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UNIVERSITY OF
Southampton
School of Medicine



**Effects of Maternal High Fat Diet and
Pharmacological Intervention on the Developmental
Origins of Metabolic & Cardiovascular Disease**

by

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Thesis Submitted for the Degree of Doctor of Philosophy

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Faculty of Medicine Health & Life Sciences

School of Medicine

Maternal, Fetal and Neonatal Physiology

Supervisors: Mark A. Hanson & Sunil K. Ohri

for my parents;

Dr Sheikh Manzoor Elahi & Mrs Fehmida Sultana

who always advised me "Maqsood search yourself".

At the time, I did n't realize what I am aiming for but I carried on. Hence in search of me, I discovered Truth. In search for Truth, I discovered Love. In search for Love,

I discovered GOD. And in GOD, I have found Everything!

I have known many different kinds of people in my life...

*I've met men who are sensitive
and caring
and men who are cruel and calculating.*

*I've known women who are sincere
and honest
and women who are jealous and hateful.*

*I've seen smiles filled with lies
and tears
wet with truths.*

*I've shared time with those who have
needed me,
and I've been by myself
when I was in need.*

*I've been associated with people who
are dreamers but not doers
and with people who make promises,
but never keep them.*

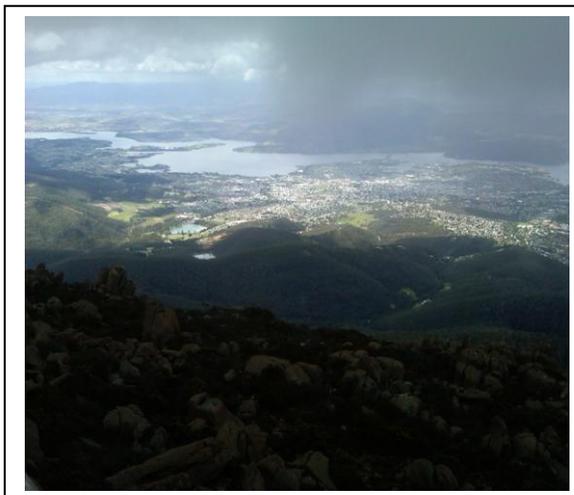
*I've found myself learning how to
understand all these personalities
and to
avoid those that cause my life sadness.*

*still believe in life's truths
of honesty, of sincerity, of compassion
and of true friendship*

*But most of all, I long to know those
few people
who really know what love is
and
how to be a loving person*

*I long for a place where people can
get together
and talk to one another about the things
that matter,
like being friends and caring about
dreams
that we all believe in.*

*I long for a time when friendship
and love
are important and the best parts of our
lives!*



I put these thoughts together on my
seven days tracking and hiking
expedition to Mount Wellington in
Tasmania from Oct 23 to Oct 30, 2010.

Signed:.....

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH & LIFE SCIENCES;

SCHOOL OF MEDICINE

Doctor of Philosophy

EFFECTS OF MATERNAL HIGH FAT DIET AND PHARMACOLOGICAL INTERVENTION ON THE DEVELOPMENTAL ORIGINS OF METABOLIC AND CARDIOVASCULAR DISEASE.

by Maqsood M Elahi

A high fat (HF) diet leads to hypercholesterolemia and predisposes the individual to developing cardiovascular disease (CVD). We hypothesised that mother's HF diet before and during pregnancy and lactation can also influence predisposition to CVD in offspring fed a similar diet. The thesis sets out to investigate whether (1) the effects of long-term consumption of a HF diet by the mother predisposes her offspring to developing a CVD/ metabolic syndrome in adult life and (2) pharmacological intervention using statin alleviates the detrimental effects of maternal HF diet on the health of the dams and their offspring.

Female C57BL/6 mice were fed either a HF diet (45% kcal fat) or standard chow (C; 21% kcal fat) from weaning through pregnancy and lactation. Pregnant C57/BL6 mice on HF diet were further given pravastatin in the drinking water (5 mg/kg of body weight per day) either short-term (2nd half of pregnancy and during lactation) or long-term (from weaning through to pregnancy and lactation) to lower cholesterol and improve post-weaning maternal blood pressure. Weaned female offspring from each group were then fed either a HF or C diets to adulthood. Body weight, blood pressure, plasma cholesterol, C-reactive protein (CRP) and bone marrow derived endothelial progenitor cells (EPC) were measured at 24, 28 and 36 weeks post-weaning in different experiments. Histology of the liver and kidneys were performed.

Offspring from hypercholesterolemic mothers on HF diet were significantly obese (bodyweight in grams; 17.2 ± 4.2 vs. 13.8 ± 4.7 ; $P < 0.05$), hypertensive (SBP mmHg; 134 ± 4.2 vs. 117 ± 3.4 ; $P < 0.001$), less active (distance in cm; 312 ± 31 vs. 563 ± 45 ; $P < 0.001$), demonstrated increased lipid laden vacuoles in liver and kidneys; and showed reduced expression of EPC ($P < 0.05$) than offspring from C dams independent of their postnatal nutrition respectively. Pravastatin therapy in HF mothers resulted in abrogation of these variables in offspring independent of post weaning nutrition ($P < 0.05$). The effects were more permanent when the dams were given long-term statin treatment.

The study demonstrates that long-term maternal HF feeding from weaning through pregnancy and lactation predisposes offspring to hypertension, raised plasma lipids, fatty liver, kidney disorders, raised CRP and inhibition of EPC numbers and expression in offspring. Pravastatin treatment of these dams inhibits these effects on the offspring and may reduce their risk of later cardiovascular pathophysiology. The findings may have implications for understanding the effects of the 'nutritional transition' to higher dietary intake of fat which could lead to increased cardiovascular disease in many societies.

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Chapter 4

Figure-4.1: “Activatory” and “inhibitory” intracellular signalling pathways regulate pravastatin-induced pleiotropic activities. Pravastatin diminishes proinflammatory effects and promote anti-inflammatory activities through the direct inhibition/activation of chemokine-, cytokine-, and acute phase reactant-induced intracellular signalling pathways in several cell types (such as leukocytes, vascular cells, adipocytes, and hepatocytes). In particular, the inhibition of ERK 1/2, rho, JAK/STAT3, or mevalonate pathways is crucial to reduce inflammation. On the other hand, statins directly activate AMPK and PI3K/Akt/NFkB pathways, thus, increasing cell survival, endothelial, and neuronal protection. (Adapted from references⁵⁰⁹⁻⁵¹⁰)

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either HF or C diets to adulthood, thus generating the dam/offspring dietary groups: HF/HF, HF-S/HF and C/C (n = 8 per group).

Figure-4.7: Overall maternal–fetal transfer likely encompasses an "uptake/influx" component for maternal lipoproteins and/or their cholesterol into the syncytiotrophoblast (STB), transport of lipoprotein-derived cholesterol (C) to the basal side of the STB, subsequent release into the villous core for passage through the extracellular matrix (ECM), uptake into ECs, and, finally, an efflux component by which cholesterol is released from ECs into the fetal circulation. To date, only the uptake and degradation of LDL and HDL in cultured trophoblasts have been described⁵³³⁻⁵³⁵ However, other lipoproteins and their respective receptors have not been analyzed, and the subsequent intraplacental transport steps are still uncharacterized.

Chapter 5

Figure-5.1: Flow diagram of the experimental protocol.

Figure-5.2: Long-term statin treatment in hypercholesterolemic mothers has beneficial effects on the body weights and blood pressure in their offspring. (a) Body weight gain males, (b) body weight gain in female (c) systolic blood pressure in male (d) systolic blood pressure in female offspring from mothers on standard chow (C), high fat-high cholesterol (HF) diet or HF diet and treated with statin during pregnancy and lactation (HF-S). Weaned offspring were then fed either HF or C diets to adulthood, thus generating the dam/offspring dietary groups: HF/HF, HF-S/HF and C/C (n = 8 per group).

Figure-5.3: Long-term statin treatment in hypercholesterolemic mothers has beneficial effects on total cholesterol and CRP in their offspring. (a) Total cholesterol levels in males, (b) Total cholesterol levels in female (c) CRP levels in male (d) CRP levels in female offspring from mothers on standard chow (C), high fat-high cholesterol (HF) diet or HF diet and treated with statin during pregnancy and lactation (HF-S). Weaned offspring were then fed either HF or C diets to adulthood, thus generating the dam/offspring dietary groups: HF/HF, HF-S/HF and C/C (n = 8 per group).

Figure 5.4: Representative photomicrographs of Oil Red O stained renal sections from (a) HF/HF and (b) HF+S/HF mice. The sections were counterstained with haematoxylin and eosin. Lipid droplets appeared as red spots that revealed the accumulation of neutral lipids in the glomerular and tubulointerstitial cells and showed an increase in HF/HF compared with HF+S/HF (n=8)

Chapter 6

Figure 6.1: Experimental protocol for Study-I

Figure-6.2: Statin treatment in hypercholesterolemic mothers during late pregnancy and lactation has beneficial effects on the cholesterol profile, blood pressure and CRP in their offspring. (a) Body weight gain, (b) systolic blood pressure (c) serum LDL-cholesterol, (d) serum CRP, (e) serum total cholesterol in offspring from mothers on standard chow (C), high fat-high cholesterol (HF) diet or HF diet and treated with statin during pregnancy and lactation (HF+S). Different letters indicate $p < 0.001$ except between bars with different letters and asterisks (*) where $p < 0.05$, showing the level of significance.

Figure 6.3: Pravastatin treatment in hypercholesterolemic mothers during late pregnancy and lactation has beneficial effects on (a) the positively stained mononuclear cells (MNCs) and (b) the number of double stained colonies in offspring from mothers high fat-high cholesterol (HF) diet. Different letters indicate $p < 0.001$ except between bars with different letters and asterisks (*) where $p < 0.05$, showing the level of significance.

Figure- 6.4: Expression of endothelial markers on EPCs. Representative photomicrographs of EPC colonies stained for endothelial markers Dil-Ac-LDL (red) and lectin (green). EPC colonies demonstrate reduced staining in HF/HF vs. C/C. Statin treatment to HF-fed dams abolished these effects in their offspring.

Figure-6.5 Correlation between the number of EPCs from HF/HF offspring with hypercholesterolaemia and total cholesterol (A) and LDL-cholesterol (B) levels

Figure 6.6: Long-term statin treatment in hypercholesterolemic mothers increased circulating endothelial progenitor cells (a) number of stained EPC colonies (b) percentage of mononuclear cells that are FITC labelled. Weaned offspring were then fed either HF or C diets to adulthood, thus generating the dam/offspring dietary groups: HF/HF, HF-S/HF and C/C (n = 8 per group).

Figure-6.7: The plot shows the linear regression results in HF/HF offspring. The *solid line* illustrates that increase in plasma CRP levels in plasma correlates with decrease in number of bone marrow derived EPC colonies expression in adult offspring. Asterisks (*) where $p < 0.05$, shows the level of significance.

Chapter 7

Figure-7.1: Adapted from Gluckman & Hanson.⁹⁵

Figure 7.2: The plot shows the linear regression results in female HF/HF offspring. The *solid line* illustrates that increase in total cholesterol correlates with increase in systolic blood pressure in 28 weeks old offspring. Similarly the *dotted line* demonstrates the correlation curve between the rises in cholesterol levels to increase in C-reactive protein values in same offspring (n=8). Asterisks (*) where $p < 0.001$, show the level of significance.

Figure-7.3: Scheme for dysfunctional activation of Kupffer cells in NAFLD. Pattern recognition receptors of Kupffer cells such as TLR4 may be increasingly exposed to exogenous and endogenous danger signals (e.g., LPS, excess fatty acids, modified lipoproteins) via the portal circulation, enhanced by lack of hepatocellular clearance. Pattern recognition pathways may intensify due to altered sorting and signalling, impaired inhibitory circuits, or amplification of redox-sensitive signalling loops. Adipokine imbalance may contribute to these events including low adiponectin levels that fail to suppress intracellular ROS generation. Fat-laden hepatocytes may compromise sinusoid microcirculation leading to entrapment of inflammatory cells. Finally, steatosis may shift away Kupffer cells from alternative activation. Solid lines, pro-inflammatory effects; dotted lines, anti-inflammatory mechanisms. Malfunction at one or more steps may promote 'second hit' responses, while cellular targeting of these checkpoints has the potential for identifying novel treatment strategies in NAFLD.

