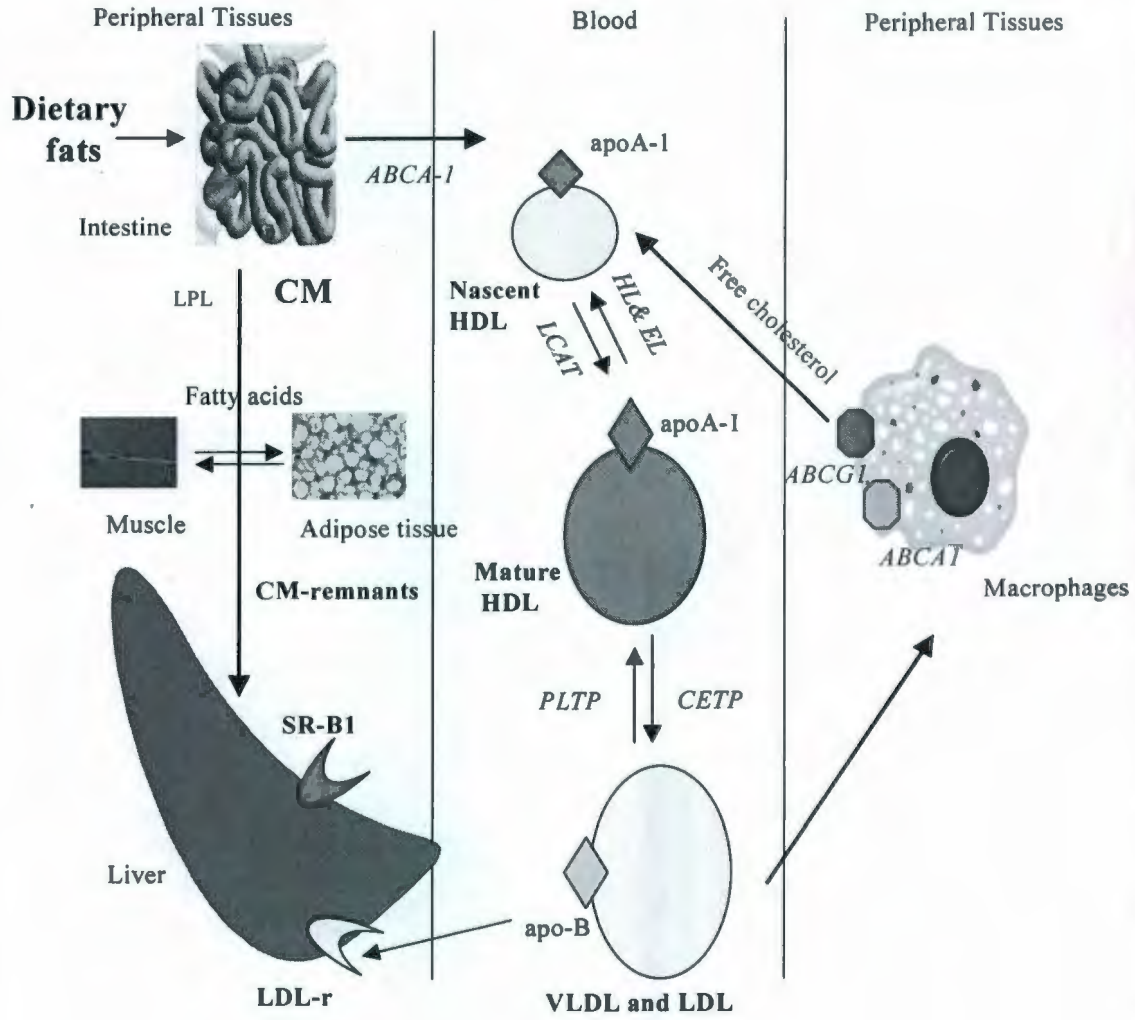

Fig.1.2 Lipid and lipoprotein metabolism

Modified from Rader and Daugherty, 2008

ABCA-1 = ATP-binding cassette transporter protein-1, Apo AI = apolipoprotein receptor AI, CETP = cholesterol ester transfer protein, CM= chylomicrons, EL= endothelial lipase, HL= hepatic lipase, SR-B1 = scavenger receptor-1, HDL= high-density lipoprotein, LPL = lipoprotein lipase, LCAT= lecithin: cholesterol acyltransferase, LDL= low-density lipoprotein, LDL-r = low-density lipoprotein receptor, PLTP= phospholipids transfer protein, VLDL = very low-density lipoprotein.

whereas intramuscular TG accumulation has been associated with insulin resistance leading to the onset of type 2DM and CVD (Phillips *et al.*, 1996; Pan *et al.*, 1997). Besides TG and cholesterol, by-products of lipid metabolism such as free fatty acids (FFA) can also play an important role in the development of various conditions associated with the onset of metabolic syndrome and CVD. Increased circulating levels of FFA may impair insulin action on various tissues such as adipose tissue, muscle and liver leading to insulin resistance. Resistance to insulin action results in the reduction of glucose uptake by skeletal muscle and adipose tissue, leading to increased levels of circulating glucose (reviewed in Le Marchand-Brustel *et al.*, 2003). In an effort to compensate for this peripheral insulin resistance, pancreatic β-cells increase basal and postprandial insulin secretion to maintain euglycemia. However, at some point, pancreatic β-cell failure occurs leading to the onset of type 2DM (reviewed in Saltiel, 2000). In addition, high concentrations of circulating FFA have been associated with visceral obesity (reviewed in Jensen *et al.*, 1989). A recent study showed that in a population of people with type 2DM, increase in BMI was related with higher serum insulin and glucose concentrations, which were further related with higher rates of cholesterol synthesis and turnover (Simonen *et al.*, 2002). Insulin resistance in adipose tissue results in the promotion of lipolysis, adding to the pool of circulating FFA. Increased levels of circulating glucose, insulin and FFA lead to an up regulation of lipogenesis in the liver, whereas impaired fat oxidation stimulates fatty acid esterification resulting in increased TG production, which may lead to the development of fatty liver. In addition, augmented synthesis of apolipoprotein (apo) B-100 and cholesterol results in

increased production and secretion of VLDL from the liver, which also causes an increase in circulating IDL and LDL levels resulting in dyslipidemia (reviewed in Ritchie and Connell, 2007).

Increased levels of circulating LDL-cholesterol can also impair endothelial function (Tamai *et al.*, 1997). In addition, defects in insulin signaling can affect endothelial cell function resulting in reduced vasorelaxation and increased vasoconstriction (reviewed in Ahmed and Goldstein, 2008). Insulin can regulate endothelial NO production *via* the phosphoinositide-3 kinase (PI-3 kinase) and Akt pathway, thereby affecting endothelial function (Wheatcroft *et al.*, 2004). Impaired endothelial function may predispose the individual to the onset of hypertension on one hand (reviewed in Taddei and Salvetti, 2002), and to the development of atheromas on the other, overall increasing the risk of developing CVD (reviewed in Bonetti *et al.*, 2003).

Taken together, the fine balance between various pathways involved in lipid metabolism plays a major role in regulating the onset of metabolic syndrome and CVD. Dietary fats are capable of modulating this balance by regulating a variety of transcription factors, which can further regulate the expression of various genes involved in these pathways, at the molecular level.

1.5.3 Dietary fat-mediated regulation of gene expression

PPAR's and sterol-regulatory element binding protein (SREBP's) are among the principal transcription factors that are known to regulate the expression of genes involved in lipid metabolism.

1.5.3.1 Dietary fats and PPAR's

PPAR's represent a family of nuclear receptors which is comprised of three subtypes, namely PPAR-α, β and γ. PPAR-α is mainly expressed in liver, kidney and heart; PPAR-β is expressed ubiquitously, whereas PPAR-γ is mainly expressed by adipose tissue, spleen and mammary glands (Braissant *et al.*, 1996). Upon ligand activation, PPAR's regulate transcription by dimerizing with the retinoid X receptor (RXR)'s and then binding to the PPAR response element (PPRE) within the regulatory regions of the target genes. The regulation of the PPAR's transcriptional pathway can occur at the levels of receptor expression and stability, post-translational modifications, ligand specificity and cofactor recruitment (reviewed in Desvergne and Wahli, 1999).

Fatty acids and fatty acid-derived compounds, such as prostaglandins and eicosanoids, are natural ligands of PPAR's (reviewed in Barbier *et al.*, 2002). In addition, hypolipidemic drugs such as fibrates and glitazones are synthetic agonists of PPAR-γ and PPAR-α, respectively (reviewed in Lehmann *et al.*, 1995 and Willson *et al.*, 2000). PPAR-α activation leads to fatty acid uptake and catabolism through upregulation of genes involved in fatty acid transport, peroxisomal proliferation and β-oxidation of fatty acids, thereby resulting in reduced TG and VLDL production by the liver (Guerre-Millo

et al., 2000). PPAR-α can also induce the expression of LPL in the liver along with inhibiting the expression of apo-CIII, which is an LPL activity and remnant lipoprotein catabolism inhibitor. These effects promote lipolysis and TG-rich lipoprotein catabolism, thus reducing plasma TG levels (reviewed in Hertz *et al.*, 1995). PPAR-α is also known to upregulate the hepatic expression of LXR-α, which subsequently upregulates the expression of cholesterol 7 alpha-hydroxylase, a rate-limiting enzyme in the conversion of cholesterol to bile acids, thereby promoting the lowering of plasma cholesterol levels (Chiang *et al.*, 2001).

Activation of PPAR-γ increases adipose tissue LPL activity leading to higher fatty acid delivery and storage of TG in adipose tissue, thereby resulting in reduced levels of FFA in the plasma and increased insulin sensitivity (Way *et al.*, 2001). In addition, all three PPAR isoforms are known to activate the expression of ABCA-1 resulting in the increased efflux of cholesterol from peripheral tissues (Chinetti *et al.*, 2001). Apo-AI, the principal apolipoprotein of HDL, is also activated by PPAR's, leading to an increase in plasma HDL concentration (Vu-Dac *et al.*, 1995). Recently, Boulay *et al.* have also shown that PPAR agonists induce the expression of phospholipid transfer protein (PLTP), an enzyme catalyzing the transfer of phospholipids from VLDL/LDL to HDL, in mice, leading to marked enlargement of HDL particles in the fibrate treated mice (Bouly *et al.*, 2001).

In general, all three PPAR isoforms bind saturated, monounsaturated and polyunsaturated fatty acids. However, their affinities for the receptor vary, which suggests a role for site-specific availability and metabolism of particular fatty acids and

differences in their affinity for specific PPAR subtypes (reviewed in Xu *et al.*, 1999a). PPAR-α has a clear preference for binding PUFA, followed by PPAR-γ and PPAR-β. EPA is a much more potent activator of PPAR-α in primary hepatocytes as compared with AA (Pawar and Jump, 2003). Similarly, LA, ALA and AA were able to activate PPAR-γ potently. Rodents fed fish oil-containing diets (at < 20% calories as fat) have also been shown to upregulate multiple hepatic genes through induction of PPAR- α (Wang *et al.*, 2006).

1.5.3.2 Dietary fats and SREBP's

SREBP's are basic-helix-loop-helix-leucine zipper transcription factors, which are bound to the membranes of the endoplasmic reticulum (ER) as inactivated precursors. Upon activation, the ER-anchored SREBP precursor undergoes a sequential two-step cleavage process to release the active domain designated as the nuclear form of SREBP. Once translocated into the nucleus, the nuclear form of SREBP promotes the expression of many genes involved in the metabolism of fatty acids and cholesterol, through binding to the sterol regulatory element binding DNA sequence (5′-TCACNCCAC-3′) present in the promoter of the target genes (reviewed in Brown and Goldstein, 1997).

Three members of the SREBP family have been identified in mammalian species: SREBP-1a and -1c and -2. Each isoform has different regulatory mechanism. SREBP-1a and -1c transcripts are produced through the use of alternative transcription start sites and differ in their first exon (exon-1a and exon-1c). SREBP-1a is known to be a more potent transcriptional activator than SREBP-1c, however, SREBP-1c is the predominant isoform

expressed in the liver, adipose tissue, skeletal muscle, adrenal gland and brain. In contrast, SREBP-1a is highly expressed in cell lines and tissues with a high capacity for cell proliferation, such as spleen and intestine (reviewed in Shimano, 2001). SREBP-1a and -1c are known to regulate the genes involved in fatty acid synthesis such as acetyl CoA carboxylase, fatty acid synthase and steroyl CoA desaturase (reviewed in Shimano, 2001). Chronic activation of SREBP-1c due to over-nutrition has been implicated in obesity-related problems. In addition, increased TG synthesis and accumulation of lipids in various organs such as liver and pancreas due to chronic activation of SREBP-1c has been shown to result in fatty liver and impaired β-cell dysfunction (Yahagi *et al.*, 2002; Takahashi *et al.*, 2005). SREBP's can also repress insulin receptor substrate-2, which is the main insulin-signaling molecule in the liver, resulting in hepatic insulin resistance (Ide *et al.*, 2004). On the other hand, SREBP-1c-mediated pancreatic β-cell dysfunction results in impaired insulin secretion (Shimano *et al.*, 2007). In addition, SREBP-1c is known to promote the differentiation and storage of TG in adipose tissue in mice, suggesting its involvement in the development of obesity (Kim and Spiegelman, 1996).

SFA can activate SREBP-1c leading to enhanced synthesis of TG, whereas ***n-3*** PUFA are known to inhibit lipogenesis and lower tissue and plasma TG through inhibition of SREBP-1c (Kim *et al.*, 1999; Yahagi *et al.*, 2002). PUFA can inhibit SREBP-1c cleavage for nuclear translocation and are also known to suppress the expression of SREBP-1c (Xu *et al.*, 1999b).

Taken together, dietary fat-mediated regulation of various isoforms of PPAR's and SREBP's plays an important role in the regulation of lipid metabolism in the body.

1.6 RATIONALE

Considering that the consumption of dietary fats is on the rise worldwide (Popkin, 1999; 2001; Popkin and Gordon-Larsen, 2004), it is essential to understand the impact of dietary fat consumption during pregnancy on the fetal origins of CVD. It has been suggested that a high-fat maternal diet during pregnancy can predispose the developing fetus to the onset of CVD in adult life. However, at present, the role played by various dietary fats in the developmental origins of CVD is not completely understood. As discussed earlier, diets rich in SFA are known to have deleterious effects, whereas diets rich in PUFA are known to have beneficial effects on the outcome of CVD and related diseases. Thus, it would be important to delineate their individual role and underlying mechanisms in the developmental origins of CVD, such that appropriate dietary recommendations can be developed for the expecting mothers, to reduce the overall burden of CVD. While focusing on nutrition during pregnancy, it would be equally important to understand its interaction with the postnatal nutrition, and to determine if postnatal nutrition has the potential to ameliorate the programming effects of maternal diet. This understanding may help us in designing the postnatal nutritional strategies to manage the 'programming effects' of maternal diet in the future.

1.7 AIMS AND OBJECTIVES

Most of the available literature dealing with the high-fat maternal diets and developmental origins of CVD is based upon studies carried out in rat models.

Considering that a number of knockout mice models such as apoE $^{-/-}$ and LDL-r $^{-/-}$ (on C57Bl/6 background) are available to study the molecular mechanisms behind altered lipid metabolism, this study sought to establish C57Bl/6 mice as an animal model to study the dietary fat-mediated programming of adult CVD. In addition, the current study investigated the role of various dietary fats, differing in quality as well as quantity, in the developmental origins of adult CVD, and further investigated some of the plausible molecular mechanisms. Offspring lipid metabolism and vascular function were chosen as study parameters, as altered lipid metabolism and endothelial dysfunction are known to be risk factors for the development of CVD. In order to determine the effects of interaction between the prenatal and postnatal nutrition on the offspring cardiovascular health, the role of various dietary fats was also studied post-weaning. The specific aims and the underlying hypotheses for the current study were:

Aim I: To investigate the effects of a high-fat maternal diet rich in SFA in the developmental origins of altered lipid metabolism and aortic vascular function in the adult offspring, and to further investigate the underlying mechanisms (Chapter 3)

Hypothesis: A diet rich in SFA is known to promote dyslipidemia and endothelial dysfunction, thereby increasing the risk of developing CVD. Thus, it was hypothesized that a diet rich in SFA, fed to the mothers during gestation and lactation, would result in higher plasma TG and cholesterol concentrations in the offspring, which would further induce endothelial dysfunction in their aortic vessels leading to an increased risk of developing CVD. In addition, an interaction between the pre- and post-weaning diets rich

in SFA was expected to exaggerate the effects of a pre-weaning diet rich in SFA, on the parameters associated with the CVD risk in the offspring.

Aim II: To investigate the effects of a high-fat maternal diet rich in *n-6* PUFA in the developmental origins of altered lipid metabolism and aortic vascular function in the adult offspring, and to further investigate the underlying mechanisms (Chapter 4).

Hypothesis: N-*6* PUFA, in general, have been shown to lower plasma TG and cholesterol levels, thereby reducing the risk of developing CVD. However, the effects of an *n-6* PUFA enriched diet, which is high in fat, are not well studied. It was hypothesized that a high-fat diet rich in *n-6* PUFA, fed to the mothers during gestation and lactation, would lower offspring plasma TG and total-cholestreol and improve their endothelial function. In addition, an interaction between the pre- and post-weaning diets rich in *n-6* PUFA was expected to exaggerate the effects of a pre-weaning diet rich in PUFA, on the parameters associated with the CVD risk in the offspring.

Aim III: To compare the effects of high-fat maternal diet rich in SFA *vs. n-6* PUFA in the developmental origins of altered lipid metabolism and aortic vascular function in the adult offspring (Chapter 5).

Hypothesis: A maternal diet rich in SFA was expected to increase plasma TG and cholesterol concentrations, and induce endothelial dysfunction in aortic vessels, in the offspring compared to a diet rich in *n-6* PUFA. It was also expected that a continuous

exposure to a diet rich in SFA would exaggerate the effects of a pre-weaning diet rich in SFA, on the parameters associated with the CVD risk in the offspring, compared to a diet rich in *n-6* PUFA.

Aim IV: To investigate the effects of pre- and post-weaning diets rich in different fatty acids on tissue fatty acid composition in the adult offspring (Chapter 6).

Hypothesis: It was hypothesized that a diet rich in SFA, fed to the mothers during gestation and lactation, would enrich the offspring liver and heart tissues with SFA, whereas a maternal diet rich in *n-6* PUFA would enrich the offspring liver and heart tissues with *n-6* PUFA.

Aim V: To investigate the beneficial effects of flax oil-feeding post-weaning on the outcome of metabolic syndrome in a rat model, and to further investigate the underlying molecular mechanisms (Chapter-7) (Appendix II).

Hypothesis: A diet rich in *n-3* PUFA is known to improve the outcome of metabolic syndrome. Since flax oil is a rich source of *n-3* PUFA, it was hypothesized that a diet rich in flax oil would lower plasma and hepatic lipids, lower oxidative stress and improve insulin sensitivity in the obese SHR/NDmcr-cp rats when compared to a diet rich in lard.

CHAPTER TWO

General Methodology and Breeding Outcomes

2.1 ANIMALS AND DIETS

2.1.1 Diets

The experimental high-fat diets were prepared using a base semi-synthetic diet obtained in powdered form with fat source omitted, designed specifically to permit the control of fat level at 20% *w/w* (MP Biomedicals, OH, USA). The macronutrient composition of the semi-synthetic diet is given in Table 2.1. Lard and safflower oil obtained from a local supermarket were used as a source of SFA and *n-6* PUFA in the experimental diets, respectively. The high-fat diets were prepared by mixing semi-synthetic diets with 1% flax oil and 19% of either lard or safflower oil, such that 20% of total fats were added per kg of the diet. Commercial rodent chow (Purina rat chow #5012; proteins=23.2%; fat = 5.0%; fiber = 3.8%; starch = 39.5%; glucose = 0.29%; fructose = 0.34%; sucrose = 3.38%; Ash = 6.6%) supplied by Agribrands Purina Inc, ON, Canada, was used as a control diet. Gas-liquid chromatography (GLC) was utilized to determine the fatty acid composition of all experimental diets (Table 2.2).

2.1.2 Breeding and maintenance of animals

All the experimental procedures were in accordance with the principles and guidelines of the Canadian Council on Animal Care and were approved by the Memorial University's Animal Care Committee.

Table 2.1 Composition of the semi-synthetic diets used for a high-fat level.

Ingredients	Semi-synthetic diets
Casein (g/Kg of diet)	200
DL-methionine (g/Kg of diet)	3
Fat (g/Kg of diet)	200
Sucrose (g/Kg of diet)	305
Corn-starch (g/Kg of diet)	190
Alphacel non-nutrtitive bulk (g/Kg of diet)	50
Vitamin mix (mg/Kg of diet)	
Thiamine hydrochloride	6.6
Riboflavin	6.6
Pyridoxine hydrochloride	7.7
Nicotinic acid	33
d-calcium pantothenate	17.6
Folic acid	2.2
d-biotin	0.22
Cyanocobalamin (vitamin B_{12})	0.011
Retinyl palmitate (vitamin A)	17.6
DL-a-tocopherol acetate	220
Cholecalciferol (vitamin D_{3})	2.75
Menaquinone (vitamin K_2)	0.055
Sucrose	10701.9
Mineral Mix (g/Kg of diet)	
Calcium phosphate dibasic	20
sodium chloride	2.96
potassium citrate monohydrate	8.8
potassium sulfate	2.08
magnesium oxide	0.96
manganese carbonate (43-48% Mn)	0.14
ferric citrate (16-17% Fe)	0.24
zinc carbonate (70% ZnO)	0.064
cupric carbonate (53-55% Cu)	0.012
potassium iodate	0.0004
sodium selenite	0.0004
chromium potassium sulfate	0.022
sucrose	4.72

‖Supplied in quantities adequate to meet requirements (National Research Council, 1995).

Table 2.2 Fatty acid composition of the experimental diets*

Fatty Acids	High-fat SFA	High-fat n-6 PUFA	Chow
C14:0 (myristic acid)	3	ND	1
C16:0 (palmitic acid)	18	8	18
C18:0 (stearic acid)	14	3	5
Σ SFA	35	11	24
C16:1 (palmitoleic acid)	3	ND	1
C18:1(oleic acid)	42	15	22
C20:1 (eicosenoic acid)	1	ND	ND
Σ MUFA	46	15	23
C18:2 (linoleic acid)	15	70	38
C18:3 (linolenic acid)	4	4	4
C 20:5 (eicopentaenoic acid)	ND	ND	1
C22:6 (docosahexaenoic acid)	ND	ND	2
Σ PUFA	19	74	45

*Given as % area covered by each fatty acid peak

ND= Not detected, Σ SFA= sum of saturated fatty acids, Σ MUFA= sum of monounsaturated fatty acids, Σ PUFA= sum of polyunsaturated fatty acids.

Source of SFA = lard; source of n-6 PUFA = safflower oil

7-weeks-old C57Bl/6 mice were purchased from Charles River Laboratories (MA, USA). Male and female animals were housed in separate cages (2 mice/cage) under controlled temperature (21± 1°C) and humidity (35 ± 5%), in a single room with a 12-hour light/12-hour dark period cycle, and were fed commercial rodent chow pellets for a period of 1 week.

After this acclimatization period, female mice were fed either high-fat diets or rodent chow according to the experimental design (detailed in respective chapters). After 2-weeks on specified diets, one male was introduced into each female cage for mating and was removed after 7-days. Pregnancy was confirmed by vaginal plug formation; pregnant female mice were then housed in individual cages until weaning of the pups. Pregnancy rate was defined as the number of pregnant mice divided by the number of female mice used for mating (Table 2.3). Mothers were provided fresh food twice per week. Litters were counted on postnatal day 1, after which the mothers and pups were not disturbed in order to prevent cannibalism. Survival rate was defined as the number of live pups at weaning divided by the number of live pups at birth (Table 2.3). At weaning (age 3 weeks), pups were housed separately according to sex (2-3 mice/cage). Sex ratio was defined as the number of male pups divided by the number of female pups obtained from a dietary group of mothers counted at the time of weaning (Table 2.3). After weaning, the dams were killed and the offspring were fed respective diets according to the experimental design (described in respective chapters). Pups were allowed free access to food and water. Body weights and food intake were recorded each week. Offspring mice were fasted overnight at 11-weeks of age, and were euthanized the next morning using

halothane. Blood and tissues were collected at the time of euthanizing the animals. Blood was used for collecting plasma, while tissues were stored at -80°C until further analysis.

2.1.2.1 Pregnancy rate, pup survival rate and sex ratio of the pups at the time of weaning

The pregnancy rate of the mice fed high-fat diets rich in SFA and *n-6* PUFA was lower (SFA, 34% and *n-6* PUFA, 36%) compared to the chow-fed mice (43%) (Table 2.3). The survival rate of pups obtained from mothers fed high-fat diets was lower (SFA, 60% and *n-6* PUFA, 71%) compared to the chow-fed mothers (83%). Moreover, the male to female ratio obtained from mothers fed a high fat diet rich in *n-6* PUFA was lower (0.56) compared to the male to female ratio obtained from mothers fed high-fat diets rich in SFA (0.86) or chow (0.97) (Table 2.3).

2.1.2.1.1 Pregnancy and pup survival rate

Reduced fertility (50%) and pup survival rate (70%) have been reported in rats fed a high-fat diet from weaning until 400-days of age (Bue *et al.,* 1989). Decreased rate of conception and delivery have also been reported in the rats fed cafeteria-style diets (Rolls *et al.,* 1980; Wehmer *et al.,* 1979). In a study involving Sprague-Dawley rats, 5-weeks of high-fat feeding was associated with higher body weight at the time of conception. Maternal obesity in these rats was associated with delivery of fewer pups and lower survival rate of the pups than the controls (Shaw *et al.,* 1997). Although the factors

Table 2.3 Pregnancy rate, pup survival rate and sex ratio of the offspring at the time of weaning

	High-fat SFA	High-fat n-6 PUFA	Chow
Pregnancy rate	53/18	33/12	40/17
Pup Suvival rate	156/80	90/62	88/73
Male/female ratio	36/42	16/29	36/37

SFA= saturated fatty acids; PUFA = polyunsaturated fatty acids.

Pregnancy rate was defined as the number of pregnant mice divided by the number of female mice used for mating; survival rate was defined as the number of live pups at weaning divided by the number of live pups at birth; sex ratio was defined as the number of male pups divided by the number of female pups obtained from a dietary group of mothers counted at the time of weaning.

responsible for reduced fertility and pup survival rate have not been established, it has been proposed that excessive diversion of fuel into storage that is seen in case of dietary obesity may render it unavailable for reproduction, leading to energetic inhibition of reproduction (Wade *et al.,* 1991). Cafeteria-style feeding in rats has been reported to induce estrous cycle irregularities that were associated with hyperphagia and increased brown adipose tissue (BAT) thermeogenesis. It was proposed that since reproductive functions are finely tuned with body temperature, an excess feeding-induced BAT thermogenesis might have caused a disruption in estrous cycle (Glick *et al.,* 1990). Additionally, both high-fat and cafeteria-style diets have been reported to negatively affect milk yield and lactation performance in rats (Rolls *et al.,* 1986; Rolls and Rowe 1982, Wehmer *et al.,* 1979). Reduced milk volume has been reported in lactating obese rats (Rolls *et al.*, 1982 and Rolls *et al.*, 1986). Feeding high-fat diets to lactating rats has been associated with diminished mammary gland lipogenesis, which could underlie the reduced milk production in these rats (Agius *et al.*, 1980; Rolls *et al.,* 1980). It has also been suggested that maternal behaviour in rats fed a high-fat diet is abnormal, leading to higher rates of cannibalism of the young (Rolls and Rowe 1982; Wehmer *et al.*, 1979). This cannibalistic maternal response may stem from either a failure of the dam to respond to the changes that occur during the transition from pregnancy to lactation, or a failure of the litter to evoke the appropriate response such as lactation.

It is apparent from a number of clinical studies that obese women are more likely to suffer from the complications of pregnancy such as pre-eclampsia, impaired glucose metabolism and gestational diabetes Moreover, other studies have indicated that obese

women experience prolonged labor and undergo unplanned cesarean sections more often than normal weight women (Calandra *et al.,* 1981; Ekblad and Grenman, 1992). Additional evidence suggests that obese women have less success initiating (Richardson, 1952), and continuing (Rutishauser and Carlin, 1992) breastfeeding. Considering that obesity is on the rise worldwide, the etiology of an unfavorable relationship between obesity and reproductive function warrants further investigation.

2.1.2.1.2 Sex ratio of the pups

Rodents, like most mammals, tend to produce roughly equivalent numbers of sons and daughters, although litters with marked imbalances in sex ratio have been reported under the conditions of food restriction and suboptimal nutrition (Labov et al., 1986; Meikle and Drickamer, 1986; Meikel and Thornton, 1995). Feeding diets deficient in essential fatty acids (Rivers and Crawford, 1974) or protein (Kwong *et al.,* 2000) have been associated with small and female-biased litters. Stresses other than food restriction, such as crowding, can also reduce the fraction of males born to rodents (Krackow, 1997). On the other hand, availability of extra energy to the mother seems to favor the birth of sons over daughters (Wauters *et al.,* 1995; Enright *et al.,* 2001). These observations have been explained based on the sex-allocation hypothesis of Trivers and Willard, which proposes that the development and subsequent reproduction of males is likely to be associated with better maternal 'condition' (e.g. size, dominance rank, and nutritional status) than the reproduction of females (Trivers and Willard, 1973).

A lard-rich diet (54% calories) fed to 4-weeks-old NIH Swiss mice till 20-weeks of age was associated with increased number of male pups over females, compared to a low-fat, high-carbohydrate diet (Rosenfeld *et al.,* 2003). The authors concluded that the source and amount of calories provided by the high-fat diets to the mature female mice on a nutritionally complete diet skewed the sex of offspring towards a male progeny, which is consistent with the sex-allocation hypothesis of Trivers and Willard (Rosenfeld *et al.,* 2003). However, in a subsequent study by the same group of authors, a diet rich in LA was found to be associated with 60% more female pups over males when compared to a diet rich in menhaden oil (Fountain *et al.,* 2008). This study clearly indicated that the type of fat instead of the amount of calories influenced the sex ratio of the progeny. Our observations are in line with the observations made by Founatin *et al.,* 2008, where a diet rich in LA led to the birth of more females than males in C57Bl/6 mice. The fatty acid composition of the diets have been shown to influence numerous events involved in the reproductive process, such as oocyte maturation and the timing of ovulation (Bilby *et al.,* 2007), the production of chemoattractants by the oocyte (Kubagawa *et al.,* 2006), prostaglandin synthesis (Handerson *et al.,* 1989; Thatcher *et al.,* 2001; Wamsley *et al.,* 2005), and properties of the reproductive tract (Pratt *et al.,* 1987). These changes may, in turn, affect the relative fertilization abilities of X and Y sperm or provide an advantage to conceptuses of one sex over the other during their development (Gutierrez-Adan et al., 1999; Martin, 1997) resulting in the birth of more females than males.

2.2 PLASMA LIPID ANALYSIS

Fasting blood was collected at necropsy using cardiac puncture into tubes containing 4.5 mM EDTA, pH 7.5. Plasma was collected after centrifugation of whole blood at 3000 *g* for 15-minutes. Plasma TG and total-cholesterol concentrations were determined using TG assay kit # 2150-101 and cholesterol assay kit # 1010-430 (Stanbio Laboratories, TX, USA). Non-HDL cholesterol was precipitated from plasma using kit # 200-26A (DCL, P.E,I, Canada) and the supernatant was utilized for measuring HDL-cholesterol using kit #1010-430. The non-HDL cholesterol concentration was calculated by subtracting the HDL-cholesterol concentration from the total-cholesterol concentration. Plasma LDL-cholesterol concentration was calculated using plasma total-, HDL-cholesterol, and TG concentration according to the method of Friedewald *et al.* (1972).

2.3 PLASMA FFA AND BLOOD GLUCOSE ANALYSIS

FFA content was determined in fasting plasma using commercially available kit # 999-34691 (Wako Chemicals Inc., USA). Glucose concentrations were measured using a commercially available glucometer (Lifescan Inc, CA, USA) in tail blood of the fasted animals at necropsy.

2.4 VASCULAR FUNCTION ANALYSIS

Thoracic descending aortas were dissected and adherent fat was removed while viewed with the aid of a dissection microscope. Rings of aortas (2 mm lengths) were mounted in separate chambers of a multi-myograph 610M (Danish Myograph Technology, Denmark). Aortas were bathed in Krebs bicarbonate buffered (pH 7.4) physiological saline solution (PSS) comprised of 114 mM NaCl, 4.7 mM KCl, 0.8 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 11 mM D-glucose at 37 °C and bubbled with a gas mixture of 5% CO_2–95% O_2. Each ring was stretched to 90% of the diameter that was estimated to produce wall stress equivalent to 100 mmHg (Mulvany and Halpern, 1977). The resting baseline tension was not found to be different among groups. After 1-hour of equilibration period, drug concentration-contractile response curves for KCl (30-120 mM), phenylephrine (PE; 1 nM to 10 μM) and thromboxane A_2 mimetic (U46619; 1 nM to 1 μM) were then constructed in separate aortic rings. Endothelium-dependent and -independent relaxation responses were measured using acetylcholine (ACh; 1 nM to 10 μM) and sodium nitroprusside (SNP; 1 nM to 10 μM), respectively, in vessels contracted submaximally with U46619 (50 - 75 % of E_{max}). All chemicals were purchased from Sigma Aldrich (ON, Canada).

2.5 GLC ANALYSIS

Lipids were extracted from diet and also from various tissues using the method of Folch *et al.* (1957). Fatty acid methyl esters were then prepared by heating the sample with 2 ml

of trans-methylation reagent (6% concentrated sulfuric acid and 94% methanol + few crystals of hydroquinone added as an anti-oxidant) for 2-hours at 65°C. Organic extractions were performed using hexane and water after which samples were placed at -20°C overnight. Samples were transferred to a new tube in order to remove any frozen water next morning, after which they were dried under N_2 gas. Samples were then dissolved in 20 ul of carbon disulfide and used for GLC analysis (Keough and Davis, 1979). Samples were run for 60 minutes on an Omegawax X 320 (30 m x 0.32 mm) column from Supleco (Sigma-Aldrich, Canada) using a flame ionization detector. The GLC parameters were set as: oven, 200°C, injector, 240°C; detector 260°C. The GLC was ignited and allowed to run overnight prior to running samples, in order to ensure that the baseline was stable. Fatty acid standards (PUFA-1, -2 and -3, Sigma-Aldrich Canada) were used for were used for identification of fatty acids by retention time.

2.6 STATISTICAL ANALYSIS

Data analysis was performed using Two-way ANOVA and Fisher' LSD *post hoc* analysis (*SYSTAT for Windows, version 12.02; SYSTAT Software Inc.*, Richmond, California). Specific details are provided in the respective chapters.