Figure 7.4: Hypercholesterolaemia plays a crucial role in the development of atherosclerotic diseases in general and CVD in particular. Statin, converts HMG-CoA to mevalonate. Inhibition of HMG-CoA reductase by statins decreases intracellular cholesterol biosynthesis, which then leads to transcriptionally upregulated production of microsomal HMG-CoA reductase and cell surface LDL receptors. This resets intracellular cholesterol homeostasis in extrahepatic tissues. The liver is the target organ for the statins,

since it is the major site of cholesterol biosynthesis, lipoprotein production and LDL catabolism, inhibits CRP metabolism and exerts a healthy effect on the bone marrow derived endothelial progenitor cells in the circulation. The beneficial effects of statin in offspring from HF dams on long term treatment may depend in part upon the degree to which statin act on extrahepatic tissues.
508-510,516,522,526,537,592-594

Figure 7.5:

Hypothesized “*Hypercholesterolemic-mediated Teratogenesis*” model originating from this thesis. This model demonstrates on one extreme (right side) the risk of adult disease and on the other extreme (left side) range of postnatal physiological settings for survival fitness. At the x-axis (bottom) five different quality of prenatal environments from very poor to very rich are presented. The red line curve represents the environment that the fetus anticipates postnatally judged from the nutritional and related signals it receives from the mother through the placenta. Provided that the postnatal environment matches the range for which the fetus has set its postnatal physiology by the processes of predictive adaptive responses, the disease risk is low. However, when it lies outside that range then the postnatal environment turns bad (indicated by the red arrow) and the disease risk increases. The fetus perceives the more restricted environment and predicts a poorer postnatal survival. But maternal high-fat dietary stress worsens the situation. Overnutritional high fat environment prenatally and postnatally shifts the postnatal survival associated with increased disease risk further to the upper limit (represented with curve black line). Maternal high fat diet causes hypercholesterolemic mediated teratogenesis with deranged lipid profile, increased oxidative stress and decreased activity of endothelial progenitor cells in the circulation of the offspring. This even further potentiates the CVD risk with early manifestation of cardiovascular disease/ metabolic syndrome in its own adult life. From an adequate nutritional environment, the offspring then lies outside the band of rich and very rich environment that can be detrimental to its survival fitness. However, administration of statin to mothers on high fat diet, even at rich nutritional upper limit, can provide a good safeguard from the disease process and maintains the survival fitness even if increasing affluence raises the richness of the mature postnatal environment.

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Declaration of the Author

I, ...Maqsood Manzoor ELAHI.....declare that the thesis entitled

EFFECTS OF MATERNAL HIGH FAT DIET AND PHARMACOLOGICAL INTERVENTION ON THE DEVELOPMENTAL ORIGINS OF METABOLIC AND CARDIOVASCULAR DISEASE.

and the work presented in it are my own. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this university or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this has always been clearly attributed;
- where I have quoted the work of others, the source is always given. With the exception of such quotations this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- parts of this work have been published as:

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Signed:.....

Date:

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Abbreviations

AcLDL	acetylated low density lipoprotein	OxLDL	oxidised low density lipoprotein
ANOVA	analysis of variance	PAR	predictive adaptive response
AHA	American Heart Association	PBS	phosphate buffered saline
BM	bone marrow	PUFA	polyunsaturated fatty acid
BMI	body mass index	QOL	quality of life
BP	blood pressure	ROS	reactive oxygen species
BNP	brain natriuretic peptide	SHR	spontaneously hypertensive rats
C	control diet of standard laboratory chow	SBP	systolic blood pressure
CVD	cardiovascular disease	SPRR 1A	small proline rich repeat protein
CAD	coronary artery disease	SuRFs	surveillance of risk factors
CDK	cyclin dependent kinases	TG	triglycerides
CRP	C-reactive protein	VCAM	vascular cell adhesion molecule
DOHaD	developmental origins of health & Disease	WHO	World Health Organization
DHA	docosahexaenoic acid		
EC	endothelial cell		
EPC	endothelial progenitor cell		
eNOS	endothelial nitric oxide synthase		
ET	exercise training		
E2	17 β estradiol		
ESRD	end stage renal disease		
FACS	fluorescence-activated cell sorting		
FAO	food and agriculture organization		
FELIC	fate of early lesions in children		
FFA	free fatty acid		
GLUT	glucose transporter		
HMG-CoA	hydroxymethylglutaryl-coenzyme A		
HF	high fat		
H₀	null hypothesis		
H_a	research hypothesis		
HDL	high density lipoprotein		
IHD	ischaemic heart disease		
INDEPTH	international network of field sites with continuous demographic evaluation of populations and their health in developing countries		
IL-6	interleukin-6		
i.p.	intraperitoneal		
LVAD	left ventricular assist device		
LA	linoleic acid		
LNA	linolenic acid		
LDL	low density lipoprotein		
MAP	mitogen activated protein		
MI	myocardial infarction		
MNC	mononuclear cell		
NAFLD	non-alcoholic fatty liver disease		
NASH	non-alcoholic steatohepatitis		
NO	nitric oxide		
NFκB	nuclear factor Kappa-B		
NCEP	national cholesterol education program		

“How lucky is that man who adopts humbleness without having scarcity, who considers himself inferior without having to beg for anything, who spends his own earned money in the right causes without disobeying God, who is kind to those having little means and who remains in the company of learned men”

The Holy Prophet of Islam Hazrat Mohummed (Peace be Upon Him).

CHAPTER 1

General Introduction

Cardiovascular disease (CVD) has become a leading cause and contributor of morbidity and mortality in this present time since the first decade of 21st century draws to a close.¹⁻² The World Health Statistics have suggested that at present, the developing countries contribute a greater share to the global burden of CVD when compared with developed countries.³⁻⁴ It is reported that 5.3 million deaths attributable to CVD occurred in the developed countries in 1990, whereas the corresponding figure for the developing countries ranged between 8 to 9 million (i.e., a relative excess of 70%).³⁻⁵ And that this difference would be even higher if the term "developed countries" is restricted to established market economies only and excludes the former socialist economies (Table-1.1).

This high, yet inadequately recognized, contribution of developing countries to the absolute burden of CVD is illustrated by the fact that 78% of the 49.9 million global deaths from all causes occurred in regions other than the established market economies or former socialist economies (Table 1.2). In addition, a greater cause for concern is the early age of CVD deaths in the developing countries compared with the developed countries e.g. the proportion of CVD deaths occurring below the age of 70 years is 26.5% in the developed countries compared with 46.7% in the developing countries³⁻⁴ and even larger for Indian subcontinent (i.e. Pakistan, India, Bangladesh & Srilanka; 52.2%).³⁻⁴ Therefore, the contribution of the developing

countries to the global burden of CVD, in terms of disability adjusted years of life lost, is 2.8 times higher than that of the developed countries (Table 1.1)¹.

Region	Population, millions	CVD Mortality, thousands	Coronary Mortality, thousands	Cerebrovascular Mortality, thousands	DALYs Lost, thousands
Developed regions	1144.0	5328.0	2678.0	1447.9	39 118
Developing regions	4123.4	9016.7	2469.0	3181.2	108 802
Established market economies	797.8	3174.7	1561.6	782.0	22 058
Former socialist economies	346.2	2153.3	1116.3	665.9	17 060
India	849.5	2385.9	783.2	619.2	28 592
China	1133.7	2566.2	441.8	1271.1	28 369
Other Asian countries and islands	682.5	1351.6	589.2	350.4	17 267
Sub-Saharan Africa	510.3	933.9	109.1	389.1	12 252
Middle Eastern Crescent	503.1	992.3	276.6	327.4	12 782
Latin America	444.3	786.7	269.1	224.1	9538

Table 1.1: Regional Differences in Burden of CVD (1990). DALY indicates disability-adjusted life year. (Adopted from Refs 1 & 4)

Region	All Causes, %	CVD, %
Established market economies	14	22
Former socialist economies	8	15
India	19	17
China	18	18
Other Asian countries and islands	11	9
Sub-Saharan Africa	10	7
Middle Eastern Crescent	9	7
Latin America	6	5
World	100	100

Table 1.2: Regional contributions to mortality (1990). Values are given as percent of world total. (Adopted from Refs 1 & 4)

Although there are inadequacies and imperfections of cause-specific mortality ascertainment methods currently used in many developing countries, the conservative assumptions made by the analysts suggest that this pattern will become even more pervasive as the CVD epidemic accelerates in many developing regions of the world, even as it retains its primacy as the leading public health problem in the developed regions.⁵⁻⁸ A considerable cause for alarm is the projected rise in both proportional and absolute CVD mortality rates in the developing countries over the next 25 years.⁶⁻⁹ Reasons for this anticipated acceleration of the epidemic include genetic factors as well as demographic factors including lifestyle changes and nutritional transitions.

1.1 Genetic Factors

It is increasingly recognized that CVD prevalence is a consequence of the interaction between the distribution of relative genotype frequencies and environmental exposures of a particular population.¹⁰⁻¹¹ It is suggested that distribution of such relative frequencies of genotypes involved in determining the distribution of individual susceptibilities to CVD is dependent on the number of segregating susceptibility genes, the number of alleles of each gene, their

relative frequencies, and the correlation between alleles of each gene and alleles of different genes.¹²⁻¹⁴ There are hundreds of genes known to have functional allelic variations that may contribute to determining an individual's susceptibility to CVD. But then all functional variations in a particular gene are not expected to be present in all populations.¹⁰⁻¹⁴ Because new DNA variations arise in isolation and their chance, selection, and migration work as "filters" in each population to modify the relative frequencies of genetic variations in evolutionary time, different populations will have different combinations of DNA variations.¹⁵⁻¹⁶ Therefore different combinations of susceptibility genes will be involved in determining CVD risk in different individuals in different families and it is always difficult to relate such different combinations of susceptibility genes to the CVD risk. However, only few genetic studies of common multifactorial diseases recognize the importance of this question.¹⁷⁻¹⁸

In 2004, the INTERHEART study¹⁹ examined the influence of nine risk factors for CVD and reported that smoking, diabetes, hypertension, obesity, diet, inactivity, alcohol intake, ApoB : ApoA1 ratio and psychosocial factors accounted for 90-94% of population-attributable risk. Based on this model, the authors¹⁹ suggested that populations with all these risk factors are 337 times more likely to suffer cardiac disease than populations with none. In addition to these risk factors (which may themselves be genetically determined), a positive family history increases coronary artery disease (CAD) and myocardial infarction (MI) risk to 2-3.9 fold.²⁰

Until recently, such attempts to identify the genetic associations used either candidate gene or linkage studies. The former examines variations in a low number of known, plausibly associated genes in affected cases and controls, and while linkage studies assess affected families/sibling pairs using microsatellite markers to define a genomic region linked to the phenotype. So far, these approaches have been applied with great success to identifying causative mutations in monogenic cardiovascular diseases, such as hypertrophic cardiomyopathy and long QT syndrome.²¹⁻²⁴ However, the complex interplay between environment and genetics demonstrated in INTERHEART made it clear that similar approaches were unlikely to identify the poorly penetrant and multiple causative genes that accounted for non-Mendelian diseases, such as CVD. Although CVD can also rarely be

inherited in a Mendelian fashion (predominantly in conditions leading to elevated LDL), this only accounts for a small proportion of incident cases,²⁵ most of which are likely to be polygenic. Linkage studies of non-Mendelian CVD have provided some biased associations,²⁶⁻³⁴ conspicuously lacked reproducibility between cohorts, suffered from poor statistical power and lacked detailed genomic mapping provided by conventional microsatellite markers.

Recent technological advancement, coupled with greater understanding of the structure of the human genome derived from genome sequencing projects,³⁵⁻³⁷ now make unbiased whole-genome association studies (GWAS) possible.³⁸ The International Haplotype Mapping project³⁹ identified hundreds of thousands of single nucleotide polymorphisms (SNPs) and assessed their degree of linkage disequilibrium (the degree to which a SNP predicts the DNA flanking it). It is reported that genotyping 0.008% of an individual's nucleotides (250 000-350 000 in total) is able to identify an individual genome.⁴⁰ This technological advance led the Wellcome Trust Case Control Consortium and others to perform SNP-based GWAS on patients with CAD compared with matched controls.⁴¹ For example the most reproducible locus conferring increased risk of CAD is situated on chromosome 9 (locus 9p21.3)⁴²⁻⁴⁴ and increases risk by approximately 1.2 for a single copy (1.5 in the 25% of the population who carry two copies).⁴⁵ Interestingly, unlike other regions associated with surrogate risk factors for CAD, such as C-reactive protein (CRP),⁴⁶ adiposity⁴⁷ and left ventricle (LV) mass,⁴⁸ the 9p21.3 locus does not affect such risk factors, suggesting that it promotes CAD in a non-canonical manner. However, studies are suggesting that SNP-based GWAS knowledge provides no additional benefit⁴⁹ despite the availability of genotyping via the internet. This may be first because loci such as 9p21.3 confer effects by altering the regulatory region of DNA⁴²; second the involved region that overlaps a non-coding RNA named ANRIL, is only conserved in primates and not other mammals or lower organisms; and third the associations of some loci (e.g. 9p21.3 locus) are also present in conditions such as dementia⁵⁰ and stroke,⁵¹ rather than specifically CAD. Therefore, incorporation of risk-conferring alleles such as 9p21.3 and others into a CAD prediction algorithm is still not clear and thus remains to be

substantiated in terms of its true importance under the current models and on clinical parameters.⁵²