CHAPTER THREE

Effects of feeding a high-fat maternal diet rich in lard (SFA) during gestation and lactation on lipid metabolism and aortic vascular function in the adult offspring

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3.1 INTRODUCTION

As described in section 1.1, recent evidence based on the 'developmental origins' hypothesis suggests that early nutrition can be critical in determining the cardiovascular health of an individual (McMillen and Robinson, 2005; McMillen *et al.*, 2008). Considering that a typical Western diet is rich in dietary fat content (Cordain *et al.*, 2005), especially SFA, some studies have investigated the role of high-fat feeding in the developmental origins of CVD (Armitage *et al.*, 2004; Armitage *et al.*, 2005; Armitage *et al.*, 2008b). These studies indicate that maternal high SFA consumption during pregnancy can induce features of metabolic syndrome including dyslipidemia (Guo and Jen, 1995; Ghosh *et al.*, 2001), insulin resistance (Taylor *et al.*, 2005) and hypertension (Khan *et al.*, 2003; Khan *et al.*, 2004) in the adult offspring. However, the underlying mechanisms of these effects are not known. Moreover, the role of interaction between the prenatal and postnatal diets on the health outcome in the offspring has yet to be determined. It is also important to evaluate if the postnatal diet can improve or worsen the "programming effects" of the maternal diet.

A diet rich in SFA causes plasma lipid abnormalities (Denke, 2006) and endothelial dysfunction (Brown and Hu, 2001), which are considered to be early predictors of the future development of CVD. SFA intake raises plasma LDL levels (Spady *et al.*, 1993; Watts *et al.*, 1996) and inhibits the expression of hepatic LDL-r (Horton *et al.*, 1993; Hayes *et al.*, 1997). Both *in vitro* and *in vivo* studies have shown that aortic endothelial function may be abnormal within a few hours of exposure to increased levels of LDL-cholesterol (Jayakody *et al.*, 1987; Liao, 1994). Furthermore SFA intake can evoke free

radical synthesis that may contribute to vascular dysfunction by decreasing endothelium derived vasodilator molecules like nitric oxide (Erdei *et al.*, 2006).

This study was designed to investigate the effects of feeding a high-fat maternal diet rich in SFA during gestation and lactation on the lipid metabolism and aortic vascular function, as biomarkers for assessing the CVD risk, in the adult offspring. In addition, the effects of interaction between the pre- and post-weaning diets rich in SFA, on offspring CVD risk, were studied. A number of previous studies have used lard as a source of SFA, while assessing the effects of maternal diet on the offspring cardiovascular health in rat models (Ghosh *et al.*, 2001, Taylor *et al.*, 2005, Khan *et al.*, 2003, Khan *et al.*, 2004). Although the lard used in the current study was rich in both SFA and MUFA (Table 2.2), keeping in line with the previous studies, it has been called a source of SFA throughout this chapter.

It was hypothesized that a diet rich in SFA, fed to the mothers during gestation and lactation, would result in higher plasma TG and cholesterol concentrations in the offspring, which would induce endothelial dysfunction in their aortic vessles, leading to an increased risk of developing CVD. In addition, an interaction between the pre- and post-weaning diets rich in SFA was expected to exaggerate the effects of a pre-weaning diet rich in SFA, on the parameters associated with the CVD risk in the offspring.

3.2 METHODS

3.2.1 Experimental design

As described in section 2.1, female C57Bl/6 mice were maintained on rodent chow for a week prior to feeding the experimental diets. After this acclimatization period, female mice were divided into two groups, and were fed the experimental diets according to the design specified in Fig. 3.1. The offspring groups were identified by *pre-/post-weaning diet* combinations as SFA/SFA (S/S), SFA/chow (S/C), chow/chow (C/C) and chow/SFA (C/S). Both male and female offspring from each dietary group were used in the current study.

3.2.2 Quantitative-PCR analysis of hepatic LDL- r

Total RNA was isolated from the liver samples using the method of Chomczynski and Sacchi, 1987. Reverse transcription of total RNA into cDNA was performed using one-step reverse transcription kit from Roche Diagnostics (PQ, Canada). The mRNA expression levels were determined on a Lightcycler 2.0 Detection System (Roche Diagnostics, PQ, Canada). An upstream primer 5'- AGGCTGTGGGCTCCATAGG-3' with a downstream primer 5'- TGCGGTCCAGGGTCATCT-3' corresponding to a coding sequence of mouse LDL-r and an upstream primer 5'- TGAAGCAGGCATCTGAGGG-3' with a downstream primer 5'- CGAAGGTGGAAGAGTGGGAG-3' corresponding to a coding sequence of mouse GAPDH cDNA were used. Briefly, standard curves were generated using the serial

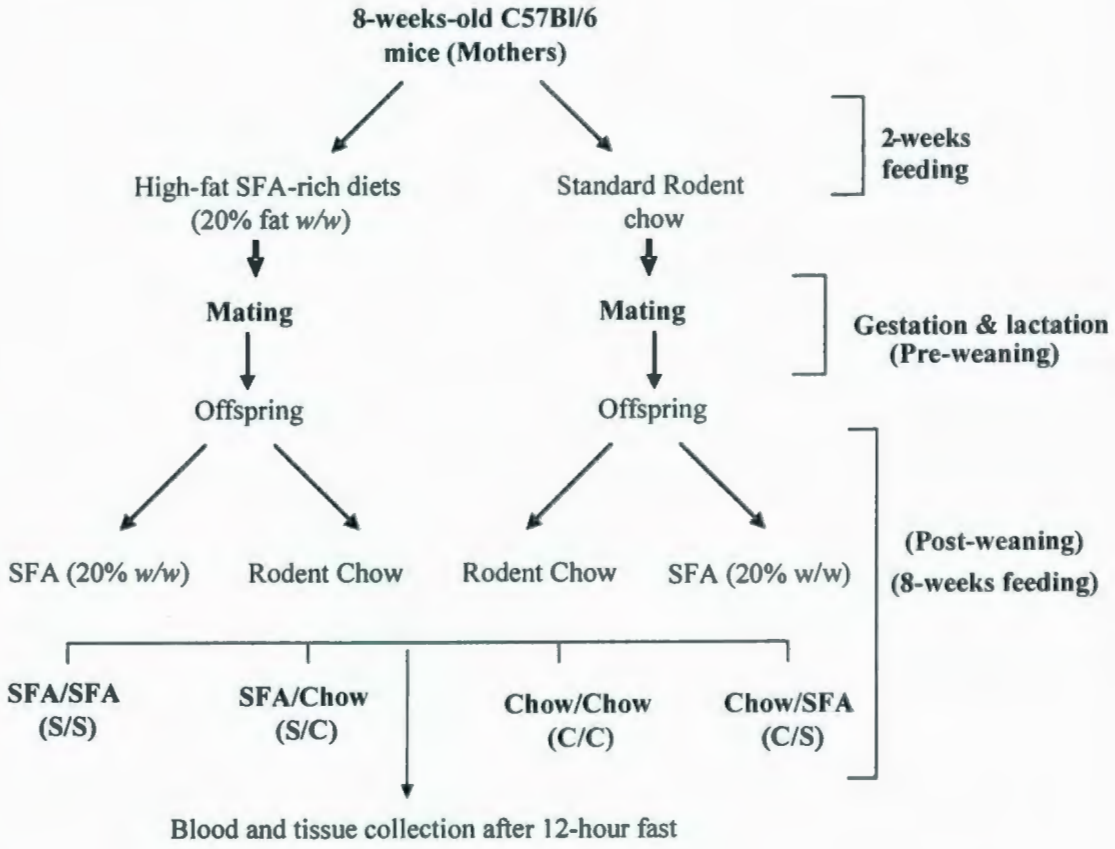

Fig. 3.1 Experimental Design.

8-week-old female C57BL/6 mice were fed either a high-fat diet containing lard (SFA) or standard rodent chow for 2-weeks. Females continued on these diets during mating, pregnancy and lactation. At weaning, the offspring obtained from each mother were divided into two groups; one group continued on the lard-rich diets and the other group was fed rodent chow. The offspring groups were identified by *pre-/post-weaning diet* combinations as: SFA/SFA (S/S), SFA/chow (S/C), chow/chow (C/C) and chow/SFA (C/S). The offspring were fed their assigned diets *ad libitum* for another 8- weeks.

dilution of a control sample for both LDL-r and GAPDH genes and the PCR efficiency for each reaction was calculated. No differences were found in the expression of GAPDH among various groups. The LDL-r expression for each sample was then calculated in relation to the expression of GAPDH, thus, normalizing and correcting the data for the differences in PCR efficiencies for each set of primers.

3.2.3 Vascular function analysis

The contractile and relaxation responses of the aorta of each mouse were studied as described in section 2.4. In addition, aortic vessels were tested for the activation of the nitric oxide synthases (NOS) system by repeating the cumulative concentration–contractile responses towards KCl and PE after treatment with the NOS inhibitor (G)-nitro-L-arginine methyl ester (L-NAME) (300 μM; 10 minutes).

3.2.4 Calculations and Statistical Analysis

Data are expressed as means ± SEM, where S/S, $n = 8$; S/C, $n = 7$; C/C, $n = 8$, and C/S, $n = 7$ for the male offspring and S/S, $n = 10$; S/C, $n = 8$; C/C, $n = 10$ and C/S, $n = 8$ for the female offspring. Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet x post-weaning diet) were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post*

hoc tests (*SYSTAT for Windows, version 12.02*; *SYSTAT Software Inc.*, Richmond, California). Differences having a $P < 0.05$ were considered significant.

Constrictor responses were reported as the force generated in response to each concentration of the agonist, and relaxant responses as the percentage reversal of U44619-induced contraction. Cumulative concentration–response curves to agonists were analyzed by fitting to a four-parameter logistic equation using non-linear regression to obtain the -log effective concentration equal to 50% of the maximal response (pEC_{50}) and maximum response (E_{max}) (Prism 3.0, GraphPAD Software Inc). pEC_{50} and E_{max} values were then compared using two-way ANOVA and Fisher's LSD *post hoc* analysis. Since the KCl-response curves were not sigmoidal, pEC_{50} values were not calculated and only maximal responses were compared among various groups.

3.3 RESULTS

3.3.1 Effects of pre- and post-weaning lard-rich diets on body weight, food and caloric intake, plasma glucose and FFA concentrations of male and female offspring

A lard-rich diet fed post-weaning was associated with a higher body weight in both male ($P < 0.001$) and female offspring ($P = 0.023$) compared to chow, at 11-week of age (Table 3.1). In contrast, a significant interaction of pre- and post-weaning diets affected the cumulative food and caloric intake of the male ($P = 0.002$) and female offspring ($P < 0.01$), respectively (Table 3.1). Multiple

Table 3.1 Body weight, food intake, caloric intake, plasma glucose and FFA concentrations of male and female offspring.

	SFA/SFA	SFA/Chow	Chow/Chow	Chow/SFA	*Pre*	*Post*	*Int*
			Male offspring				
BW (g)	24.3 ± 1.2	18.6 ± 0.8	21.7 ± 0.2	24.1 ± 0.7	NS	$P < 0.001$	NS
FI (g)	3.4 ± 0.3^{b}	3.2 ± 0.1^{b}	4.2 ± 0.4^{a}	2.5 ± 0.2^{b}	NS	$P = 0.008$	$P = 0.002$
CI (g)	17.1 ± 1.5^{a}	13.1 ± 0.6^{b}	17.3 ± 1.6^{a}	12.6 ± 0.9^{b}	NS	NS	$P = 0.002$
Glucose (mM)	11.7 ± 1.0	9.4 ± 0.5	8.5 ± 0.4	10.4 ± 1.1	NS	$P = 0.016$	NS
FFA (mEq/L)	1.2 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	NS	NS	NS
			Female offspring				
BW (g)	20.3 ± 0.8	17.1 ± 0.7	17.5 ± 0.5	18.8 ± 0.8	NS	$P = 0.023$	NS
FI (g)	3.1 ± 0.3^{b}	3.2 ± 0.1^{b}	4.1 ± 0.2^{a}	2.8 ± 0.2^{b}	NS	$P = 0.002$	$P = 0.005$
CI (g)	15.7 ± 1.1^{ab}	13.1 ± 0.4^{b}	17.0 ± 1^{a}	14.1 ± 1.3^{ab}	NS	NS	$P = 0.010$
Glucose (mM)	9.8 ± 0.8^{ab}	8.9 ± 0.5^{bc}	8.1 ± 0.4^{c}	11.8 ± 0.3^{a}	NS	$P = 0.002$	$P = 0.045$
FFA (mEq/L)	1.7 ± 0.2^{a}	1.1 ± 0.2^{b}	1.0 ± 0.1^{b}	0.9 ± 0.1^{b}	$P = 0.009$	NS	$P = 0.046$

Values are expressed as means ± SEM, n = 7-10 (specified in section 3.2.4). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Superscripts represent significant differences having $P < 0.05$. BW, body weight; FI, food intake; CI, caloric intake; FFA, free fatty acids; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction; NS, not significant.

comparisons further revealed that the both male ($P < 0.05$) and female C/C offspring ($P < 0.01$) exhibited the highest food intake compared to all other offspring (Table 3.1). In addition, a lard-rich diet fed pre-weaning was associated with lower caloric intake in both male and female S/C offspring compared to the C/C offspring ($P < 0.05$) (Table 3.1).

A lard-rich diet fed post-weaning was associated with higher fasting plasma glucose concentration in the male offspring compared to chow ($P = 0.016$) (Table 3.1). However, a significant interaction of the pre- and post-weaning diets affected the fasting plasma glucose concentration in the female offspring ($P = 0.045$) (Table 3.1). In addition, female C/S offspring had higher plasma glucose concentration compared to S/C and C/C offspring (S/C *vs.* C/S, $P = 0.015$; C/S *vs.* C/C, $P = 0.001$) (Table 3.1). No differences were observed in plasma FFA concentration among various groups of male offspring. However, a significant interaction of the pre- and post-weaning diets affected the plasma FFA concentration in the female offspring ($P = 0.046$) (Table 3.1). Moreover, a continuous exposure to the lard-rich diet during pre- and post-weaning time periods was associated with higher FFA concentration in the female S/S offspring compared to all other offspring (S/S *vs.* C/C, $P = 0.005$; S/S *vs.* S/C, $P = 0.019$; S/S *vs.* C/S, $P = 0.003$) (Table 3.1).

3.3.2 Effects of pre- and post-weaning lard-rich diets on plasma lipid levels of male and female offspring

Two-way ANOVA revealed that a lard-rich diet fed both during pre-weaning ($P = 0.004$) and post-weaning time period ($P = 0.049$) was associated with higher plasma TG concentration in the male offspring compared to chow (Fig. 3.2A). However, no differences were observed among various groups of female offspring for their plasma TG concentrations (Fig. 3.2A). A lard-rich diet fed post-weaning was associated with higher plasma total-cholesterol concentration in the male offspring ($P < 0.001$) compared to chow (Fig. 3.2B). On the other hand, a significant interaction of the pre- and post-weaning diets affected the plasma total-cholesterol concentration in the female offspring ($P = 0.007$). Multiple comparisons further revealed that a lard-rich diet fed pre-weaning was associated with higher plasma total-cholesterol concentration in the female S/C offspring compared to the C/C offspring ($P = 0.029$), while a lard-rich diet fed post-weaning was associated with higher plasma total-cholesterol concentration in the C/S offspring compared to the C/C offspring ($P < 0.001$) (Fig 3.2B).

Similar to the plasma total-cholesterol, a lard-rich diet fed post-weaning was associated with higher plasma LDL-cholesterol concentration in the male offspring compared to chow ($P < 0.001$) (Fig. 3.3A). On the other hand, a significant interaction of the pre- and post-weaning diets affected the plasma LDL-cholesterol concentration in the female offspring ($P = 0.046$) (Fig.3.3A). A lard-rich diet fed post-weaning was associated

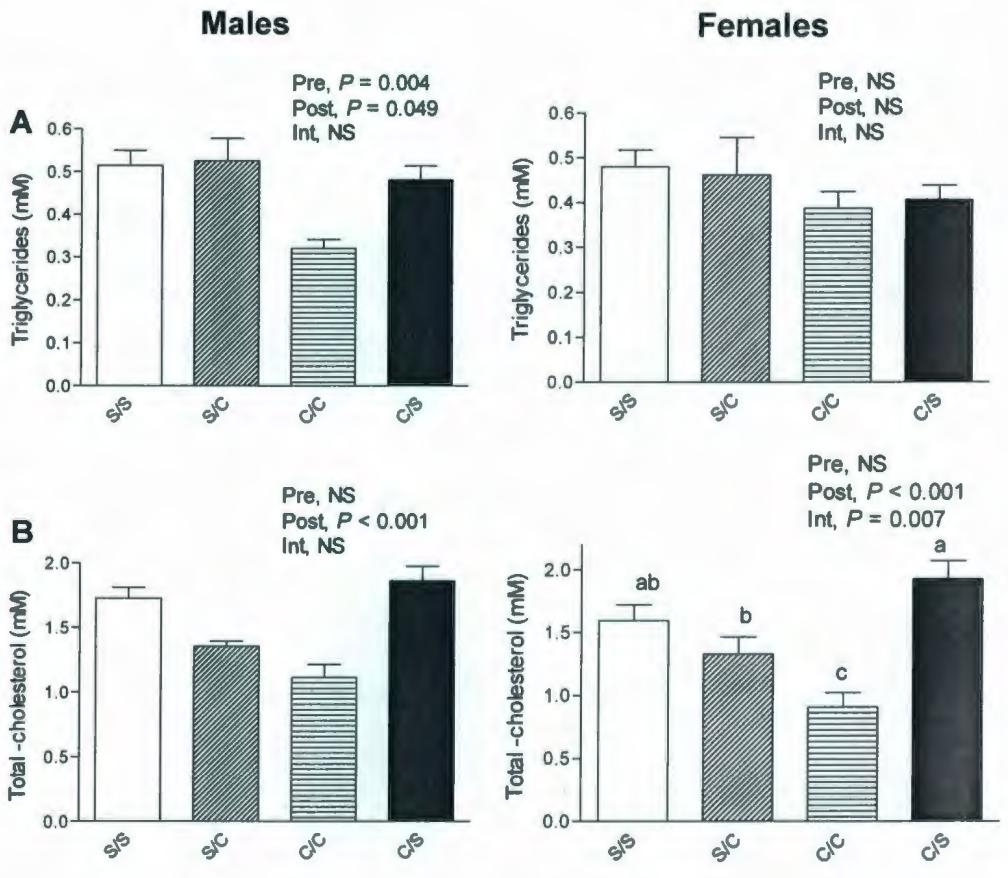

Fig. 3.2 Plasma concentrations of (*A*) triglycerides and (*B*) total-cholesterol in the male and female offspring.

Values are expressed as means ± SEM, n = 7-10 (specified in section 3.2.4). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Letters represent significant differences having $P < 0.05$. NS, non significant; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction.

with higher plasma LDL-cholesterol concentration in female S/S and C/S offspring compared to the S/C offspring (S/S *vs.* S/C, $P = 0.020$) and C/C offspring (C/S *vs.* C/C $P < 0.001$), respectively (Fig. 3.3A). Although no differences were observed between S/S and C/S offspring, a lard-rich diet fed pre-weaning was also associated with higher plasma LDL-cholesterol concentration in the female S/C offspring compared to the C/C offspring ($P = 0.021$) (Fig. 3.3A).

A significant interaction of pre- and post-weaning diets affected the plasma HDL-cholesterol concentration in both male ($P = 0.037$) and female offspring ($P = 0.001$) (Fig 3.3B). Multiple comparisons further revealed that a lard-rich diet fed post-weaning was associated with higher plasma HDL-cholesterol in both male and female C/S offspring compared to the C/C offspring ($P = 0.033$ and $P = 0.001$, respectively) (Fig. 3.3B). In addition, female S/S offspring exhibited lower HDL-cholesterol concentration compared to the C/S offspring ($P = 0.002$) (Fig. 3.3B).

A lard-rich diet fed post-weaning was associated with higher plasma non-HDL cholesterol concentration in both male ($P < 0.001$) and female offspring ($P < 0.001$) compared to chow (Fig. 3.4A). The LDL/HDL-ratio was not different among various groups of male offspring (Fig. 3.4B). However, a lard-rich diet fed both during pre- ($P = 0.031$) and post-weaning time periods ($P < 0.001$) was associated with higher LDL/HDL-ratio in the female offspring compared to chow (Fig. 3.4B).

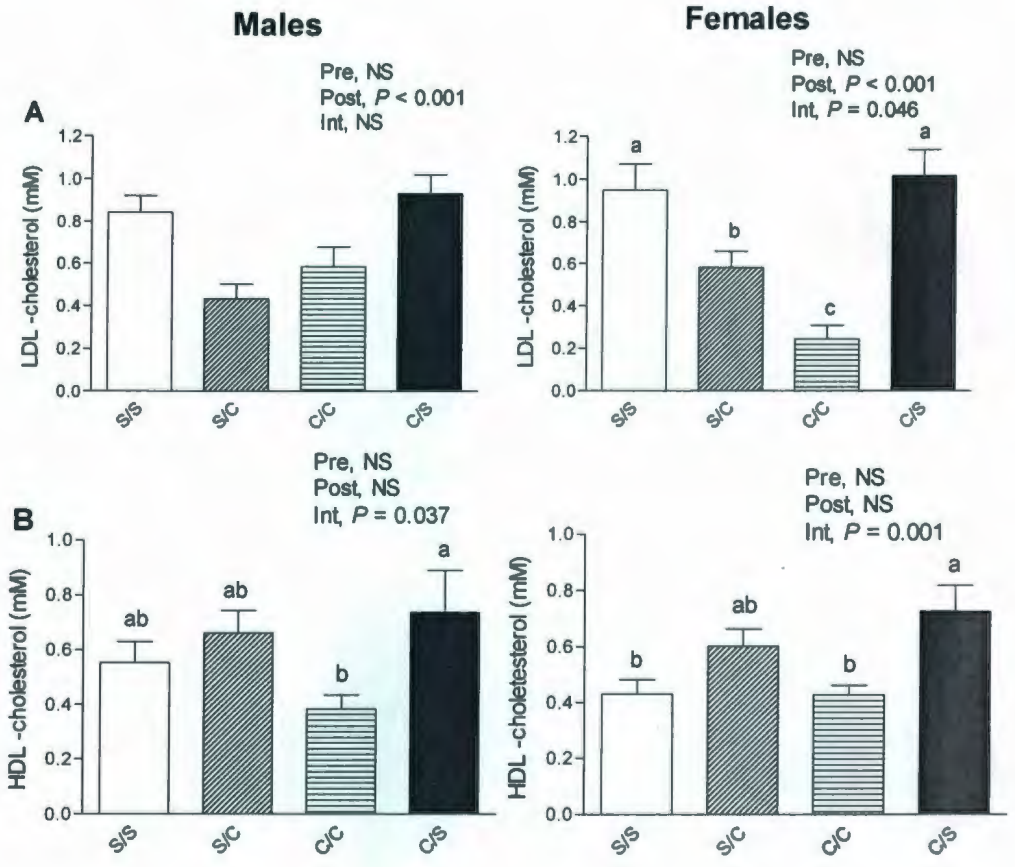

Fig. 3.3 Plasma concentrations of (*A*) LDL-cholesterol and (*B*) HDL-cholesterol in the male and female offspring.

Values are expressed as means ± SEM, n = 7-10 (specified in section 3.2.4). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Letters represent significant differences having $P < 0.05$. NS, non significant; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction.

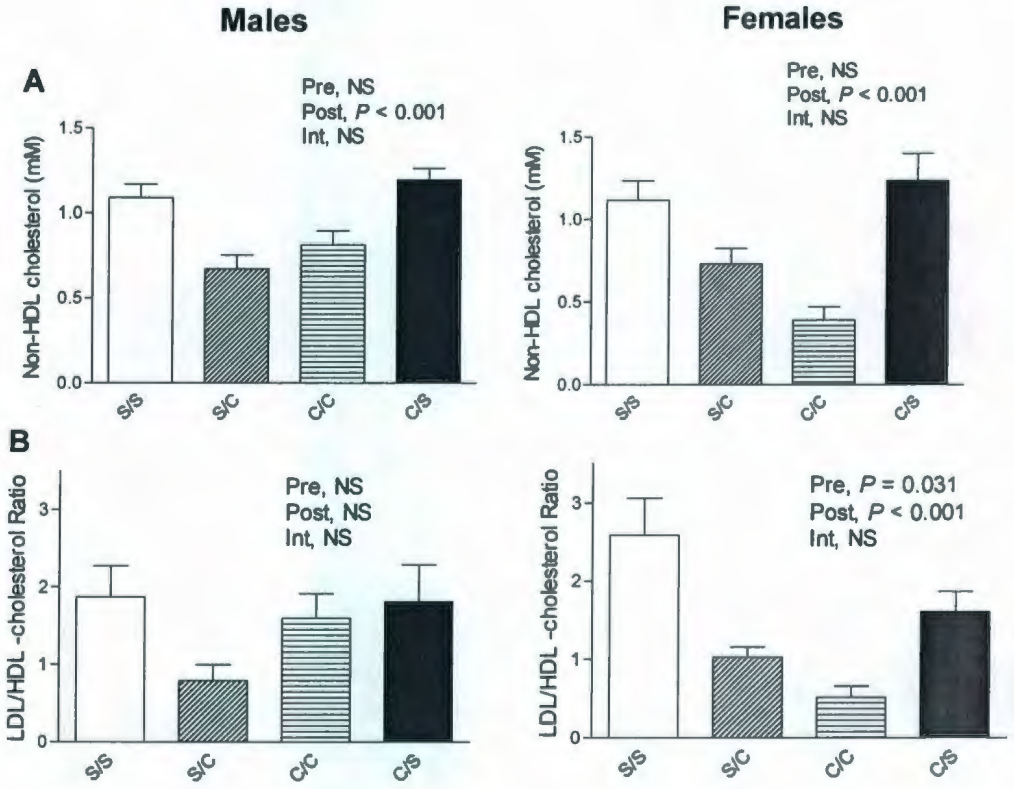

Fig. 3.4 Plasma concentrations of (*A*) non-HDL cholesterol and (*B*) LDL/HDL-cholesterol ratio in the male and female offspring.

Values are expressed as means ± SEM, n = 7-10 (specified in section 3.2.4). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Letters represent significant differences having $P < 0.05$. NS, non significant; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction.

3.3.3 Effects of pre- and post-weaning lard-rich diets on hepatic LDL-r mRNA expression in the male and female offspring

Hepatic LDL-r mRNA expression was not significantly different among various groups of male offspring (Fig. 3.5). However, a significant interaction of the pre and post-weaning diets affected the hepatic LDL-r expression in the female offspring ($P = 0.024$) (Fig. 3.5). Multiple comparisons further revealed that a lard-rich diet fed post-weaning was associated with lower expression of hepatic LDL-r in the female S/S and C/S offspring compared to the S/C offspring (S/S *vs.* S/C, $P < 0.001$) and C/C offspring (C/S *vs.* C/C, $P < 0.001$), respectively (Fig. 3.5). In addition, a lard-rich diet fed pre-weaning was associated with lower expression of hepatic LDL-r in the female S/C offspring compared to the C/C offspring ($P = 0.001$) (Fig. 3.5). A correlation analysis further revealed a significant and inverse relationship between the plasma LDL-cholesterol concentrations and the hepatic mRNA expression of LDL-r in the female S/S ($r = -0.98$, $P = 0.004$), S/C ($r = -0.93$, $P = 0.023$), C/C ($r = -0.97$, $P = 0.008$) and C/S offspring ($r = -0.94$, $P = 0.019$) (Fig. 3.6 A-D).

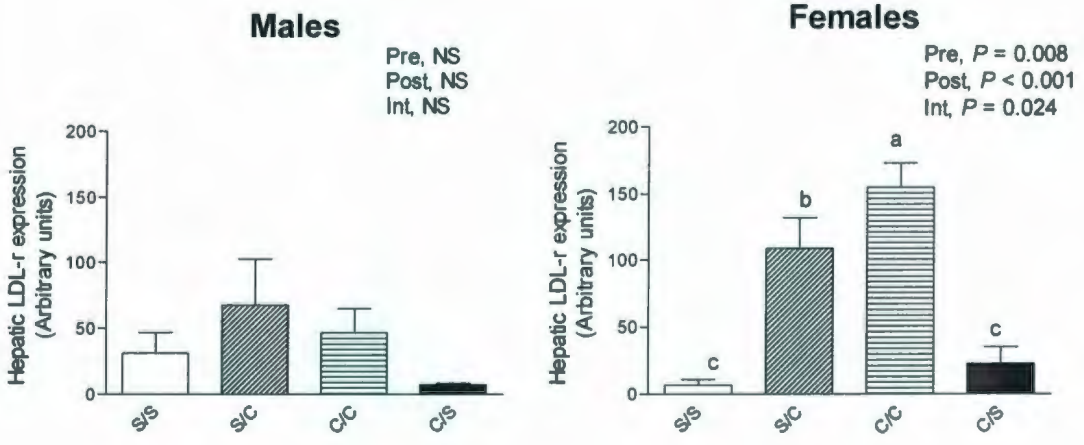

Fig. 3.5 Real-time PCR analysis of hepatic LDL-r mRNA levels in the male and female offspring.

Values are expressed as means ± SEM, $n = 5$. The mRNA expression of LDL-r was calculated in relation to the expression of GAPDH, which was not found to be different among groups. Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Letters represent significant differences having $P < 0.05$. NS, non significant; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction.

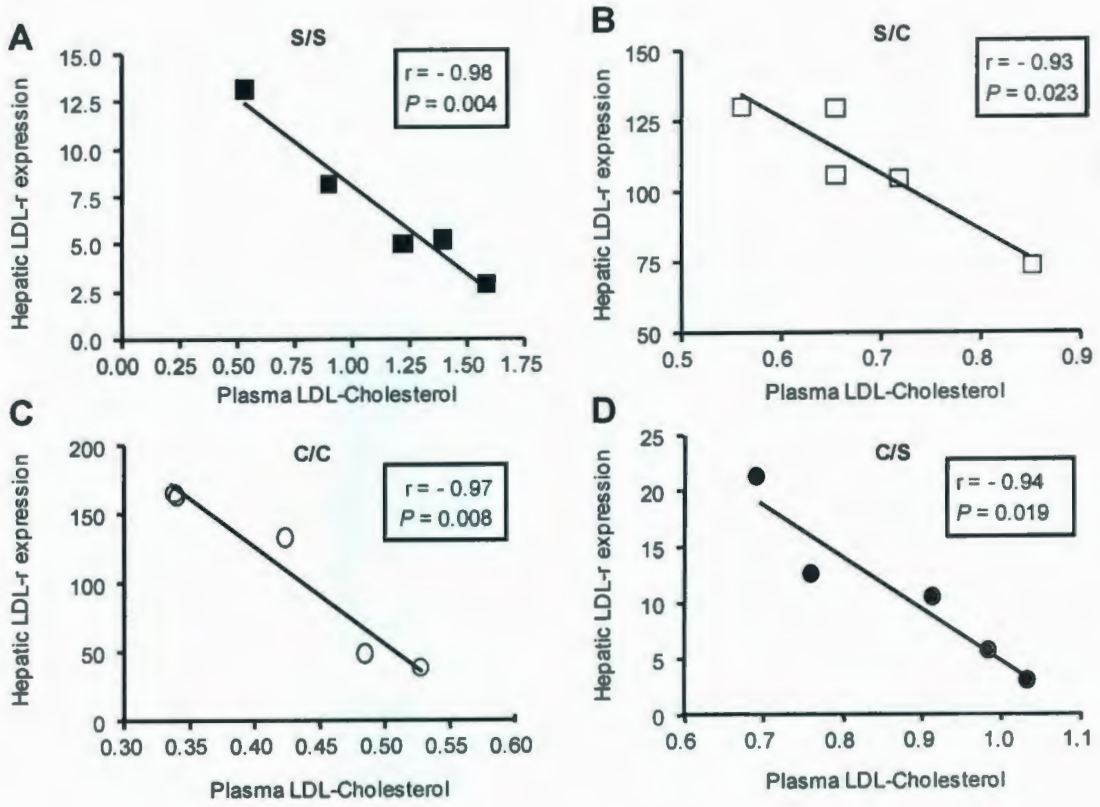

Fig. 3.6 Correlational analysis between hepatic LDL-r mRNA expression and plasma LDL-cholesterol concentration in the female *(A)* SFA/SFA; S/S *(B)* SFA/chow; S/C *(C)* chow/SFA; C/S and *(D)* chow/chow; C/C offspring.

Correlation coefficient (r) was determined using Graphpad Prism software.

3.3.4 Effects of pre- and post-weaning lard-rich diets on contractile responses of the male and female offspring aortas

A lard-rich diet fed pre-weaning was associated with lower contractile responses to KCl in the aortic vessels of male offspring compared to chow ($P < 0.001$) (Fig. 3.7A). On the other hand, a significant interaction of the pre-weaning and post-weaning diets affected the contractile responses to KCl in the female offspring ($P = 0.039$) (Fig. 3.7A). A lard-rich diet fed either pre-weaning or post-weaning was associated with lower contractile responses towards KCl in the female S/S, S/C and C/S offspring compared to the C/C offspring (S/S *vs.* C/C, $P = 0.017$; S/C *vs.* C/C, $P = 0.023$; C/S *vs.* C/C, $P = 0.008$) (Fig.3.7A).

A lard-rich diet fed pre-weaning was associated with lower E_{max} to PE in the aortas of male offspring compared to chow ($P = 0.007$) (Fig. 3.7B). On the other hand, a significant interaction of the pre-weaning and post-weaning diets affected the E_{max} of the aortas to PE in the female offspring ($P = 0.033$) (Fig. 3.7B). A lard-rich diet fed either pre-weaning or post-weaning was associated with lower E_{max} to PE in the aortas of female S/S, S/C and C/S offspring compared to the C/C offspring (S/S *vs.* C/C, $P < 0.001$; S/C *vs.* C/C, $P = 0.001$; C/S *vs.* C/C, $P = 0.004$) (Fig. 3.7B). No differences were observed for the pEC_{50} values to PE among various groups of male and female offspring (Table 3.2).