1.2 Demographic Factors

There was a major surge in life expectancy experienced by most developing countries in the second half of the twentieth century.⁵³ For example, the life expectancy in India rose from 41.2 years in 1951–1961 to 61.4 years in 1991–1996 as reported by Reddy & Yusuf¹. The authors¹ further explained that this is due to a decline in deaths occurring in infancy, childhood, and adolescence. This was also related to more effective public health responses to perinatal, infectious, and nutritional deficiency disorders and to improved economic indicators such as per-capita income and social indicators such as female literacy in some areas¹. Although much remains to be done in these areas, the demographic shifts have augmented the ranks of middle-aged and older adults. The increasing longevity provides longer periods of exposure to the risk factors of CVD resulting in a greater probability of clinically manifested CVD events.⁵⁴ The concomitant decline of infectious and nutritional disorders (competing causes of death) further enhances the proportional burden due to CVD and other chronic lifestyle-related diseases. This shift, representing a decline in deaths from infectious diseases and an increase in those due to chronic diseases, is often referred to as the modern epidemiological transition.⁸⁻⁹

It is reported that the ratio of deaths due to pretransitional diseases (related to infections and malnutrition) to those caused by post-transitional diseases (e.g., CVD and metabolic disorder) varies among regions and between countries, depending on factors such as the level of economic development and literacy as well as availability and access to health care¹. The direction of change towards a rising relative contribution of post-transitional diseases is, however, common to and consistent among the developing countries.^{9,55} The experience of

urban China, in which the proportion of CVD deaths rose from 12.1% in 1957 to 35.8% in 1990, is illustrative of this phenomenon.⁵⁶

The United Kingdom itself is a diverse society with 7.9% of the population from minority ethnic groups (Africa, Middleeast, Indian Subcontinent, South America and Chinese region)⁵⁷. The causes of the excess CVD and metabolic disorder morbidity and mortality in minority ethnic groups are incompletely understood by socio-economic factors. However, the role of classical CVD risk factors is clearly important despite the patterns of these risks factors varying significantly by ethnic group. Moreover, the CVD epidemiology of African Americans does not represent well the morbidity and mortality experience seen in black Africans and black Caribbeans, both in Britain and in their native African countries⁵⁸. In particular, atherosclerotic disease and CAD are still relatively rare in the latter groups. This is unlike the South Asian Diaspora, who has prevalence rates of CVD in epidemic proportions both in the Diaspora and on the subcontinent⁵⁵⁻⁵⁷.

Data for population surveillance of CVD and metabolic disorders are limited in many countries. The World Health Organization (WHO) has set up a range of projects aimed at improving the amount and quality of relevant data. The Surveillance of Risk Factors (SuRFs) project, launched in 2003, presents chronic disease risk factor profiles from 170 WHO member states. These data include patterns of physical inactivity, low fruit/vegetable intake, obesity, blood pressure, cholesterol, and diabetes.⁵⁸ The most recent report SuRF2 enables country comparisons for these data⁵⁹⁻⁶⁰. Figure-1.1 shows data on the percentage of adults in the different countries of Southeast Asian nations with body mass index (BMI) $>30 \text{ kg/m}^2$. The variation is marked and it is interesting to note that two of the poorest countries in the region, Laos and Myanmar, have severe obesity rates comparable with some of the wealthiest. On the other hand, Singapore, the most developed country in the region does not suffer from obesity epidemic.

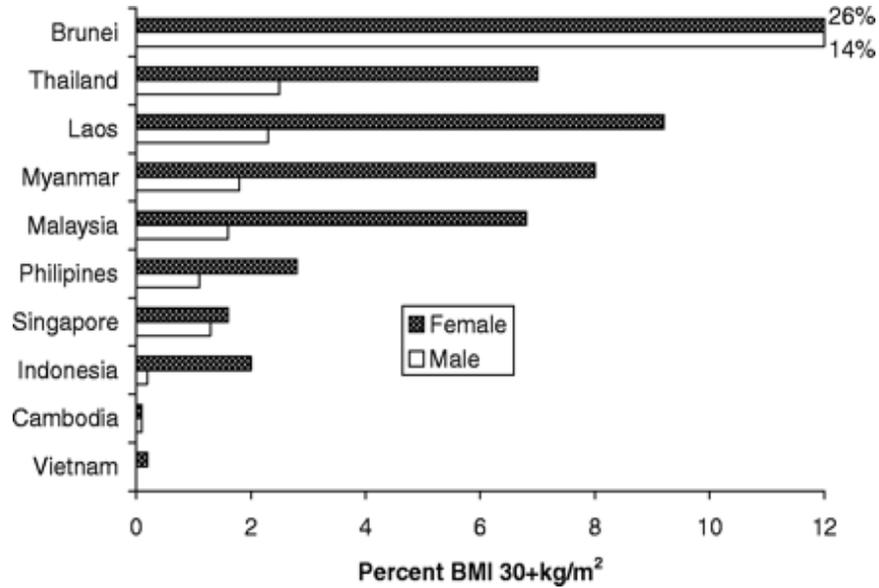


Figure 1.1: Use of WHO web Global InfoBase.⁵⁸⁻⁵⁹ Obesity (BMI \geq 30 kg/m²) in the Association of Southeast Asian Nations in 2002

Although the biological determinants of CVD and metabolic disorders in low and middle income countries are likely to be similar to those in affluent countries,⁶⁰ the drivers of these determinants are likely to differ. For example, rural–urban migration may be an important factor in promoting the adoption of Western dietary habits and activity patterns, leading to an increased CVD risks.

Similarly, socioeconomic patterns of CVD disease risk, though well established in affluent countries, are more complex in some low and middle income countries.⁶⁰⁻⁶² For example It has been the historical experience of the developed countries that the CVD epidemic usually commences in members of the higher social classes, who are the first to change from a low-risk to a high-risk lifestyle, which is characterized by diets rich in fat and calories, sedentariness, and smoking. But today, the risk permeates across the social spectrum, affecting all classes. The higher-social classes are, again, the first to respond to the knowledge of risk factors and the message of prevention. The CVD rates begin to decline in them, with the present pattern of higher CVD rates among the lower social classes becoming increasingly established. In most developing countries, there has been an initial preponderance of CVD in

the higher socioeconomic strata. However, the pattern observed in the developed countries, in which the burden of disease shifts progressively to the lower social classes, is likely to be replicated as the epidemic advances^{1,60-61}. Indeed, recent reports from India (described in reference 55), based on community surveys and case-control studies, suggest that poor educational or economic status is associated with higher risk of CHD in some regions.⁵⁵ Hence, with the complete reverse of the social gradient, progressively larger numbers of poor individuals will become its victims and will be unable to obtain the necessary health care.

New opportunities to use large demographic surveillance projects as tools to study CVD and metabolic disorders are emerging rapidly as part on the work of INDEPTH (International Network of field sites with continuous Demographic Evaluation of Populations and their Health in developing countries).⁶³⁻⁶⁴ Even with such studies, the understanding of determinants for rising CVD/ metabolic disorder epidemic and explaining such differences as to how rural–urban migration increases risks of obesity, diabetes, and CVD will still be not possible. One important caveat to looking at such data is to study the role of impaired early growth, resulting from fetal and infant nutrition operating at different stages of the life course,⁶⁴⁻⁶⁵ an issue that particularly applies when defining the causality of this problem.

1.3 Nutritional Transition and Life Style Changes

Another concern is that if population levels of CVD risk factors rise as a consequence of adverse lifestyle changes accompanying industrialization and urbanization, the rates of CVD mortality and morbidity could rise even higher than the rates predicted solely by demographic changes. It is suggested that both the degree and the duration of exposure to CVD risk factors would increase due to higher risk factor levels coupled with a longer life expectancy. An increase in body weight (adjusted for height), blood pressure, and cholesterol levels in Chinese population samples aged 35 to 64 years, between the two phases of the Sino-MONICA study (1984 to 1986 and 1988 to 1989) and the substantially higher levels of CVD risk factors in

urban population groups compared with rural population groups in Indian subcontinent provide evidence of such trends.^{54-55,66-67} A cross-sectional survey of urban Delhi and its rural environs revealed a higher prevalence of CAD in urban Delhi.⁵⁴ This is reported to be associated with higher levels of body mass index, blood pressure, fasting blood lipids (total cholesterol, ratio of cholesterol to HDL cholesterol, triglycerides), and diabetes.⁵⁴ The increasing use of tobacco in a number of developing countries also translates into higher mortality rates of CVD, CAD and other tobacco-related diseases.⁶⁸⁻⁷⁰

As reviewed by Drewnowski and Popkin,⁷¹ the global availability of cheap vegetable oils and fats has resulted in greatly increased fat consumption among many countries. The transition now occurs at lower social classes than previously and is further accelerated by rapid urbanization. For example, the proportion of upper-income persons who were consuming a relatively high-fat diet (>30% of daily energy intake) rose from 22.8% to 66.6% between 1989 and 1993 in China. The lower- and middle-income classes also showed a rise (from 19% to 36.4% in the former and from 19.1% to 51.0% in the latter).⁶⁸ These countries, with a diet that is traditionally high in carbohydrates and low in fat, have shown an overall decline in the proportion of energy from complex carbohydrates along with the increase in the proportion of fat.⁷¹ The globalization of food production and marketing is also contributing to the increasing consumption of energy-dense foods poor in dietary fibre and several micronutrients.⁷¹

1.4 The Complexity of the Problem

The prior discussion in sections 1.1-1.3 hence shows that CVD has a complex multifactorial aetiology leading to a reappraisal of the ways in which three key factors—genome, development and environment—influence the adult phenotype, including the individual's susceptibility to disease. Neither genetic makeup nor exposures to adverse environments predict with certainty the onset, progression, or severity of CVD. Disease develops as a consequence of interactions between the "initial" conditions, coded in the genotype, and

exposures to environmental agents indexed by time and space⁷²⁻⁷⁴ that are integrated by dynamic, regulatory networks at levels above the genome.⁷⁵

The interaction of an individual's environmental experiences with her/his genotype determines the history of her/his multidimensional phenotype, beginning at conception and continuing through adulthood (Figure 1.2).

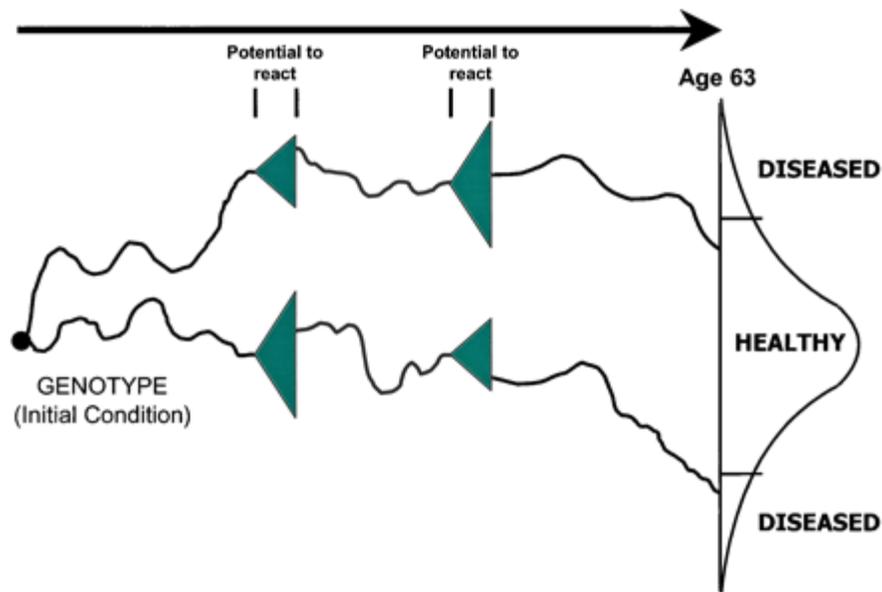


Figure 1.2: From Sing *et al.*⁷⁷ with permission. At a particular point in time, each genotype has a range of possible phenotypes determined by the range of possible environmental histories. The phenotype of an individual to react to contemporary environments in a particular environmental niche, at a particular point in time, is influenced by the phenotype produced by previous genotype-phenotype combination. The figure illustrates this relationship, by collapsing an individual's phenotype into single dimension, showing two of the many possible phenotype histories for a given genotype.

The consequence of these interactions with exposures to environmental agents indexed by time and space is that many individuals who have a genotype that predicts an increased risk of CVD will remain healthy because of exposures to compensatory environments. The converse will also be true: individuals who have a genotype that has a low risk of CVD might develop disease because of an adverse environmental history.

CVD research has revealed tens of high-risk environmental factors and hundreds of genes, each with many variations that influence disease risk. The phenotypic measures of health are constantly being shaped, changed, and transposed as a consequence of epigenetic mechanisms of cellular and organismal dimensions that change over the lifetime of the individual. At the level of the cell, these mechanisms influence DNA methylation and repair; they also serve to organize coordinated responses to heat-shock, oxygen deprivation, and other environmental changes.⁷⁷ The relationships between these subsystems influence the trajectory of an individual's phenotype to influence the expression of the participating genes⁷⁸⁻⁸⁰ (Figure 1.3).