Neither the contractions nor the pEC_{50} values of aortas to U46619 were different amongst various groups of male offspring (Fig. 3.7C and Table 3.2). However, a

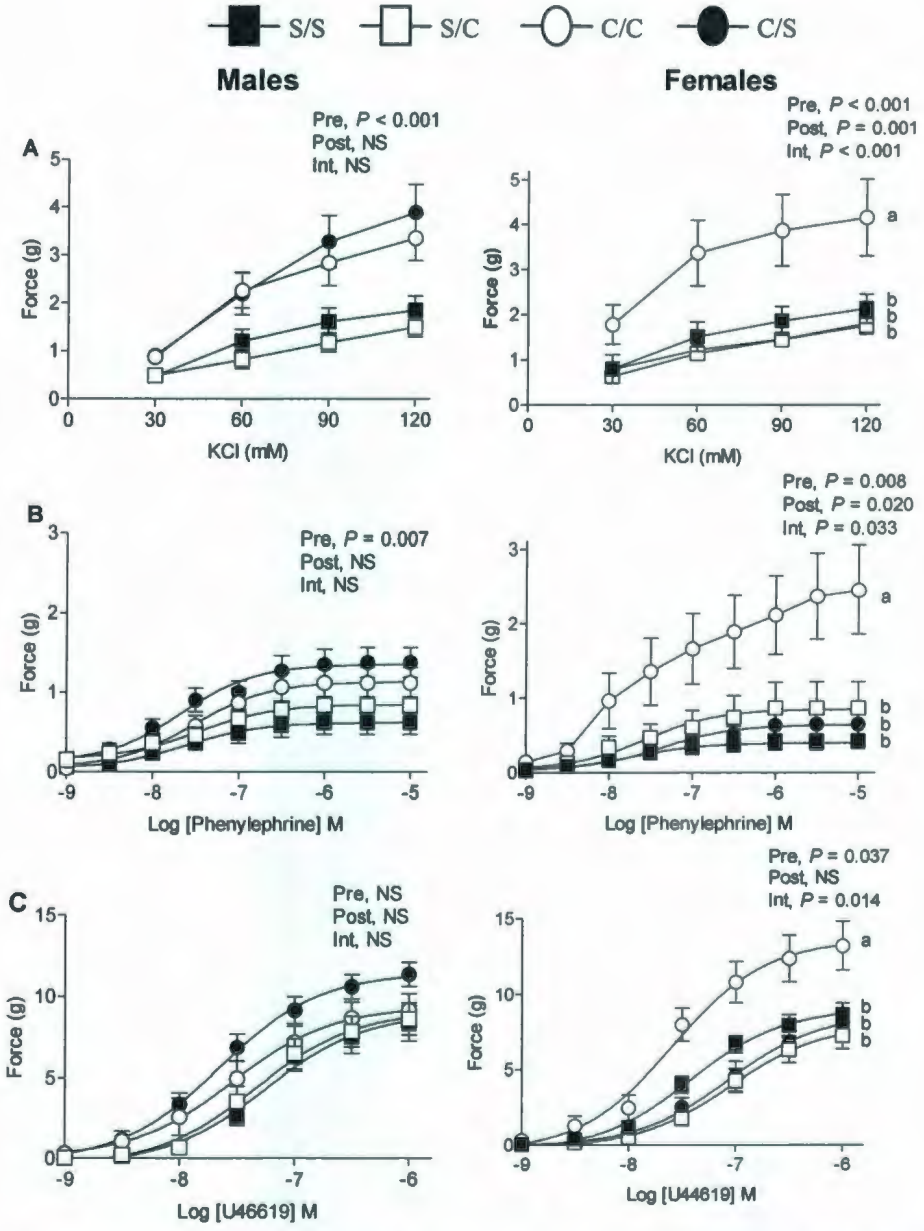

Fig. 3.7 Dose-contraction responses of aortas from the male and female offspring to (*A*) KCl, (*B*) phenylephrine and (*C*) thromboxane mimetic U46619.

Values are expressed as means ± SEM, n = 7-10 (specified in section 3.2.4). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) on E_{max} were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Letters represent significant differences having $P < 0.05$. NS, non significant; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction.

significant interaction of the pre-weaning and post-weaning diets affected the E_{max} (P = 0.014) (Fig. 3.7C) and the pEC_{50} values ($P < 0.001$)] of the aortas to U44619 in the female offspring (Table 3.2). A lard-rich diet fed either pre-weaning or post-weaning was associated with lower E_{max} to U44619 in the aortas of female S/S, S/C and C/S offspring compared to the C/C offspring (S/S *vs.* C/C, $P = 0.008$; S/C *vs.* C/C, $P = 0.001$ and C/S *vs.* C/C, $P = 0.012$) (Fig. 3.7C). Similarly, both S/C and C/S offspring aortas exhibited lower sensitivity to U44619 compared to the C/C and S/S offspring (S/C *vs.* C/C, $P <$ 0.001; S/C *vs.* S/S, $P = 0.010$; C/S *vs.* C/C, $P = 0.002$; C/S *vs.* S/S, $P = 0.027$), (Table 3.2).

3.3.5 Effects of pre- and post-weaning lard-rich diets on endothelium -dependent and -independent relaxation responses of the male and female offspring aortas

The maximal relaxations induced by ACh of aortas were not different among males (Fig. 3.8A). However, a lard-rich diet fed post-weaning was associated with higher sensitivity to ACh in the male offspring compared to chow ($P = 0.032$) (Table 3.2). In females, neither ACh-induced relaxations (Fig. 3.8A) nor their pEC_{50} values were different amongst various offspring (Table 3.2).

SNP-induced maximal relaxations of aortas in various groups of male offspring were not different (Fig. 3.8B), although a significant interaction of the pre- and post-weaning diets affected their sensitivity to SNP ($P = 0.009$) (Table 3.2). Multiple comparisons further revealed that a lard-rich diet fed either pre- or post-weaning was associated with higher

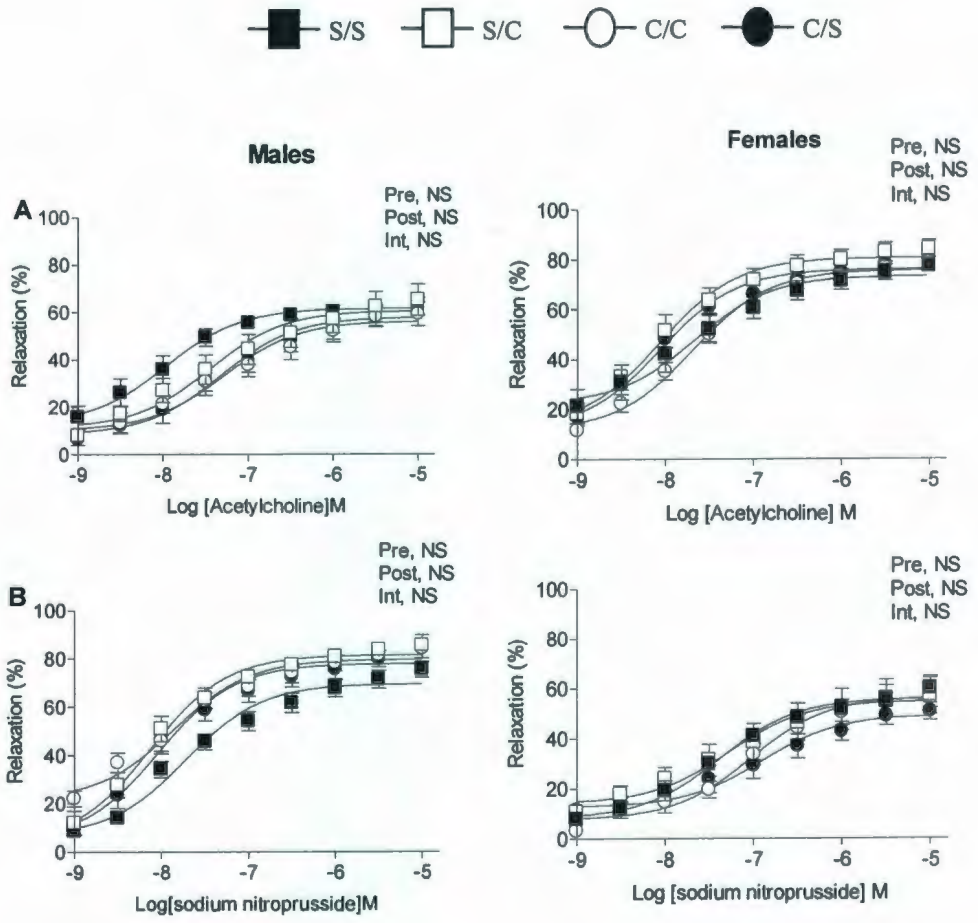

Fig. 3.8 Dose-relaxation responses of aortas pre-constricted with U46619, from the male and female offspring to (*A*) acetylcholine and (*B*) sodium nitroprusside.

Values are expressed as means ± SEM, n = 7-10 (specified in section 3.2.4). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) on E_{max} were assessed using two-way ANOVA. NS, non significant; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction.

Table 3.2 Half-maximal dose concentrations (pEC_{50}) of the male and female offspring aortas towards various drugs.

	SFA/SFA	SFA/Chow	Chow/Chow	Chow/SFA	*Pre*	*Post*	*Int*
			Male offspring				
PE	7.7 ± 0.2	7.6 ± 0.1	7.5 ± 0.2	7.7 ± 0.2	NS	NS	NS
U44619	7.2 ± 0.1	7.3 ± 0.1	7.6 ± 0.2	7.6 ± 0.1	NS	*NS*	NS
ACh	8.1 ± 0.1	7.4 ± 0.2	7.3 ± 0.2	7.4 ± 0.2	NS	$P = 0.032$	NS
SNP	8.2 ± 0.1^{a}	7.7 ± 0.1^{b}	7.9 ± 0.1^{ab}	8.0 ± 0.1^{b}	NS	NS	$P = 0.009$
			Female offspring				
PE	7.7 ± 0.1	7.6 ± 0.1	7.6 ± 0.2	7.3 ± 0.1	NS	NS	NS
U44619	7.5 ± 0.1^{a}	7.0 ± 0.1^{b}	7.7 ± 0.1^{a}	7.0 ± 0.2^{b}	NS	NS	$P < 0.001$
ACh	7.4 ± 0.1	7.2 ± 0.2	7.1 ± 0.1	6.9 ± 0.2	NS	NS	NS
SNP	8.1 ± 0.2	8.1 ± 0.1	7.7 ± 0.1	8.0 ± 0.1	NS	NS	NS

Values are expressed as means ± SEM, n = 7-10 (specified in section 3.2.4). Cumulative concentration–response curves to agonists were analyzed by fitting to a four-parameter logistic equation using non-linear regression to obtain the -log effective concentration equal to 50 % of the maximal response (pEC_{50}). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) were then assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Superscripts represent significant differences having $P < 0.05$. PE, phenylephrine; ACh, acetylcholine; SNP, sodium nitroprusside; NS, non significant; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction.

sensitivity to SNP in the aortas of male S/S offspring compared to the C/S offspring (P = 0.027) and S/C offspring (P = 0.004), respectively (Table 3.2). Unlike males, no differences were found in either maximal relaxation or pEC_{50} values in response to SNP amongst females (Fig. 3.8B and Table 3.2).

3.3.6 Effect of L-NAME on contractile responses of aortas in the male and female offspring

L-NAME treatment was associated with significantly higher E_{max} to KCl in the aortas of both male and female S/C offspring compared to the untreated S/C group (P = 0.003 and P = 0.008, respectively) (Fig. 3.9A). L-NAME treatment was also associated with significantly higher aortic E_{max} to KCl in female C/S offspring compared to the untreated C/S group (P = 0.01) (Fig. 3.9A). The aortic E_{max} to PE was increased by L-NAME treatment only in male S/C group compared to the untreated S/C group (P = 0.045), whereas no differences were observed amongst female offspring (Fig. 3.9B). L-NAME treatment had no effect on contractions to U46619 in either males or females (data not shown).

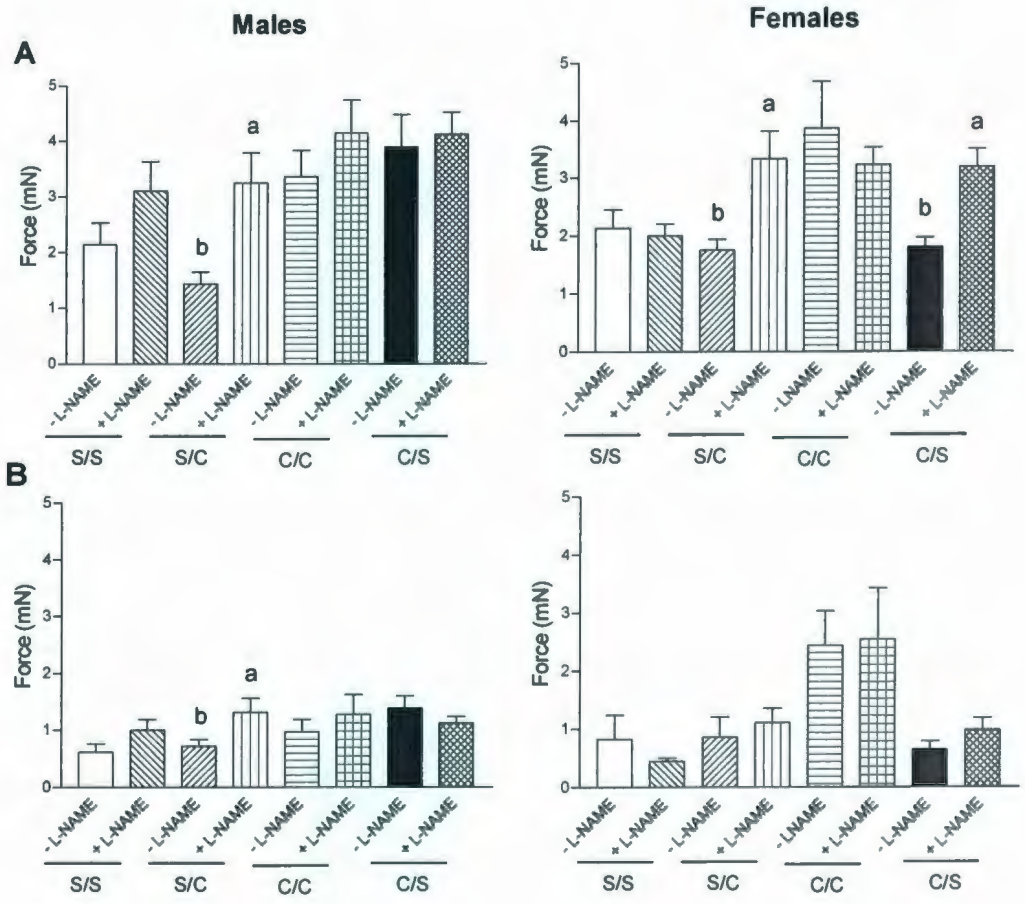

Fig. 3.9 E_{max} of aortas from various male and female offspring groups, before and after treatment with L-NAME, to (*A*) KCl and (*B*) phenylephrine.

Values are expressed as means ± SEM, n = 7-10 (specified in section 3.2.4). Effects of L-NAME treatment on each dietary group were evaluated using paired t-tests. Superscripts represent significant differences between the treated and untreated groups where $P < 0.05$.

3.4 DISCUSSION

This study evaluated the effects of feeding a lard-rich diet during pre- and post-weaning time periods on several cardiovascular risk factors in the adult offspring of C57Bl/6 mice. It was demonstrated that feeding a lard-rich diet to the mothers during gestation and lactation induced sex-dependent features of dyslipidemia and aortic contractile dysfunction in their offspring. Furthermore, this study established that maternal intake of a lard-rich diet can 'program' the expression of hepatic LDL-r mRNA in their offspring, resulting in an increase in the offspring plasma LDL-cholesterol concentrations. The suppression of hepatic LDL-r gene expression may prove to be a key factor in the pathogenesis of cardiovascular abnormalities observed in these offspring.

Consumption of a lard-rich diet post-weaning was associated with higher body weight in both male and female offspring. However, both male and female offspring fed a lard-rich diet post-weaning consumed less food than the C/C offspring. Rodents, like humans, can regulate their food intake and energy expenditure in order to maintain a set body weight (Keesey and Hirvonen, 1997). Considering that the lard-rich diet used in the current study was energy-dense as compared to chow, the mice exposed to a lard-rich diet could have reduced their food intake to maintain their energy balance. However, since the lard-rich diet derived most of its calories from fats (40%) as opposed to chow (10%), excess fat-derived calories could have contributed to a higher body weight in these mice compared to chow-fed mice. Moreover, quite interestingly both male and female S/C offspring also consumed less food and calories than C/C offspring. It appears that the consumption of a lard-rich diet by mothers during pregnancy had a "programming effect"

on the developing fetus to lower their appetite after birth. Maternal nutrition during pregnancy has previously been shown to program the appetite-regulation in the offspring (Muhlhausler *et al.*, 2006; Ferezou-Viala *et al.*, 2007; Morris and Chen, 2009), which is proposed to involve leptin. Leptin, an adipocyte-derived hormone, has been reported to influence the appetite-controlling centers in the brain and hence proposed to play an important role in the regulation of food intake (Muhlhausler *et al.*, 2006; Vickers, 2007). A diet rich in SFA has been reported to increase plasma leptin concentrations both in mice as well as in humans (Frederich *et al.*, 1995; Chu *et al.*, 2001). It is likely that feeding a lard-rich diet to the mothers during pregnancy caused an increase in leptin concentrations, which in turn would have programmed the developing fetus to consume less food after birth. Plasma leptin concentrations, however, were not measured in the current study.

Plasma lipids were higher in the female S/C offspring as compared to C/C offspring, indicating that a maternal lard-rich diet fed during pregnancy induced features of dyslipidemia in the female offspring only. However, a post-weaning lard-rich diet was associated with higher plasma lipids in both male and female offspring. The deleterious effects of consuming a lard-rich diet on the plasma lipid parameters were in line with the proposed effects of SFA intake. It has been established that a high SFA intake increases the risk of CVD by causing dyslipidemia (Mensink, 1993). Some, but not all of the previous studies investigating the role of maternal high-fat feeding in the 'fetal programming' of adult disorders have reported features of dyslipidemia in the offspring (Ghosh *et al.*, 2001; Khan *et al.*, 2003; Khan *et al.*, 2005). An increase in plasma total-

and LDL-cholesterol was reported only in the female offspring in response to a maternal diet rich in lard, thus it can be concluded that gender-associated mechanisms are involved in the 'programming' of offspring dyslipidemia.

The ratio of LDL- to HDL-cholesterol concentration is considered to be a marker for the future development of CVD (Lemieux *et al.*, 2001; Panagiotakos *et al.*, 2003). A diet rich in SFA has been shown to increase plasma LDL-cholesterol and decrease HDL-cholesterol concentrations (Mensink, 1993), resulting in higher LDL/HDL-ratio. Female S/S offspring exhibited the highest LDL/HDL-ratio compared to all other offspring, suggesting that the additive effects of pre- and post-weaning diets rich in lard contributed to their plasma lipid levels.

A possible explanation for the higher circulating levels of plasma LDL-cholesterol can be related to its reduced clearance from the circulation. Liver LDL-r removes LDL-cholesterol from circulation, thereby maintaining cholesterol homeostasis (Brown and Goldstein, 1984). A high SFA intake during the postnatal time period has been reported to lower the expression of hepatic LDL-r in several animal models (Srivastava *et al.*, 1991; Hennessy *et al.*, 1992; Horton *et al.*, 1993; Dorfman and Lichtenstein, 2006) and in human studies (Hayes *et al.*, 1997). The LDL-r mRNA expression was significantly lower in the female S/S and C/S offspring. Moreover, female S/C offspring had significantly lower LDL-r expression than the C/C offspring, which correlated negatively with their plasma LDL-cholesterol concentrations. Thus, a decrease in hepatic LDL-r expression supported the hypothesis that in female offspring, an increase in LDL-cholesterol was a result of 'programming effects' of a maternal diet enriched with lard.

This is the first study to report the 'programming' of hepatic LDL-r mRNA expression in the offspring by a lard-rich maternal diet, providing a mechanistic link between programming of dyslipidemia and CVD.

The experimental design of the current study further allowed the investigation of whether "programming" effects due to the maternal diet could be reversed by the post-weaning diet of the offspring. The offspring obtained from mothers fed a lard-rich diet during gestation and lactation and fed chow post-weaning (*i.e.* S/C) had higher expression of hepatic LDL-r expression than the S/S offspring. These findings suggest that although maternal intake of a lard-rich diet programmed the hepatic expression of LDL-r in the female offspring, chow feeding post-weaning was, in part, able to reverse the programming effects of the maternal diet.

Endothelial dysfunction has been considered to be another marker for the onset of CVD. It has been reported earlier that endothelial function may be abnormal within a few hours of exposure to increased levels of LDL cholesterol (Jayakody *et al.*, 1987; Cohen *et al.*, 1988; Liao, 1994). Thus, vascular reactivity was expected to change in the presence of high levels of LDL-cholesterol, especially in the female offspring. Increased vascular contractility and decreased vasodilator capacity have been observed in adult models of maternal over-nutrition and CVD (Koukkou *et al.*, 1998; Gerber *et al.*, 1999; Khan *et al.*, 2005). Feeding a lard-rich diet to the mothers during gestation and lactation caused a reduction in vascular contractility, whereas vasodilator capacity was unaffected in the current study. A lard-rich diet fed pre-weaning was associated with lower aortic contractile responses towards KCl and PE in the male offspring, indicating that maternal

intake of a lard-rich diet may have altered the components involved in voltage-gated ion channel or α_1-adrenergic activation and/or sensitivity of the contractile apparatus to calcium ions in the offspring (Ozaki *et al.*, 2001). In females, the contractile responses to all of the agents *i.e.* KCl, PE and U46619, were reduced in S/S, S/C and C/S offspring as compared to the C/C offspring, suggesting that female aortic vessels were more affected by feeding of lard-rich diets, irrespective of the time of exposure being pre-or post-weaning. Ozaki *et al.,* 2001 have previously reported a reduction in the maximal contractile responses to PE in the femoral arteries of 20-day-old offspring and an increase in the maximal vasoconstriction to U46619 and sensitivity to KCl in 200-day-old offspring of mothers subjected to 30% caloric food restriction during pregnancy. Although it is difficult to exclude the possibility of specific defects in the sympathetic and thromboxane responsiveness of these vessels, a simultaneous reduction in all components of the contractile apparatus points towards a more general effect of lard-rich diet feeding, irrespective of the pre- or post-weaning nutrition, especially in the female offspring.

The principal vasodilating agent generated in conduit vessels is currently thought of as being NO (Loscalzo and Welch, 1995). It was proposed that an increased production of basal NO would lower the contractions to all of the agonists used in this study. This hypothesis was tested by assessing the effects of NOS inhibition on contractile responses of the aortic vessels, using a NOS inhibitor. L-NAME caused a small increment in the contractile responses to KCl and PE in S/C males and to KCl in the S/C females, which would be consistent with the proposed hypothesis. Previous studies dealing with maternal

over-nutrition during pregnancy have indicated an impairment of ACh-induced vasorelaxation, albeit using smaller caliber resistance type arteries, where the endothelial-derived relaxing factors (EDRFs) differ from those present in aorta *e.g.* EDHF's (Koukkou *et al.*, 1998; Ghosh *et al.*, 2001; Khan *et al.*, 2003). Female offspring did not show any alterations in the relaxation responses to ACh and SNP. In contrast, the aortas of male S/S were more sensitive to ACh and SNP yet showed no changes in the maximal relaxations. An increased sensitivity to ACh and SNP may be considered consistent with the findings of increased basal NO activity, perhaps linked to an insulin-mediated increase in NO availability in these animals. Insulin has been shown to increase the basal NO availability through the PI-3 kinase and protein kinase B-mediated phosphorylation and hence activation of endothelial NOS (Wheatcroft *et al.*, 2004). Maternal high SFA intake during pregnancy has been shown to induce hyperglycemia and hyperinsulinemia (Gerber *et al.*, 1999) and whole body insulin resistance in the 24-week-old rat offspring (Taylor *et al.*, 2005). The age of the offspring in the current study then would be a discriminating factor associated with the observed differences to other studies, such that at later ages these mice may become insulin resistant, and exhibit the impairment of endothelium-dependent vasodilation associated with worsened CVD status. The insulin resistance status of these animals was not determined, but female S/S showed an increase in plasma FFA concentration, which suggests that these females could become insulin resistant later in life.

Maternal high-fat intake has also been reported to generate reactive oxygen species (ROS) and oxidative stress, which has been linked to vascular disorders in the offspring

(Koukkou *et al.*, 1998; Gerber *et al.*, 1999). It has been reported that increased ROS generation can lead to increased production of hydrogen peroxide in vascular cells, that can act as an EDRF and can induce the vasodilation of aortic vessels in response to ACh (Matoba *et al.*, 2000), despite the presence of endothelial dysfunction (Noronha *et al.*, 2005). Although, the ROS production in the aortic vessels was not determined in the current study, it is likely that consuming a lard-rich diet during pre- or post-weaning time period caused an increase in the oxidative stress that may underlie the reduced aortic contractile responsiveness of these offspring.

In addition, membrane fatty acids, especially those of the endothelial cells, play a crucial role in the manifestation of the signaling cascades that are responsible behind the agonist-induced contractile and relaxation responses of the vascular tissue (Saraswathi *et al.*, 2004). Alterations in the aortic fatty acid composition can also affect the fluidity of the membrane, and hence its function (Bing *et al.*, 1993). The total fatty acid composition of the aortic vessels from the male and female offspring was altered by the exposure to the lard-rich diets during pre- and post-weaning time periods (Appendix I), which could affect the receptor-mediated signaling events involved behind the aortic contractile responses to KCl, PE or U44619. Moreover, an increased accumulation of SFA was observed in the offspring aortic vessels (Appendix I), which could affect membrane saturation leading to reduced elasticity, ultimately resulting in the aortic contractile dysfunction of these vessels.

In conclusion, the current study reported that a lard-rich diet fed to the mothers during gestation and lactation was associated with higher plasma total- and LDL-

cholesterol concentrations and lower hepatic LDL-r mRNA expression in the female offspring. In addition, reduced aortic contractile reactivity towards various agonists was observed in the female offspring exposed to lard-rich diets, irrespective of the time of exposure being pre- or post-weaning. On the other hand, male offspring of the mothers fed a lard-rich diet during gestation and lactation exhibited higher plasma TG concentration and lower contractile reactivity to KCl and PE, pointing towards the gender-associated differences of dietary fat-mediated programming. In addition to the maternal diet, the current study also revealed an effect for interaction between the pre- and post-weaning lard-rich diets on the overall physiological outcome in the offspring, suggesting the importance of both prenatal and postnatal environments.

3.5 LIMITATION OF THE STUDY

The current study involved the comparison of a semi-purified diet enriched with lard (20% *w/w)* with rodent chow, during the pre- and post-weaning time periods, on the cardiovascular biomarkers in the adult offspring. Although the primary conclusions of the current study have been drawn with respect to the SFA content of the lard-rich diet, it would be reasonable to acknowledge that the semi-purified high-fat diet differs in various respects from the rodent chow, used in the current study. Thus, all or some of those factors may have contributed to the outcomes of the current study. Some of the reasonable differences in macronutrients would involve the source of protein, amount of sucrose.

Although the amount of protein was similar in both chow and the semipurified-diet enriched with lard, the source of protein was different. The source of protein in the lard-rich diet was casein, whereas the protein in the rodent chow comes from ingredients such as soybean meal and fishmeal. Both soy-proteins and fish-proteins have been attributed as having cholesterol-lowering effects when compared to casein as the source of protein. More specifically, soy-proteins have been shown to lower LDL-cholesterol and LDL/HDL-ratio in human studies (Pipe *et al.,* 2009, Forsathye *et al.,* 1986). In addition, soy-proteins and fish-proteins have been reported to increase the hepatic LDL-r expression in animal and human studies (reviewed in Sirtori, 2009). Presence of soy- and fish- proteins may explain the reversal of hepatic LDL-r expression in the female S/C offspring compared to the S/S offspring in the current study. In addition, a number of clinical studies have reported an improvement in the endothelial function with feeding diets supplemented with soy-proteins (Cuevas *et al.,* 2003; Dubroff *et al.,* 1999; Yildirir *et al.*, 2001).

As compared to the chow, lard-rich diet also contained a higher amount of sucrose (3.38% *vs.* 30%), thereby making the lard-rich diets rich in both fat and sugar. High-fat high-sucrose diet has been shown to induce an increase in body weight, plasma total-and HDL-cholesterol in C57Bl/6 mice (Nascimento *et al.,* 2010). Feeding a diet rich in lard (10%) and sucrose (30%) for 36-weeks was associated with impaired glucose clearance and increased adiposity in the male Japanese rabbits compared to rabbits fed chow (Zhao *et al.,* 2008). Another study demonstrated dyslipidemia, hyperinsulinemia and hepatic steatosis in Wistar rats fed a high-sucrose diet (70% calories from sucrose) for a period of

5-weeks (Huang *et al.,* 2007). In addition, reduced vasodilator capacity has been reported in the aortic rings of Sprague-Dawley rats fed a high-fat high-sucrose diet (20% fat and 45% sucrose) for a period of 4-weeks compared to the rats fed chow (Bourgoin *et al.,* 2008).

Thus, it can be concluded that besides the fat content, other factors present in the semi-purified diets may have affected the outcome of the current study. These discrepancies can be tackled in future studies, where the diets are specifically formulated to control the amount and source of various macronutrients. Ideally, a semi-purified diet rich in 20% fat should be compared with a semi-purified diet designed for a 5% fat level.

Lastly, the lard-rich diet used in the current study contained 46% MUFA and 35% SFA (Table 2.2). A diet rich in MUFA is considered to be beneficial on the outcome of CVD as compared to a diet rich in SFA (Kris-Etherton, 1999). Moreover, it has been associated with lowering plasma cholesterol levels (Kris-Etherton, 1999), and the prevention of endothelial dysfunction in humans when compared to a diet rich in SFA (Fuentes *et al.,* 2004). Thus, it can be argued that the presence of MUFA in the lard-rich diet would offset the effects of SFA, suggesting that the observations of the current study might be even exaggerated, if a diet purely rich in SFA was used instead of the lard-rich diet. This viewpoint can be explored in the future studies.

CHAPTER FOUR

Effects of feeding a high-fat maternal diet rich in safflower oil (n-6 PUFA) during gestation and lactation on lipid metabolism and aortic vascular function in the adult offspring

A version of this chapter is under review for publication in the *British Journal of Nutrition*

4.1 INTRODUCTION

It is well known that besides the quantity of dietary fat intake, its quality reflected by the type of fatty acids, also plays a significant role in the development of CVD. While an increased consumption of SFA has been associated with higher incidence of CVD (Artaud-Wild *et al.*, 1993; Keys, 1997; Denke, 2006), a diet rich in PUFA is reported to lower the risk of developing CVD (Dolecek, 1992; Demaison and Moreau, 2002). Therefore, the current recommendation is to replace dietary SFA with PUFA (Sanders, 2000; Russo, 2009), which has led to an excessive average intake of PUFA by the general population. Among the PUFA, the *n-3* PUFA in comparison to *n-6* PUFA, are well known to possess beneficial effects on the outcome of CVD (Demaison and Moreau, 2002). Although the role of PUFA intake during adult life is relatively well studied, the role of PUFA intake *in utero* and its effects on the development of CVD in adult life, remain to be defined.

A handful of studies dealing with the role of maternal PUFA intake have focused on *n-3* PUFA status during pregnancy. It has been reported that *n-3* PUFA deficiency during the perinatal period was associated with higher mean arterial blood pressure in the offspring (Weisinger *et al.*, 2001; Armitage *et al.*, 2003), which was further associated with a 30% reduction in the brain DHA levels in the offspring (Armitage *et al.*, 2003). In addition, *n-3* PUFA feeding during pregnancy was associated with increased pancreatic islet number in the adult offspring (Siemelink *et al.*, 2002). Overall, these studies suggest a beneficial role of *n-3* PUFA intake during pregnancy on the health outcome in the offspring. However, the role of *n-6* PUFA intake during pregnancy and its impact on the

offspring cardiovascular health has not yet been addressed. Ailhaud *et al* have argued that increased exposure to *n-6* PUFA during early life is responsible for the epidemic of obesity in the European population (Ailhaud *et al.*, 2006; Ailhaud *et al.*, 2008). Similarly, *n-6* PUFA has been reported to constitute ~85% of the total dietary PUFA intake in North American diet (Simopoulos *et al.*, 1999). This prevalence would also point towards an increased intake of *n-6* PUFA during pregnancy, thus emphasizing the need to determine the role of high *n-6* PUFA consumption during pregnancy on the cardiovascular health outcome in the offspring.

A diet rich in *n-6* PUFA is known to lower plasma TG and total-cholesterol in adults, thereby reducing CVD risk. It was hypothesized that a high-fat diet, enriched in *n-6* PUFA, fed to the mothers during gestation and lactation would lower the risk of developing CVD by the offspring. The current study tested this hypothesis by investigating the effects of feeding a diet rich in safflower oil to the mothers during gestation and lactation on the lipid metabolism and aortic vascular reactivity, as biomarkers for assessing CVD risk in their adult offspring. In addition, the effects of interaction between the pre- and post-weaning diets on the risk of developing CVD in the offspring were studied.

4.2 METHODS

4.2.1 Experimental Design

As described in section 2.1, female C57Bl/6 mice were maintained on rodent chow for a week prior to feeding the experimental diets. After this acclimatization period, females

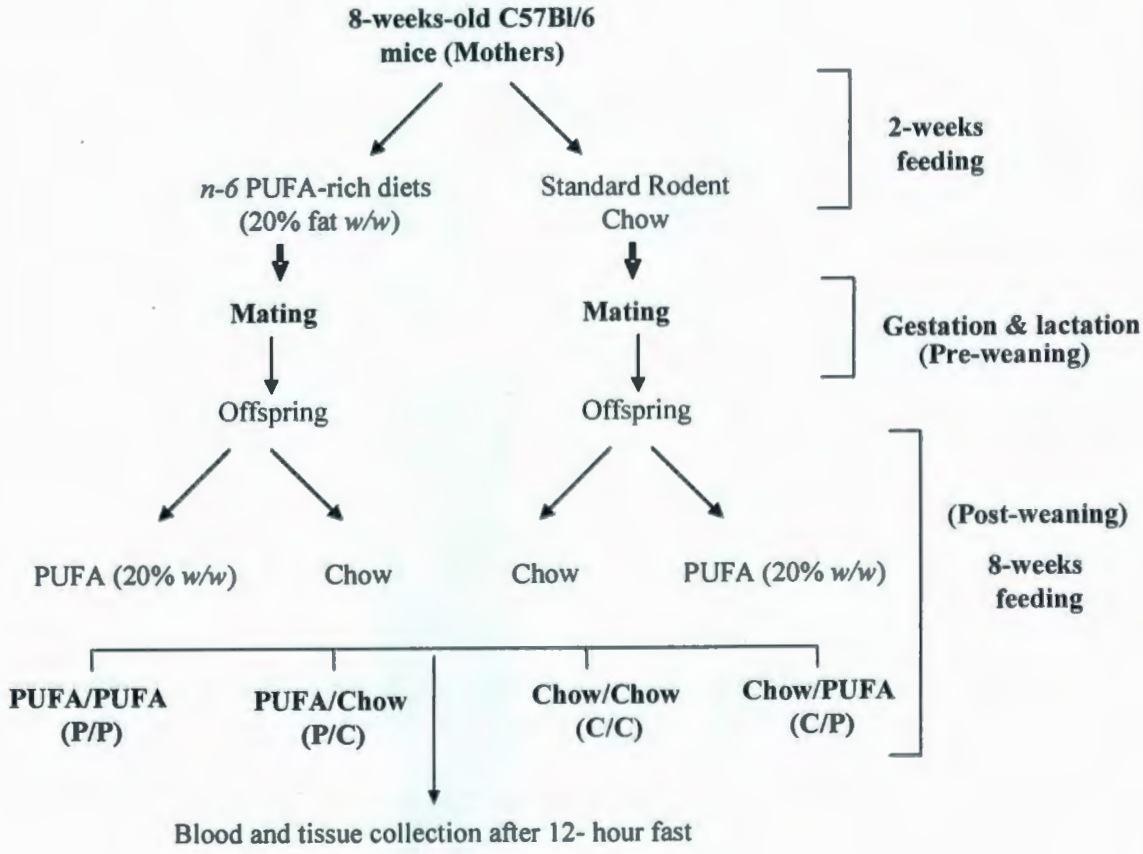

Fig. 4.1 Experimental Design.

8-week-old female C57BL/6 mice were fed either a high fat diet rich in *n-6* PUFA (safflower oil) or standard rodent chow for 2-weeks. Females continued on these diets during mating, pregnancy and lactation. At weaning, the offspring obtained from each mother were divided into two groups; one group continued on safflower oil-rich diet and the other group was fed rodent chow. The offspring groups were identified by *pre-/post-weaning diet* combinations as: PUFA/PUFA (P/P), PUFA/chow (P/C), chow/chow (C/C) and chow/PUFA (C/P). The offspring were fed their assigned diets *ad libitum* for another 8-weeks.

were divided into two groups and were fed the experimental diets according to the design specified in Fig. 4.1. The offspring groups were identified by *pre-/post- weaning diet* combinations as: PUFA/PUFA (P/P), PUFA/chow (P/C), chow/chow (C/C) and chow/PUFA (C/P). Due to skewed sex ratio and poor survival of the male offspring born to the mothers fed a diet rich in *n-6* PUFA, only female offspring from each dietary group were used for the current study.

4.2.2 Quantitative-PCR analysis of hepatic LCAT and SR-B1 mRNA expression

Total RNA was isolated from liver as previously described (Chomczynski and Sacchi, 1987). Reverse transcription of total RNA into cDNA was performed using one-step reverse transcription kit from Roche Diagnostics (PQ, Canada). The mRNA expression levels of LCAT and SR-B1 were determined on a Lightcycler 2.0 Detection System (Roche Diagnostics, PQ, Canada). Briefly, standard curves were generated using the serial dilution of a control sample for both target (LCAT and SR-B1) and GAPDH genes and the PCR efficiency for each reaction was calculated. No differences were found in the expression of GAPDH among various groups. The expression of LCAT and SR-B1 for each sample was then calculated in relation to the expression of GAPDH, thus, normalizing and correcting the data for the differences in PCR efficiencies for each set of primers. The sequence of the primers used for the q-PCR analysis is given in Table 4.1.

4.2.3 Calculations and Statistical Analysis

Data were expressed as means ± SEM, where PUFA/PUFA (P/P), $n = 8$; PUFA/chow (P/C), $n = 6$; Chow/chow (C/C), $n = 10$ and Chow/PUFA (C/P), $n = 6$. Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet x post-weaning diet) were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests (*SYSTAT for Windows, version 12.02*; *SYSTAT Software Inc.*, Richmond, California). Differences having a $P < 0.05$ were considered significant.

Constrictor responses were reported as the force generated in response to each concentration of the agonist, and relaxant responses as the percentage reversal of U44619-induced contraction. Cumulative concentration–response curves to agonists were analyzed by fitting to a four-parameter logistic equation using non-linear regression to obtain the -log effective concentration equal to 50% of the maximal response (pEC_{50}) and maximum response (E_{max}) (Prism 3.0, GraphPAD Software Inc). pEC_{50} and E_{max} values were then compared using two-way ANOVA and Fisher's LSD *post hoc* analysis. Since the KCl-response curves were not sigmoidal, pEC_{50} values were not calculated and only maximal responses were compared among various groups.

Table. 4.1 Sequence of the primers used for the quantitative PCR analyses

Gene	Primers
SR-B1 (S)	5' TTTGGAGTGGTAGTAAAAAGGGC-3'
SR-B1 (AS)	5'-TGACATCAGGGACTCAGAGTAG-3'
LCAT (S)	5'- GTAACCACACACGGCCTGTC-3'
LCAT (AS)	5'- TCTTACGGTAGCACATCCAGTT-3'
GAPDH (S)	5'-TGAAGCAGGCATCTGAGGG-3'
GAPDH (AS)	5'-CGAAGGTGGAAGAGTGGGAG-3'

SR-B1 = Scavenger receptor-B1, LCAT = Lecithin: cholesterol acyltransferase, GAPDH= Glyceraldehyde 3-phosphate dehydrogenase

4.3 RESULTS

4.3.1 Effects of pre- and post-weaning safflower oil-rich diets on offspring body weight, food and caloric intake, and plasma concentrations of glucose and FFA

A significant interaction between the pre- and post-weaning diets affected the offspring body weight at 11-weeks of age ($P = 0.002$) (Table 4.2). Multiple comparisons further revealed that a continuous exposure to the safflower oil-rich diet during pre- and post-weaning periods was associated with higher body weight in the P/P offspring compared to the all other offspring (P/P *vs.* C/P, $P = 0.013$; P/P *vs.* C/C, $P = 0.024$; P/P *vs.* P/C, $P < 0.001$). In contrast, a safflower oil-rich diet fed pre-weaning was associated with lower body weight in P/C offspring compared to the C/C offspring ($P = 0.038$) (Table 4.2). A significant interaction between the pre- and post-weaning diets also affected the food ($P = 0.028$) and caloric intake ($P = 0.037$) in the female offspring (Table 4.2). Multiple comparisons further revealed that a safflower oil- rich diet fed post-weaning was associated with lower food and caloric intake in C/P and P/P offspring compared to C/C ($P < 0.001$) and P/C offspring ($P < 0.001$), respectively (Table 4.2). In addition, a safflower oil-rich diet fed pre-weaning was associated with a lower food and caloric intake in the P/C offspring compared to the C/C offspring ($P < 0.05$) (Table 4.2). No differences were observed among the offspring for either fasting plasma glucose or FFA concentrations (Table 4.2).

Table 4.2 Body weight, food and caloric intake, plasma glucose and FFA concentrations of various offspring at the time of sacrifice

	P/P	P/C	C/C	C/P	*Pre*	*Post*	*Int*
BW (g)	21.3 ± 0.8^{a}	16.7 ± 0.3^{c}	18.6 ± 0.8^{b}	18.3 ± 0.4^{bc}	NS	$P = 0.014$	$P = 0.002$
FI (g/day)	2.5 ± 0.2^{c}	3.6 ± 0.1^{b}	4.1 ± 0.2^{a}	2.3 ± 0.0^{c}	NS	$P = 0.000$	$P = 0.028$
CI (kcal/day)	12.8 ± 1.0^{c}	14.7 ± 0.3^{b}	17.0 ± 1.0^{a}	11.7 ± 0.2^{c}	NS	$P = 0.000$	$P = 0.037$
Glucose (mM)	7.4 ± 0.7	8.5 ± 0.6	8.3 ± 0.4	8.5 ± 0.4	NS	NS	NS
FFA (mEq/L)	1.4 ± 0.3	1.2 ± 0.0	1.0 ± 0.1	1.4 ± 0.1	NS	NS	NS

Values are expressed as means ± SEM, n = 6-10 (specified in section 4.2.3). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Superscripts represent significant differences having $P < 0.05$. BW, body weight; FI, food intake; CI, caloric intake; FFA, free fatty acids; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction; NS, not significant, P, PUFA; C, chow.

4.3.2 Effects of pre- and post-weaning safflower oil-rich diets on offspring plasma lipid levels

A significant interaction between the pre- and post-weaning diets was observed for the offspring plasma TG ($P = 0.014$) and total-cholesterol concentrations ($P = 0.005$) (Fig. 4.2 A&B). In addition, a safflower oil-rich diet fed post-weaning was associated with higher plasma total-cholesterol in the C/P offspring compared to all other offspring (P/P *vs.* C/P, $P = 0.007$; P/C *vs.* C/P, $P = 0.047$; C/C *vs.* C/P, $P = 0.001$) (Fig. 4.2B). No differences were observed for the plasma LDL-cholesterol concentrations among various offspring (Fig. 4.2 C). However, a significant interaction between the pre- and post-weaning diets affected plasma HDL-cholesterol concentration ($P < 0.001$) (Fig. 4.2D). Multiple comparisons further revealed that a safflower oil-rich diet fed either pre- or post-weaning was associated with lower plasma HDL-cholesterol concentration in the P/P offspring compared to C/P offspring ($P = 0.018$) and P/C offspring (P/P *vs.* P/C, $P < 0.001$). In addition, a safflower oil-rich diet fed pre-weaning was associated with higher plasma HDL-cholesterol concentration in the P/C offspring compared to the C/C offspring ($P < 0.001$) (Fig. 4.2D).

A safflower oil-rich diet fed post-weaning was associated with higher plasma non-HDL cholesterol concentrations in the offspring compared to chow ($P = 0.001$) (Fig. 4.3A). However, a significant interaction between the pre- and post-weaning diets affected offspring plasma LDL/HDL- ratio ($P = 0.043$) (Fig. 4.3B). Multiple comparisons further revealed that a continuous exposure to the safflower oil-rich diets during pre- and

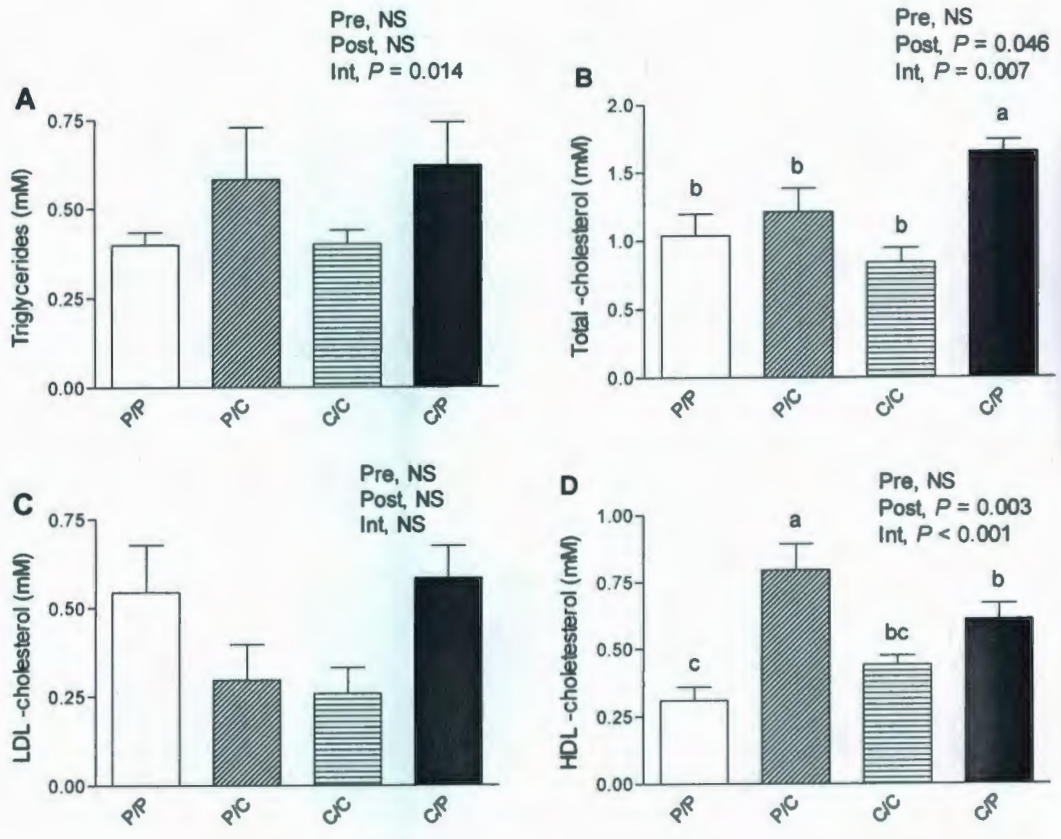

Fig. 4.2 Plasma analysis of various offspring for (*A*) triglycerides, (*B*) total-cholesterol, (*C*) LDL-cholesterol and (*D*) HDL-cholesterol.

Values are expressed as means ± SEM, n = 6-10 (specified in section 4.2.3). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Letters represent significant differences having $P < 0.05$. NS, non significant; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction.

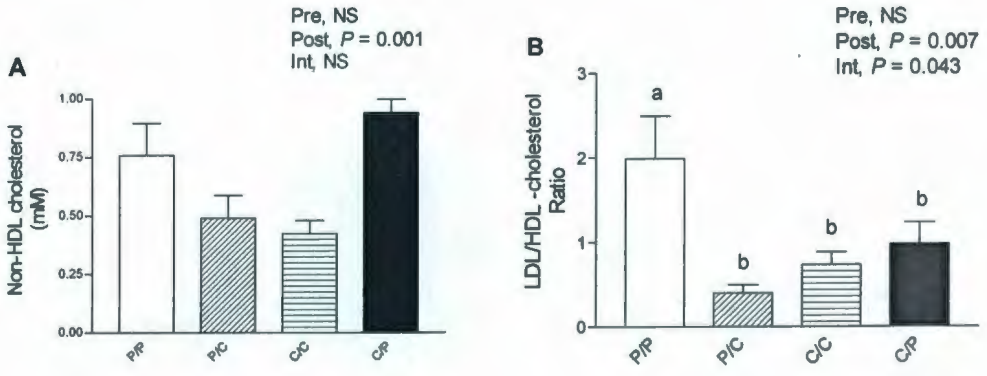

Fig. 4.3 Plasma analysis of various offspring for (*A*) non-HDL cholesterol and (*B*) LDL/HDL-cholesterol ratio.

Values are expressed as means ± SEM, n = 6-10 (specified in section 4.2.3). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Letters represent significant differences having $P < 0.05$. NS, non significant; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction

post-weaning time periods was associated with higher plasma LDL/HDL-ratio in the P/P offspring compared to all other offspring (P/P *vs.* C/P, $P = 0.036$; P/P *vs.* P/C, $P = 0.002$; P/P *vs.* C/C, $P = 0.004$) (Fig. 4.3B).

4.3.3 Effects of pre- and post-weaning safflower oil-rich diets on offspring hepatic mRNA expression of LCAT and SR-B1

A safflower oil-rich diet fed post-weaning was associated with lower hepatic mRNA expression of SR-B1 in the offspring compared to chow ($P < 0.001$) (Fig. 4.4 A). In contrast, a significant interaction between the pre- and post-weaning diets affected the hepatic mRNA expression of LCAT in the offspring ($P = 0.043$) (Fig. 4.4B). Multiple comparisons further revealed that the P/C offspring exhibited the highest mRNA expression of hepatic LCAT compared to all other offspring (P/C *vs.* P/P, $P = 0.003$; P/C *vs.* C/C, $P = 0.018$; P/C *vs.* C/P, $P = 0.002$) (Fig. 4.4B).

4.3.4 Effects of pre- and post-weaning safflower oil-rich diets on the contractile responses of the offspring aortas

A significant interaction between the pre- and post-weaning diets affected the maximal contractile responses to KCl in the offspring aortas ($P = 0.002$) (Fig. 4.5A). A safflower oil-rich diet fed either pre or post-weaning was associated with lower E_{max} to

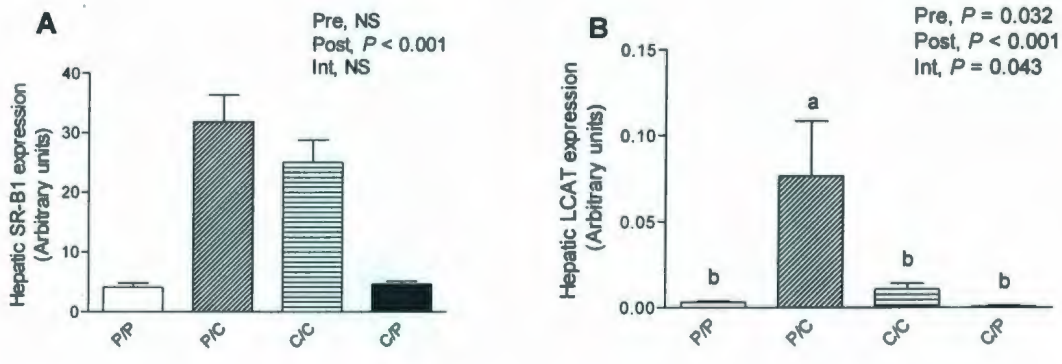

Fig. 4.4 Real-time PCR analysis of offspring hepatic (*A*) SR-B1 and (*B*) LCAT mRNA expression.

Values are expressed as means ± SEM, $n = 6$. The mRNA expression of LCAT and SR-B1 was calculated in relation to the expression of GAPDH, which was not found to be different among groups. Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Letters represent significant differences having $P < 0.05$. SR-B1, Scavenger receptor-B1; LCAT, Lecithin: cholesterol acyltransferase; NS, non significant; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction.

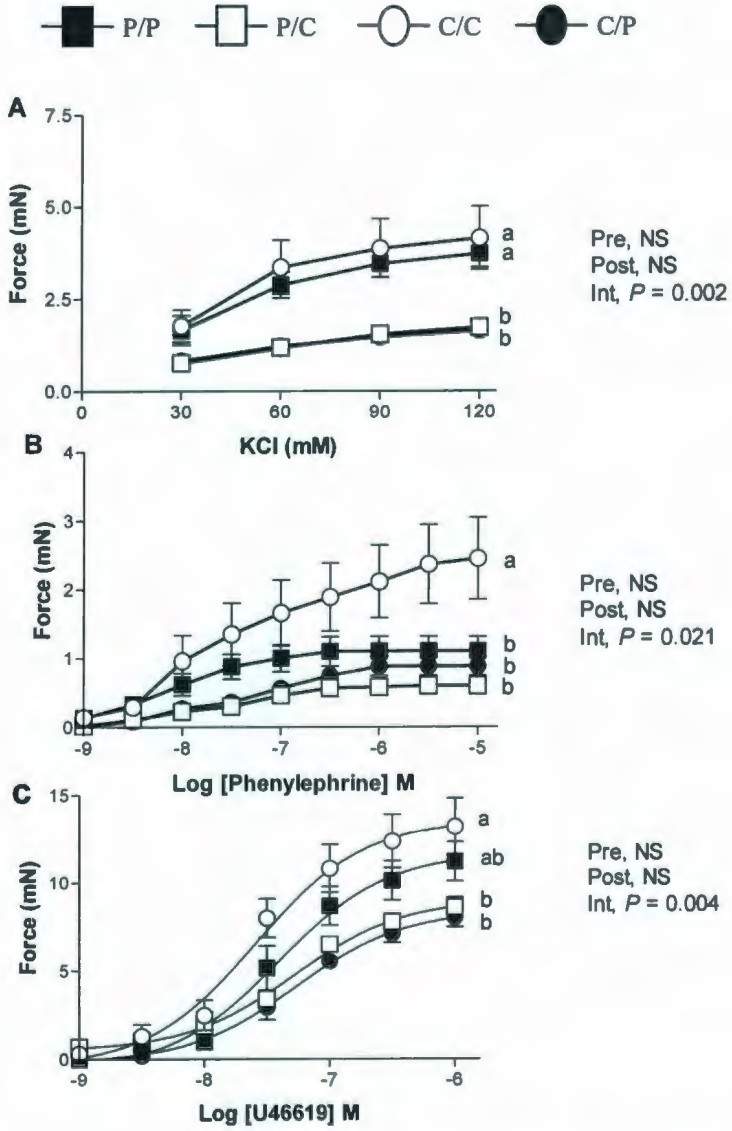

Fig. 4.5 Dose-contraction responses of the offspring aortas to (*A*) KCl, (*B*) phenylephrine and (*C*) thromboxane mimetic U46619.

Values are expressed as means ± SEM, n = 6-10 (specified in section 4.2.3). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) on E_{max} were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Letters represent significant differences having $P < 0.05$. NS, non significant; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction.

KCl in the aortas of the P/C and C/P offspring compared to the C/C (P/C *vs.* C/C, P = 0.018; C/P *vs.* C/C, P = 0.014) and with a higher E_{max} to KCl in the aortas of P/P offspring compared to the C/P (P = 0.022) and the P/C offspring (P = 0.028), respectively (Fig. 4.5A).

A significant interaction between the pre- and post-weaning diets also affected the maximal contractile responses to PE in the offspring aortas (P = 0.021) (Fig. 4.5B). A safflower oil-rich diet fed either pre- or post-weaning was associated with lower E_{max} to PE in the aortas of P/P, P/C and C/P offspring compared to the C/C offspring (P/P *vs.* C/C, P = 0.017; P/C *vs.* C/C, P = 0.004 and C/P *vs.* C/C, P = 0.014, respectively) (Fig. 4.5B). In addition, a safflower oil-rich diet fed pre-weaning was associated with higher sensitivity to PE in the offspring aortas compared to chow (P = 0.009) (Table 4.3). Similar to PE, an interaction between the pre- and post-weaning diets affected the E_{max} to U46619 in the offspring aortas (P = 0.004) (Fig. 4.5C). A safflower oil-rich diet fed either pre- or post-weaning was associated with lower E_{max} to U44619 in the aortas of P/C and C/P compared to the C/C offspring (P/C *vs.* C/C, P = 0.016 and C/P *vs.* C/C, P = 0.007), respectively (Fig. 4.5C). The sensitivity to U44619 however was not different among various offspring (Table 4.3).

4.3.5 Effect of pre- and post-weaning safflower oil-rich diets on the endothelium-dependent and – independent relaxation responses of the offspring aortas

A significant interaction between the pre- and post-weaning diets affected the relaxation responses to ACh in the offspring aortas ($P = 0.002$) (Fig. 4.6A). In addition, the C/P offspring aortas exhibited the lowest relaxation responses to ACh compared to all other offspring (P/P *vs.* C/P, $P < 0.001$; C/C *vs.* C/P, $P < 0.001$; P/C *vs.* C/P, $P = 0.003$) (Fig. 4.6A). In contrast, a safflower oil-rich diet fed pre-weaning was associated with a higher sensitivity to ACh in the offspring aortas compared to chow ($P = 0.012$) (Table 4.3). A pre-weaning diet rich in safflower oil was also associated with higher sensitivity to SNP in the offspring aortas compared to chow ($P = 0.014$) (Table 4.3), however no differences were observed for the SNP-induced maximal relaxation responses of the aortas in various offspring groups (Fig. 4.6B).

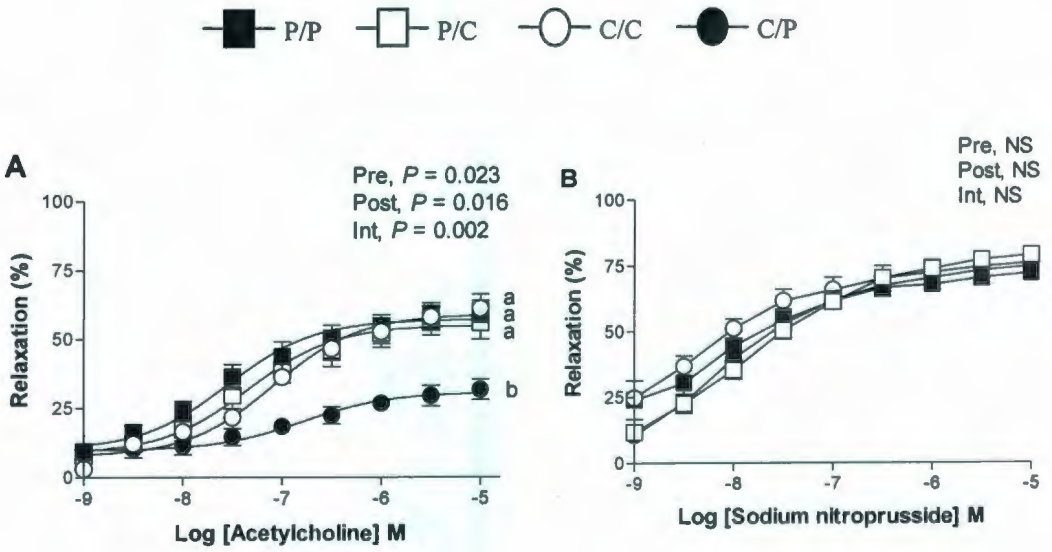

Fig. 4.6 Dose-relaxation response of aorta pre-constricted with U46619 from various offspring, to (*A*) acetylcholine and (*B*) sodium nitroprusside.

Values are expressed as means ± SEM, n = 6-10 (specified in section 4.2.3). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) on E_{max} were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Letters represent significant differences having $P < 0.05$. NS, non significant; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction.

Table 4.3 Half-maximal dose concentrations (pEC50) of the offspring aortas towards various drugs.

	P/P	P/C	C/C	C/P	*Pre*	*Post*	*Int*
PE	8.2 ± 0.1	7.8 ± 0.2	7.6 ± 0.2	7.3 ± 0.1	$P = 0.009$	NS	NS
U44619	7.4 ± 0.1	7.4 ± 0.2	7.7 ± 0.1	7.3 ± 0.1	NS	NS	NS
ACh	7.4 ± 0.2	7.3 ± 0.1	7.1 ± 0.1	6.9 ± 0.1	$P = 0.012$	NS	NS
SNP	8.1 ± 0.1	8.1 ± 0.1	7.7 ± 0.1	7.9 ± 0.1	$P = 0.014$	NS	NS

Values are expressed as means ± SEM, n = 6-10 (specified in section 4.2.3). Cumulative concentration–response curves to agonists were analyzed by fitting to a four-parameter logistic equation using non-linear regression to obtain the -log effective concentration equal to 50 % of the maximal response (pEC_{50}). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) were then assessed using two-way ANOVA. PE, phenylephrine; ACh, acetylcholine; SNP, sodium nitroprusside; NS, non significant; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction.

4.4 DISCUSSION

The objective of the current study was to test the hypothesis that a maternal diet rich in *n-6* PUFA fed during gestation and lactation would lower the risk of developing CVD in the adult offspring. Results indicate that although a safflower oil-rich diet fed to the mothers during gestation and lactation was associated with higher plasma HDL-cholesterol and a higher hepatic mRNA expression of LCAT in the offspring, these offspring also exhibited an attenuation of aortic contractility in response to the thromboxane and adrenergic receptor agonists and high K^+, which may not be beneficial for their cardiovascular health. The current study further reported that an exposure to a safflower oil-rich diet during post-weaning alone was associated with higher plasma total-cholesterol, reduced mRNA expression of hepatic SR-B1, an attenuation of the aortic contractile responses to all the agonists along with reduced aortic relaxation to ACh in the adult offspring. Moreover, a significant interaction between the pre- and post-weaning diets was observed for offspring lipid levels and aortic contractile reactivity, underscoring the importance of both pre- and post-weaning diets in affecting the cardiovascular health of the offspring in adult life.

A safflower oil-rich diet fed to the mothers during gestation and lactation was associated with lower body weight in the offspring fed chow post-weaning compared to the offspring exposed to chow throughout (*i.e.* P/C *vs.* C/C), whereas an exposure to a safflower oil-rich diet during post-weaning alone did not affect the body weight in the offspring. On the other hand, a continuous exposure to a safflower oil-rich diet during the pre- and post-weaning time periods was associated with higher body weight in the P/P

offspring compared to all other offspring. These observations indicate that a maternal high-fat diet rich in safflower oil can lead to a higher body weight in the offspring, only when the offspring continues on a high-fat diet. A similar observation was made in Chapter-3, where a continuous exposure to high-fat diet enriched with lard during the pre- and post-weaning time periods was associated with higher body weight in the adult female offspring (Chechi *et al.*, 2009). Besides lard, an increased intake of LA has previously been shown to enhance adiposity in humans and rodent models (Dayton *et al.*, 1966; Cleary *et al.*, 1999; Prentice, 2001; Massiera *et al.*, 2003). It has also been proposed that an increased intake of LA, especially from breast milk during early postnatal development, contributed to an increased incidence of childhood obesity in humans (Ailhaud *et al.*, 2008). The safflower oil-rich diet used in the current study was specifically rich in LA, thus suggesting that an interaction of pre- and post-weaning diets rich in LA may have caused a higher body weight in the P/P offspring. As discussed in chapter-3, besides the LA, the high sucrose content of the semi-purified diets could have also contributed to a higher body weight in these offspring. Previous studies have reported that high-fat high-sucrose diets can lead to higher body weight and obesity in rodents (Sato Mito *et al.*, 2009; Murase *et al.*, 2001) and humans (Raben *et al.*, 1997). Moreover, soy protein compared to casein, as the source of protein was associated with lower body weight in the obese rats fed calorically restricted diets (Aoyama *et al.*, 2000). Thus, the presence of casein as the source of protein may have also added to increased body weight observed in these offspring.

An increased body weight observed in the case of the P/P offspring may have resulted from an increase in their food intake, however a post-weaning diet rich in safflower oil was associated with reduced food intake in both P/P and C/P offspring compared to the P/C and C/C offspring fed chow post-weaning. Rodents, as do humans, have been found to regulate their food intake and energy expenditure in order to maintain a set body weight (Keesey and Hirvonen, 1997). Since the high-fat diets enriched with safflower oil were energy-dense compared to the chow, the offspring mice fed a safflower oil-rich diet may have consumed less food compared to the offspring fed chow post-weaning, in order to maintain their caloric intake. Interestingly however, a safflower oil-rich diet fed to the mothers during gestation and lactation was also associated with a reduction of food intake in the P/C offspring compared to the C/C offspring. Recently, leptin was proposed to be the active factor in the human breast milk that is responsible for conferring protection against adulthood obesity in the suckling infant (Palou *et al.*, 2009). Moreover, it has been proposed that the presence of leptin in the mother's milk may play an important role in the development of the hypothalamic centers involved in the regulation of food intake by the offspring (Pico *et al.*, 2007; Palou *et al.*, 2009). It was previously proposed that a maternal diet rich in SFA (lard) could have a 'leptin-mediated' programming component behind the appetite regulation in the offspring (Chapter-3) (Chechi *et al.*, 2009). A diet rich in *n-6* PUFA has been shown to increase leptin levels compared to SFA enriched diets in the Sprague-Dawley rats (Cha and Jones, 1998). Moreover feeding high-fat high-sucrose diets for 5-weeks have been shown to increase plasma leptin levels in rats (Lindqvist *et al.*, 2005). While requiring further testing, it is

hypothesized that safflower oil-rich maternal diets may also employ a 'leptin-mediated' programming mechanism behind the regulation of food intake in the offspring.

A safflower oil-rich diet fed to mothers during gestation and lactation did not affect plasma TG, total- or LDL-cholesterol concentration, but it was associated with higher HDL-cholesterol concentration in the offspring fed chow post-weaning, indicating its beneficial effects. HDL-cholesterol is involved in the reverse cholesterol transport pathway, thus it is considered to be anti-atherogenic in nature. A diet rich in LA has previously been shown to increase HDL-cholesterol concentration, which was associated with reduced aortic atherosclerotic lesion area in apo-E deficient mice (Sato *et al.*, 2005). In addition, diets rich in MUFA and PUFA have previously been associated with higher HDL-cholesterol concentrations in C57Bl/6 mice (Gallou-Kabani *et al.*, 2007). However, the current study would be the first to report that a high-fat maternal diet rich in *n-6* PUFA was associated with higher HDL-cholesterol concentration in the adult offspring of C57Bl/6 mice. A maternal diet rich in lard was reported to be associated with higher total- and LDL-cholesterol concentrations in the offspring fed chow post-weaning compared to the C/C offspring (chapter-3) (Chechi *et al.*, 2009). A maternal high-fat diet rich in safflower oil was not associated with higher LDL-cholesterol, whereas it was found to be associated with higher offspring HDL-cholesterol concentration in the current study, suggesting that a maternal safflower oil-rich diet utilized different mechanisms to regulate offspring plasma cholesterol levels as compared to a maternal diet rich in lard.

LCAT and SR-B1 play an important role in the reverse cholesterol transport pathway and have been shown to be up-regulated by the diets rich in PUFA (Spady *et al.*,

1999). Thus, in an attempt to pinpoint the plausible underlying mechanisms of an increase in HDL-cholesterol by maternal intake of safflower oil-rich diets, the hepatic gene expression of LCAT and SR-B1 was assessed. A safflower oil-rich diet fed to the mothers during gestation and lactation was associated with higher hepatic mRNA expression of LCAT in the offspring fed chow post-weaning (*i.e.* P/C) compared to all other offspring. The hepatic mRNA expression of LCAT has been shown to increase plasma HDL-cholesterol concentration in rats (Aizawa and Inakuma, 2006). Since LCAT is responsible for converting free cholesterol into cholesterol esters in the HDL particles, thereby assisting in further removal of cholesterol from the peripheral cells (Chen *et al.*, 2009), an increased expression of LCAT may be contributing to the higher plasma HDL-cholesterol concentrations in these offspring.

The SR-B1 plays an important role in the selective uptake of HDL-cholesterol by the liver (Kinoshita *et al.*, 2004). A safflower oil-rich diet fed post-weaning was associated with reduced expression of hepatic SR-B1, and a concomitant increase in non-HDL cholesterol concentrations, in the adult offspring as compared to the offspring fed chow post-weaning, irrespective of the maternal diet. Besides clearing the HDL particles, hepatic SR-B1 has been reported to act as a remnant receptor and bind to LDL, VLDL and apo-B containing lipoprotein-particles resulting in their clearance from the plasma (Fu *et al.*, 2003). It is likely that down-regulation of hepatic SR-B1 expression by safflower oil-rich diets resulted in an increase in the plasma non-HDL cholesterol concentration of the offspring exposed to these diets post-weaning.

An increase in plasma HDL-cholesterol concentration has been associated with improved endothelial function in humans (Hovingh *et al.*, 2004) and in mice (Terasaka *et al.*, 2008). Since a maternal diet rich in safflower oil fed during gestation and lactation was associated with higher HDL-cholesterol concentration in the offspring fed chow post-weaning, it was hypothesized that safflower oil-rich diet would have beneficial effects on the aortic smooth muscle and endothelium function in the offspring. Offspring exposed to a safflower oil-rich diet either during pre- or post-weaning time periods (*i.e.* P/C and C/P) exhibited reduced aortic contractions in response to KCl, PE and U44619, whereas P/P offspring exhibited reduced contractile responses to PE only. In Chapter-3, it was shown that exposure to a lard-rich diet, irrespective of the time of exposure being pre- or post-weaning, was associated with reduced contractions of aortas by these three agonists in the adult offspring of C57Bl/6 mice (Chechi *et al.*, 2009). Therefore, it can be suggested that consumption of these semi-synthetic high-fat diets was generally associated with reduced contractions of aortas in the female offspring, irrespective of the type of fat involved. The effect of high-fat feeding on vascular contractile responses has been controversial with reports of both increased and decreased contractile responses (Freiman *et al.,* 1986; Galle *et al.,* 1991, Ibengwe and Suzuki, 1986; Wroblewski and Witanowska, 1982). Recently, Ellis *et. al.,* 2008 reported reduced aortic contractility to α_1-adrenoreceptor agonist in male C57Bl/6 mice fed a high-fat Western-style diet for a period of 8-weeks, while Jiang *et. al,* 2001 reported reduced aortic contractile responses to high K^+ and U44619 in apo $E^{-/-}$ mice fed a high-fat diet. Both studies attributed the presence of hyperlipidemia behind the reduced aortic contractility in these mice.