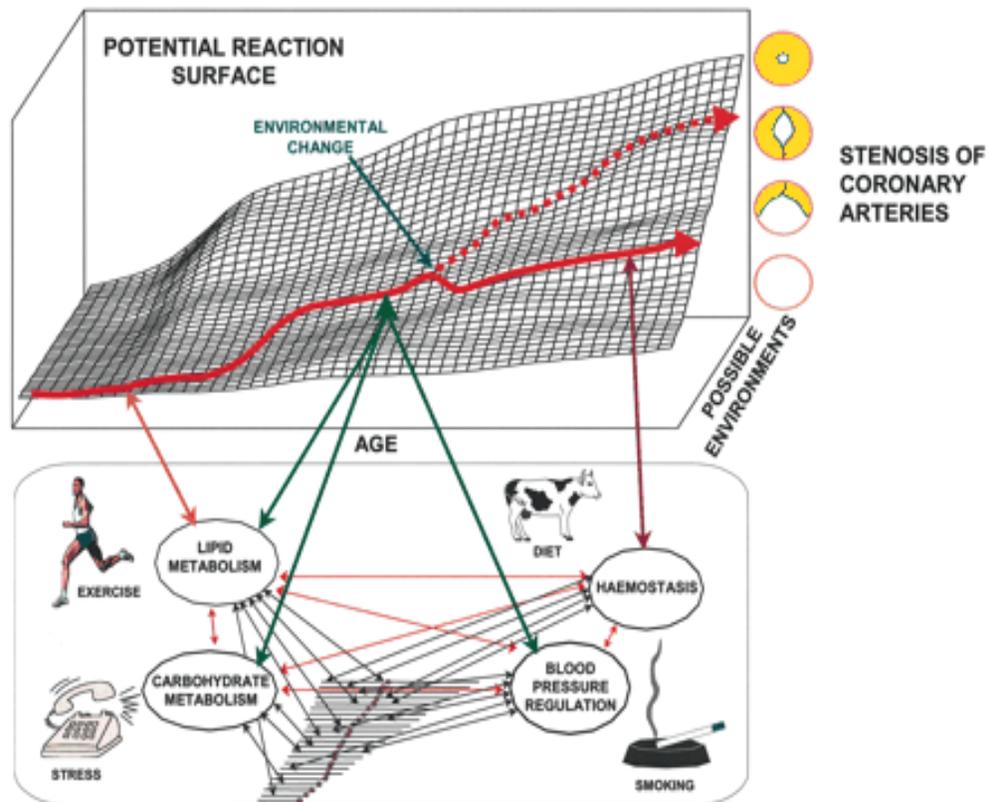


Figure 1.3: From Sing *et al.*⁷⁷ with permission. A model for an individual's propensity to develop CVD such as coronary artery disease. This figure shows how a particular multigene genotype is connected to the domain of potential CVD phenotypes through the primary biochemical and physiological subsystems. The important role that biochemistry and physiology play in the connections between the genome and disease phenotypes brings into question the utility of the overused, simplistic view that the genome produces an independent, isolated, and fixed one-way flow of information from genome to phenotype.

However, how differences in neonatal human epigenotype may be linked to a functional outcome in later life is still in its infancy. Yet, it suggests that the developmental component of phenotype determination is very important in humans and provides mechanistic proposal to explain the variable risk of individuals living in obesogenic world. Given the epigenetic changes are both reversible and can be influenced by maternal state, the potential for interventions during early development to reduce the risks of metabolic and CVD in the modern obesogenic environment warrants greater attention.

Studies have suggested that different ethnic groups that live in the same geographic areas and share similar environmental risks have different profiles of disease markers and prevalence, which may propose a genetic cause for differences in disease susceptibility.⁸¹⁻⁸² Yet, with some notable exceptions,⁸³ few ancestry-specific alleles have been discovered that can explain particular pathologies. Other explanations of both inter-individual and ethnic differences in disease risk, therefore, need to be considered. Of note, high incidences of metabolic disease are found in those ethnic groups in which the average birth weight is low⁸⁴ or the rates of gestational diabetes and maternal obesity are high.⁸⁵

Untangling the effects of genes from those of environmentally determined developmental processes is not straightforward. Importantly, fetal nutrition does not equate to maternal food intake, but rather is dependent on maternal metabolism, cardiovascular function and, particularly, placental function.⁸⁶ The long-lasting changes in developmental trajectory that underpin altered susceptibility to disease may arise, at least in part, from epigenetically mediated alterations in gene expression. Whereas compelling evidence supports both the developmental origins of health and disease and the underlying epigenetic mechanisms,⁸⁷ many features of the latter remain insufficiently understood. These elements include the differences among epigenetic mechanisms across species and between patterns of epigenetic modifications on paternal and maternal genomes, the mechanisms that regulate the establishment, stability and flexibility of epigenetic changes, and the precise connection

between an epigenetic change, altered gene expression and the resultant phenotype for CVD epidemic in countries⁷²⁻⁸⁰. The hypothesis is currently recognised as "Developmental origins of Health and Disease" (DOHaD) and requires particular understanding before proceeding further with the subject.

1.5 Developmental Origins of Health and Disease

DOHaD hypothesis states that adverse intrauterine influences such as poor maternal nutrition lead to impaired fetal growth, resulting in low birth weight, short birth length, and small head circumference. These adverse influences are postulated to also induce the fetus to develop adaptive metabolic and physiological responses. These responses, however, may lead to disordered reactions to environmental challenges as the child grows, with an increased risk of glucose intolerance, hypertension, and dyslipidaemia in later life and adult CVD as a consequence.⁸⁸⁻⁹⁴ Although some supportive evidence for the hypothesis has been provided by observational studies,⁹⁵⁻⁹⁹ it awaits further evaluation for a causal role. If it does emerge as an important risk factor for CVD, the populations of developing countries will be at an especially enhanced risk because the vast numbers of poorly nourished infants who have been born in the past several decades now suffer a threat through an over-nourished rich environment. The steady improvement in child survival will lead to a higher proportion of such infants surviving to adult life, when their hypothesized susceptibility to vascular disease may manifest itself¹⁰⁰⁻¹⁰⁹.

1.5.1 Origins of the hypothesis- Historical perspective

The "early or fetal" origin of adult disease hypothesis was originally put forward by David Barker and colleagues in Southampton⁸⁹. The authors suggested that environmental factors, particularly nutrition, act in early life to program the risks for the early onset of cardiovascular and metabolic disease in adult life and premature death^{89,93-94,110-112}. Before the fetal origins hypothesis was articulated, an association between early life events and later CVD had been proposed on more than one occasion. In 1934, Kermack *et al*¹¹³ demonstrated that death rates

from all causes in the United Kingdom and Sweden fell between 1751 and 1930. The authors concluded that this was the result of better childhood living conditions during this period. Subsequently, Forsdahl¹¹⁴ reported that there was a correlation within different geographical regions of Norway between coronary heart disease in 1964–1967 and infant mortality rates some 70 years earlier. Forsdahl¹¹⁴ postulated that poverty may act through a nutritional deficit to result in a life-long vulnerability to disease with a more affluent adult life-style. In 1985, Wadsworth *et al.*¹¹⁵ in the United Kingdom reported that adult blood pressure was inversely related to birth weight in men and women born in 1946. In 1986, Barker and colleagues suggested that poor health and physique of mothers were important determinants of the risk of stroke in their offspring⁹⁴. Soon afterwards, it is proposed that environmental influences, that impair growth and development in early life, result in an increased risk for CVD^{87-93;95-112}. This then led to a worldwide series of epidemiological studies that extended the initial observations on the association between pre- and postnatal growth and CVD to include associations between early growth patterns and an increased risk for hypertension, impaired glucose tolerance, non-insulin-dependent or type-2 diabetes, insulin resistance, and obesity in adult life¹¹⁵⁻¹²⁴.

1.5.2. The thrifty phenotype hypothesis, developmental plasticity and predictive adaptive responses

To explain the biological basis of the associations observed between early growth patterns and health outcomes in the epidemiological studies, number of mechanistic frameworks such as “*thrifty genotype*”¹²⁵⁻¹²⁶ and then “*thrifty phenotype*”¹²⁷ derived from thrifty genotype, were proposed. “Thrifty genes” were proposed to be selected during evolution at a time when food resources were scarce and they resulted in a “fast insulin trigger” and thus an enhanced capacity to store fat, which placed the individual at risk of insulin resistance and type-2 diabetes¹²⁶. In contrast the thrifty phenotype hypothesis suggested that when the fetal environment is poor, there is an adaptive response, which optimizes the growth of key body organs to the detriment of others and leads to an altered postnatal metabolism, which is designed to enhance postnatal survival under conditions of intermittent or poor nutrition¹²⁸⁻¹²⁹.

It was proposed that these adaptations only became detrimental when nutrition was more abundant in the postnatal environment, than it had been in the prenatal environment¹²⁷.

Lucas suggests that there are embryonic and fetal adaptive responses to a suboptimal intrauterine environment which result in permanent adverse consequences either via the induction, deletion, or impaired development of a permanent somatic structure or the physiological system.¹³⁰ In fact closing the critical window early in development allows the preservation of maternal strategy in offspring phenotype, which in humans benefits the mother by constraining offspring demand after weaning. The offspring gains by being buffered against environmental fluctuations during the most sensitive period of development, allowing coherent adaptation of organ growth to the state of the environment. The critical window is predicted to close when offspring physiology becomes independent of maternal physiology, the timing of which depends on offspring trait¹³⁰⁻¹³³. All this highlights the relationship between intrauterine nutritional experiences and subsequent health outcomes¹³⁴⁻¹³⁵

Researchers working with humans and animal models of human diseases often view the effects of early life events as developmental plasticity. This embodies the idea that developmental plasticity is the ability of a single genotype to produce more than one alternative form of structure, physiological state, or behaviour in response to environmental conditions¹³⁶⁻¹³⁸. Consistent with this, it is thought that CVD may be a consequence of fetal adaptations to undernutrition that are beneficial for short-term survival, even though they are detrimental to health in postreproductive life¹¹². Although some effects of nutrition may be direct consequences of alterations in substrate availability, McCance and Widdowson demonstrated that early undernutrition had a permanent effect on the subsequent growth of rats, whereas later undernutrition only had a transient effect¹³⁹.

It is clear from a range of diverse fields including evolutionary ecology and molecular biology that a given genotype can give rise to different phenotypes, depending on environmental conditions¹³⁹⁻¹⁴¹. There are many different species where the impact of an environment experienced by one generation determines the development and behaviour of the next generation. Female birds are able to alter many aspects of the composition of the egg in response to a range of environmental factors including food availability, levels of sibling competition, and the quality of their mates¹⁴⁰. Such maternal effects can result in the effects of a specific environmental factor persisting across several generations^{136-137,141}. If the effects of the past conditions produce mismatches with current, changed conditions, however, then developmental plasticity may have a detrimental effect on survival and reproductive success¹⁴². Thus Bateson *et al.*¹⁴² proposed that for individuals whose early environment has predicted a high level of nutrition in adult life and who develop a large phenotype, the better the postnatal conditions the better will be their adult health. For individuals whose conditions in fetal life predicted poor adult nutrition and who develop a small phenotype, the expected outcomes may vary, although they are predicted to be worse off when there is a relative excess of nutrition in postnatal life. There has also been a proposal to separate those homeostatic responses that represent fetal adaptations to changes in the intrauterine environment and that may have long-term consequences, from those which need not confer immediate advantage but are induced in the expectation of future adaptive changes¹⁴³; this latter group of responses has been defined as "predictive adaptive"^{90,92,137,141}. In this model of predictive adaptive response, selection across generations operates to favour protection of those predictive adaptive responses that aid survival to reproductive age. The programmed or plastic responses made during development that have immediate adaptive advantage might also act to limit the range of postnatal adaptive responses to a new environment and would be considered to be "inappropriate" predictive adaptive responses. This general model is therefore consistent with the original thrifty phenotype hypothesis which stated that fetal adaptations to a poor intrauterine environment may have adverse consequences if there is a relative excess of nutrition available in adult life.

The use of the term “predictive adaptive response” (PAR) must be clarified because it is used in two very different ways in the literature. In a physiological context it refers to adjustments made by an individual in response to current conditions. For example, in conditions of severe intrauterine deprivation, there is the capacity to lose structural units such as nephrons, cardiomyocytes, or pancreatic β -cells within developing organ systems. Such decreases in structural and hence the life-long functional capacity of an organ system may be an inadvertent consequence of a decrease in energy supply across the placenta or a selective trade off to maintain the development of more important tissues, such as the brain¹⁴⁴⁻¹⁴⁶.

In an evolutionary context it refers to changes in the characteristics of populations or species resulting from natural selection, mainly promoting Darwinian fitness and adaptive according to the evolutionary criteria of enhancing survival or reproductive success¹⁴⁷. In case of fetal origins of disease, this would require that environmental conditions present early in life are predictive of the conditions the individual will encounter in the future over a range of timescales (Figure 1.4).