Although the mechanisms behind these observations remain to be determined, one could interpret the reduction in contractile responsiveness to these agonists as being predictive of a good cardiovascular adaptation for their health, possibly to counter other effects of a high-fat diet. For example, high-fat diets may induce changes in the nature and contribution of endothelium-derived relaxing and contracting factors resulting in altered receptor expression or receptor desensitization. An attenuation of PE mediated increase in the α_1-adrenergic coronary vascular resistance was reported in the isolated heart preparations of high-fat fed C57BL6 mice, which by testing further with endothelin (ET) receptor antagonists was suggested to result from ET-A mediated desensitization of α_1 (Bender and Klabunde, 2007). Conversely, the more generalized nature (no apparent specificity to agonist) of the reduced contractile responses may predict vascular dysfunction; if in vitro observations are extended to other vasculature in vivo, the loss of vasoconstrictor tone would be detrimental to cardiovascular homeostasis. Reductions in contractile responses to KCl, thrombin and norepinephrine have been reported in the human radial artery with macroscopically evident atherosclerotic lesions, suggesting that changes in the vascular tone may predict atherosclerosis progression (Stähli *et al.*, 2004). In the current study, the diet regimen was terminated at an earlier age (11-weeks of age), thus further time course studies are warranted to investigate the trend and mechanisms that produce changes in vascular smooth muscle phenotype by these high-fat diets, and to further explore the association between reduced aortic vascular reactivity and CVD progression.

The relaxation response towards ACh was found to be lower only in the case of the offspring exposed to a safflower oil-rich diet post-weaning. These offspring had higher total-cholesterol concentrations, so these findings are consistent with observations of endothelial cell dysfunction in other hypercholesteremic models; albeit at much lower levels of cholesterol than in apo $E^{-/-}$mice (Shi *et al.*, 2005; Xiang-Qun Yang, 2009). A diet rich in LA has been found to inhibit the basal endothelial NOS activity in an immortalized human endothelial cell line (Couloubaly *et al.*, 2007). In addition, LA-rich diets have been shown to increase urinary 8-iso-prostaglandin F2α, which was also associated with reduced urinary NO metabolites in healthy young individuals (Turpeinen *et al.*, 1998), suggesting that LA-mediated increase in oxidative stress may be one of the causative mechanisms behind the reduced availability of basal NO. The safflower oil-rich diet used in the current study was particularly rich in LA, which may be the active candidate that induced endothelial cell dysfunction observed in the case of C/P offspring. Moreover, the presence of high sucrose content may have contributed to higher plasma total-cholesterol and endothelial dysfunction in these offspring as discussed in Chapter-3. An objective of future investigations should be to determine the effect of these dietary components on the vascular contractile and endothelial function separately.

In conclusion, this study reported that a safflower oil-rich diet fed to the mothers during gestation and lactation was associated with higher plasma HDL-cholesterol concentration in the offspring, and was also associated with an increase in the hepatic mRNA expression of LCAT, which can be expected to lower their CVD risk. However, a safflower oil-rich maternal diet was also associated with reduced aortic contractile

reactivity towards various agonists in the adult offpsirng, which may not be entirely beneficial for their cardiovascular health. This study further provided the evidence to support the role for an interaction between the pre- and post-weaning dietary environments in the overall physiological outcome in the offspring.

CHAPTER FIVE

Comparison of a high-fat maternal diet rich in lard vs. safflower oil fed during gestation and lactation on lipid metabolism and aortic vascular function in the adult offspring

A version of this chapter is published in *Experimental and Clinical Cardiology* (2006) 11:129-135

5.1 INTRODUCTION

The previous studies (Chapters-3 and 4) compared the effects of high-fat diets rich in either lard or safflower oil with rodent chow, fed during pre- and post-weaning time periods, on the CVD risk in the adult offspring. A lard-rich (SFA) diet fed to the mothers during gestation and lactation was associated with higher LDL-cholesterol in the S/C offspring compared to the C/C offspring (Chapter-3). In contrast, a safflower oil-rich (*n-6* PUFA) diet fed to the mothers during gestation and lactation was associated with higher HDL-cholesterol in the P/C offspring compared to the C/C offspring (Chapter-4). These studies indicated that the fatty acid composition of the maternal high-fat diets played an important role in the offspring lipid metabolism. However, as discussed previously, the high-fat semi-purified diets differed in more than one respect from chow, thus, other factors (*eg.* sucrose content, source of protein) could also have contributed to these observations. The current study was therefore designed to investigate the role of fatty acid composition of the maternal high-fat diets fed during gestation and lactation on the CVD risk in the adult offspring.

To this end, the effects of maternal high-fat diets rich in either lard or safflower oil fed during gestation and lactation on offspring lipid metabolism and aortic vascular function were compared, as biomarkers to assess their CVD risk. It was hypothesized that a maternal diet rich in lard would increase plasma TG and cholesterol concentrations, and induce endothelial dysfunction in the offspring compared to a diet rich in safflower oil. It

was also expected that a continuous exposure to a diet rich in SFA would increase the risk of developing CVD in the offspring compared to a diet rich in *n-6* PUFA.

5.2 METHODS

5.2.1 Experimental Design

As described in section 2.1, female C57Bl/6 mice were maintained on rodent chow for a week prior to feeding the experimental diets. After this acclimatization period, mother mice were divided into two groups and were fed the experimental diets according to the design specified in Fig. 5.1. The offspring were identified by *pre-/post-weaning diet* combinations as: SFA/SFA (S/S), SFA/PUFA (S/P) PUFA/PUFA (P/P) and PUFA/SFA (P/S). Due to skewed sex ratio and poor survival of the male offspring born to the mothers fed a diet rich in *n-6* PUFA, only female offspring from each dietary group were used for the current study. The S/S and P/P offspring groups were shared between the current and previous studies (Chapters 3 and 4)

5.2.2 Calculations and Statistical Analysis

Data were expressed as means ± SEM, where S/S, $n = 10$, S/P, $n = 8$; P/P, $n = 8$ and P/S, $n = 8$. Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet x post-weaning diet) were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests (*SYSTAT*

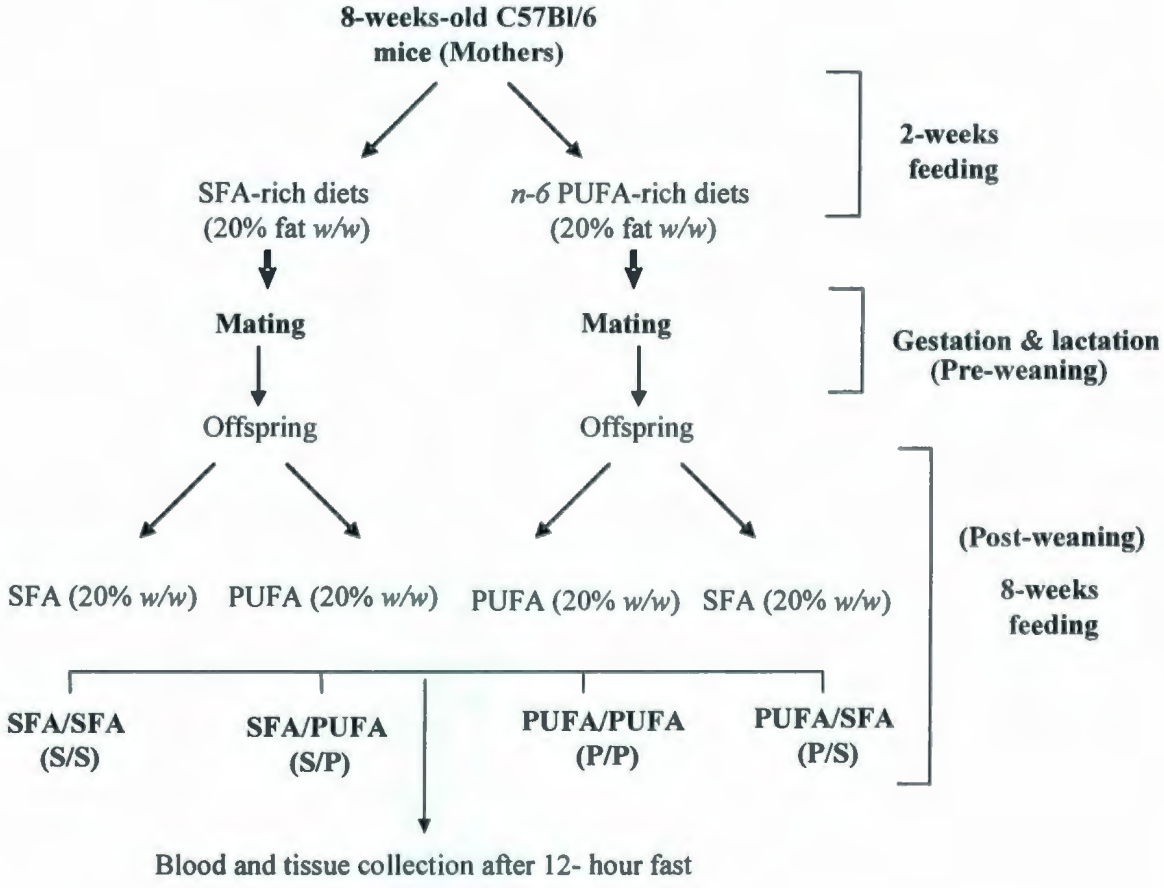

Fig. 5.1 Experimental Design.

8-week-old female C57BL/6 mice were fed high-fat diets rich in either lard or safflower oil for 2-weeks. The females continued on these diets during mating, pregnancy and lactation. At weaning, the offspring obtained from each mother were divided into two groups; one group continued on a lard-rich diet and the other group was fed the safflower oil-rich diet. The offspring were identified by *pre-/post-weaning diet* combinations as: SFA/SFA (S/S), SFA/PUFA (S/P), PUFA/PUFA (P/P) and PUFA/SFA (P/S). The offspring were fed their assigned diets *ad libitum* for another 8-week.

for Windows, version 12.02; *SYSTAT Software Inc.*, Richmond, California). Differences having a $P < 0.05$ were considered significant.

Constrictor responses were reported as the force generated in response to each concentration of the agonist, and relaxant responses as the percentage reversal of U44619-induced contraction. Cumulative concentration–response curves to agonists were analyzed by fitting to a four-parameter logistic equation using non-linear regression to obtain the -log effective concentration equal to 50% of the maximal response (pEC_{50}) and maximum response (E_{max}) (Prism 3.0, GraphPAD Software Inc). pEC_{50} and E_{max} values were then compared using two-way ANOVA followed by Fisher's LSD *post hoc* analyses. Since the KCl-response curves were not sigmoidal, pEC_{50} values were not calculated and only maximal responses were compared among various groups.

5.3 RESULTS

5.3.1 Effects of pre- and post-weaning high-fat diets enriched with lard or safflower oil on offspring body weight, food and caloric intake, and plasma glucose concentrations

Body weights at the age of 11-weeks were not different among various offspring groups (Table 5.1). However, there was a significant interaction between the pre- and post-weaning diets on their food and caloric (P = 0.005), respectively (Table 5.1). Multiple comparisons further revealed that a continuous exposure to a lard-rich diet during pre- and post-weaning time periods was associated with higher food and caloric

Table 5.1 Offspring body weight, food intake, caloric intake and plasma glucose concentrations

	S/S	S/P	P/P	P/S	*Pre*	*Post*	*Int*
BW (g)	20.3 ± 0.8	19.8 ± 0.3	21.3 ± 0.8	20.9 ± 1.0	NS	NS	NS
FI (g/day)	3.1 ± 0.3^{a}	2.2 ± 0.2^{b}	2.5 ± 0.2^{b}	2.2 ± 0.2^{b}	NS	NS	$P = 0.005$
CI (kcal/day)	15.7 ± 1.3^{a}	11.1 ± 0.8^{b}	12.8 ± 1.0^{b}	11.4 ± 0.8^{b}	NS	NS	$P = 0.005$
Glucose (mM)	9.8 ± 0.8^{b}	12.9 ± 0.6^{a}	7.4 ± 0.7^{c}	10.9 ± 0.6^{b}	$P = 0.006$	NS	$P < 0.001$

Values are expressed as means ± SEM, n = 8-10 (specified in section 5.2.2). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) were assessed using two-way ANOVA. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Superscripts represent significant differences having $P < 0.05$. BW, body weight; FI, food intake; CI, caloric intake; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction; NS, not significant.

intake in the S/S offspring compared to the all other offspring (S/S *vs.* S/P $P = 0.002$; S/S *vs.* P/P, $P = 0.047$; S/S *vs.* P/S, $P = 0.005$) (Table 5.1).

An interaction of the pre- and post-weaning diets also affected offspring plasma glucose concentrations ($P < 0.001$) (Table 5.1). A lard-rich diet fed either during pre- and/or post-weaning time periods was associated with higher plasma glucose concentrations in the S/S, S/P and P/S offspring compared to the P/P offspring (S/S *vs.* P/P, $P = 0.025$; S/P *vs.* P/P, $P < 0.001$; P/S *vs.* P/P, $P = 0.003$) (Table 5.1). In contrast, a lard-rich diet fed post-weaning was associated with lower plasma glucose concentration in the S/S offspring compared to the S/P offspring ($P = 0.005$) (Table 5.1).

5.3.2 Effects of pre- and post-weaning high-fat diets enriched with lard *vs.* safflower oil on offspring plasma lipid levels

No differences were observed for the plasma TG concentration among various offspring (Fig. 5.2A). However, a post-weaning diet rich in lard was associated with higher plasma total-cholesterol ($P = 0.002$), LDL-cholesterol ($P = 0.016$) and HDL-cholesterol ($P = 0.002$) concentrations in the offspring compared to a diet rich in safflower oil (Fig. 5.2 B, C & D). In addition, a pre-weaning diet rich in lard was associated with higher plasma LDL-cholesterol concentration in the offspring compared to a diet rich in safflower oil ($P = 0.040$) (Fig. 5.2 B).

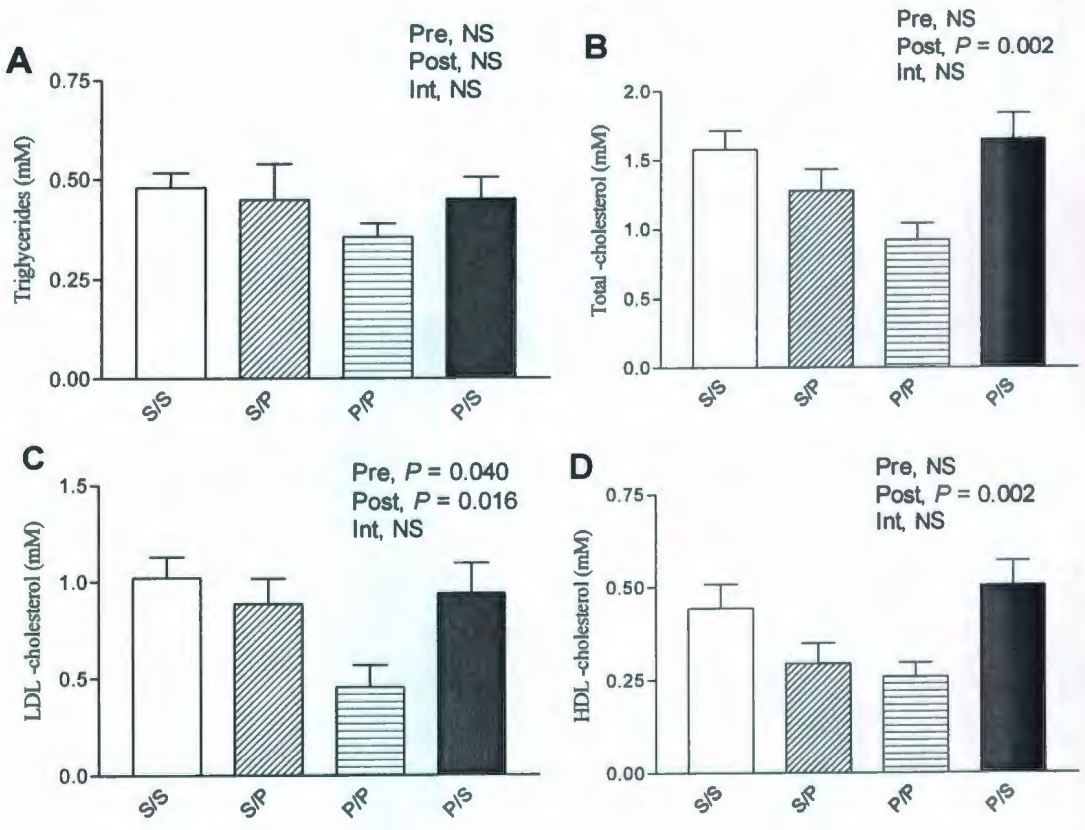

Fig. 5.2 Plasma concentrations of (*A*) triglycerides, (*B*) total-cholesterol, (*C*) LDL-cholesterol and (*D*) HDL-cholesterol in various offspring.

Values are expressed as means ± SEM, n = 8-10 (specified in section 5.2.2). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) were assessed using two-way ANOVA. NS, non significant; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction.

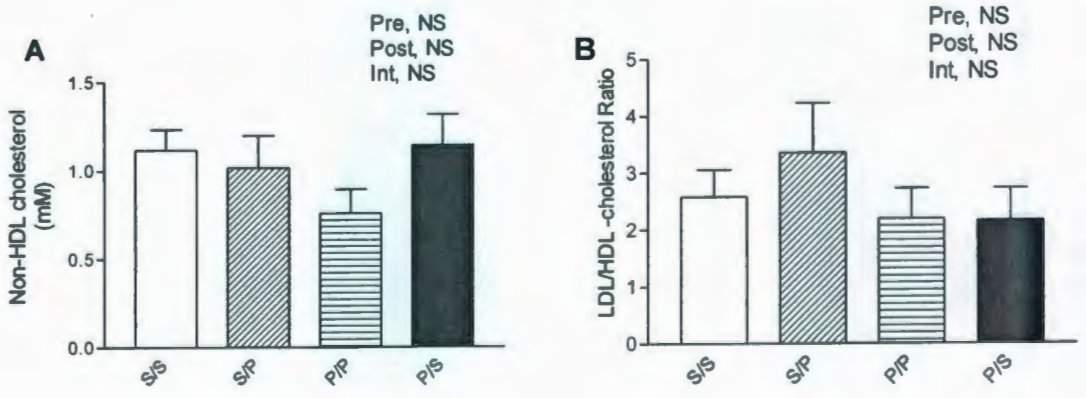

Fig. 5.3 Plasma concentrations of (A) non-HDL cholesterol and (B) LDL/HDL-cholesterol ratio in various offspring.

Values are expressed as means ± SEM, n = 8-10 (specified in section 5.2.2). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) were assessed using two-way ANOVA. NS= non significant, Pre = pre-weaning diet, Post = post-weaning diet, Int = pre-weaning X post-weaning diet interaction.

No differences were observed for the non HDL-cholesterol concentrations and the LDL/HDL-cholesterol ratio among various offspring (Fig. 5.3A & B).

5.3.3 Effects of pre- and post-weaning high-fat diets enriched with lard *vs.* safflower oil on the contractile and relaxation responses of the offspring aortas

A pre-weaning diet rich in lard was associated with lower E_{max} to KCl ($P = 0.020$) in the offspring aortas compared to a safflower oil-rich diet (Fig. 5.4A). Similarly, a lard- rich diet fed pre-weaning was associated with lower E_{max} ($P = 0.006$) and pEC_{50} ($P = 0.037$) to PE in the offspring aortas compared to the safflower oil-rich diet (Fig. 5.4B & Table 5.2). However, no differences were observed in the E_{max} or pEC_{50} to U44619 in the aortas of various offspring (Fig. 5.4C & Table 5.2).

In contrast to the contractile responses, a lard-rich diet fed pre-weaning was associated with higher maximal relaxation response (E_{max}) ($P = 0.019$) and higher pEC_{50} ($P = 0.045$) to ACh in the offspring aortas compared to the safflower oil-rich diet (Fig. 5.5A & Table 5.2). However, no differences were observed for the aortic E_{max} and pEC_{50} to SNP among various offspring groups (Fig. 5.5B & Table 5.2).

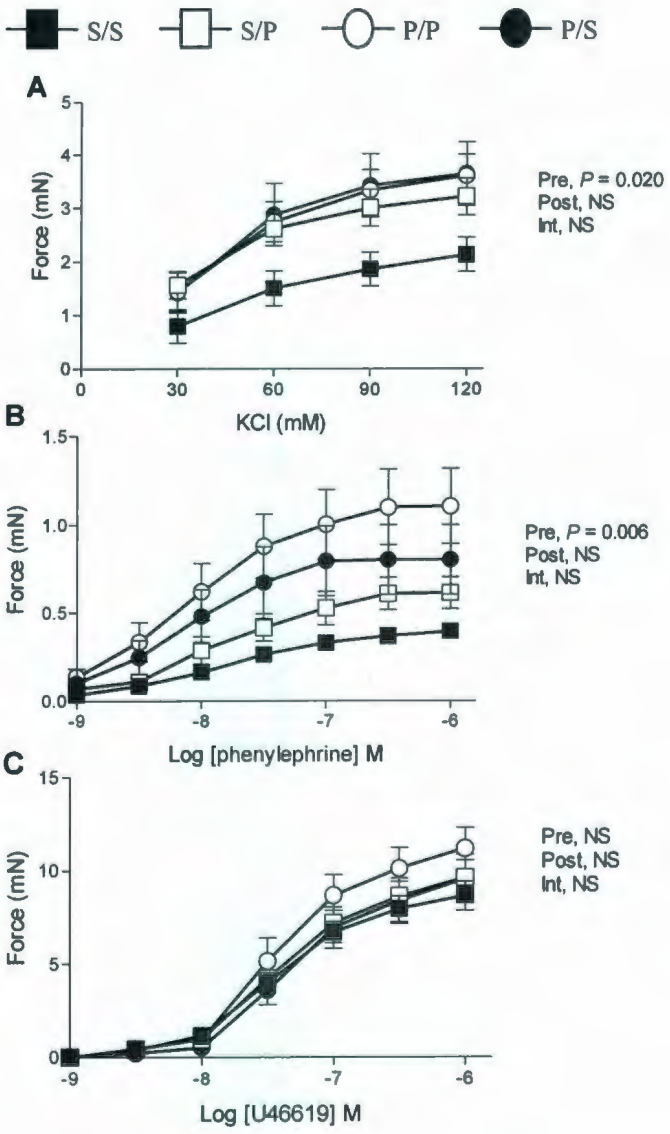

Fig. 5.4 Dose contraction responses of the offspring aortas to (A) KCl, (B) phenylephrine and (C) U46619.

Values are expressed as means ± SEM, n = 8-10 (specified in section 5.2.2). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) on E_{max} were assessed using two-way ANOVA. NS= non significant, Pre = pre-weaning diet, Post = post-weaning diet, Int = pre-weaning X post-weaning diet interaction.

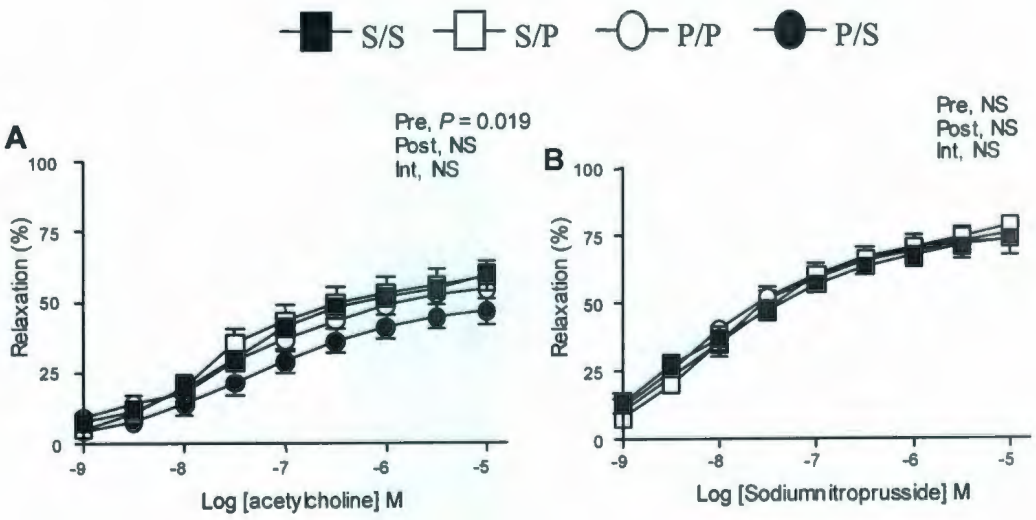

Fig. 5.5 Dose-relaxation responses of the offspring aortas pre-constricted with U46619, to (*A*) acetylcholine and (*B*) sodium nitroprusside.

Values are expressed as means ± SEM, n = 8-10 (specified in section 5.2.2). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) on E_{max} were assessed using two-way ANOVA. NS= non significant, Pre = pre-weaning diet, Post = post-weaning diet, Int = pre-weaning X post-weaning diet interaction.

Table 5.2 Half-maximal dose concentration (pEC_{50}) of the offspring aortas towards various drugs.

	S/S	S/P	P/P	P/S	*Pre*	*Post*	*Int*
PE	7.8 ± 0.1	7.9 ± 0.2	8.2 ± 0.1	8.0 ± 0.1	$P = 0.037$	NS	NS
U44619	7.4 ± 0.1	7.4 ± 0.0	7.4 ± 0.1	7.3 ± 0.1	NS	NS	NS
ACh	7.4 ± 0.1	7.6 ± 0.1	7.2 ± 0.2	7.3 ± 0.1	$P = 0.045$	NS	NS
SNP	7.8 ± 0.2	7.8 ± 0.1	8.1 ± 0.2	7.9 ± 0.1	NS	NS	NS

Values are expressed as means ± SEM, n = 8-10 (specified in section 5.2.2). Cumulative concentration–response curves to agonists were analyzed by fitting to a four-parameter logistic equation using non-linear regression to obtain the -log effective concentration equal to 50% of the maximal response (pEC_{50}). Main effects of pre-weaning diet, post-weaning diet and their interaction (pre-weaning diet X post-weaning diet) were then assessed using two-way ANOVA. PE, phenylephrine; ACh, acetylcholine; SNP, sodium nitroprusside; NS, non significant; Pre, pre-weaning diet; Post, post-weaning diet; Int, pre-weaning X post-weaning diet interaction.

5.4 DISCUSSION

This study evaluated the effects of feeding diets rich in either lard or safflower oil during pre- and post-weaning time periods on plasma lipids and aortic vascular function, as biomarkers to assess the CVD risk, in the 11-week-old offspring. A lard-rich diet fed pre-weaning was associated with higher plasma LDL-cholesterol and lower aortic contractile reactivity to KCl and PE in the offspring. On the other hand, a safflower oil-rich diet fed pre-weaning was associated with lower aortic maximal relaxation responses to ACh in the offspring. These observations clearly indicate that fatty acid composition of the maternal diets fed during gestation and lactation has significant effects on offspring lipid levels and aortic vascular reactivity during adulthood.

Body weights at the age of 11-weeks were not different among various offspring. However, the offspring exposed to a lard-rich diet during pre- and post-weaning time periods (*i.e.* S/S) exhibited higher food and caloric intake than all other offspring. Rodents are known to maintain a set body weight by regulating their food intake and energy expenditure, which may explain the absence of weight gain, despite an increase in the food and caloric intake by the S/S offspring (Keesey and Hirvonen, 1997). As discussed in section 1.4.1.4, fatty acid composition of the high-fat diets has been reported to affect the expression of hypothalamic neuro-peptides involved in the regulation of food intake. Feeding a high-fat diet rich in lard for 6-weeks was reported to induce hyperphagia and weight gain in adult Wistar rats when compared to a high-fat diet rich in sunflower oil. This study further reported a significant reduction in the hypothalamic

expression of POMC in the lard-fed rats (Dredzic *et al.*, 2007). Similarly, a pre-weaning diet rich in lard has been shown to induce hyperphagia (Kozak *et al.*, 2000), hyperleptinemia (Férézou-Viala *et al.*, 2007) and changes in the expression of hypothalamic peptides involved in the regulation of food intake (Chen *et al.*, 2008; Morris and Chen, 2009). Moreover, a recent study indicated that a continuous exposure to a SFA-rich diet during pre- and post-weaning time periods was associated with hyperphagia and changes in the expression of hypothalamic neuro-peptides in 120-day-old Sprague-Dawley rats compared to the offspring exposed to chow throughout (Page *et al.*, 2009). Our observations are in line with these previous studies. However, it is interesting to note that in the current study, an interaction of lard-rich diets fed pre- and post-weaning was associated with hyperphagia in comparison to a diet rich in safflower oil, thereby suggesting the importance of maternal dietary fatty acid composition in regulating offspring food intake.

Plasma total-, LDL- and HDL-cholesterol concentrations were found to be higher in the offspring fed a lard-rich diet post-weaning compared to the safflower oil rich diet. It is well known that diets rich in SFA can increase (Mensink, 1993; Denke, 2006), while diets rich in *n-6* PUFA can lower, plasma cholesterol levels (Hayes, 2000). Thus, the observations from the current study are in line with the reported effects of dietary SFA and *n-6* PUFA. However, it is interesting to note that a maternal diet rich in SFA was associated with higher plasma LDL-cholesterol concentration in the offspring, even when the offspring continued on a diet rich in *n-6* PUFA post-weaning, pointing towards the programming effects of lard-rich maternal diets.

Previous studies (Chapters 3 and 4) described that in comparison to the offspring exposed to chow during pre- and post-weaning time periods (*i.e.* C/C), high-fat diets rich in either lard or safflower oil were associated with reduced aortic contractile responses towards various agonists in the offspring, irrespective of the time of exposure being pre- or post-weaning. Based on these observations, it was proposed that consumption of the high-fat semi-synthetic diets was generally associated with reduced aortic contractility in the offspring. However, the current study reported that a pre-weaning diet rich in lard was associated with lower aortic contractile responses to KCl and PE in offspring compared to the safflower oil-rich diet. These observations indicate that the fatty acid composition of the pre-weaning diets play a significant role in affecting the aortic contractile properties of the offspring. A previous study reported that an exposure to a high-fat diet rich in lard from early developmental period to 90-days of age, was associated with lower maximal responses to NE and KCl in the aortas of Sprague-Dawley rats compared to a corn oil-rich diet (Hodgkin *et al.,* 1991). Although the underlying mechanisms remain largely unknown, it may be proposed that the fatty acid composition of the lard-rich diets may alter the aortic membrane properties in a way that leads to its reduced contractile responsiveness. For instance, changes in the lipid environment of the membrane phospholipids may affect receptor efficacy, activity or expression leading to changes in the vascular function. It is well known that various agonists used in the study utilize different mechanisms to induce vascular contractility. While KCl's effects are mediated by depolarization, PE is a α_1-adrenergic receptor agonist and U46619 is a thromboxane A_2 mimetic. Since no differences were observed for the aortic contractile responses

towards U46619 among various offspring, whereas KCl and PE contractile responses were affected, it can be suggested that the components involved in α_1 adrenergic system or KCl-mediated contractions are more prone to the changes in dietary fat composition than the components involved behind the thromboxane-mediated contractile responses. Dietary lipid modulation has previously been reported to affect the myocardial adrenoreceptor function (Hoffman *et al.,* 1982; McLennan *et al.,* 1987). Moreover, impairment of the sympathetic neuronal processes of NE storage and release has been demonstrated in Sprague-Dawley rats fed diets rich in coconut oil (Panek *et al.,* 1985). An increase in the sensitivity to PE in the offspring aortas as observed in the current study may futher point towards changes in the receptor expression, efficacy or affinity due to the changes in the maternal dietary fatty acid composition. Besides the receptor-dependent effects, changes in the lipid environment may alter the mechanical properties of the vascular wall resulting in its reduced contractility. Increased stiffness, reduced smooth muscle cell number and endothelial cell volume has been reported in the aortas of the offspring exposed to a lard-rich diet pre-weaning (Armitage et al., 2005). Moreover, we (Chechi *et al.,* 2009) (Appendix I), and others (Ghosh *et al.,* 2001) have reported an increased accumulation of SFA in the aortas of the offspring obtained from mothers fed lard-rich diets during gestation and lactation. Reduced vascular compliance due to these changes may lead to the reduced contractility of the aortic vessels.

It is interesting to note that although the aortic contractile responsiveness was reduced in the offspring due to the maternal lard-rich diets, no effects were observed on their relaxation properties. In contrast, safflower oil-rich maternal diet was associated

with lower relaxation responses to ACh in the offspring aortas compared to the lard-rich diets. As discussed in Chapter-4, higher plasma cholesterol concentrations may play a role in inducing endothelial dysfunction in the offspring aortic vessels. However, the safflower oil-rich diets fed pre-weaning were associated with lower plasma cholesterol concentrations than the lard-rich diets. Thus, it may be concluded that the endothelial dysfunction observed in these vessels was independent of their plasma cholesterol levels. Alternatively, the presence of LA in the safflower oil-rich diets may have accounted for the endothelial dysfunction observed in these offspring (discussed in chapter-4). However, a lower sensitivity to ACh would further reflect upon changes in the receptor-mediated signaling events of the endothelial cells that may have been affected by the fatty acid composition of the maternal diets.

In conclusion, the current study provided the evidence to support the hypothesis that the fatty acid composition of the maternal diets fed during gestation and lactation plays an important role in regulating offspring plasma lipid levels and aortic vascular reactivity. While a lard-rich diet fed pre-weaning diet was associated with higher plasma LDL-cholesterol concentration and reduced aortic contractility to KCl and PE in the offspring, a safflower oil-rich maternal diet was associated with reduced relaxation responses to ACh in the offspring aortic vessels. Similar to the reduced relaxation, reduced aortic contractility may also affect offspring cardiovascular homeostasis. Thus, it can be concluded that both lard- and safflower oil-rich high-fat maternal diets may increase the risk of developing CVD in the offspring. In addition, the interaction between the pre- and post-weaning diets reported for the offspring food intake and plasma glucose

levels indicates the importance of each dietary environment in offspring cardiovascular health in later life.

CHAPTER SIX

Effects of pre- and post-weaning diets rich in different fatty acids on tissue fatty acid composition in the adult offspring

A version of this chapter is accepted for publication in *Prostaglandins, Leukotrienes and Essential Fatty acids 2010 (in press).*

6.1 INTRODUCTION

Fatty acids play an important role in regulating the structure and function of various tissues. Tissue fatty acids may occur in various lipids such as phospholipids, mono-, di-, tri-acylglycerols and FFA. While TG and FFA can be utilized as a source of metabolic energy, fatty acids in the form of phospholipids serve as structural components of various membranes. Moreover, fatty acids can act as ligands for several nuclear receptors such as PPAR's and SREBP's, which are known to participate in the sub-cellular control of a number of metabolic pathways (Wolfrum *et al.*, 2001). Since fatty acids are involved in the regulation of a variety of cellular activities, changes in the fatty acid composition have been associated with a number of pathological conditions such as insulin resistance (Borkman *et al.*, 1993), obesity (Phinney *et al.*, 1994), NAFLD (Pawlosky and Salem, 2004), hypertension and CVD (Seidelin, 1995; Zheng *et al.*, 1999; Tremblay *et al.*, 2004).

It is well known that the fatty acid composition of serum and adipose tissue TG can reflect the dietary fat intake over the previous weeks and years, respectively (Katan *et al.*, 1997). In addition, animal studies have indicated changes in both liver and heart fatty acid composition in a diet-specific manner (Murphy *et al.*, 2004; Okada *et al.*, 2008). All of these studies have focused on the influence of postnatal dietary fat intake on tissue fatty acid composition, however, to date, there are only a handful of studies suggesting an effect of maternal dietary fat consumption during gestation and lactation on offspring tissue fatty acid composition in adult life. An exposure to *n-3* PUFA-deficient diet during perinatal time period was associated with a reduction in hypothalamic DHA levels in the

24-week-old rat offspring (Armitage *et al.*, 2003). Similarly, a reduction in the AA and DHA levels was reported in the aortas of 160-day-old Sprague-Dawley rat offspring that were obtained from mothers fed a high-fat diet rich in SFA during gestation and lactation (Ghosh *et al.*, 2001). We have also reported changes in the aortic fatty acid composition of the 11-week-old C57Bl/6 mice offspring that were exposed to lard-rich diets during pre- and/or post-weaning time periods (Chechi *et al.*, 2009) (Appendix I).

Previous studies (Chapters 3, 4 and 5) described changes in offspring lipid levels and aortic vascular reactivity in response to the maternal exposure to different high-fat diets during gestation and lactation. The objective of the current study was to investigate whether the quantity and the quality of fat present in these high-fat diets affected the tissue fatty acid composition of the adult offspring. Any changes in the tissue fatty acid composition may in turn affect offspring cellular metabolism, in a way that could result in the programming of adult disease. To this end, the effects of pre- and post-weaning high-fat diets rich in lard or safflower oil were compared with each other, and with the rodent chow, on the liver and heart fatty acid composition in the 11-week-old offspring of C57Bl/6 mice. In addition, the role of interaction between the high-fat pre- and post-weaning diets on offspring tissue fatty acid composition was assessed. It was hypothesized that a lard-rich diet fed to the mothers during gestation and lactation would enrich the offspring liver and heart tissues with SFA and MUFA, whereas a safflower oil-rich maternal diet would enrich the offspring liver and heart tissues with *n-6* PUFA.

6.2 METHODS

6.2.1 GLC analysis

Liver and heart tissues from offspring mice obtained during each set of experiments (*i.e.* Chapters 3, 4 and 5) were used for lipid extraction, using the method by Folch *et al.* (1957). Fatty acid composition was then determined using GLC as described in section 2.5.

6.2.2 Statistical analysis

The fatty acid data are reported as percent of total extracted fatty acids. Data were arcsine transformed prior to statistical analysis. The transformed data sets from dietary groups of each experimental set were then compared using two-way ANOVA, in an attempt to identify the main effects of pre-weaning diets, post-weaning diets and their interaction. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests (*SYSTAT for Windows, version 12.02*; *SYSTAT Software Inc.*, Richmond, California).

6.3 RESULTS

6.3.1 Effects of pre- and post-weaning high-fat diets rich in lard *vs.* chow on offspring hepatic fatty acid composition

Two-way ANOVA revealed that a post-weaning diet rich in lard was associated with higher hepatic content of palmitic acid (PA, 16:0) ($P = 0.003$), oleic acid (OA, 18:1) ($P < 0.001$), total MUFA ($P < 0.001$), alpha-linolenic acid (ALA, 18:3 *n-3*) ($P = 0.001$) and lower content of linoleic acid (LA, 18:2 *n-6*) ($P < 0.001$), total *n-6* PUFA ($P < 0.001$), docosapentaenoic acid (DPA, 22:5 *n-3*) ($P = 0.008$), DHA ($P < 0.001$), and total *n-3* PUFA ($P < 0.001$) in the offspring compared to chow (Table 6.1). An interaction of pre- and post-weaning diets was observed for the hepatic content of palmitoleic acid (PTA, 16:1) ($P = 0.01$) and EPA ($P < 0.001$) in the offspring (Table 6.1). Multiple comparisons further revealed that the C/S offspring had the highest hepatic content of PTA ($P < 0.05$) and S/C offspring had the highest hepatic content of EPA ($P < 0.001$) as compared to all other offspring ($P < 0.05$) (Table 6.1).

6.3.2 Effects of pre- and post-weaning high-fat diets rich in safflower oil *vs.* chow on offspring hepatic fatty acid composition

Two-way ANOVA revealed that a pre-weaning diet rich in safflower oil was associated with lower hepatic content of OA ($P = 0.006$) and total MUFA ($P = 0.014$) in the offspring compared to chow (Table 6.2). In contrast, a post-weaning diet rich in safflower oil was associated with higher hepatic content of LA ($P < 0.001$), total *n-6* PUFA ($P <$

Table 6.1 Hepatic fatty acid composition of the offspring exposed to a high-fat diet rich in lard *vs.* chow during pre- and post-weaning time periods*

Fatty acids	SFA/SFA	SFA/chow	Chow/chow	Chow/SFA	*Pre*	*Post*	*Int*
C14:0 (MA)	0.45 ± 0.08	0.48 ± 0.08	0.36 ± 0.07	0.52 ± 0.04	NS	NS	NS
C16:0 (PA)	23.93 ± 0.26	19.48 ± 1.65	21.78 ± 0.53	23.50 ± 0.70	NS	$P = 0.003$	NS
C18:0 (SA)	8.72 ± 0.48	11.63 ± 1.65	11.0 ± 1.14	9.32 ± 0.71	NS	NS	NS
Σ SFA	33.10 ± 0.49	31.59 ± 3.72	33.15 ± 1.29	33.34 ± 1.31	NS	NS	NS
C16: 1 *n9* (PTA)	2.00 ± 0.16^{b}	1.86 ± 0.41^{bc}	1.17 ± 0.09^{c}	2.82 ± 0.30^{a}	NS	$P = 0.001$	$P = 0.010$
C18: 1 (OA)	30.24 ± 1.07	17.17 ± 2.28	19.73 ± 1.95	30.77 ± 1.42	NS	$P < 0.001$	NS
C20: 1*n9* (EA)	0.29 ± 0.02	0.31 ± 0.06	0.32 ± 0.06	0.35 ± 0.09	NS	NS	NS
Σ MUFA	32.53 ± 1.21	19.34 ± 2.66	21.21 ± 2.0	33.94 ± 1.80	NS	$P < 0.001$	NS
C18: 2 *n6* (LA)	13.22 ± 0.37	17.69 ± 2.30	19.18 ± 0.69	11.55 ± 0.58	NS	$P < 0.001$	NS
C20: 4 *n6* (AA)	7.13± 0.44	8.73± 1.40	9.24 ± 1.06	9.13 ± 0.37	NS	NS	NS
Σ *n-6* PUFA	20.36 ± 0.55	26.41 ± 1.41	28.42 ± 0.43	20.67 ± 0.79	NS	$P < 0.001$	NS
C18:3 n*3* (ALA)	1.01 ± 0.08	0.67 ± 0.17	0.43 ± 0.01	0.91 ± 0.13	NS	$P = 0.001$	NS
C20:5 *n3* (EPA)	0.57 ± 0.02^{c}	0.97 ± 0.07^{a}	0.65 ± 0.06^{bc}	0.72 ± 0.02^{b}	NS	$P = 0.003$	$P < 0.001$
C22:5 *n3* (DPA)	0.53 ± 0.03	0.67± 0.07	0.61 ± 0.05	0.42 ± 0.08	NS	$P = 0.008$	NS
C22:6 *n3* (DHA)	6.55 ± 0.38	10.97 ± 1.41	10.09 ± 0.41	8.10 ± 0.22	NS	$P < 0.001$	NS
Σ *n-3* PUFA	8.66 ± 0.38	13.28 ± 1.33	11.788 ± 0.38	10.01 ± 0.35	NS	$P < 0.001$	NS

*Data are expressed as percentage of the total extracted fatty acids. Statistical analysis was performed after transforming the data using arcsine equation. Values are expressed as mean ± SEM, n = 7-10 (specified in section 3.2.4). Two-way ANOVA was used to compare the main effects of pre-weaning diet, post-weaning diet and their interaction. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Superscripts represent significant differences where $P < 0.05$ was considered significant.

MA, myristic acid; PA, palmitic acid; SA, stearic acid; SFA,saturated fatty acids; PTA, palmitoleic acid; OA, oleic acid; EA, eicosaenoic acid; MUFA, monounsaturated fatty acids; LA, linoleic acid; AA, arachidonic acid; PUFA, polyunsaturated fatty acids; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; pre, pre-weaning diet; post, post-weaning diet, Int, pre- and post-weaning diet interaction; NS= not significant.

Table 6.2. Hepatic fatty acid composition of the offspring exposed to a high-fat diet rich in safflower oil *vs.* chow during pre- and post-weaning time periods*

Fatty acids	PUFA/PUFA	PUFA/Chow	Chow/Chow	Chow/PUFA	*Pre*	*Post*	*Int*
C14:0 (MA)	0.36 ± 0.04	0.49 ± 0.08	0.36 ± 0.07	0.40 ± 0.02	NS	NS	NS
C16:0 (PA)	21.22 ± 0.71^{a}	16.24 ± 0.95^{b}	21.78 ± 0.53^{a}	19.74 ± 0.64^{a}	$P = 0.013$	NS	$P < 0.001$
C18:0 (SA)	12.53 ± 0.53^{a}	7.76 ± 0.33^{c}	11.0 ± 1.14^{ab}	8.97 ± 0.35^{bc}	NS	NS	$P = 0.001$
Σ SFA	33.3 ± 1.5^{a}	24.48 ± 0.89^{b}	33.15 ± 1.29^{a}	29.11 ± 0.60^{ab}	NS	NS	$P = 0.002$
C16: 1 *n9* (PTA)	0.90 ± 0.13	1.46 ± 0.17	1.17 ± 0.09	1.06 ± 0.11	NS	$P = 0.032$	NS
C18: 1 (OA)	11.02 ± 0.98	14.36 ± 1.55	19.72 ± 1.95	13.31 ± 0.55	$P = 0.006$	$P = 0.001$	NS
C20: 1*n9* (EA)	0.17 ± 0.01	0.33 ± 0.04	0.32 ± 0.06	0.10 ± 0.07	NS	$P = 0.001$	NS
Σ MUFA	12.09 ± 1.10	16.15 ± 1.63	21.21 ± 2.00	14.10 ± 0.45	$P = 0.014$	$P < 0.001$	NS
C18: 2 *n6* (LA)	31.27 ± 2.56	18.23 ± 1.31	19.18 ± 0.69	35.13 ± 1.21	NS	$P < 0.001$	NS
C20: 4 *n6* (AA)	11 ± 1.02	10.47 ± 0.93	9.24 ± 1.06	10.43 ± 0.56	NS	NS	NS
Σ *n-6* PUFA	43.8 ± 1.5	31.3 ± 0.4	30.1 ± 0.5	47.6 ± 0.9	NS	$P < 0.001$	NS
C18:3 n3 (ALA)	0.62 ± 0.13^{bc}	0.71 ± 0.08^{ab}	0.43 ± 0.01^{c}	0.99 ± 0.07^{a}	NS	$P = 0.028$	$P = 0.002$
C20:5 *n3* (EPA)	0.12 ± 0.01^{d}	1.12 ± 0.09^{a}	0.65 ± 0.06^{b}	0.23 ± 0.02^{c}	NS	$P < 0.001$	$P < 0.001$
C22:5 *n3* (DPA)	0.22 ± 0.02^{c}	1.15 ± 0.07^{a}	0.61 ± 0.05^{b}	0.27 ± 0.02^{c}	$P < 0.001$	$P < 0.001$	$P < 0.001$
C22:6 *n3* (DHA)	5.8 ± 0.56^{c}	16.34 ± 1.53^{a}	10.1 ± 0.41^{b}	6.99 ± 0.18^{c}	$P = 0.025$	$P < 0.001$	$P < 0.001$
Σ *n-3* PUFA	6.8 ± 0.5^{d}	19.3 ± 1.6^{a}	11.8 ± 0.4^{b}	8.5 ± 0.2^{c}	$P = 0.009$	$P < 0.001$	$P < 0.001$

*Data are expressed as percentage of the total extracted fatty acids. Statistical analysis was performed after transforming the data using arcsine equation. Values are expressed as mean ± SEM, n = 6-10 (specified in section 4.2.3). Two-way ANOVA was used to compare the main effects of pre-weaning diet, post-weaning diet and their interaction. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Superscripts represent significant differences where $P < 0.05$ was considered significant.

MA, myristic acid; PA, palmitic acid; SA, stearic acid; SFA,saturated fatty acids; PTA, palmitoleic acid; OA, oleic acid; EA, eicosaenoic acid; MUFA, monounsaturated fatty acids; LA, linoleic acid; AA, arachidonic acid; PUFA, polyunsaturated fatty acids; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; pre, pre-weaning diet; post, post-weaning diet, Int, pre- and post-weaning diet interaction; NS= not significant.

0.001) but lower hepatic content of PTA (P =0.032), OA (P = 0.001), EA, (P < 0.001) and total MUFA (P < 0.001) in the offspring compared to chow (Table 6.2). An interaction of pre- and post-weaning diets was observed for the hepatic content of PA (P < 0.001), SA (P = 0.001), total SFA (P < 0.001), ALA (P = 0.002), EPA (P < 0.001), DPA (P < 0.001), DHA (P < 0.001) and total *n-3* PUFA (P < 0.001) in the offspring (Table 6.2). Multiple comparisons further revealed that P/C offspring had the lowest content of hepatic PA (P < 0.05) and the highest content of hepatic EPA (P < 0.05), DPA (P < 0.05), DHA (P < 0.001), and total *n-3* PUFA (P < 0.001) compared to all other offspring (Table 6.2).

6.3.3 Effects of pre- and post-weaning high-fat diets rich in lard *vs.* safflower oil on the offspring hepatic fatty acid composition

Two-way ANOVA revealed that a pre-weaning diet rich in lard was associated with lower hepatic content of SA (P = 0.033) in the offspring compared to a safflower oil-rich diet. In contrast, a post-weaning diet rich in lard was associated with lower hepatic content of SA (P = 0.047) but higher hepatic content of PTA (P < 0.001), ALA (P < 0.001), DPA (P < 0.001), DHA (P < 0.039) and total *n-3* PUFA (P < 0.001) in the offspring compared to the safflower oil-rich diet (Table 6.3). In addition, an interaction of pre- and post-weaning diets was observed for the hepatic content of PA (P = 0.001), total SFA (P = 0.047), OA (P = 0.001), EA (P < 0.001), total MUFA (P = 0.001), LA (P = 0.006), total *n-6* PUFA (P = 0.003) and EPA (P < 0.001) in the offspring (Table 6.3).

Multiple comparisons further revealed that S/P offspring had the lowest hepatic content of PA ($P < 0.005$) and total SFA ($P < 0.05$). The P/P offspring had lowest hepatic content of OA ($P < 0.001$), EA ($P < 0.005$), total MUFA ($P < 0.001$), EPA ($P < 0.001$), but the highest content of LA ($P < 0.05$) and total *n-6* PUFA ($P < 0.05$) compared to all other offspring (Table 6.3).

6.3.4 Effects of pre- and post-weaning high-fat diets rich in lard *vs.* chow on offspring heart fatty acid composition

Two-way ANOVA revealed that a pre-weaning diet rich in lard was associated with lower content of SA ($P = 0.023$) and AA ($P = 0.031$) in the offspring heart compared to chow (Table 6.4). In addition, an interaction of pre- and post-weaning diets was observed for the content of MA ($P = 0.002$), PA ($P = 0.004$), total SFA ($P < 0.001$), PTA ($P = 0.001$), OA ($P = 0.001$), EA ($P < 0.001$), total MUFA ($P < 0.001$), DPA ($P < 0.001$), DHA ($P < 0.001$) and total *n-3* PUFA ($P < 0.001$) in the offspring heart (Table 6.4). Multiple comparisons further revealed that S/S offspring heart had the highest content of MA ($P < 0.001$), PA ($P < 0.05$), PTA ($P < 0.001$), OA ($P < 0.001$), and total MUFA ($P < 0.001$), but the lowest content of DHA ($P < 0.001$) and total *n-3* PUFA ($P < 0.001$) compared to all other offspring. In contrast, the S/C offspring heart had the lowest content of PA ($P < 0.05$) and total SFA ($P < 0.001$) compared to all other offspring (Table 6.4). The C/S offspring heart had the lowest content of EA ($P < 0.05$) and the highest content of DPA ($P < 0.01$) compared to all other offspring (Table 6.4).

Table 6.3. Hepatic fatty acid composition of the offspring exposed to a high-fat diet rich in lard *vs.* safflower oil during pre- and post-weaning time periods*

Fatty acids	SFA/SFA	SFA/PUFA	PUFA/PUFA	PUFA/SFA	*Pre*	*Post*	*Int*
C14:0 (MA)	0.45± 0.08	0.31 ± 0.02	0.36 ± 0.04	0.34 ± 0.04	NS	NS	NS
C16:0 (PA)	23.93 ± 0.26^{a}	19.15 ± 0.38^{c}	21.22 ± 0.71^{b}	22.68 ± 0.34^{a}	NS	$P < 0.001$	$P = 0.001$
C18:0 (SA)	8.72 ± 0.48	9.79 ± 0.60	12.53 ± 0.53	9.9 ± 0.63	$P = 0.033$	$P = 0.047$	NS
Σ SFA	33.10 ± 0.49^{a}	29.25 ± 0.93^{b}	33.33 ± 1.53^{a}	32.93 ± 0.79^{a}	NS	NS	$P = 0.047$
C16: 1 *n9* (PTA)	2.0 ± 0.16	0.67± 0.07	0.90 ± 0.13	1.78 ± 0.18	NS	$P < 0.001$	NS
C18: 1 (OA)	30.24 ± 1.07^{a}	28.57 ± 4.19^{a}	11.02 ± 0.98^{b}	31.06 ± 1.94^{a}	$P = 0.001$	$P < 0.001$	$P = 0.001$
C20: 1*n9* (EA)	0.29 ± 0.02^{a}	0.40 ± 0.05^{a}	0.17 ± 0.01^{b}	0.35 ± 0.04^{a}	$P = 0.012$	NS	$P < 0.001$
Σ MUFA	32.53 ± 1.21^{a}	29.63 ± 4.31^{a}	12.09 ± 1.10^{b}	33.19 ± 2.11^{a}	$P = 0.002$	$P < 0.001$	$P = 0.001$
C18: 2 *n6* (LA)	13.22 ± 0.37^{c}	20.17 ± 3.60^{b}	31.27 ± 2.56^{a}	12.12 ± 0.32^{c}	$P = 0.027$	$P < 0.001$	$P = 0.006$
C20: 4 *n6* (AA)	7.13 ± 0.44	7.67 ± 1.00	11 ± 1.02	8.16 ± 0.50	$P = 0.020$	NS	NS
Σ *n-6* PUFA	20.36 ± 0.55^{c}	27.84 ± 4.06^{b}	42.27 ± 1.70^{a}	20.28 ± 0.76^{c}	$P = 0.003$	$P < 0.001$	$P = 0.003$
C18:3 n*3* (ALA)	1.01 ± 0.0	0.51 ± 0.08	0.62 ± 0.13	0.86 ± 0.07	NS	$P < 0.001$	NS
C20:5 *n3* (EPA)	0.57 ± 0.08^{a}	0.27 ± 0.03^{b}	0.12 ± 0.01^{c}	0.64 ± 0.04^{a}	$P = 0.004$	$P < 0.001$	$P < 0.001$
C22:5 *n3* (DPA)	0.53 ± 0.02	0.24 ± 0.01	0.22 ± 0.02	0.48 ± 0.04	NS	$P < 0.001$	NS
C22:6 *n3* (DHA)	6.56 ± 0.03	5.28 ± 0.23	5.84 ± 0.56	6.44 ± 0.50	NS	$P = 0.039$	NS
Σ *n-3* PUFA	8.66 ± 0.38	7.96 ± 0.20	6.80 ± 0.48	8.42 ± 0.52	NS	$P < 0.001$	NS

*Data are expressed as percentage of the total extracted fatty acids. Statistical analysis was performed after transforming the data using arcsine equation. Values are expressed as mean ± SEM, n = 8-10 (specified in section 5.2.2). Two-way ANOVA was used to compare the main effects of pre-weaning diet, post-weaning diet and their interaction. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Superscripts represent significant differences where $P < 0.05$ was considered significant.

MA, myristic acid; PA, palmitic acid; SA, stearic acid; SFA,saturated fatty acids; PTA, palmitoleic acid; OA, oleic acid; EA, eicosaenoic acid; MUFA, monounsaturated fatty acids; LA, linoleic acid; AA, arachidonic acid; PUFA, polyunsaturated fatty acids; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; pre, pre-weaning diet; post, post-weaning diet, Int, pre- and post-weaning diet interaction; NS= not significant.

Table 6.4. Heart fatty acid composition of the offspring exposed to a high-fat diet rich in lard *vs.* chow during pre- and post-weaning time periods*

Fatty acids	SFA/SFA	SFA/Chow	Chow/chow	Chow/SFA	Pre	Post	Int
C14:0 (MA)	1.07 ± 0.22^{a}	0.16 ± 0.02^{c}	0.26 ± 0.03^{bc}	0.40 ± 0.40^{b}	NS	$P < 0.001$	$P = 0.002$
C16:0 (PA)	18.23 ± 1.29^{a}	11.54 ± 0.30^{c}	14.23 ± 0.47^{b}	15.7 ± 0.57^{b}	NS	$P < 0.001$	$P = 0.004$
C18:0 (SA)	13.50 ± 1.82	15.80 ± 0.49	18.36 ± 0.49	16.45 ± 0.25	$P = 0.023$	NS	NS
Σ SFA	32.80 ± 0.48^{a}	27.50 ± 0.42^{b}	32.85 ± 0.52^{a}	32.56 ± 0.76^{a}	$P < 0.001$	$P < 0.001$	$P < 0.001$
C16: 1 *n9* (PTA)	2.69 ± 0.68^{a}	0.26 ± 0.05^{b}	0.56 ± 0.11^{b}	0.71 ± 0.09^{b}	NS	$P < 0.001$	$P = 0.001$
C18: 1 (OA)	29.92 ± 3.50^{a}	9.29 ± 1.13^{b}	10.98 ± 0.78^{b}	12.77 ± 0.47^{b}	$P = 0.002$	$P < 0.001$	$P < 0.001$
C20: 1*n9* (EA)	0.58 ± 0.07^{a}	0.46 ± 0.07^{a}	0.57 ± 0.03^{a}	0.24 ± 0.06^{b}	NS	NS	$P = 0.001$
Σ MUFA	33.19 ± 4.14^{a}	10.01 ± 1.18^{b}	12.03 ± 0.87^{b}	13.67 ± 0.53^{b}	$P = 0.002$	$P < 0.001$	$P < 0.001$
C18: 2 *n6* (LA)	10.56 ± 0.36	12.52 ± 0.80	12.53 ± 0.54	11.02 ± 0.35	NS	$P = 0.003$	NS
C20: 4 *n6* (AA)	5.56 ± 1.27	6.18 ± 0.32	6.78 ± 0.12	8.16 ± 0.08	$P = 0.031$	NS	NS
Σ *n-6* PUFA	16.12 ± 1.56	18.7 ± 0.94	19.30 ± 0.49	19.18 ± 0.42	NS	NS	NS
C18:3 n3 (ALA)	0.67 ± 0.09	ND	0.12 ± 0.02	0.29 ± 0.02	NA	NA	NA
C20:5 *n3* (EPA)	0.071 ± 0.02	0.58 ± 0.23	0.13 ± 0.03	ND	NA	NA	NA
C22:5 *n3* (DPA)	0.84 ± 0.19^{c}	1.22 ± 0.04^{b}	0.94 ± 0.12^{bc}	1.96 ± 0.04^{a}	$P = 0.013$	NS	$P < 0.001$
C22:6 *n3* (DHA)	9.75 ± 2.18^{b}	29.11 ± 1.26^{a}	29.25 ± 0.96^{a}	25.66 ± 0.89^{a}	$P < 0.001$	$P < 0.001$	$P < 0.001$
Σ *n-3* PUFA	11.34 ± 2.29^{b}	30.92 ± 1.25^{a}	30.41 ± 0.93^{a}	27.91 ± 0.91^{a}	$P < 0.001$	$P < 0.001$	$P < 0.001$

*Data are expressed as percentage of the total extracted fatty acids. Statistical analysis was performed after transforming the data using arcsine equation. Values are expressed as mean ± SEM, n = 7-10 (specified in section 3.2.4). Two-way ANOVA was used to compare the main effects of pre-weaning diet, post-weaning diet and their interaction. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Superscripts represent significant differences where $P < 0.05$ was considered significant.

MA, myristic acid; PA, palmitic acid; SA, stearic acid; SFA,saturated fatty acids; PTA, palmitoleic acid; OA, oleic acid; EA, eicosaenoic acid; MUFA, monounsaturated fatty acids; LA, linoleic acid; AA, arachidonic acid; PUFA, polyunsaturated fatty acids; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; pre, pre-weaning diet; post, post-weaning diet, Int, pre- and post-weaning diet interaction; NS= not significant.

6.3.5 Effects of pre- and post-weaning high-fat diets rich in safflower oil *vs.* chow on offspring heart fatty acid composition

Two-way ANOVA revealed that a pre-weaning diet rich in safflower oil was associated with lower content of EA ($P = 0.039$), DHA ($P = 0.014$) and total *n-3* PUFA ($P = 0.021$) in the offspring heart compared to chow (Table 6.5). Similarly, a post-weaning diet rich in safflower oil was associated with lower content of DHA ($P < 0.001$) and total *n-3* PUFA ($P < 0.001$) in the offspring heart compared to chow (Table 6.5). In addition, an interaction of pre- and post-weaning diets was observed for the heart content of LA ($P = 0.005$), AA ($P = 0.035$), total *n-6* PUFA ($P = 0.016$), ALA ($P = 0.019$) and DPA ($P = 0.015$) in the offspring (Table 6.5). Multiple comparisons further revealed that P/C offspring heart had the lowest content of LA ($P < 0.05$), AA ($P < 0.001$) and total *n-6* PUFA ($P < 0.001$), whereas P/P offspring heart had the lowest content of DPA ($P < 0.05$) and C/C offspring heart had the lowest content of ALA ($P < 0.001$) compared to all other offspring (Table 6.5).

6.3.6 Effects of pre- and post-weaning high-fat diets rich in lard *vs.* safflower oil on offspring heart fatty acid composition

Two-way ANOVA revealed that a pre-weaning diet rich in lard was associated with higher content of MA ($P = 0.006$), OA ($P < 0.001$), total MUFA ($P < 0.001$), but lower content of SA ($P = 0.005$) and total SFA ($P = 0.037$) in the offspring heart compared to a

safflower oil-rich diet. Similarly, a post-weaning diet rich in lard was associated with higher content of MA ($P = 0.002$), total SFA ($P = 0.001$), OA ($P = 0.001$) and total MUFA ($P < 0.001$) but lower content of SA ($P < 0.042$) in the offspring compared to a safflower oil-rich diet (Table 6.6). In addition, an interaction of pre- and post-weaning diets was observed for the content of PA ($P = 0.015$), PTA ($P = 0.030$), LA ($P = 0.003$), AA ($P = 0.020$), ALA ($P = 0.006$), DPA ($P = 0.001$), DHA ($P < 0.001$) and total *n-3* PUFA ($P < 0.001$) in the offspring (Table 6.6). Multiple comparisons further revealed that S/S offspring heart had the highest content of PA ($P < 0.001$), PTA ($P < 0.01$), ALA ($P < 0.001$) and the lowest content of AA ($P < 0.001$), DHA ($P < 0.05$) and total *n-3* PUFA ($P < 0.05$), whereas P/P offspring heart had the highest content of LA ($P < 0.001$) and P/S offspring heart had the highest content of DPA ($P < 0.001$) compared to all other offspring (Table 6.6).

Table 6.5. Heart fatty acid composition of the offspring exposed to a high-fat diet rich in safflower oil *vs.* chow during pre- and post-weaning time periods*

Fatty acids	PUFA/PUFA	PUFA/Chow	Chow/chow	Chow/PUFA	*Pre*	*Post*	*Int*
C14:0 (MA)	0.24 ± 0.07	0.39 ± 0.10	0.26 ± 0.03	0.26± 0.03	NS	NS	NS
C16:0 (PA)	12.24 ± 0.60	14.19± 2.24	14.23 ± 0.47	12.03 ± 0.69	NS	NS	NS
C18:0 (SA)	19.20 ± 0.60	18.0 ± 2.39	18.36 ± 0.49	17.28± 0.84	NS	NS	NS
Σ SFA	32.81 ± 0.72	32.58 ± 4.92	32.85 ± 0.52	29.57 ± 0.44	NS	NS	NS
C16: 1 *n9* (PTA)	0.62 ± 0.33	0.66 ± 0.19	0.56 ± 0.11	0.47 ± 0.15	NS	NS	NS
C18: 1 (OA)	8.61 ± 0.85	9.94 ± 1.43	10.98 ± 0.78	8.70 ± 0.55	NS	NS	NS
C20: 1*n9* (EA)	0.37 ± 0.03	0.36 ± 0.03	0.57 ± 0.03	0.49 ± 0.15	$P = 0.039$	NS	NS
Σ MUFA	9.45 ± 1.05	10.96 ± 1.61	12.03 ± 0.87	9.65 ± 0.67	NS	NS	NS
C18: 2 *n6* (LA)	26.15 ± 1.14^{a}	9.73 ± 1.16^{c}	12.53 ± 0.54^{b}	23.30 ± 0.58^{a}	NS	$P < 0.001$	$P = 0.005$
C20: 4 *n6* (AA)	9.79 ± 0.41^{a}	5.44 ± 0.50^{c}	6.78 ± 0.12^{b}	9.63 ± 0.32^{a}	NS	$P < 0.001$	$P = 0.035$
Σ *n-6* PUFA	35.94± 1.07^{a}	16.59 ± 1.68^{c}	19.87 ± 0.54^{b}	33.50± 0.86^{a}	NS	$P < 0.001$	$P = 0.016$
C18:3 n3 (ALA)	0.29 ± 0.02^{a}	0.27± 0.04^{a}	0.12 ± 0.02^{b}	0.26 ± 0.02^{a}	$P = 0.002$	$P = 0.005$	$P = 0.019$
C20:5 *n3* (EPA)	ND	0.76 ± 0.30	0.13 ± 0.03	ND			
C22:5 *n3* (DPA)	0.63 ± 0.05^{b}	1.04 ± 0.10^{a}	0.94 ± 0.12^{a}	0.98 ± 0.04^{a}	NS	$P = 0.055$	$P = 0.015$
C22:6 *n3* (DHA)	14.49± 1.22	27.42 ± 3.18	29.25 ± 0.96	20.84 ± 0.31	$P = 0.014$	$P < 0.001$	NS
Σ*n-3* PUFA	15.41 ± 1.27	29.50 ± 3.32	30.41 ± 0.93	22.08 ± 0.34	$P = 0.021$	$P < 0.001$	NS

*Data are expressed as percentage of the total extracted fatty acids. Statistical analysis was performed after transforming the data using arcsine equation. Values are expressed as mean ± SEM, n = 8-10 (specified in section 4.2.3). Two-way ANOVA was used to compare the main effects of pre-weaning diet, post-weaning diet and their interaction. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Superscripts represent significant differences where $P < 0.05$ was considered significant.

MA, myristic acid; PA, palmitic acid; SA, stearic acid; SFA,saturated fatty acids; PTA, palmitoleic acid; OA, oleic acid; EA, eicosaenoic acid; MUFA, monounsaturated fatty acids; LA, linoleic acid; AA, arachidonic acid; PUFA, polyunsaturated fatty acids; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; pre, pre-weaning diet; post, post-weaning diet, Int, pre- and post-weaning diet interaction; NS= not significant.

Table 6.6. Heart fatty acid composition of the offspring exposed to a high-fat diet rich in lard *vs.* safflower oil during pre- and post-weaning time periods*

	SFA/SFA	SFA/PUFA	PUFA/PUFA	PUFA/SFA	*Pre*	*Post*	*Int*
C14:0 (MA)	1.07 ± 0.22	0.33 ± 0.06	0.24± 0.07	0.39 ± 0.09	$P = 0.006$	$P = 0.002$	NS
C16:0 (PA)	18.23 ± 1.29^{a}	11.85 ± 0.49^{c}	12.24 ± 0.60^{c}	14.23 ± 0.78^{b}	$P = 0.048$	$P = 0.000$	$P = 0.015$
C18:0 (SA)	13.50 ±1.82	17.98 ± 0.85	19.20 ± 0.60	19.07 ± 0.80	$P = 0.005$	$P = 0.042$	NS
Σ SFA	32.80 ± 0.48	30.16 ±0.74	31.68 ± 0.72	33.69 ± 0.52	$P = 0.037$	$P = 0.001$	NS
C16: 1 *n9* (PTA)	2.70 ± 0.68^{a}	0.50 ± 0.14^{b}	0.62 ± 0.33^{b}	0.84 ± 0.26^{b}	$P = 0.039$	$P = 0.003$	$P = 0.030$
C18: 1 (OA)	29.92 ±3.5	17.45± 2.67	8.61 ± 0.85	14.57 ±2.14	$P < 0.001$	$P = 0.001$	NS
C20: 1*n9* (EA)	0.58 ± 0.07	0.44 ± 0.08	0.37± 0.03	0.46 ± 0.04	NS	NS	NS
Σ MUFA	33.19 ±4.14	18.4± 2.82	9.45 ± 1.05	15.87 ± 2.41	$P < 0.001$	$P < 0.001$	NS
C18: 2 *n6* (LA)	10.6 ± 0.36^{c}	16.34 ± 2.12^{b}	26.15 ± 1.14^{a}	11.27 ± 0.69^{c}	$P = 0.001$	$P < 0.001$	$P = 0.003$
C20: 4 *n6* (AA)	5.56 ± 1.27^{b}	9.21 ± 0.35^{a}	9.80 ± 0.41^{a}	9.37 ± 0.41^{a}	$P = 0.004$	$P = 0.006$	$P = 0.020$
Σ *n-6* PUFA	16.12 ± 1.56	25.56 ± 2.35	35.94 ± 1.07	20.64 ± 0.94	$P < 0.001$	$P < 0.001$	NS
C18:3 *n3* (ALA)	0.67 ± 0.09^{a}	0.26 ± 0.04^{b}	0.29 ± 0.02^{b}	0.37 ± 0.06^{b}	$P = 0.039$	$P < 0.001$	$P = 0.006$
C20:5 *n3* (EPA)	ND	ND	ND	ND			
C22:5 *n3* (DPA)	0.84 ± 0.19^{b}	0.85 ± 0.08^{b}	0.63 ± 0.05^{b}	1.60 ± 0.14^{a}	NS	$P = 0.002$	$P = 0.001$
C22:6 *n3* (DHA)	9.75 ± 2.18^{c}	17.68 ± 0.73^{ab}	14.49 ± 1.22^{b}	20.0 ± 1.93^{a}	$P = 0.030$	NS	$P < 0.001$
Σ *n-3* PUFA	11.34 ± 2.29^{c}	18.79 ± 0.77^{ab}	15.41 ± 1.27^{b}	21.96 ± 1.98^{a}	$P = 0.038$	NS	$P < 0.001$

*Data are expressed as percentage of the total extracted fatty acids. Statistical analysis was performed after transforming the data using arcsine equation. Values are expressed as mean ± SEM, n = 8-10 (specified in section 5.2.2). Two-way ANOVA was used to compare the main effects of pre-weaning diet, post-weaning diet and their interaction. Effects of significant interaction were further analyzed using Fisher's LSD *post hoc* tests. Superscripts represent significant differences where $P < 0.05$ was considered significant.