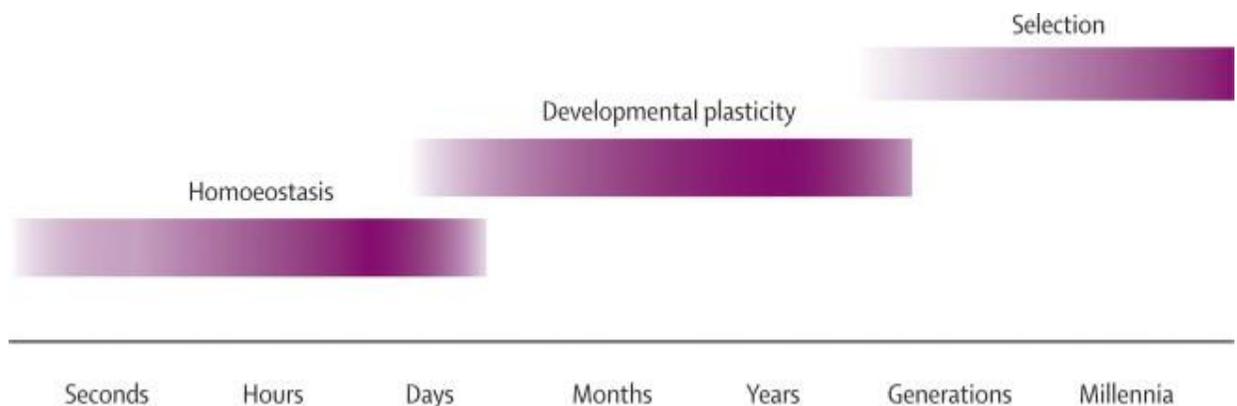


Figure 1.4: From Gluckman *et al.*¹³⁷ with permission. Modes of human adaptability.

It is suggested that at one extreme, rapid and reversible homoeostatic mechanisms counter an

immediate challenge. Then, stressors or exposures during critical developmental periods can affect growth, tissue differentiation, and physiological set-points, affecting responses to environmental challenges for life. New evidence suggests that epigenetic mechanisms could contribute to such challenges¹⁴⁸⁻¹⁴⁹. On a long timescale, the genomes of populations can change over many generations as a result of selection or drift, and there are many examples of responses to environmental change becoming integrated into the human genome^{141,150-152}. Clinical medicine and public health research have focused largely on causation and intervention at the short-term end of this spectrum. In this context consideration of the outcomes of developmental plasticity acting over the intermediate timescale is now important¹⁴¹. In humans, development plasticity can induce responses that have short-term benefits for the mother or the fetus but on longer term reduced fitness and increased disease process¹⁵²⁻¹⁵³. It is suggested that when environmental conditions change strikingly between conception and adulthood, as has happened in most current human populations, the potential for a substantial mismatch is especially great, and this difference contributes to increased disease risk¹³⁵.

1.5.3 Environmental cues affecting human development

These broad considerations are relevant to understanding of some critical variations such as developmental adaptations that permanently change structure, physiology, and metabolism, thereby predisposing individuals to cardiovascular, metabolic, and endocrine disease in adult life.¹⁵⁴⁻¹⁵⁵ The human baby responds to undernutrition, placental dysfunction and other adverse influences by changing the trajectory of his or her development and slowing growth. Although the fetus was thought to be well-buffered against fluctuations in its mother's condition, a growing body of evidence suggests that the morphology and physiology of the human baby is affected by the state of the mother¹⁵⁶⁻¹⁵⁷. It is possible therefore, that human development may involve induction of particular patterns of development by cues that prepare the developing individual for the type of environment in which he or she is likely to live. Individuals may be affected

adversely if the environmental prediction provided by the mother and the conditions of early infancy prove to be incorrect¹¹³.

Thus, people whose birth weights were towards the lower end of the normal range and who subsequently grows up in affluent environments are at increased risk of developing CAD, type-2 diabetes and hypertension^{39-40,156,158}. Those born as heavier babies and brought up in affluent environments enjoy a much reduced risk. The long-term influences may arise from cues acting from before conception to infancy¹⁵⁹. The ill effects of being small, which in the short term include high death rates and childhood illness, are usually treated as yet another inevitable consequence of adversity. However, a functional and evolutionary approach suggests that the pregnant women in poor nutritional condition may signal to her unborn baby that help it to cope with a shortage of food. When sufficiently high levels of nutrition are available after the development of a small phenotype has been initiated, marginal benefits of rapid growth may offset the costs¹⁶⁰, but they may also trigger the health problems arising in later life. This concept is illustrated in Figure 1.5.

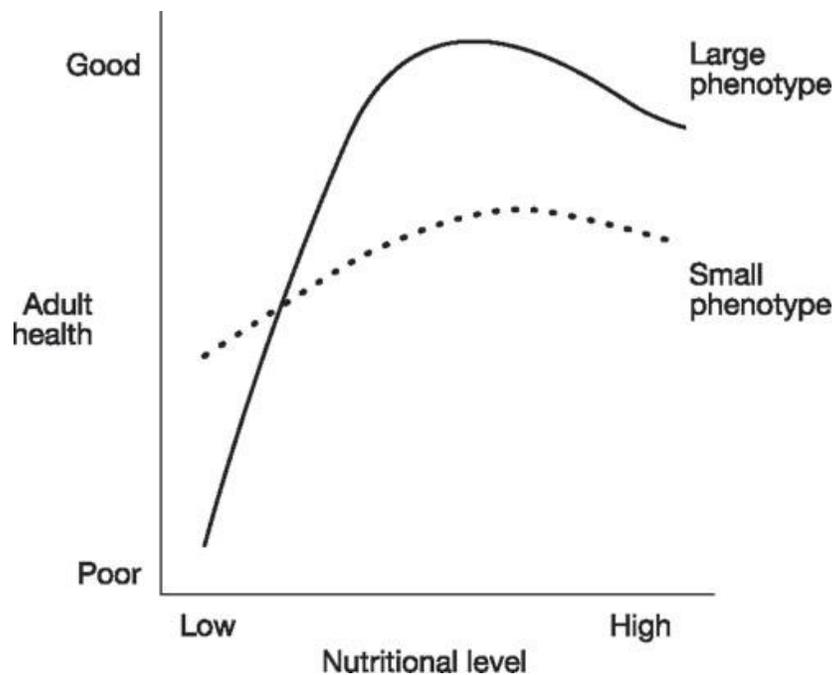


Figure 1.5 The hypothetical relationship between adult health and nutritional level during later development for two extreme human phenotypes that were initiated by cues received by the fetus. From Bateson *et al.*¹⁴² with permission.

Although adaptive responses may explain some variation in human development, it would be implausible to argue that all responses to the environment should be explained in these terms. Undernutrition, stress or hypoxia may impair normal development. Babies with low birth weight have a reduced functional capacity and fewer cells¹⁶¹. The latter may be part of a general reduction in cell numbers or a selective trade-off in the development of tissues that are less important to the baby, such as the kidney¹⁶². Reduced numbers of nephrons at birth is a life-long deficit, as all nephrons are formed during a sensitive period of development in late gestation. The resulting increased functional demand on each individual nephron, for example by increased blood flow through each nephron, may lead to acceleration of the nephron death that accompanies normal ageing, with a consequent rise in blood pressure¹⁶³⁻¹⁶⁵.

The diversity in past and present ecological conditions of humans is also likely to introduce complexity into the relationship between developmental prediction and later health outcome. For example, some populations may have adapted genetically to conditions of nutritional stress, especially seasonal food shortages, over a long time span, while others will have been buffered from such local evolutionary effects. The sharp increase in glucose intolerance leading to type-2 diabetes might arise from genetic differences between populations¹⁶⁶⁻¹⁶⁷. The possibility of a thrifty genotype well adapted to harsh conditions is not incompatible with the plastic induction of thrifty phenotypes from a pool of uniform genotypes. However, the hypothesis that differences in susceptibility to diabetes are explained by genetic differences would not readily account for the evidence from the Dutch famine of 1944–45 that glucose intolerance is induced by maternal malnutrition during the final three months of pregnancy¹⁶⁷. However, persisting into adult life, insulin resistance leads to increasing blood glucose and type-2 diabetes develops, especially in people who have become overweight. 'Thrifty' handling of sugar becomes maladaptive if undernutrition in the womb is followed by excess in later

life¹⁶⁸. Conversely, individuals with large bodies may be particularly at risk in harsh environments such as prison camps or during famines.^{153,168} Especially striking is the evidence from a famine-exposed Ethiopian population, where the incidence of rickets was nine times greater in children who had been reported as having high birth weights than in age-matched control children¹⁶⁹. No such differences were found in children with normal birth weights.

1.6 DOHaD Approach to Cardiovascular Disease

Lower birth weight and weight at 1 year of age were associated with an increased risk of death from CVD and stroke¹⁷⁰⁻¹⁷¹. Early criticisms of the association of death rate from CVD with lower birth weight raised questions about completeness of follow-up¹⁷²⁻¹⁷³. This criticism was largely answered in a Swedish study of 15,000 men and women with a 97% follow-up over a period of more than 50 yr. The study suggested that death rates from ischemic heart disease (IHD) were increased in individuals in the lower quartiles of birth weight compared with those in the highest quartile¹⁷⁴. Also of note was the Nurses Study in the United States which includes 121,700 women who have been followed since 1976. In one retrospective study of more than 70,000 women from this group, there were strong negative trends between self-reported birth weight and nonfatal coronary heart disease and stroke¹⁷⁵. The association of cardiovascular death and birth weight has now been reported in many studies¹⁷⁶⁻¹⁷⁹ and this raises the importance of developmental environmental influences.

It has also been shown that cardiovascular disorders may be greatest in males born to mothers who were obese¹⁷⁹. Short birth length of female infants followed by increase in height was also associated with increased risk of death from coronary heart disease¹⁸⁰. These women tended to have tall mothers¹⁸¹, which suggests that prenatal growth of these female infants was constrained. Thus the most adverse cardiovascular disease risk profile is found in men and women who were small at birth but became obese in adult life¹⁸², and the effects of adult BMI

on high blood pressure, type-2 diabetes, and insulin resistance were greater in individuals of low birth weight¹⁸¹. Whatever the mechanisms, the possibility of a biological link between birth weight and adult body mass index (BMI) complicates interpretation of studies that link birth weight and adult BMI to other adult outcomes such as CVD, hypertension, and metabolic syndrome.

Soon afterwards, the association between low birth weight and high blood pressure in adult life was extended to include the normal range of birth weight¹⁸³. In addition to raised blood pressure at age 10, children living in areas with higher rates of death from CVD also had high resting pulse rates. The children and their mothers were shorter than those living in areas with low cardiovascular mortality, raising the possibility of environmental or inherited/genetic effects. A small number of studies have also examined the contribution of the placenta to the association between size at birth and CVD later in life. In cohorts in Preston in the United Kingdom and in Adelaide in Australia, blood pressure increased with decreasing birth weight and increasing placental weight¹⁸⁴⁻¹⁸⁵. In the Adelaide cohort, however, the effect of placental weight was no longer apparent when the blood pressure was measured and when the subjects were nearly 20 yr of age. In this group, the amplification of blood pressure from 8–20 yr of age was greatest in those with lower birth weights¹⁸⁵. No support for an effect of placental size or birth weight was found in the New Zealand cohort followed from birth to 18 yr of age or for other indices of fetal or placental growth in 8-yr-old Australians¹⁸⁶.

During the past two decades there has been a marked increase in the global prevalence of adult and childhood obesity, and currently >50% of all adults in the United States and the United Kingdom are overweight, i.e., have a BMI of >25 kg/m²¹⁸⁷⁻¹⁸⁹. An increase in the prevalence of obesity (BMI >30 kg/m²) is associated with an increase in a range of co-morbidities including type-2 diabetes, high blood pressure, and CVD¹⁹⁰⁻¹⁹³. A range of epidemiological studies have shown that there is a relationship between increased BMI (obesity) in childhood and CVD in adult life¹⁹⁰⁻¹⁹¹. In this context it is of particular interest that epidemiological,

clinical, and experimental studies have shown that there is a relationship between the prenatal nutritional environment and patterns of postnatal growth and adult adiposity including CVD and dyslipidemia¹⁹¹⁻¹⁹³. There is also an interaction between lesser prenatal growth and postnatal weight gain¹⁸⁹. Thus, the risk of developing CVD is greatest in those who are small or thin at birth and who then develop relative obesity postnatally. Individuals at greatest risk have an earlier pre-pubertal adiposity rebound and gain weight faster in the late pre-pubertal period¹⁹⁰⁻¹⁹². The weight of epidemiological evidence is substantial, though controversial because the studies were criticized as retrospective, confounded by other variables (e.g. socioeconomic status) and as biologically implausible. Critics questioned the size of the relationship between birth weight and surrogate measures of cardiovascular disease risk (e.g. blood pressure). However, stronger and robust relationships exist between birth size and the incidence of clinical disease (e.g. heart disease or clinical hypertension). Furthermore, prospective clinical and experimental observations demonstrate clear relationships between measures of the fetal environment and risk of developing CVD. It is, therefore important to consider in populations of the world that are undergoing the nutritional and epidemiologic transition to Western styles of diet, sedentary behaviour and obesity, the ominous pattern that Baker *et al.*, identified- lower birth weight followed by excess weight gain in childhood- is both common and liable to persist for the foreseeable future^{191-192,194-195}. It is these environmental factors that induce change in metabolic and cardiovascular development in our changing world. Several models have been developed to explain these effects¹⁹⁵⁻²⁰⁰. Experiments in several species show that it is easy to induce insulin resistance and other manifestations of the CVD, including hypertension and endothelial dysfunction, by manipulating maternal nutrition or exposing the mother to synthetic glucocorticoids²⁰¹⁻²⁰⁴. Following maternal under-nutrition in rats, pups develop fasting hyperinsulinemia, hyperleptinemia, hyperphagia and have increased lethargy; they also develop central obesity and have reduced muscle mass²⁰⁴⁻²⁰⁶. In similar experiments, changes in hypothalamic appetite regulatory peptides and a preference for fatty food consumption are reported²⁰⁷⁻²⁰⁸.

1.7 Mechanistic Basis of Responses

Relatively, few studies into the aetiology of the DOHaD have attempted to establish the mechanistic basis of this phenomenon. Whereas most research has involved pathways through which under-nutrition during development translates into subsequent metabolic disorder and ultimately CVD, studies are beginning to focus on the role of excess nutrition, excess exposure to hyperglycaemia *in utero* and whose mothers are obese, at increase risk of developing metabolic disorders and CVD²⁰⁸⁻²¹⁴. One possible mechanism under investigation is that maternal nutrition prior to or during pregnancy can program fetal development and adult CVD via heritable epigenetic mechanisms involving additional interactions between non-transcribed RNAs and covalent modifications to associated histone proteins (discussed in ref 210). Our work in animal models has demonstrated that the adverse effects of impaired early-life nutrition and the associated epigenetic changes can be prevented or reversed by nutritional interventions (such as folate supplementation) or endocrinological interventions (such as neonatal leptin administration)^{212, 215}. Further support for such a mechanistic base has come from studies in rats, where Zambrano *et al* answered the question of (a) reversibility of maternal obesity by altered dietary intake commencing before conception and (b) the adverse metabolic effects of maternal obesity on offspring metabolic phenotype, and that outcomes and reversibility vary by tissue affected²¹⁶. Other studies with rodents have reported both epigenetic and phenotypic effects in offspring of dams transiently exposed to endocrine disrupting compounds during pregnancy²¹⁷⁻²¹⁹. Moreover, it is reported that epigenetic processes are involved in some of the immediate manifestations. For example, decreased histone H3 methylation (an epigenetic marker associated with repression of transcription), together with increased expression of proinflammatory genes, has been proposed to underlie the sustained proinflammatory phenotype of vascular smooth muscle cells that is seen in diabetic animals even after normalization of glycemia²²⁰⁻²²⁵. Similarly, transient hyperglycemia causes persistent expression of proatherogenic genes, which is underpinned by specific changes in histone H3 methylation in vascular endothelial cells²¹⁸⁻²¹⁹. Whilst such phenomena would not normally be considered in the context of developmental origins of CVD, they do serve to illustrate the environmental liability of acquired epigenetic modifications induced by maternal over-nutrition that may contribute to transgenerational amplification of obesity in humans²²⁶.

The second possible mechanism involves altered tissue differentiation. For example, adult nephron numbers are reduced both in humans who are born small and in animals manipulated experimentally *in utero*²²⁷⁻²²⁸. This might be the result of a biological trade-off to conserve energy in response to deprivation during a crucial developmental window, having immediate but no long-term adaptive value. Alternatively, it might simply be a result of developmental disruption with no adaptive value, analogous to teratogenesis. Similarly, in the rat, the developing pancreatic islet undergoes a wave of developmental apoptosis during the perinatal period, presumed to reflect a transition from a fetal to a postnatal form of islet cell with a different regulatory profile²²⁹. The rate of β -cell apoptosis is increased in infant rats whose mothers are fed a low-protein diet²³⁰, perhaps because of an increased sensitivity to cytokines²³¹. Inhibition of this apoptosis in the neonatal period prevents the development of subsequent diabetes²³².

The third mechanism involves altered homeostatic processes. Offspring of rats fed a low-protein diet have an altered ratio of periportal to perivenous hepatocytes, leading to an altered ratio of phosphoenolpyruvate carboxykinase to glucokinase-enriched cells and greater hepatic glucose production²³³⁻²³⁴. The expression of enzymes involved in lipid homeostasis, including carnitine palmitoyl transferase, is suppressed²³⁵. Skeletal muscle from growth-retarded infant rats is relatively resistant to both insulin and insulin-like growth factor I²³⁶. There is also mitochondrial dysfunction and reduced expression of protein kinase C- ζ , which might lead to altered glucose transporter 4 (GLUT-4) mobilization in muscle²³⁶⁻²³⁸. Cardiac muscle also shows reduced synthesis of GLUT-1, GLUT-4 and hexokinase II²³⁹ in such settings.

1.8 Proposed Model of Over-nutrition under Current Conditions

It is quite clear from the subject presented so far that there is a growing recognition of the importance of environmental factors acting on the genotype throughout the life cycle of an individual to progressively modify its phenotype, thus leading to metabolic disorder and CVD in later life in an unpredicted environment. Several models have been developed to explain these effects and are discussed earlier. Clearly there are windows of time in the lifecycle when the susceptibility of the genome to such an influence is very high. The periconceptual and intrauterine period seem to be the most crucial, when a small change in environment could have a large effect on the phenotype. Any preventive intervention will therefore have to start *in utero*, and improving the health of future mothers will be a very important aspect of such an approach.

Since the discovery that low birth weight is associated with increased cardiovascular mortality rate and the development of type-2 diabetes mellitus and the metabolic syndrome²⁴⁰, numerous epidemiologic and experimental studies have confirmed these associations. The adverse effects of low birth weight are increased if it is followed by accelerated weight gain after the age of two years²⁴¹⁻²⁴². These findings have led to the concept that an undernourished mother produces a small (thin-fat) insulin resistant baby. If this baby remains undernourished in postnatal life, the cycle is propagated. If the thin-fat insulin resistant baby is over-nourished, it becomes obese, hyperglycaemic and possibly hypercholesterolemic. An obese and hypercholesterolemic mother then produces a baby at higher risk of obesity, hyperglycemia and dyslipidemia. Thus, this intergenerational cycle of metabolic like syndrome is propagated through a girl child. Rapid transition shifts the balance from undernutrition to overnutrition and contributes to escalation of the metabolic/ CVD epidemic, a current situation of our new world. Improving health of a girl child is of paramount importance in controlling this epidemic. This proposed model is illustrated in Figure 1.6.

1.9 Mechanistic Studies and their Failure in High Fat Overnutrition Model

It is becoming increasingly clear that the evidence from historical and prospective work so far published contains major apparent contradictions²⁴³⁻²⁵⁶. Whilst some researchers believe that fetal and infant undernutrition is key to the developmental induction of adult disease²⁰², others argue that overnutrition in the very same time periods is fundamental²⁰³. This controversy polarises research and detracts from the ability to develop a more holistic model of environmental impacts on disease risk. So far published literature (referenced above) has led to controversy driven primarily from inconsistency in research outcomes, terminology, study designs and the interpretation of statistics. This approach has, hence, made difficult to understand the concept of overnutrition during early life in increasing risk of common set of diseases.

Fetal programming in rapid transition

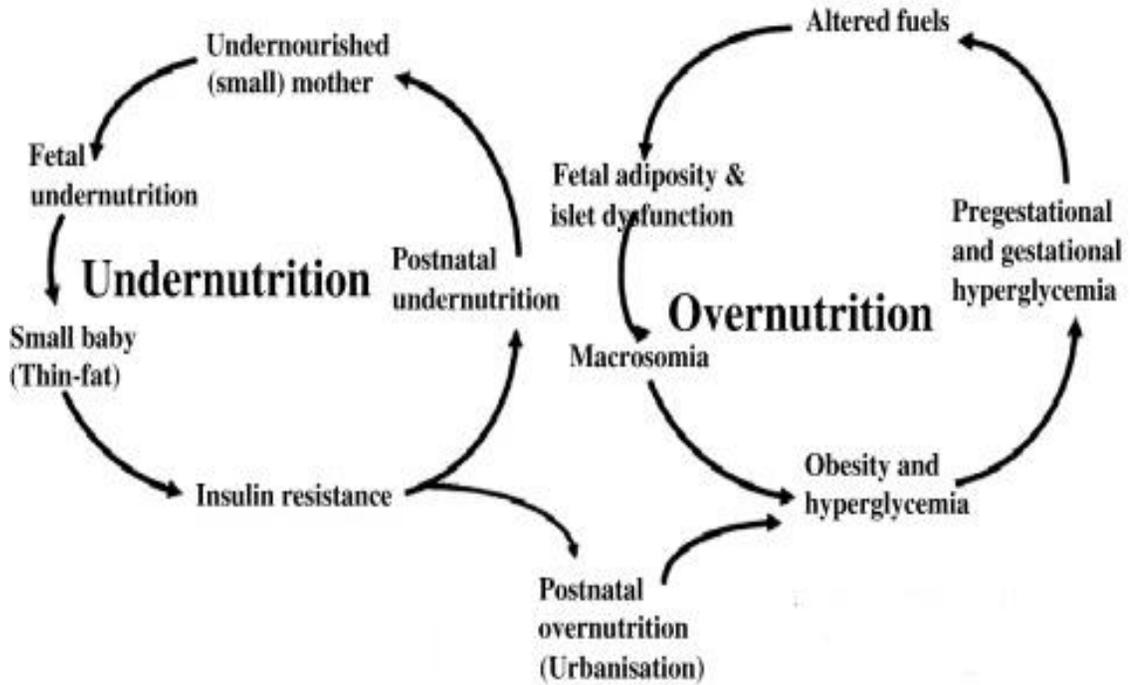


Figure-1.6: Proposed model of fetal programming in rapid transition. The model shows the interrelationship of two major maternal factors (undernutrition and overnutrition) in fetal programming. An undernourished mother produces a small (thin fat) baby. If the baby remains undernourished in a postnatal life, the cycle is propagated. If the thin fat baby is overnourished, it becomes obese and hyperglycaemic and produce metabolic syndrome like phenotype. An obese and metabolic syndrome mother produces a “macrosomic” baby at higher risk of obesity and hyperglycemia. Thus the intergenerational cycle is propagated through a girl child. Rapid transition shifts the balance from undernutrition to overnutrition and contributes to escalation of the CVD and metabolic epidemic. Improving health of a girl child is of paramount importance in controlling this epidemic.^{85,88,89,111,112,194}

So far the interpretation of associations between maternal overnutrition and later disease risk in offspring remains controversial. This is because the statistical significance for these associations tends to depend on adjustment for adult weight. It has also been argued that change in size between birth and adulthood is the primary determinant of risk, implicating postnatal growth rather than *in utero* development²⁰⁵. For example, data from some published work consistently demonstrate associations between increased infant growth rate and poorer

subsequent metabolic profile^{206, 257-258}, paradoxically suggesting increased risk of the same diseases that have been associated in observational cohort studies with infant undernutrition.

Given the prominence of CVD risk in offspring, it is helpful to begin by exploring the notion that adequacy of fetal nutritional supply reflects variability during pregnancy and/or lactation. Genetic factors undoubtedly influence fetal growth; however, studies are relatively consistent in attributing a minority to genetic factors²⁰⁷⁻²⁰⁸, indicating that most can be attributed to environmental factors. Logic behind the Dutch famine data suggests that those individuals exposed during the third trimester provide support for the paradoxical ‘U’-shaped association between prenatal environment and subsequent risk of obesity, as categorised by BMI²⁰⁰, and diabetes²⁰⁹. Nevertheless, a recent study using ultrasound measurements to identify growth faltering in each trimester of pregnancy has revealed unexpected findings²⁵⁹, that early faltering may result in a degree of catch-up growth *in utero* and that large birth weight need not necessarily indicate a lack of fetal growth faltering.

A second critical issue are apparent inconsistencies in the literature reported with different outcomes assessed. Several studies have shown that a maternal diet rich in fat and cholesterol during pregnancy can induce obesity, vascular dysfunction, impaired skeletal muscle development, sedentary behaviour, and gender-specific hypertension in the offspring^{213, 217-219, 246-247, 254}. However, these studies have been confined to short-term modifications in the maternal diet, such as during pregnancy and/or lactation periods only. This disparity in research outcomes is a major contributing factor to conceptual confusion, for two related reasons. First, it is difficult to integrate the different outcomes within a simple model of disease. Second, study findings may on occasion conflict drastically, according to the age of the participants.