MA, myristic acid; PA, palmitic acid; SA, stearic acid; SFA, saturated fatty acids; PTA, palmitoleic acid; OA, oleic acid; EA, eicosaenoic acid; MUFA, monounsaturated fatty acids; LA, linoleic acid; AA, arachidonic acid; PUFA, polyunsaturated fatty acids; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; pre, pre-weaning diet; post, post-weaning diet, Int, pre- and post-weaning diet interaction; NS= not significant.

6.4 DISCUSSION

This study sought to establish whether maternal dietary fat intake during gestation and lactation would have an impact on the offspring liver and heart fatty acid composition. In addition, the effects of interaction between pre- and post-weaning dietary fat intake on the tissue fatty acid composition in the adult offspring were assessed. As expected and based on previous studies (Murphy *et al.*, 2004; Okada *et al.*, 2008), liver and heart tissue fatty acid composition reflected the composition of the post-weaning diet of the offspring. However, the current study also demonstrated lasting effects of maternal diet fed during gestation and lactation on the offspring tissue total fatty acid composition at 11-weeks of age. The fatty acid composition of total extracted lipids may not indicate whether these changes are reflected specifically in the phospholipids or the TG pool of the liver and the heart tissue. Considering that the fatty acids of the phospholipids pool are generally more involved in the control of cellular activities than the fatty acids of the TG pool, it is acknowledged that the observations of the current study are limited in their interpretations.

A lard-rich diet fed to the mothers during gestation and lactation was associated with significantly higher hepatic content of EPA in the offspring compared to chow (S/C *vs.* C/C, Table 6.1). As described in section 2.1.1, both the lard-rich diet and the chow used in the current study contained 4% ALA (Table 2.2). However, since the lard-rich diet contained 20% of fat *w/w* as opposed to 5% fat *w/w* contained in chow, it is apparent that lard-rich diet contained much higher amounts of ALA than chow. ALA is a precursor for the synthesis of EPA, DPA and DHA, thus its availability in the maternal diet during

gestation and lactation may have contributed to the increased content of EPA in the offspring liver. It is important to note that EPA represented less than 1% of total hepatic fatty acids, while DHA represented the majority of *n-3* PUFA content of offspring hepatic tissue, which was not affected by the maternal diet (S/C *vs.* C/C, Table 6.1).

A pre-weaning diet rich in lard was associated with higher hepatic content of OA and EA compared to a safflower oil-rich diet, even when the offspring continued on a safflower oil-rich diet post-weaning (S/P *vs.* P/P, Table 6.3). Since the lard-rich diet contained higher amounts of OA and EA than the safflower oil-rich diet (Table 2.2), an increase in these fatty acids reflects upon the long-lasting effects of maternal diet on the offspring tissue fatty acid composition. In addition, S/P offspring exhibited lower hepatic content of PA, LA, AA and higher content of hepatic EPA as compared to the P/P offspring (Table 6.3). Similar to the lard-rich diet, the safflower oil-rich diet used in the current study also contained 4% ALA (Table 2.2). However, the safflower-oil rich diet also contained a higher content of LA than the lard-rich diet. Thus, an increased availability of LA compared to ALA in the safflower oil-rich maternal diet may have led to an increased incorporation of LA in the offspring tissues *via* fetal circulation, thereby explaining the increased proportions of LA and AA, while reduced proportions of EPA observed in the liver of P/P offspring compared to S/P offspring (Table 6.3) (Lands *et al.*, 1990). A reduction in the PA content of the S/P offspring, on the other hand could have resulted due to the increased metabolism of SFA into MUFA in the offspring liver, or it could be a result of the interaction between the pre-weaning lard-rich diets and post-weaning safflower oil-rich diets.

In contrast to the lard-rich diet, a safflower oil-rich diet fed to the mothers during gestation and lactation was associated with reduced proportions of hepatic PA, SA, OA and significantly higher proportions of ALA, EPA, DPA and DHA in the offspring compared to chow (P/C *vs.* C/C, Table 6.2). Considering that the safflower oil-rich diet was low in SFA and MUFA and had the highest proportions of LA (Table 2.2.), a reduction of SFA and MUFA with an increase in the content of LA and its derivatives was expected in the offspring liver, in response to a maternal diet rich in safflower oil. However, maternal feeding of safflower oil was associated with the enrichment of offspring hepatic tissues with *n-3* PUFA instead of *n-6* PUFA. It has been shown previously that the presence of both *n-6* PUFA and *n-3* PUFA in the diet was associated with higher levels of DHA in the retinal phospholipids than a diet rich in *n-6* PUFA alone (Schnebelen *et al.,* 2009). Thus, presence of both LA and ALA in the diet may have led to an increase in their hepatic *n-3* PUFA levels, however the exact mechanism behind this observation remains to be determined.

Opposite to the maternal diet, a continuous exposure to the safflower oil-rich diets during pre- and post-weaning time periods was associated with reduced proportions of EPA and *n-3* PUFA in the offspring (*i.e.* P/P) compared to all other offspring (Table 6.2). These observations suggest that enrichment of offspring liver by a maternal diet rich in safflower oil does not continue if the offspring is exposed to a safflower oil-rich diet post-weaning, due to the interaction between the pre- and post-weaning diets. It is known that PUFA of *n-6* and *n-3* series compete with each other during absorption, plasma transport, metabolism through desaturation-chain elongation, and as substrates of the

downstream cyclooxygenase and lipooxygenase pathways (Holman and Mohrhauer, 1963). An increased availability of *n-6* PUFA compared to the *n-3* PUFA in post-weaning diet may explain the observed reduction in the hepatic *n-3* PUFA content in the offspring exposed to a safflower oil-rich diet post-weaning, which is further reinforced by the observation that the hepatic content of LA and total *n-6* PUFA was significantly higher in these offspring (*i.e.* P/P and C/P offspring *vs.* P/C and C/P offspring, Table 6.2). However, it remains to be determined how the interaction between the pre- and post-weaning safflower oil diets resulted in the reduction of *n-3* PUFA content in the P/P offspring compared to the C/P offspring (Table 6.2).

Although no changes were observed in the hepatic DHA content among various offspring that were exposed to high-fat diets both during pre- and post-weaning time periods (*i.e.* S/S, S/P, P/P and P/S) (Table 6.3), both S/S and P/P offspring had lower hepatic DHA levels than the C/C offspring (Table 6.1 and Table 6.2). It is well established that *n-3* PUFA, especially DHA, plays an important role in the etiology of liver disease. A previous study in Rhesus monkeys has shown that feeding an experimental diet low in *n-3* PUFA predisposed these animals to develop alcoholic fatty liver and fibrosis, which was associated with reduced content of DHA in their liver phospholipids (Pawlosky and Salem, 2004). Another study reported that fish oil supplementation suppressed the development of acute hepatitis and prolonged the survival of the Long-Evans Cinnamon rats, an experimental model of Wilson disease, which was also found to be associated with an increase in the total content of hepatic DHA in these rats (Chunyan *et al.*, 2004). Moreover, a recent study reported that *in vitro*

supplementation of DHA significantly reduced oxidative stress and hydrogen peroxide-induced DNA damage in cultured murine hepatocytes, while dietary supplementation of mice with DHA ameliorated carbon tetrachloride-induced necro-inflammatory damage of liver tissue *in vivo* (Gonzalez-Periz *et al.*, 2006). Thus, reduced proportions of hepatic DHA as a result of a continuous exposure to high fat diets during pre- and post-weaning time periods may have deleterious effects on liver physiology of these offspring in later life.

In contrast to the liver fatty acid composition, a lard-rich diet fed to the mothers during gestation and lactation was associated with lower PA in the offspring heart compared to chow (S/C *vs.* C/C, Table 6.4). Since the lard-rich diets contained higher proportions of PA than chow, it may be proposed that offspring heart did not incorporate the excess PA coming from the maternal diet. Alternatively, PA can be metabolized to form ceramide in the heart (Sparagn *et al.*, 2001), which may have led to the lower content of PA in the offspring heart. As opposed to the pre-weaning exposure to a lard-rich diet, a continuous exposure to a lard-rich diets during pre- and post-weaning time periods was associated with a higher content of MA, PA, PTA, OA and lower content of DHA in the S/S offspring heart compared to all other offspring (Table 6.4). Heart tissue contains high levels of DHA, which is known to affect the membrane fluidity of cardiomyocytes, thereby altering their contractile properties (Leifert *et al.*, 2000), whereas enrichment of heart tissues with SFA has been associated with arrhythmic effects (Honen and Saint, 2002), Thus, presence of higher SFA and lower DHA due to a continuous exposure to the lard-rich diets during pre- and post-weaning time periods may

cause dysfunction of the offspring heart later in life. A continuous exposure to lard-rich diets during pre- and post-weaning time periods was also associated with higher PA, PTA and reduced proportions of AA and DHA in the offspring heart compared to the safflower oil-rich diet. These findings suggest that the alterations in heart fatty acid composition induced by a lard-rich maternal diet may prove to be more harmful to the function of the heart than a safflower oil-rich diet (Table 6.6).

The safflower oil-rich diet contained higher proportions of LA than chow, so a maternal diet rich in safflower oil was expected to result in higher proportions of LA and AA in the offspring heart. However, P/C offspring exhibited lower content of LA and AA in the offspring heart than the C/C offspring (Table 6.5). These data indicated that increased availability of LA and its metabolic product AA, in the maternal circulation may have down-regulated the synthesis and/or incorporation of these fatty acids in the developing fetal heart, which persisted into adulthood. It has been demonstrated that AA production was reduced in the enterally or parenterally fed infants receiving corn or safflower oil as a predominant source of fatty acids (Innis, 1991; Simopoulos, 1991; Herrera, 2002). Studies have also shown that a high-fat diet rich in LA was able to down-regulate the endogenous synthesis of AA compared to a low-fat diet in female Sprague Dawley rats (Rodríguez-Cruz *et al.*, 2009). AA is known to play an important role in the intracellular signaling pathways associated with the contraction of heart muscle cells (Huang *et al.*, 1997; Qiu and Quilley, 1999). A reduction in AA content, illustrates the potential for altered cardiac function in the offspring resulting from diet-programming effects.

A pre-weaning safflower oil-rich diet exposure was associated with reduced proportions of DHA in the heart of P/P offspring than C/P offspring. Moreover, both P/P and C/P offspring heart had lower DHA content than the P/C and C/C offspring (Table 6.5). An increased availability of LA in the safflower-oil rich diet fed post-weaning would explain the competition between *n-6* and *n-3* PUFA for incorporation into heart tissue, as reflected by an increase in LA and AA, with a concomitant decrease in their heart DHA content. Thus, a reduction in the heart DHA content of the offspring obtained from mothers fed a high *n-6* PUFA diet and fed chow post-weaning was expected. However, no differences were observed in the DHA content of the P/C and C/C offspring (Table 6.5). Owing to its essentiality for fetal development, DHA has been reported to be preferentially taken up from maternal circulation by the placental fatty acid binding proteins in order to enrich the fetal circulation (Haggarty *et al.*, 1999). Thus, preferential uptake of DHA from maternal circulation may protect the developing fetus from the deficiency of DHA that may occur due to low maternal dietary intake of *n-3* PUFA or due to an increased intake of dietary *n-6* PUFA during pregnancy to a certain extent.

In conclusion, the current study reported for the first time that the maternal diet during pregnancy and lactation could have long lasting effects on the offspring heart and liver fatty acid composition. While a high-fat maternal diet rich in safflower oil was associated with an increase in offspring heart DHA proportions, a continuous exposure to both safflower oil and lard-rich-diets during pre- and post-weaning time periods was associated with a reduction in offspring liver and heart DHA proportions, indicating that

the interaction between the pre- and post-weaning dietary fat intake plays an important role in regulating tissue fatty acid composition in the adult offspring.

CHAPTER SEVEN

Comparison of high-fat diets rich in flax oil vs. lard on the outcome of parameters associated with metabolic syndrome in adult SHR/NDmcr-cp rat, a genetic model of metabolic syndrome

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7.1 INTRODUCTION

Metabolic syndrome, a constellation of co-morbidities that includes visceral obesity, hypertension, glucose intolerance, and dyslipidemia, is a highly predisposing condition for CVD (Bricker and Greydanus, 2008). As discussed earlier, high intake of dietary SFA is associated with increased incidence of CVD (Renaud and de Lorgeril, 1989; Artaud-Wild *et al.*, 1993; Keys, 1997; Denke, 2006), whereas a high intake of PUFA is known to reduce the incidence of CVD (Dolecek, 1992). Among PUFA, the *n-3* PUFA such as EPA and DHA commonly found in fish and fish oil are well known to possess cardioprotective effects. The beneficial effects of *n-3* PUFA are attributed to their hypolipidemic, antithrombotic and antiarrhythmic properties (Demaison and Moreau, 2002). Recently flax oil derived from flax seed (*Linum usitatissimum*) has gained a lot of attention as an important dietary source of *n-3* PUFA, especially among the vegetarian populations (Davis and Kris-Etherton, 2003).

Flax oil is a rich source of an essential *n-3* PUFA, ALA, which is converted to EPA and DHA by the (Δ^6-Δ^5) elongase and desaturase enzyme systems in the body. Although there is a debate regarding the efficacy of the conversion of ALA into EPA and DHA in the human body (Davis and Kris-Etherton, 2003), ALA consumption by itself has been reported to possess beneficial effects on the clinical outcomes of renal failure, multiple sclerosis, cancer, hypertension as well as CVD (Kelley *et al.*, 1991; Mantzioris *et al.*, 1994). A number of studies have shown that flax oil supplementation can reduce serum TG and cholesterol concentrations (Cunnane *et al.*, 1993; Craig, 1999). Moreover, flax oil supplementation has been shown to improve NAFLD by reducing the lipid

content of the liver (Murase *et al.*, 2005). Furthermore, it has been proposed that the *n-3* PUFA of flaxseed oil have anti-inflammatory properties that are mediated by the production of anti-inflammatory cytokines (Cohen *et al.*, 2005).

SHR/NDmcr-cp rats, which represent a genetic model of metabolic syndrome, are derived from a cross between the spontaneously hypertensive rat (SHR) and the obese Koletsky rat (Junko *et al.*, 2005). Due to the genetic background from the SHR rats, they exhibit hypertension whereas the nonsense mutation in the leptin receptor derived from the Koletsky rats results in severe obesity. Moreover, obese SHR/NDmcr-cp rats (-/-) carrying the homozygous mutation in the leptin receptor exhibit most of the abnormalities associated with metabolic syndrome including hyperglycaemia, hyperinsulinemia, hyperlipidemia and fatty liver, when compared with their lean (+/+) counterparts (Yasui *et al.*, 2007). Oxidative stress is also increased in the obese SHR/NDmcr-cp rats, which is similar to observations in patients of metabolic syndrome (Yamaguchi *et al.*, 2006). Obese SHR/NDmcr-cp rats thus exhibit most of the metabolic derangements observed in patients with metabolic syndrome, making them one of the most suitable animal models of metabolic syndrome.

In an attempt to identify the beneficial effects of flax oil, diets enriched with either flax oil or lard were fed to both obese and lean SHR/NDmcr-cp rats and various parameters related to metabolic syndrome, *i.e.* hyperlipidemia, hyperglycaemia, hyperinsulinemia and oxidative stress, were measured. In addition, the mRNA expression of various transcription factors involved in the regulation of lipid metabolism was also studied. It was hypothesized that flax oil-rich diet would lower plasma lipids, hepatic

lipids, oxidative stress and improve the insulin sensitivity of the obese rats as compared to the lard-rich diets.

7.2 METHODS

7.2.1 Animals and diets

All the animals used in this study were treated in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Mukogawa Women's University (Nishinomiya, Japan). Male obese SHR/NDmcr-cp rats (-/-) and lean (+/+) SHR/NDmcr-cp rats, aged 7-weeks, were provided by the Disease Model Cooperative Research Association (Kyoto, Japan). Rats were kept on rodent chow for a period of 1 week prior to feeding the experimental diets. After this acclimatization period, both obese as well as lean SHR/NDmcr-cp rats were divided into two groups: one group was fed a high-fat diet enriched with flax oil whereas the other group was fed a high-fat diet enriched with lard. The groups were designated according to their genetic composition and diet as: *flax oil-fed obese rats (FO); flax oil-fed lean rats (FL); lard-fed obese rats (LO) and lard-fed lean rats (LL).* All the groups were continued on the specified diets for 4-weeks. Fat free semi-synthetic sterilized (10KGy) diet containing (per kg) casein 20 g, DL-methionine 0.3 g, sucrose 30.5 g, corn starch 19 g, fiber 5 g, vitamin mix 1.1 g and mineral mix 4 g was obtained from Funabashi Farms Co. Ltd. (Chiba, Japan). Lard and soybean oil were obtained from a local supermarket (Japan), whereas lignan-free flax oil was a gift from the Flaxseed Association of Japan (Tokyo). To prepare the experimental diets, 5% soybean oil and 10% of either flax oil or lard were

added to the fat-free semi-synthetic diet and stored at -80°C after flushing with N_2 gas. Animals were given fresh diets every day. Fatty acid composition of the high-fat diets was determined using GLC (Keough and Davis, 1979) and is given in Table 7.1.

The animals were housed in a single room with a 12-hour light/ 12-hour dark period cycle. The temperature and humidity were maintained at 21°C and 35 ± 5%, respectively. Body weights and food consumption of the animals were recorded weekly. At 12-weeks of age, the animals were kept in metabolic cages for 24-hours, a day prior to sacrificing, and their food intake, water intake and urinary excretion were recorded. Rats were fasted for 12-hour overnight, and then sacrificed by anaesthetizing with ether vapor in a closed chamber the next morning. Blood and tissues were collected, weighed and then snap frozen at –80°C until further analyses.

7.2.2 Serum glucose and FFA analysis

Blood was collected and centrifuged at 3000 g for 15 minutes to separate the serum. Fasting serum glucose concentration was measured using a commercially available kit, Glucose-C2 # 439-90901 (Wako, Osaka, Japan). Fasting serum FFA concentrations were measured using kit # 279-75401 (Wako, Osaka, Japan). Fasting serum insulin concentration was measured using kit # AK RIN-010T (Shibayagi Co. Ltd, Gunma, Japan).

Table. 7.1 Fatty acid composition of the experimental diets*

Fatty acids	Flax oil-rich diet	Lard-rich diet
14: 0 (MA)	0.18	1.04
16: 0 (PA)	9.3	17.31
16: 1 (PTA)	0.25	1.49
18 :0 (SA)	3.25	8.07
18:1 *n-9* (OA)	16.6	27.22
18:2 *n-6* (LA)	33.25	36.43
18:3 *n-3* (ALA)	33.69	5.23
20:5 *n-3* (EPA)	0.32	0.38
22:6 *n-3* (DHA)	0.48	0.53
Σ SFA	12.73	26.42
Σ MUFA	16.85	28.71
Σ PUFA	67.74	42.57

*Given as percentage of total extracted fatty acids.

MA, myristic acid; PA, palmitic acid; SA, stearic acid; SFA, saturated fatty acids; PTA, palmitoleic acid; OA, oleic acid; MUFA, monounsaturated fatty acids; LA, linoleic acid; PUFA, polyunsaturated fatty acids; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

7.2.3 Serum and hepatic lipid analysis

The cholesterol and TG content of fasting serum and its various lipoprotein fractions were determined using HPLC by Liposearch, Skylight Biotech Inc., Tokyo, Japan (Usui *et al.*, 2002). Liver lipids were extracted as described previously (Folch *et al.*, 1957) and were analyzed using enzymatic kit methods for cholesterol (kit # 439-17501, Wako Chemicals, Japan) and TG (kit # 432-40201, Wako Chemicals, Japan).

7.2.4 Quantitative-PCR analysis

Total RNA was isolated from the liver samples using RNeasy Mini kit #74104 (Qiagen, Japan) and first-strand cDNA was synthesized using SuperScript-III Reverse Transcriptase #18080-044 (Invitrogen, Japan). Synthesized cDNA was mixed with Power SYBR Green PCR Master Mix #4367659 (Applied Biosystems, Japan) and gene-specific sense (S) and antisense primers (AS) (Table 7.2). Samples were subjected to real-time PCR quantification using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Japan). GAPDH was used as a housekeeping gene, and no differences were found in the expression of GAPDH among various groups. Briefly, standard curves were generated using serial dilution of a control sample for PPAR-α, PPAR-γ, SREBP-1c and GAPDH, which were used to calculate the PCR efficiency for each reaction. The expression of each gene per sample was then calculated in relation to the expression of GAPDH, thus, normalizing and correcting the data for the differences in PCR efficiencies for each set of primers. All reactions were performed in duplicate.

Table 7.2 Sequence of the primers used for the quantitative PCR analysis

Gene	Primers
PPAR- (S)	TCACACAATGCAATCCGTTT
PPAR- (AS)	GGCCTTGACCTTGTTCATGT
PPAR- (S)	CTTGGCCATATTTATAGCTGTCATTATT
PPAR- (AS)	AGCAGGTTGTCTTGGATGTCCT
SREBP-1c (S)	TGGACTACTAGTGTTGGCCTGCTT
SREBP-1c (AS)	ATCCAGGTCAGCTTGTTTGCGATG
GAPDH (S)	GGCATTGCTCTCAATGACAA
GAPDH (AS)	ATGTAGGCCATGAGGTCCAC

PPAR= peroxisome proliferators-activated receptor, SREBP= sterol-regulatory element binding protein, GAPDH= glyceraldehyde 3-phosphate dehydrogenase

7.2.5 Oxidative stress analysis

Urinary thiobarbituric acid reactive substances (TBARS) concentrations were used as a marker of oxidative stress (Yagi, 1998). TBARS levels, expressed as malondialdehyde (MDA) levels, were determined in the urine samples collected for 24-hours in the metabolic cages, using commercially available kit # 10009055 (Cayman Chemicals, MI, USA).

7.2.6 Statistical analysis

Data are expressed as means ± SD for $n = 5$, in each group. Two-way ANOVA was used to identify the main effects of genotype, diet and their interaction (genotype X diet). Effects of a significant interaction were further analyzed using Fisher's LSD *post hoc* tests (*SYSTAT for Windows, version 12.02*; *SYSTAT Software Inc.*, Richmond, California). Differences having $P < 0.05$ were considered significant. All assay measurements were made in duplicates.

7.3 RESULTS

7.3.1 Effects of high-fat diet rich in flax oil *vs.* lard on body weight, organ weights, food and caloric intake in obese and lean SHR/NDmcr-cp rats

After 4-weeks of dietary treatment, both flax-fed and lard-fed obese SHR/NDmcr-cp rats weighed significantly heavier than their lean counterparts indicating a main effect for

genotype ($P < 0.001$, two-way ANOVA) (Table 7.3). Similarly, obese rats consumed higher food and calories compared to the lean rats, irrespective of their diet ($P = 0.003$) (Table 7.3). An obese genotype was also associated with significantly higher liver weight, mesenteric-, epididymal- and renal fat-pad weights in both flax-fed and lard-fed obese SHR/NDmcr-cp rats compared to their lean counterparts ($P < 0.001$) (Table 7.3).

Both flax oil-fed and lard-fed obese rats had higher serum glucose and FFA compared to their lean counterparts, irrespective of their diet ($P < 0.001$) (Table 7.4). However, a significant interaction of genotype and diet was observed for the serum insulin concentrations ($P < 0.001$) (Table 7.4). Multiple comparisons further revealed that both flax oil-fed and lard-fed obese rats had higher serum insulin concentrations compared to their lean counterparts (FO *vs.* FL, $P < 0.001$; LO *vs.* LL, $P < 0.001$) (Table 7.4). However, in addition, flax oil feeding was associated with lower serum insulin concentration in obese rats compared to the lard fed obese rats (FO *vs.* LO, $P < 0.001$) (Table 7.4).

Table 7.3 Body weight, organ weights, food and caloric intake in obese and lean SHR/NDmcr-cp rats fed high-fat diets rich in flax-oil *vs.* lard.

	FO	LO	FL	LL	*Gen*	*Diet*	*Int*
Body weight (g)	383 ± 31.5	386.3 ± 35.8	312.8 ± 35.9	295.2 ± 22.5	$P < 0.001$	NS	NS
Food intake (g/day)	21.9 ± 1.4	24.2 ± 1.8	19.8 ± 1.8	20.4 ± 2.3	$P = 0.003$	NS	NS
Caloric Intake (kj/day)	459.1 ± 30.1	508.3 ± 37.6	416.7 ± 37.2	428.5 ± 48.4	$P = 0.003$	NS	NS
Tissue weight (%)*							
Liver	4.4 ± 0.7	4.8 ± 0.4	3.0 ± 0.2	3.1 ±0.4	$P < 0.001$	NS	NS
Mesenteric fat	2.2 + 0.5	2.1 ± 0.3	0.9 ± 0.1	0.7 ± 0.1	$P < 0.001$	NS	NS
Epidydymal fat	2.3 ± 0.1	2.4 ± 0.1	1.2 ± 0.1	1.5 ± 0.1	$P < 0.001$	NS	NS
Renal fat	2.9 ± 0.2	3.3 ± 0.2	1.6 ± 0.4	1.7 ± 0.2	$P < 0.001$	NS	NS

Values are expressed as means ± SD, $n = 5$. Main effects of genotype, diet and their interaction (genotype X diet) were identified using two-way ANOVA, where $P < 0.05$ was considered significant. FF, Flax oil-fed obese rats; FL, flax oil-fed lean rats; LO, lard-fed obese rats; LL, lard-fed lean rats; Gen, genotype; Int, Interaction between genotype and diet.

*Tissue weight/body weight x 100

Table 7.4 Fasting serum glucose, FFA and insulin concentrations in obese and lean SHR/NDmcr-cp rats fed high-fat diets rich in flax oil *vs.* lard.

	FO	LO	FL	LL	*Gen*	*Diet*	*Int*
Glucose (mM)	13.58 ± 1.65	12.08 ± 2.46	8.68 ± 1.14	8.19 ± 0.79	$P < 0.001$	NS	NS
FFA (mEq/L)	1.5 ± 0.3	1.4 ± 0.2	0.7 ± 0.1	0.8 ± 0.1	$P < 0.001$	NS	NS
Insulin (mM)	16.4 ± 3.4^{b}	43.8 ± 7.9^{a}	3.6 ± 1.3^{c}	1.7 ± 0.7^{c}	$P < 0.001$	$P < 0.001$	$P < 0.001$

Values are expressed as means ± SD, $n = 5$. Main effects of genotype, diet and their interaction (genotype X diet) were identified using two-way ANOVA. Significant interaction was further analyzed using Fisher's LSD *post hoc* tests, where superscripts represent significant differences of $P < 0.05$. FF, Flax oil-fed obese rats; FL, flax oil-fed lean rats; LO, lard-fed obese rats; LL, lard-fed lean rats; FFA, free fatty acids Gen, genotype; Int, Interaction between genotype and diet.

7.3.2 Effects of high-fat diets rich in flax oil *vs.* lard on TG and cholesterol concentrations in serum and its lipoprotein fractions of obese and lean SHR/NDmcr-cp rats

An obese genotype was associated with significantly higher TG and cholesterol concentrations in the serum, VLDL, LDL and HDL fractions of the flax oil-fed and lard-fed obese rats compared to their lean counterparts, irrespective of their diet ($P < 0.001$) (Figs. 7.1 and 7.2A-D). In addition, flax oil feeding was associated with lower LDL-cholesterol concentration in the lean rats compared to the lard-fed lean rats ($P = 0.006$) (Fig.7.2C).

7.3.3 Effects of high-fat diets rich in flax oil *vs.* lard on hepatic TG and cholesterol concentrations in obese and lean SHR/NDmcr-cp rats

Two-way ANOVA revealed a main effect for both genotype and diet on liver TG and cholesterol concentrations (Fig. 7.3). While an obese genotype was associated with higher liver TG ($P = 0.003$) and cholesterol concentrations ($P = 0.025$) in the lard-fed obese rats compared to the lard-fed lean rats, flax oil feeding was associated with significantly lower concentrations of both liver TG ($P = 0.012$) and cholesterol ($P = 0.001$) in the obese rats compared to the lard-fed obese rats (Fig. 7.3 A&B). The reduction in the liver TG and cholesterol concentration by flax oil feeding was significant

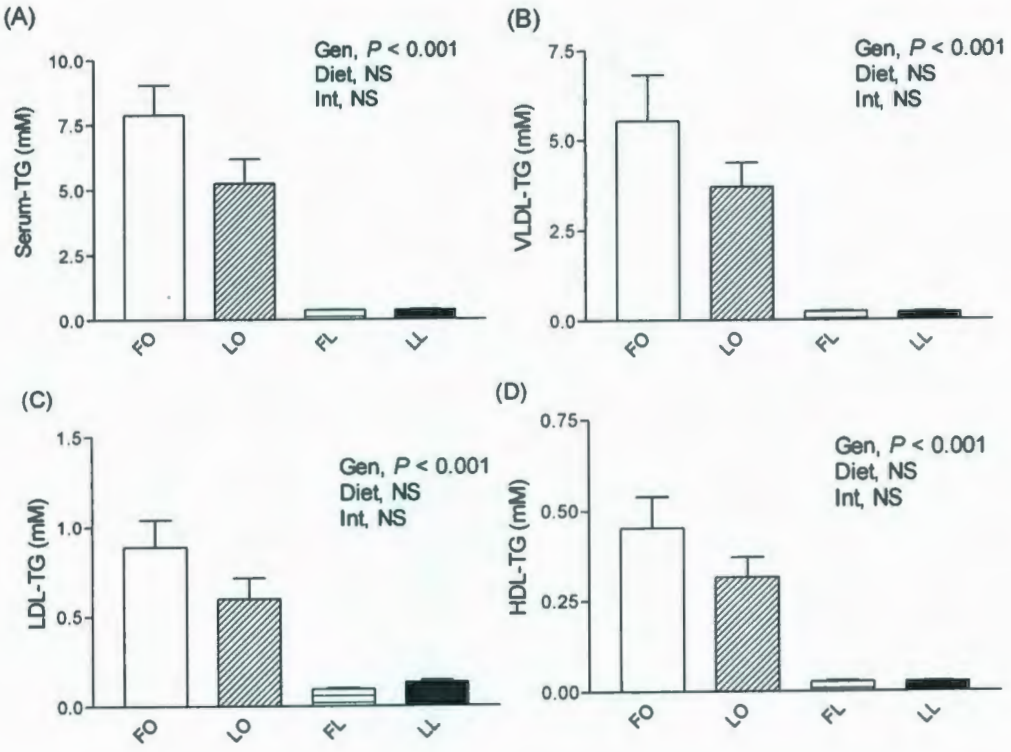

Fig 7.1 TG concentrations in (*A*) whole serum (*B*), VLDL (*C*), LDL and (*D*) HDL fractions of obese and lean SHR/NDmcr-cp rats fed high-fat diets rich in flax oil *vs.* lard diet for 4-weeks.

Values are expressed as means ± SD, $n = 5$. Main effects of genotype, diet and their interaction (genotype X diet) were identified using two-way ANOVA, where $P < 0.05$ was considered significant. FF, Flax oil-fed obese rats; FL, flax oil-fed lean rats; LO, lard-fed obese rats; LL, lard-fed lean rats, TG, triglycerides, Gen, genotype; Int, Interaction between genotype and diet.

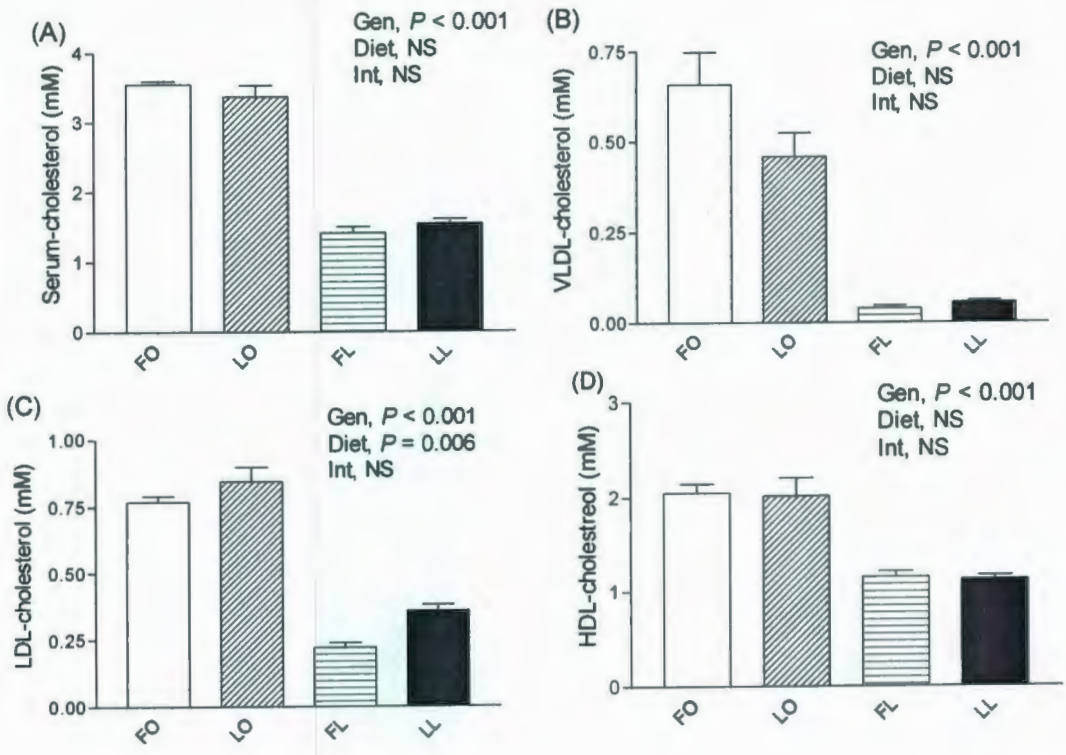

Fig. 7.2 Cholesterol concentrations in (*A*) whole serum (*B*), VLDL (*C*), LDL and (*D*) HDL fractions of obese and lean SHR/NDmcr-cp rats fed high-fat diets rich in flax oil *vs.* lard diet for 4-weeks.

Values are expressed as means ± SD, $n = 5$. Main effects of genotype, diet and their interaction (genotype X diet) were identified using two-way ANOVA, where $P < 0.05$ was considered significant. FF, Flax oil-fed obese rats; FL, flax oil-fed lean rats; LO, lard-fed obese rats; LL, lard-fed lean rats; Gen, genotype; Int, Interaction between genotype and diet.

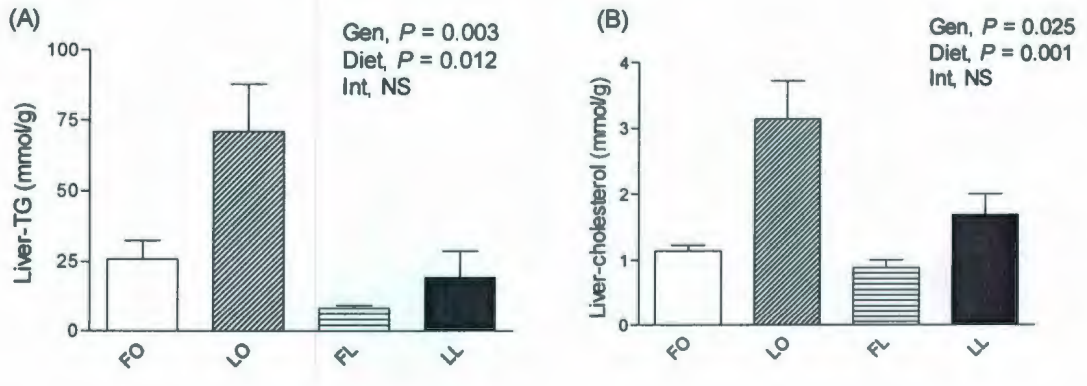

Fig. 7.3 Hepatic (*A*) TG and (*B*) cholesterol concentrations in obese and lean SHR/NDmcr-cp rats fed high-fat diets rich in flax oil *vs.* lard diet for 4-weeks.