Defining the fetal environment is complex. One in which the offspring’s immediate environment comprises maternal physiology, and a subsequent one in which the offspring is

directly exposed to the external environment²⁶⁰. Organogenesis occurs primarily within the first period, hence this critical period of development could be argued to be induced not by the external environment but by maternal phenotype²⁶⁰⁻²⁶¹. The duration of this maternal induction varies according to the trait in question. During pregnancy maternal and fetal haemodynamics interact, generating differential growth of peripheral vs. central organs¹¹¹. The offspring can respond adaptively, but its strategy is also open to maternal manipulation^{260,263}. This opportunity for direct haemodynamic interaction ceases at parturition. In contrast, offspring metabolism remains sensitive to maternal phenotype during the window of lactation, through hormonal effects on milk output and composition. Physiological traits such as nephron number²⁶⁴, cardiac structure²⁶⁵ and pancreatic β -cell mass¹²⁷ are therefore strongly (although not necessarily exclusively) associated with fetal experience, whereas insulin metabolism is further sensitive to infant experience²⁶⁶. It has been argued, from an evolutionary perspective, that the offspring adapts to the ‘niche’ of maternal metabolism and that the mother manipulates her offspring to optimise her own reproductive strategy²⁶⁰⁻²⁶¹. As many aspects of organ phenotype are essentially fixed from birth or early infancy onwards, as a result of the majority of rounds of cell division having been achieved²⁶⁷, aspects of phenotype induced during this developmental period track subsequently into adulthood.

Thus, reference can be made to the ‘maternal induction’ of offspring ‘metabolic capacity’, which is closely associated with certain aspects of organ phenotype. The influence of maternal phenotype on offspring development must inevitably weaken with offspring age, but the schedule of this change depends on ecological factors. Until the development of agriculture, early postnatal growth would likewise have been strongly influenced by maternal phenotype, via lactation. Even after weaning, the tendency for human offspring to be provisioned by their mother would maintain a link between maternal phenotype and offspring growth rate²⁶⁰. Such extended maternal care confers coherence on offspring development throughout the period of pregnancy, infancy and childhood²⁶¹.

Postnatal growth contrasts with prenatal growth by increasingly impacting on tissue size rather than fundamental structure, especially from late infancy. Postnatal growth, the sum of both size and somatic tissue (lean and fat), may thus be conceptualised primarily as generating 'metabolic load'. The relative magnitude of growth, and the load it imposes, is associated with homeostatic adaptations in metabolic traits. For example, blood pressure increases systematically during the growth process²⁶⁷, influenced independently by both somatic size (lean mass)²⁶⁸ and adiposity²⁶⁹, with each contributing to the total metabolic load. Even weight gain in the first 3 months demonstrates such effects on blood pressure by 1 year of age²⁷⁰. These increases in blood pressure have been attributed to larger bodies imposing a greater load on the kidneys²⁶⁷, invoking haemostatic changes in order to maintain renal homeostasis. In other words, variability in blood pressure during childhood and adulthood reflects the normalisation of metabolic load for a given metabolic capacity²⁶⁸.

This process of normalisation, evident for blood pressure is merely one example of a broader pattern whereby metabolic traits mediate the impact of growth-generated metabolic load on metabolic capacity. For example, insulin resistance and insulin secretion are closely related, such that in healthy individuals insulin resistance can be accommodated by increases in insulin secretion to maintain glycaemic control²⁶⁶. However, the understanding of this remains incomplete.

The notion that disease is the result of disparity between metabolic capacity and metabolic load was essentially described in the thrifty phenotype hypothesis¹²⁶, and this conceptual model remains capable of integrating the findings of diverse developmental origins research studies. What has not been sufficiently appreciated as yet is the shift most notably in industrialised populations, the possible maternal high fat influence to the offspring exposing the offspring to stochastic growth patterns as early as 30 weeks post conception in those born. It is this coherence in nutritional experience that appears most strongly to predict subsequent disease.

Differentiating growth into periods targeting high fat metabolic capacity or metabolic load clarifies in several ways the apparent controversy between the ‘low birth weight’ and ‘growth acceleration’ schools. First, it is easier to appreciate apparent inconsistencies relating to outcomes. Birth weight has been associated with risk of diabetes¹⁵², while rapid infant growth has been associated with insulin resistance²⁵⁷. However, since diabetes represents a ‘two hit’ phenomenon, in which insulin resistance is accompanied by β -cell defect preventing compensations in insulin secretion^{236,239,241,242, 258}, the epidemiology of diabetes need not be identical to the epidemiology of insulin resistance. For example, the life-course induction of physiology (e.g. β -cell mass, nephron number) differs from that of physiological function (e.g. glycaemic control, blood pressure), which in turn differs from that of disease (e.g. diabetes, hypertension). Whether the insulin resistance induced by faster infant growth²⁵⁷ actually leads to diabetes in later life is likely to be mediated by β -cell function, and hence by fetal developmental experience.

Second, it is possible to resolve apparent inconsistencies relating to statistical models. Studies of older adults with CVD show that, holding current weight constant, those with low birth weight have an increased disease risk²⁰², which implicates the induction of metabolic capacity in early life through strong effects on traits such as nephron number or β -cell mass, as discussed earlier. Studies of younger adults show that rapid infant weight gain is associated with increased physiological markers of risk, regardless of birth weight^{206,257}, which implies the environmental induction of increased metabolic load. The greatest risk for metabolic load and metabolic capacity occurs in those individuals who are born small and become large, for whom the risk of CVD and the metabolic syndrome is greatly increased^{241, 271-272}. Recent studies further suggest that different periods of childhood growth impact differentially on the risk of specific diseases²⁷³⁻²⁷⁴. Each statistical approach may therefore be considered merely to emphasise one component of the dynamic process whereby metabolic load is superimposed on metabolic capacity.

Third, it is beneficial to differentiate between study designs and possible sensitive periods of growth. The findings of intervention studies conducted in both preterm and full-term infants during the immediate postnatal period have been used to argue that postnatal growth is implicated as the key ‘critical window’ in which susceptibility to CVD is induced²⁰³. However, such studies only demonstrate the magnitude of effect of an intervention conducted during a specific period. This interpretation does not therefore support the model that CVD is essentially induced by undernutrition prenatal and postnatal experience alone, as suggested previously²⁰³. Although it is claimed that the hypothesis of ‘undernutrition’ (being harmful) is supported by data from a range of animal species, the vast majority of such animal studies describe catch-up growth following initial nutritional insult, while the remaining studies involve genetically variant animals²⁰⁶ and so cannot attribute adult phenotypic variability to early-life environmental variability. Again, these animal studies implicate an inherent link between different growth periods in the aetiology of later disease, one earlier period exerting deleterious effects on metabolic capacity and the other imposing an increased metabolic load on that capacity.

Fourth, it is possible to differentiate alternative pathways whereby public health policies might beneficially impact on health. The close association between birth weight and subsequent lean mass,²⁷⁵ and the fact that organogenesis is largely completed by birth, suggests that interventions on the mother, during her own development or during pregnancy, may represent the optimum approach for benefitting offspring metabolic capacity. Birth-weight supplementation studies tend to have limited efficacy,²⁶⁰⁻²⁶¹ hence nutritional intervention earlier in the maternal life course may be most successful. Animal studies, for example, show that maternal effects continue to act on offspring phenotype across several generations.²⁷⁶ Alternatively, during pregnancy maternal metabolic control may represent a better candidate for intervention than maternal diet,²⁶⁰ as it is the nutrient concentration gradients between fetus and mother that determine fetal supply. In contrast, postnatal interventions may prove most beneficial in preventing the induction of excess metabolic load.

Prompted by animal studies, the proposed over-nutrition model (section 1.8) changes perspectives on how to intervene in the “life-style” disease epidemic. It shows that lifestyle interventions alone may be only partially effective in that those who are most affected by inappropriate PAR and may be difficult to manage with life-style interventions in adulthood. The proposed model suggests that improving maternal and fetal health will allow humans to cope better with current postnatal nutritional conditions—conditions that we do not evolve to inhabit. There is increasing focus on the periconceptual period being the critical period when programming cues are most effective. If that is the case, then the focus will have to be on the health of women before pregnancy and their nutritional status at conception and early pregnancy. The DOHaD paradigm is clearly important in these evolution patterns of human disease and intervention studies with strict experimental design to test the importance of maternal nutrition on offspring phenotype need to be initiated. But before discussing the specific hypothesis and overall aims of this thesis, it would be advantageous to elaborate on the aetiology of the CVD in the following paragraphs.

1.10 Factors that Promote Early Cardiovascular Disease

The underlying cause of early CVD is characterized by a long lag time between onset and clinical manifestation. The prodromal stages of CVD lesions are suggested to be already formed during fetal development²⁷⁷⁻²⁷⁸. Once initiated, progression of the CVD is influenced by classical risk factors that promote vascular inflammation²⁷⁹⁻²⁸². These inflammatory changes in vasculature results in remodelling with a well established consequence of perturbations in blood pressure or flow. These perturbations are affected by the secretion of vascular smooth muscle elastin, reported to occur during development and thus induce effects on vascular compliance²⁸³. In turn, this results in increased numbers of macrophages and lymphocytes, release of hydrolytic enzymes, cytokines, chemokines, and growth factors²⁸⁴ that could induce further damage and eventually lead to focal necrosis²⁸⁵. Endothelial cell activation, results in poor synthesis of NO and increased expression of cell adhesion molecules

considered to provide further pro-atherogenic stimulus. It is suggested that poor endothelial function implicated in insulin resistance, observed in metabolic syndrome; and leads to reduced glucose uptake. This impaired vascular growth in the early development is linked to prematurely reduced compliance in adulthood²⁸⁶ by a few investigators working on CVD lesions during fetal development.

1.10.1 Maternal lipid profile

Napoli and co-workers observed that maternal hypercholesterolemia was associated with greatly enhanced fatty streak formation in human fetal arteries²⁷⁸. These results hence, suggested that during the earlier stages of pregnancy, maternal hypercholesterolemia promoted lesion formation in the fetus.²⁷⁸ However, conventional risk factors assessed in children and their mothers remained unable to explain this difference.

Studies have so far reported a direct evidence describing a correlation of maternal hypercholesterolemia with the plasma concentrations of oxidized fatty acids and lipid peroxidation by-products in offspring of hypercholesterolemic and cholestyramine-treated mothers.²⁸⁷⁻²⁹⁰ Fetal arterial cells exposed to significant oxidized low density lipoprotein (OxLDL) from maternal hypercholesterolemia demonstrated a pattern of atherogenesis distribution similar to that in early lesions of adults and animal models.²⁹¹⁻²⁹² In humans, it remains to be established how much of the accelerated atherogenesis in offspring of hypercholesterolemic mothers is caused by *in utero* programming events and how much is due to inherited genetic differences. Nevertheless, numerous signalling pathways downregulated in fetuses by increased oxidation of LDL in hypercholesterolemic mothers²⁹³⁻²⁹⁶ suggested that fetal pathogenic events increased the postnatal susceptibility to atherosclerosis and this led to early CVD.

1.10.2 Acute phase response- C-reactive protein

Pregnancy is generally associated with an increase in circulating cytokine and C-reactive protein (CRP) concentrations²⁹⁷ and this increase strongly correlates to the decrease in insulin sensitivity in pregnancy.²⁹⁸ In rodents, it is demonstrated that prenatal exposure to cytokines leads to marked increase in adipose tissue mass in male and female offspring.²⁹⁹ The metabolic consequences of this excess abdominal fat mass manifests in reduced insulin sensitivity in male offspring and hyperandrogenism in female offspring. Hence, pregnancy-induced elevations in cytokines especially CRP may have a role to play in fetal programming.

CRP is an acute-phase reactant pathologically produced during infection, inflammatory disease, cancer, and tissue injury.³⁰⁰ It consists of five identical nonglycosylated 21-kDa subunits that are synthesized mainly in the liver³⁰¹ and is present at a low level (below 10 µg/ml) in humans under normal conditions. In response to acute-phase stimuli, plasma CRP concentration can increase significantly up to 1,000-fold and return to normal levels (≤ 1 µg/ml) with resolution of the disease.³⁰¹ It is reported that in patients at increased risk of CVD, the CRP levels are elevated and remains elevated for many months to years.³⁰² According to the recently issued guidelines for cardiovascular risk assessment, patients with CRP levels between 1 and 3mg/L are considered to be at intermediate risk and those with levels above 3mg/L at high risk³⁰³.

It is suggested that CRP directly causes endothelial dysfunction through reducing the expression and bioactivity of endothelial nitric oxide synthase (eNOS), and inhibits *in vitro* angiogenic functions as measured by endothelial cell migration.³⁰⁴⁻³⁰⁵ Increased CRP concentration in the plasma significantly associates with the presence of macrophages and T lymphocytes in plaques in high-grade carotid stenosis patients.³⁰⁶ CRP is also shown to accelerate aortic atherosclerosis development in apolipoprotein-E deficient mice, which points out that CRP is an active player in CVD process. CRP also contributes to the formation of

foam cells in atherosclerotic lesions by causing the aggregation of LDL molecules that are then taken up by macrophages through a CD32-independent pathway.³⁰⁶⁻³⁰⁷

In a synchronous fashion, CRP has been shown to stimulate endothelin-1 and interleukin-6 release, upregulates adhesion molecules, and stimulates monocyte chemoattractant protein-1 while facilitating macrophage LDL uptake³⁰⁸. CRP potently downregulates endothelial nitric oxide synthase (eNOS) transcription and destabilizes eNOS mRNA in the vascular endothelium that decreases both basal and stimulated nitric oxide (NO) release³⁰⁹ (Figure-1.7).