Values are expressed as means ± SD, $n = 5$. Main effects of genotype, diet and their interaction (genotype X diet) were identified using two-way ANOVA, where $P < 0.05$ was considered significant. FF, Flax oil-fed obese rats; FL, flax oil-fed lean rats; LO, lard-fed obese rats; LL, lard-fed lean rats, TG, triglycerides, Gen, genotype; Int, Interaction between genotype and diet.

enough (67% reduction) such that no differences were observed between the flax oil-fed obese and lean SHR/NDmcr-cp rats.

7.3.4 Effects of high-fat diet rich in flax oil *vs.* lard on hepatic gene expression in obese and lean SHR/NDmcr-cp rats

A significant interaction of the genotype and diet affected the hepatic mRNA expression of PPAR-α ($P = 0.018$) (Fig. 7.4A). Multiple comparisons further revealed that an obese genotype was associated with significantly higher mRNA expression of hepatic PPAR-α in the flax oil-fed and lard-fed obese rats compared to their lean counterparts (FO *vs.* FL, $P < 0.05$; LO *vs.* LL, $P < 0.001$) (Fig. 7.4A). In addition, flax oil-fed lean rats had significantly higher hepatic expression of PPAR-α compared to the lard-fed lean rats ($P = 0.007$) (Fig. 7.4A).

Similar to the hepatic PPAR-α, an obese genotype was associated with significantly higher mRNA expression of PPAR-γ in the flax oil-fed and lard-fed rats compared to their lean counterparts ($P < 0.001$) (Fig. 7.4B). In addition, a flax oil-rich diet was associated with significantly higher hepatic mRNA expression of PPAR-γ compared to a lard-rich diet in both obese and lean rats ($P < 0.001$) (Fig. 7.4B). No differences were observed for the hepatic mRNA expression of SREBP-1c among various dietary groups of obese and lean SHR/NDmcr-cp rats (Fig. 7. 4C).

Since flax oil feeding was specifically associated with an increase in PPAR-γ mRNA expression in both obese and lean SHR/NDmcr-cp rats, its correlation analysis with the hepatic TG and cholesterol concentrations in each dietary group was performed. Interestingly, the hepatic PPAR-γ mRNA expression correlated negatively with the hepatic TG concentration ($r = -0.98$, $P < 0.05$) in flax oil-fed obese SHR/NDmcr-cp rats (Fig. 7.5A). On the other hand, the hepatic PPAR-γ mRNA expression correlated negatively with the hepatic cholesterol concentration in both flax oil-fed obese ($r = -0.99$, $P < 0.001$) and flax oil-fed lean rats ($r = -0.97$, $P < 0.05$) (Fig. 7.5B).

7.3.5 Effects of high-fat diets rich in flax oil *vs.* lard on urinary TBARS levels in obese and lean SHR/NDmcr-cp rats

A significant interaction of the genotype and diet affected the urinary TBARS concentration ($P = 0.016$) (Fig. 7.6). Multiple comparisons further revealed that while lard-fed obese rats had higher urinary TBARS concentration than the lard-fed lean rats ($P = 0.001$), flax-oil-fed obese SHR/NDmcr-cp rats had significantly lower urinary TBARS concentration than the lard-fed obese rats ($P = 0.001$) (Fig. 7.6). No differences were observed between the flax oil-fed obese and lean SHR/NDmcr- cp rats for their TBARS concentrations (Fig. 7.6).

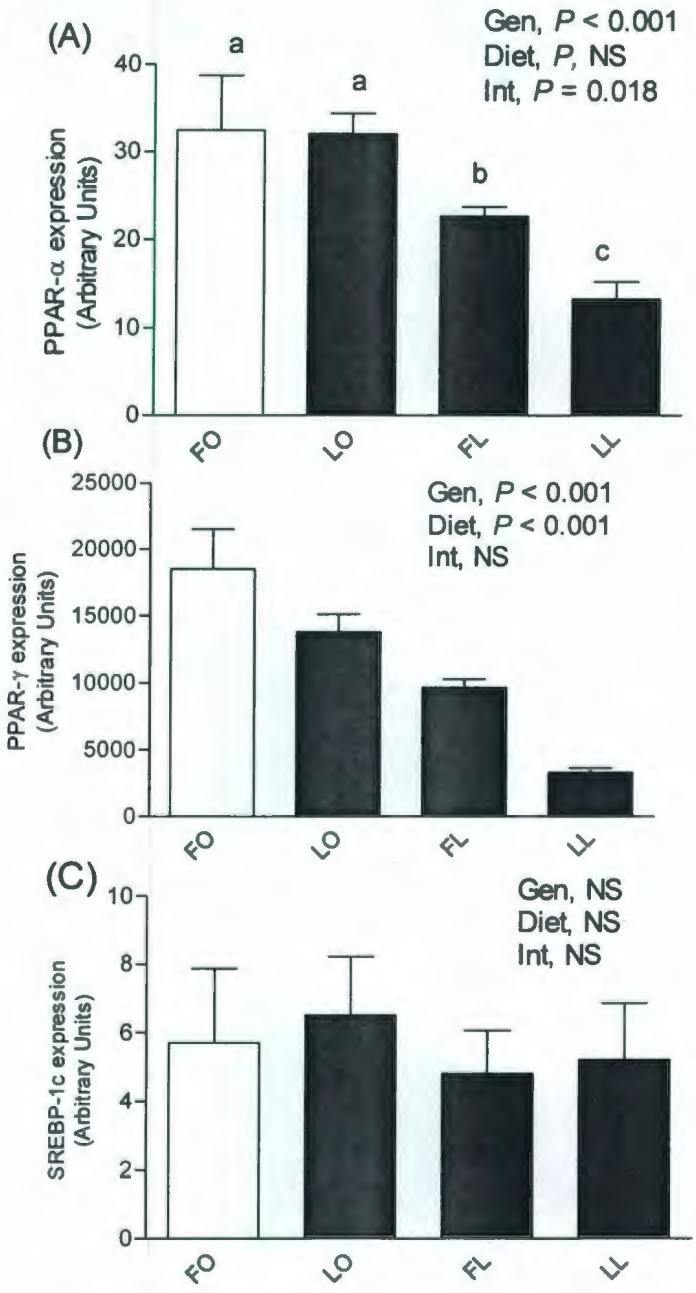

Fig. 7.4 Hepatic mRNA expression of (*A*) PPAR-α (*B*) PPAR-γ and (*C*) SREBP-1c in obese and lean SHR/NDmcr-cp rats fed high-fat diets rich in flax oil *vs.* lard diet for 4-weeks.

Values are expressed as means ± SD, $n = 5$. Main effects of genotype, diet and their interaction (genotype X diet) were identified using two-way ANOVA. Significant interaction was further analyzed using Fisher's LSD *post hoc* tests, where superscripts represent significant differences of $P < 0.05$. FF, Flax oil-fed obese rats; FL, flax oil-fed lean rats; LO, lard-fed obese rats; LL, lard-fed lean rats, Gen, genotype; Int, Interaction between genotype and diet.

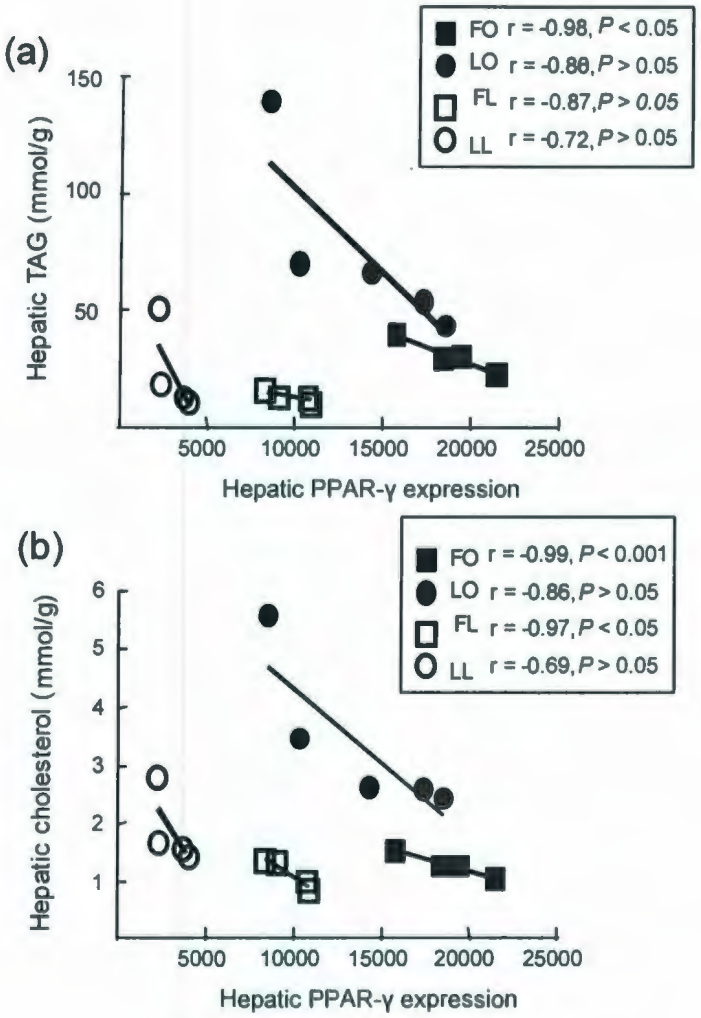

Fig. 7.5 Correlation analysis of hepatic PPAR-γ mRNA expression with (*A*) hepatic TG and (*B*) cholesterol concentration in obese and lean SHR/NDmcr-cp rats fed high-fat diets rich in flax oil *vs.* lard diet for 4-weeks.

FF, Flax oil-fed obese rats; FL, flax oil-fed lean rats; LO, lard-fed obese rats; LL, lard-fed lean rats.

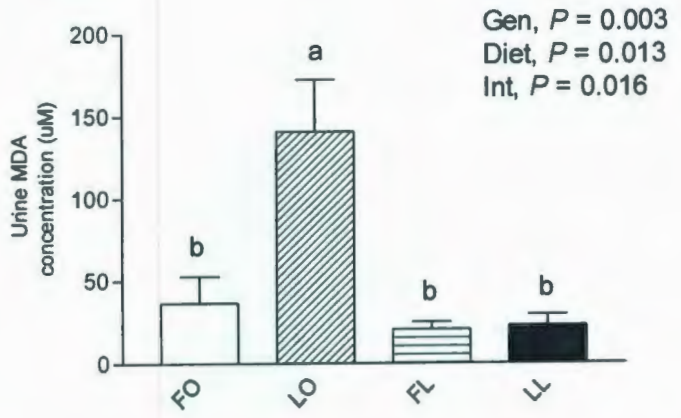

Fig. 7.6 Urinary TBARS concentration, measured as MDA, in obese and lean SHR/NDmcr-cp rats fed high-fat diets rich in flax oil *vs.* lard diet for 4-weeks. Values are expressed as means ± SD, $n = 5$. Main effects of genotype, diet and their interaction (genotype X diet) were identified using two-way ANOVA Significant interaction was further analyzed using Fisher's LSD *post hoc* tests, where superscripts represent significant differences of $P < 0.05$. FF, Flax oil-fed obese rats; FL, flax oil-fed lean rats; LO, lard-fed obese rats; LL, lard-fed lean rats, TG, triglycerides, Gen, genotype; Int, Interaction between genotype and diet.

7.4. DISCUSSION

The obese SHR/NDmcr-cp rats represent a genetic model of metabolic syndrome exhibiting obesity, insulin resistance, hepatic steatosis and enhanced oxidative stress. Recent studies have shown that the consumption of flax oil, rich in ALA, an *n-3* PUFA, has beneficial effects on insulin resistance, dyslipidemia, hypertension and fatty liver disease (Chan et al., 1991; Cunnane et al., 1993; Ghafoorunissa et al., 2005; Murase et al., 2005). The current study was therefore, designed to investigate the effects of flax oil feeding on various parameters related to the metabolic syndrome such as obesity, hypelipidemia, insulin resistance and oxidative stress in obese and lean SHR/NDmcr-cp rats. It was demonstrated that flax oil feeding was associated with significantly lower hepatic concentrations of TG and cholesterol, along with lower fasting insulin and 24-hour urinary TBARS levels in obese SHR/NDmcr-cp rats. Flax oil feeding was also associated with significantly higher expression of hepatic PPAR-γ mRNA in the obese SHR/NDmcr-cp rats, which correlated negatively with their hepatic TG and cholesterol levels.

As discussed in previous chapters, liver plays a central role in regulating lipid and cholesterol metabolism in the body. The synthesis, uptake and secretion of various lipids by the liver not only regulates hepatic lipid levels, but also maintains the serum lipid levels. Disturbances in any of these pathways can lead to accumulation of lipids in the liver, which is characterized as NAFLD, a condition very commonly associated with metabolic syndrome (Kotronen *et al.*, 2007). According to the "two-hit hypothesis" explaining the development of NAFLD (Gan *et al.*, 2008), it is proposed that the "first

hit" results from the conditions leading to TG accumulation in the hepatocytes such as central obesity and insulin resistance. Other factors, such as increased *de novo* lipogenesis and increased post-prandial TG delivery, along with reduced mitochondrial as well as peroxisomal oxidation, further enhance the hepatic accumulation of lipids (Chitturi *et al.*, 2002). The "second hit" in the hypothesis involves the emergence and progression of inflammation, which leads to the development of non-alcoholic steatohepatitis (NASH). These mechanisms include the pre-inflammatory and pro-apoptotic effects of oxidative stress and other factors including cytokines and endoplasmic stress (Browning and Horton, 2004; Ozcan *et al.*, 2004).

In the current study, flax oil-fed obese SHR/NDmcr-cp rats exhibited a significant reduction in the hepatic concentration of TG as well as cholesterol, when compared with the lard-fed obese SHR/NDmcr-cp rats, suggesting that flax oil feeding may prove to be an important nutritional tool in the prevention of the "first hit" behind the development of NAFLD. Interestingly, flax oil feeding was also associated with a significant reduction in the urinary TBARS levels in the obese SHR/NDmcr-cp rats, a marker for systemic oxidative stress. Although the oxidative stress in the liver of these rats was not evaluated, the reduction in the overall oxidative stress levels indicate that flax oil feeding could also prevent the "second hit" *i.e.* inflammatory development of NASH.

A number of previous studies offering a preventive role for flax oil supplementation/feeding in the development of NAFLD have attributed its beneficial effects to its much higher content of ALA (Morise *et al.*, 2005; Morise *et al.*, 2006; Vijaimohan *et al.*, 2006). It is reported that ALA-rich-flax oil can act as a better substrate

for mitochondrial and peroxisomal β-oxidation, thus stimulating increased oxidation of lipids in the liver (Ide *et al.*, 2000; Murase *et al.*, 2005). In addition, flax oil is also proposed to suppress fatty acid synthesis, thus inhibiting the accumulation of lipids in the liver (Murase *et al.*, 2005). The regulation of hepatic lipid metabolism is mediated by a variety of transcription factors such as SREBP-1c, PPAR-α and PPAR-γ. The SREBP-1c regulates the expression of genes involved in the synthesis of fatty acids and cholesterol (Osborne, 2000). Flaxseed lignan-, but not flax oil- supplementation, has previously been reported to lower the hepatic expression of SREBP-1c, thus inhibiting fatty acid and cholesterol synthesis by the liver and hence reducing the hepatic lipid levels (Fukumitsu *et al.*, 2008). No differences were, however, observed in the hepatic expression of SREBP-1c levels among flax oil- or lard fed-SHR/NDmcr-cp rats, suggesting that flax oil feeding was not involved with the down-regulation of SREBP-1c expression in the obese SHR/NDmcr-cp rats.

PPAR-α is known to regulate the expression of genes involved in the peroxisomal proliferation and β-oxidation of fatty acids (Braissant *et al.*, 1996; Shalev *et al.*, 1996). An up-regulation of PPAR-α can lead to increased peroxisomal oxidation of fatty acids which can reduce the accumulation of lipids in the liver, a mechanism that has been previously proposed for the lipid-lowering effects of flax oil supplementation (Vijaimohan *et al.*, 2006). A significant increase in the mRNA expression of liver PPAR-α was observed in the flax oil-fed lean SHR/NDmcr-cp rats compared to the lard-fed lean rats, but not in flax oil-fed obese rats. These findings would indicate that PPAR-α

expression was not associated with reduced hepatic lipid levels in the case of obese SHR/NDmcr-cp rats.

Another member of the PPAR family of transcription factors, PPAR-γ, is predominantly expressed in adipose tissue, while having a very low expression in liver (Braissant *et al.*, 1996). Although PPAR-γ is principally expressed in adipose tissue, there is increasing evidence for its up-regulation in the liver, which is associated with obesity and the fatty liver condition (Vidal-Puig *et al.*, 1996; Burant *et al.*, 1997; Edvardsson *et al.*, 1999; Bedoucha *et al.*, 2001; Rahimian *et al.*, 2001). However, it remains to be established whether the increased expression of PPAR-γ in the liver causes fatty liver or whether it is the fatty liver condition that turns on the expression of PPAR-γ as a restorative mechanism. A significant increase in the hepatic mRNA expression of PPAR-γ was observed in flax oil-fed obese SHR/NDmcr-cp rats as compared to lard-fed obese rats. A similar up-regulation was also observed in the flax oil-fed lean SHR/NDmcr-cp rats compared to the lard-fed lean rats. The reduction in the hepatic TG and cholesterol levels observed in obese SHR/NDmcr-cp rats was selectively correlated with an up-regulation of their hepatic PPAR-γ mRNA expression, thus pointing to an association between the hepatic expression of PPAR-γ and reduction in liver lipids, which may prove beneficial in the prevention of NAFLD.

A recent study reported a significant reduction in the hepatic lipids along with a significant up-regulation in the hepatic mRNA expression of PPAR-γ in *ob/ob* mice, when treated with troglitazone (Memon *et al.*, 2000). Interestingly, the hepatic expression

of PPAR-γ in this study was much more pronounced than the hepatic expression of PPAR-α, similar to the observations of the current study. Reduction of liver lipid content in *ob/ob* mice by troglitazone treatment suggests that PPAR-γ activators may increase the utilization of lipids in the liver of obese diabetic mice. In addition, other PPAR-γ activators such as candesartan have also been shown to improve insulin resistance through promoting the expression of PPAR-γ in liver and adipose tissue in Wistar rats (Yan *et al.*, 2008). Furthermore, a recent study by Kelly *et al.,* (2009) reported that flax oil was associated with the prevention of conjugated linoleic acid-induced insulin resistance and fatty liver in C57Bl/6 mice. Thus, it seems plausible that flax oil mediates its beneficial effects by activating hepatic PPAR-γ expression, which perhaps enhances the insulin sensitivity of the liver as well as of the peripheral tissues, similar to the glitazone family of insulin sensitizers. Flax oil feeding was also associated with a significant reduction in plasma insulin concentration in the obese SHR/NDmcr-cp rats compared to lard fed-obese rats, which appears to support this proposal.

In addition, flax oil feeding was associated with lower LDL-cholesterol concentration in lean SHR/NDmcr-cp rats compared to the lard-fed lean rats. However, no effects of flax oil feeding were observed for serum and lipoproteins TG and cholesterol concentrations in the obese rats. These observations are in line with the previous observations where flax oil did not affect the serum and lipoprotein TG and cholesterol concentrations in humans (Schwab *et al.*, 2006 and Kaul *et al.*, 2008) and hypercholesterolemic rabbits (Lee and Prasad, 2003). On the other hand, flaxseed, as opposed to flax oil, supplementation was shown to lower serum TG and cholesterol

concentrations in humans (Lucas *et al.,* 2008). Flaxseed contains both omega-3 fatty acids and lignans; flaxseed lignans alone have previously been shown to lower serum lipids in hyperlipidemic rats (Felmlee *et al.,* 2009). Thus, the presence of lignans could have contributed to the lipid lowering properties of flaxseed supplementation.

In conclusion, the current study reported that flax oil feeding was associated with a reduction in hepatic lipid accumulation in obese SHR/NDmcr-cp rats, which represent a genetic model of metabolic syndrome. An increase in hepatic PPAR-γ mRNA expression by flax oil feeding showed a negative correlation with hepatic lipid levels in the obese SHR/NDmcr-cp rats. Furthermore, flax seed oil feeding also lowered serum insulin levels and systemic oxidative stress in obese SHR/NDmcr-cp rats. Thus, it may be proposed that flax oil mediated activation of PPAR-γ and its insulin sensitizing effects resulted in the reduction of hepatic lipid accumulation in the obese rats. Future studies would be required to prove this proposal.

CHAPTER EIGHT

Summary and Conclusions

8.1 SUMMARY

Current statistics report that CVD and related diseases are the number one cause of mortality and morbidity across the globe (Reddy and Yusuf, 1998). All sections of the society, including people of all ages (*i.e.* children, young and old people), both sexes (*i.e.* males and females) and various socioeconomic status (*i.e.* developing and developed world) are being affected by CVD (Murray and Lopez, 1996). Among various risk factors associated with the development of CVD, diet and nutrition have long been identified as the key players. It is apparent at the global level that 'nutrition transition' towards the higher-fat Western style diet has paralleled the rise in the incidence of CVD (Popkin and Gordon-Larsen, 2004). In addition to the quantity of fat, the quality of fat is also known to affect the development of CVD. Whilst an increased consumption of SFA has largely been associated with higher incidence of CVD (Keys, 1997), a diet rich in PUFA has been considered to be beneficial (Dolecek, 1992). Among the PUFA, the *n-3* PUFA, in comparison to *n-6* PUFA, are well known to possess beneficial effects on the outcome of CVD (Demaison and Moreau, 2002). While it is apparent that nutrition during adult life is important, recent evidence based on the concept of developmental programming suggests that *in utero* nutrition can also be critical in determining the cardiovascular health of an individual in later life (Armitage *et al.*, 2004). As a result, there has been tremendous interest in understanding the role of maternal dietary fat intake in the developmental origins of CVD.

The objective of the current dissertation was to examine the role of quantity and quality of dietary fat intake in the developmental origins of CVD, and to further investigate the underlying mechanisms. Considering that dietary fat intake can directly affect lipid metabolism, it was proposed that maternal dietary fat intake induced 'programming' of offspring lipid metabolism would affect their risk of developing CVD in adult life. Moreover, an altered lipid metabolism was expected to affect offspring vascular function, which was also studied to assess their risk of developing CVD.

8.1.1 Key Observations

1. A "lard-rich" diet fed to the mothers during gestation and lactation was associated with higher plasma LDL-cholesterol, lower hepatic mRNA expression of LDL-r and lower aortic contractile reactivity to various agonists, in their adult female offspring. Based on these observations, it was proposed that maternal diet-induced a reduction in offspring LDL-r and led to an increase in their plasma LDL-cholesterol, which in turn may induce insulin resistance, oxidative stress and alterations in the NOS pathways. This may work in concert with the deleterious effects of higher LDL-cholesterol, to increase their risk of developing CVD. Thus, maternal diet-induced reduction in offspring LDL-r expression may serve as a plausible mechanism of the dietary fat-induced programming of CVD (Chapter-3).

(Chechi K, McGuire JJ and Cheema SK. *American Journal of Physiology, Regulatory, Integrative and Comparative Physiology* 296: R1029-40, 2009)

2. Gender-associated differences were observed in the programming effects of a "lard-rich" diet fed to the mothers during gestation and lactation, where alterations in the regulation of metabolic pathways were more obvious in females than males. These observations point towards the hormonal regulation of maternal diet-induced programming effects, which can be explored in the future studies (Chapter-3).

   (Chechi K, McGuire JJ and Cheema SK. *American Journal of Physiology, Regulatory, Integrative and Comparative Physiology* 296: R1029-40, 2009)

3. A safflower oil-rich diet fed to the mothers during gestation and lactation was associated with higher plasma HDL-cholesterol and higher hepatic mRNA expression of LCAT in the female offspring. It was proposed that the safflower oil-rich maternal diet-induced increase in the offspring LCAT led to an increase in their plasma HDL-cholesterol levels, which would be expected to lower their risk of developing CVD. However, these offspring also exhibited reduced aortic contractile reactivity to various agonists. Considering that reduced aortic contractility may affect offspring cardiovascular homeostasis, it might also affect their risk of developing CVD in later life (Chapter-4).

(Chechi K, McGuire JJ and Cheema SK. Ready to be submitted in *British Journal of Nutrition*)

4. A continuous exposure to high-fat diets rich in lard and safflower oil during pre- and post-weaning time periods was associated with higher body weight and higher LDL/HDL-cholesterol ratio, which was not seen in case of an exposure to either lard or safflower oil -rich diets during pre-weaning or post-weaning time period alone. These findings highlighted that the effects of post-weaning nutrition could be influenced by the pre-weaning diets of the offspring and the interaction of pre- and post-weaning diets played an important role in affecting the metabolic outcome in the offspring (Chapters- 3 and 4).

5. A comparison between the continuous exposure to high-fat diets during pre- and post-weaning time periods revealed higher plasma LDL-cholesterol and lower aortic contractile reactivity to KCl and PE, in the offspring exposed to a lard-rich diet as compared to the safflower oil-rich diet. It was therefore concluded that continuous exposure to lard-rich diets may prove to be more deleterious than diets rich in safflower oil for the offspring cardiovascular health (Chapter-5).

   (Chechi K and Cheema SK. *Experimental and Clinical Cardiology* 11:129-135, 2006).

6. A safflower oil-rich diet fed pre-weaning was associated with enrichment of offspring liver tissue with *n-3* PUFA, especially DHA. In contrast, a safflower oil-rich diet fed pre-weaning was associated with lower DHA in the offspring heart. In addition, a continuous exposure to lard-rich diet during pre- and post-weaning time periods was associated with even lower DHA levels in the offspring heart compared to a safflower oil-rich diet. These observations indicated that fatty acid composition of the maternal diet could have long-lasting effects on the offspring liver and heart fatty acid composition. Moreover, the interaction between the pre- and post-weaning diets affected the offspring tissue fatty acid composition (Chapter-6).

   (Chechi K, Herzberg G and Cheema SK. *Prostaglandins, Leukotrienes and Essential fatty acids, under review*)

7. The last study reported the beneficial effects of flax oil feeding on the parameters associated with metabolic syndrome in the adult obese SHR/NDmcr-cp rats, for a period of 4-weeks. Feeding a diet rich in flax oil was associated with lower hepatic TG and cholesterol concentrations in the obese SHR/NDmcr-cp rats, as compared to a diet rich in lard. In addition, flax oil feeding was associated with lower plasma insulin and urinary TBARS concentrations along with higher hepatic mRNA expression of PPAR-γ in these rats. Based on these observations, it can be proposed that an activation of hepatic PPAR-γ may increase insulin sensitivity leading to increased utilization of the hepatic lipids in the flax oil-fed obese rats.

(Chechi K, Yasui N, Ikeda K, Yamori Y and Cheema SK. *British Journal of Nutrition, under review*).

In conclusion, results of the current dissertation support the proposed hypothesis that maternal dietary fat intake during gestation and lactation can have significant effects on offspring lipid metabolism and aortic vascular function in adult life. Maternal semi-synthetic high-fat diets, irrespective of the type of fat, were found to be associated with reduced aortic contractile reactivity when compared to chow. However, in a high-fat environment, lard-rich diets were associated with higher plasma LDL-cholesterol and reduced aortic contractility compared to the diets rich in safflower oil. Although it remains to be determined whether reduced aortic contractility would increase the risk of developing CVD in the offspring, these observations point to the disturbances in the offspring cardiovascular homeostasis, due to the high-fat maternal diets. In addition, the current thesis demonstrated the effects of interaction between the pre- and post-weaning high-fat diets on the parameters associated with offspring cardiovascular health, underscoring the importance of healthy nutrition at each stage of life.

In addition, our observations of the beneficial aspect of flax oil, a source of *n-3* PUFA, may help in its promotion as an important nutritional tool for the management of metabolic syndrome. Furthermore, it would be highly relevant to investigate the effects of *n-3* PUFA-rich diets such as flax oil in the developmental origins of adult CVD

8.2 IMPLICATIONS AND FUTURE DIRECTIONS

Besides the fat content, other constituents of the semi-synthetic diets may have contributed to the observations of the current thesis. However, comparing the observations of Chapters- 3 and 4, it can be stated that fatty acid composition of the maternal high-fat diets clearly affected offspring lipid metabolism. While a maternal lard-rich diet was associated with higher plasma LDL-cholesterol, a maternal safflower oil-rich diet was associated with higher plasma HDL-cholesterol in the offspring. These observations were further corroborated by the observations of Chapter-5, where a continuous exposure to lard-rich diets was associated with higher LDL-cholesterol compared to the safflower oil-rich diets. Thus, it can be concluded that the type of fat in the maternal diet plays an important role in programming the offspring lipid metabolism, which may affect their CVD risk in adult life. However, future studies with better controls should be conducted where high-fat semi-synthetic diets are compared with the low-fat semi-synthetic diets, in order to isolate the programming effects of the amount of fat in the maternal diet. Moreover, diets can be specifically formulated to be enriched with SFA, MUFA, *n-3* PUFA and *n-6* PUFA, such that the specific programming effects of each class of dietary fats can be evaluated. The observations of the current dissertation have further established C57BL/6 mice as yet another animal model for such studies. Since a number of knockout models are available on the C57Bl/6 mice background, these could be utilized in the future to explore the role of lipid metabolism in the developmental programming of adult CVD.

For the current dissertation, the offspring were studied at an age of 11-weeks, which is a relatively young age for mice. Feeding high-fat diets for 6-months has been shown to induce obesity, hyperglycemia and hyperinsulinemia in the C57Bl/6 mice (Surwit *et al.,* 1988). Thus, longer-term studies can be planned to evaluate the programming effects of dietary fats on the development of end points such as atherosclerotic plaque, hypertension or diabetes in the offspring. Longer-term studies can also be utilized to study the reversal of programming effects of maternal high-fat diets. On the other hand, studying the offspring during fetal development may provide further insights into the "programming" mechanisms.

We used 8-week-old mice mothers that were subjected to a high-fat diet for two weeks prior to breeding. However from the context of the human population, two-weeks is a relatively short time span for exposure to a high-fat diet. Thus, future studies can be designed where mothers are subjected to high-fat diets for a longer period of time before breeding. In addition, it will be important to investigate whether mothers become obese on high-fat diets, which will further influence the outcome of CVD in the offspring. Moreover, we subjected the mothers to high-fat diets both during gestation and lactation. In context of the human population, a number of babies are fed formula diets after birth, which creates a different postnatal nutritional environment for them. Thus, future studies can be planned such that the programming effects of gestation and lactation can be segregated by employing a formula fed pup model.

We observed altered hepatic gene expression (LDL-r and LCAT) in the offspring of mothers subjected to the high-fat semi-synthetic diets during gestation and lactation.

Epigenetic changes are proposed to play an important role in the programming of adult disease, thus hepatic DNA methylation patterns (global or gene- specific) should also be studied in these offspring to ascertain the role of epigenetics in our model.

Besides the programming effects of high-fat diets, the current thesis highlighted that high-fat semi-synthetic diets were associated with poor reproductive performance in the C57Bl/6 mice. The rate of conception, survival of pups and the sex ratio of the offspring were affected by the high-fat diets. Although there is some evidence linking high-fat environment to reproductive dysfunction, this relationship is poorly understood (discussed in section 2.1.2.1). Thus, future studies can be planned to investigate the association between dietary fat intake and reproductive dysfunction.

It is well established that the reduced vascular relaxation responses led to the future development of CVD; however this relationship is less studied with respect to vascular contractile responses. We observed that feeding high-fat semi-synthetic diets was associated with reduced aortic contractility in the C57Bl/6 mice, irrespective of the time of exposure of diets being pre- or post-weaning. Considering that both contractility and relaxation properties of blood vessels are important for maintaining the cardiovascular homeostasis, these observations should be extended further to understand the relationship between the high-fat diets and vascular contractile responses.

Overall, the findings from the current thesis support the role of dietary fats in the fetal origins of CVD. We have developed a mouse model that is relevant to the understanding of the fetal programming in Western populations. Future studies could utilize the knockouts based on our model to explore the molecular mechanisms

underlying the developmental origins of CVD. A better understanding of the concept of fetal programming can prove invaluable for the development of preventive strategies, which can help in reducing the burden of CVD.

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APPENDIX I

Table 1. Effects of pre- and post-weaning lard-rich diets on the aortic fatty acid composition of various male and female offspring groups (Chapter-3).

	SFA/SFA	SFA/Chow	Chow/Chow	Chow/SFA
Male Offspring				
C14:0	2.6	2.2	2.4	2.2
C16:0	40.1	31.2	24.1	33
C18:0	30.6	21.3	16.8	20.3
C16:1	0.9	1.2	1.5	2
C 18:1	10.9	17.4	14.8	26.2
C18:2	2.1	9	6.3	6.4
C18:3	0.6	1.3	1.9	0.5
C 20:4	1.1	5.4	5.8	4.2
C20:5	ND	1.7	2.7	ND
C22:6	ND	1.9	1.6	1.4
Σ SFA	73.3	54.6	43.3	55.5
Σ MUFA	11.8	18.6	16.3	28.2
Σ PUFA	3.76	19.24	18.3	12.6
Female Offspring				
C14:0	1.7	2.4	1.8	2.2
C16:0	26.9	24.1	20.4	31.6
C18:0	19.6	16.8	16.3	22.8
C16:1	3.2	1.5	0.4	1.6
C 18:1	9.7	14.8	4.7	24.6
C18:2	1.8	6.3	1.5	7
C18:3	4.7	2	1.2	0.5
C 20:4	11.8	4.3	2.7	4.2
C20:5	9.2	2.7	1.9	ND
C22:6	0.6	1.6	0.9	1.6
Σ SFA	48.2	43.3	38.5	56.5
Σ MUFA	12.9	16.3	5.1	26.2
Σ PUFA	28	16.9	8.2	13.2

Total fatty acid composition was determined after pooling aortic tissues (n = 8-10) (specified in section 3.2.4) for each offspring group, followed by lipid extraction and GLC analysis as described in section 2.5. Fatty acids are expressed as a percentage of the total extracted fatty acids. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA; polyunsaturated fatty acids; S/C

APPENDIX II

This study was conducted as part of a Matsumane International Foundation scholarship conducted for a period of 6 months at Mukogawa Women's University, Japan. The study was originally designed to investigate the effects of diets rich in *n-3* PUFA, *i.e.* flax oil, in the fetal programming of metabolic syndrome. However, there were challenges to breed the SHR/NDmcr-cp rats on high-fat diets; the presence of obesity, in adition to high-fat diet feeding, resulted in complete failure of breeding. As a result, the study was switched to investigate the effects of flax oil supplementation on metabolic syndrome during postnatal time period.