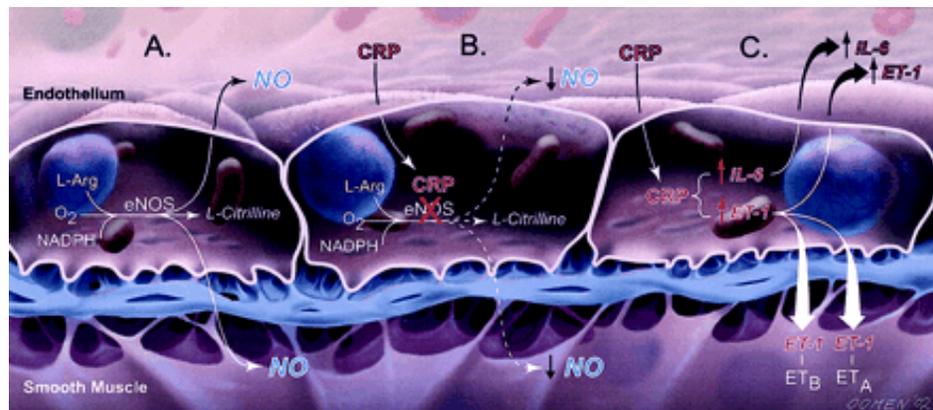


Figure-1.7: Human recombinant CRP at concentrations known to predict adverse cardiovascular events directly interacts with the endothelium to decrease the production of the multifactorial vasoactive peptide NO. This effect appears to occur in part via decreased eNOS mRNA stability. In a synchronous fashion, CRP promotes the EC release of the potent endothelium-derived vasoconstrictor, ET-1, and a key inflammatory cytokine, IL-6. These actions of CRP induce EC dysfunction and promote a proinflammatory and proatherosclerotic phenotype. CRP also promotes EC apoptosis and inhibits angiogenesis via decreasing NO production.³⁰⁹

CRP has also been shown to facilitate endothelial cells (EC) apoptosis, inhibits angiogenesis and upregulates nuclear factor- κ B (NF κ B; a key nuclear factor that facilitates the transcription of numerous proatherosclerotic genes).³⁰⁷⁻³¹⁰ The direct proatherogenic effects of CRP extends beyond the endothelium to the vascular smooth muscle, where it directly upregulates

angiotensin type-1 receptors and stimulates vascular smooth muscle migration, proliferation, neointimal formation, and reactive oxygen species (ROS) production.^{309,311,312} This detrimental effect of CRP is also reported to extend to bone marrow derived endothelial progenitor cells (EPCs) that contribute to postnatal neovascularisation (Figure-1.8).

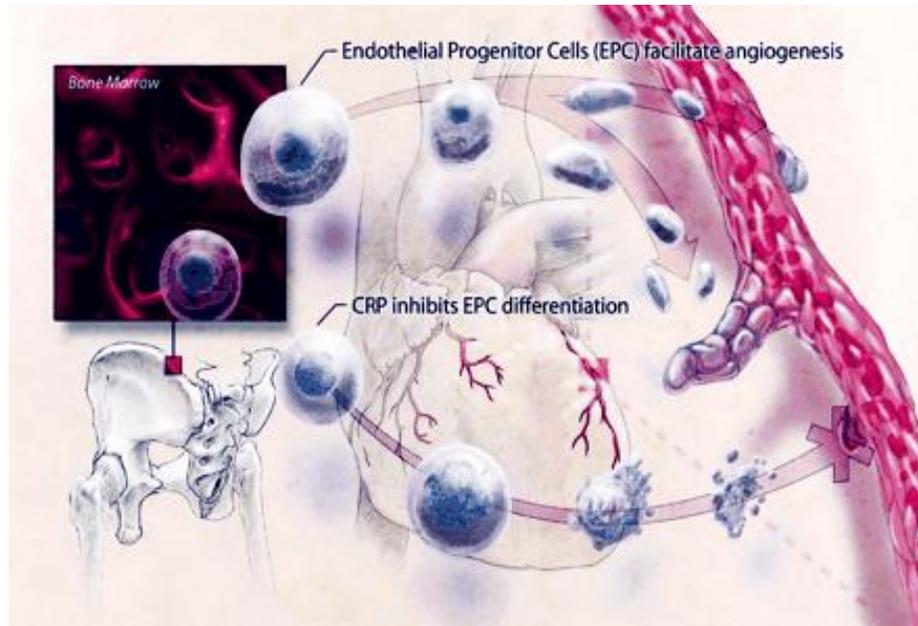


Figure-1.8: Bone marrow–derived EPCs contribute to postnatal neovascularization. CRP, at concentrations known to predict cardiovascular disease, inhibits EPC survival, differentiation, and function, thereby inhibiting an important mechanism of angiogenesis and compensatory response in chronic ischemia.³⁰²

1.10.3 Role of bone marrow derived endothelial progenitor cells

Altered angiogenesis is a key component of the later development of vascular dysfunction and CVD, while the endothelium plays a major role in the development of the vasculature. The adherent endothelium undergoes a continuous renewal from tissue and circulating progenitor cells that originate in the bone marrow. In turn, circulating endothelial cells and microparticles are released from the adherent endothelium.³¹¹⁻³¹⁷ Even hyperglycemia has been shown to alter

angiogenesis in various experimental models,³¹⁸ possibly through decreased proliferation and increased apoptosis of endothelial cells and dysregulation of the angiogenic factor VEGF. Early endothelial dysfunction may well pave the way for later hypertension, as reduced vascular density and increased vascular resistance are considered to be among the early alterations that lead to hypertension³¹⁹.

Ingram *et al*³¹⁷ previously showed that endothelial progenitor cells (EPCs) from the offspring of diabetic mothers display altered angiogenic functions. Such early endothelial dysfunction consists of reduced colony formation and self-renewal capacity and capillary-like tube formation due to reduced proliferation and accelerated senescence.³¹⁷ High glucose concentrations *in vitro* are responsible for similar effects. Concentrations of circulating soluble markers of endothelial function, such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin, have been found to be increased in offspring of type-1 diabetic mothers compared with offspring from non-diabetic pregnancies³¹⁸.

Differentiation of mesodermal cells to angioblasts and subsequent endothelial differentiation were believed to exclusively occur in embryonic development. This dogma was overturned in 1997, when Asahara and colleagues³¹⁹ published that purified CD34⁺ hematopoietic progenitor cells from adults can differentiate *ex vivo* to an endothelial phenotype. These cells were named "endothelial progenitor cells" (EPCs) and showed expression of various endothelial markers, and incorporated into neovessels at sites of ischemia. Rafii's group³²⁰ in 1998 also reported the existence of "circulating bone marrow-derived endothelial progenitor cells" (CEPCs) in the adult. Again, a subset of CD34⁺ hematopoietic stem cells is shown to differentiate to the endothelial lineage and incorporated Dil-Ac-LDL, Acetylated Low Density Lipoprotein, labeled with 1,1'-dioctadecyl - 3,3,3',3'-tetramethyl-indocarbocyanine perchlorate. Because CD34⁺ is not exclusively expressed on hematopoietic stem cells but, albeit at a lower level, also on mature endothelial cells, further studies used the more immature hematopoietic stem

cell marker CD133³²⁰ and demonstrate that purified CD133⁺ cells can differentiate to endothelial cells *in vitro*.³²¹

EPCs reflect the phenotype of embryonic angioblasts, which are migratory endothelial cells with the capacity to circulate, proliferate, and differentiate into mature endothelial cells, but which have neither acquired characteristic markers of mature endothelium nor formed lumina.³²¹⁻³²² Although there is abundant evidence of the existence of angioblasts during embryonic development, the isolation and characterization of EPCs from adult circulation has been hampered by the absence of specific endothelial markers and functional assays. However, it is suggested that certain hematopoietic cells are able to incorporate acetylated LDL (AcLDL) and to bind lectins such as *ulex europaeus*, which are usually considered endothelial specific.³¹⁸⁻³²⁰

Several studies have attempted to identify EPCs by performing bone marrow (BM) transplantation studies in which recipient and donor cells could be distinguished by endothelial-specific markers. One study³²¹ has suggested that BM-derived VEGFR2⁺ endothelial progenitor cells could be detected in the peripheral circulation and contribute to vasculogenesis. However, although 27% of freshly isolated CD34⁺ cells were VEGFR2⁺ as determined by flow cytometry, the authors of this study were not able to detect VEGFR2 mRNA in the first 7 days of culture using RT-PCR, perhaps because the isolated cells were not, in fact, of endothelial origin. In subsequent studies, populations of cells enriched for EPCs were derived from murine peripheral blood cells or BM and labelled with DiI to track the mobilization and recruitment of these cells into ischemic limbs.³²²⁻³²⁹ However, given that subsets of hematopoietic cells have the capacity to express CD31, Tie-2, and incorporate AcLDL, it is not clear whether the mobilized cells and the cells incorporated into the vessel wall were of primarily hematopoietic or endothelial origin.³³⁰⁻³³¹

Rafii *et al* have shown that allogeneic sex-mismatched BM transplantation results in the transfer of endothelial cells to recipient dogs.³³² Replacement of the aorta of the recipient dogs months after transplantation with impervious Dacron grafts resulted in graft endothelialization arising exclusively from the transplanted BM. In humans, evidence for EPCs originates from patients implanted with a left ventricular assist device (LVAD). It has been demonstrated colonization of the flow surface of the titanium housing of LVADs with CD34⁺ endothelial-like cells 6 months after the devices were removed³³². These studies suggest that transplanted EPCs residing in the bone marrow have the capacity to be mobilized to the peripheral circulation and incorporate in the sites of active angiogenesis. However, because transplantation of whole bone marrow results in transfer of both mature bone marrow endothelium and putative EPCs, none of these studies have conclusively demonstrated the existence of a phenotypically and functionally distinct population of EPCs. Given the significant contribution of EPCs to vascular homeostasis, it may not be surprising that EPC has emerged as a contributing risk factor to developing adult CVD.

1.11 Therapeutic Strategies to Reverse Early Cardiovascular Disease

The early identification of factors that play a role in preclinical CVD and the understanding of the plausible mechanisms are important to implement strategies for reversing this situation, if possible, in early life

1.11.1 Lifestyle modification

Increased physical activity and fitness are established inverse risk factors for CVD. Exercise training is associated with both enhanced endothelium-dependent dilatation and increased NO production in young men of average fitness.³³³ Cardiovascular fitness is also positively

correlated with endothelial function in males with known CVD.³³⁴ The beneficial effect of exercise can be noted much earlier even by the first decade of life. However, the intensity and frequency of the exercise-training programme should be validated for a long-term clinical benefit. Other lifestyle modifications such as weight loss, reduction in obesity and smoking cessation have also been shown to have a beneficial effect on markers of inflammation and endothelial activation.³³⁵ We recently proposed that pathophysiology of several conditions including CVD is partly attributable to a failure of the cell energy metabolism and that exercise training (ET) improves quality of life (QOL) and is beneficial in terms of reduction of symptoms, mortality and duration of hospitalization³³⁶. However, the mechanisms underlying the beneficial effects of ET are far less understood and do not address the point that if problem arises in development, what stands the role of exercise in adults. Hence this requires further evaluation.

1.11.2 Nutrient supplements—dietary modification

Dietary L-arginine supplementation has been shown to improve endothelial function in the brachial artery of young people with hypercholesterolemia.³³⁷ Other nutrient supplements such as folic acid restore endothelial function in high-risk children for CVD.³³⁸ Although folic acid supplementation is originally related to decreased levels of homocysteine, a novel risk factor for atherosclerosis, further evidence suggests a direct effect of folate on the vascular endothelium.³³⁹ Diets low in fat and high in olive oil, vegetables and fruits equivalent to Mediterranean diet has been recommended to decrease cardiovascular risk. Different components of this diet can also have beneficial effects on endothelial function. Studies have evidenced that lifestyle modification in individuals at increased cardiovascular risk can delay and possibly prevent the progression of atherosclerotic disease.³³⁹⁻³⁴⁰

With regard to antioxidant therapy it has been shown that vitamins C and E restores endothelial function in hyperlipidemic children (EARLY trial)³⁴¹ and has synergistic antioxidant actions in improving endothelial vasodilatory response and decreased serum

inflammatory and thrombotic/fibrinolysis markers.³⁴¹⁻³⁴² Although large randomized clinical trials failed to demonstrate so far a beneficial effect of antioxidant vitamins for disease prevention and treatment of late CVD stages, the above mentioned positive results derived from observational studies provide some evidence for a potential role of antioxidants in the early phase of the disease.³⁴³

However, lifestyle modifications/ interventions alone may be partially effective in that those who are already affected by inappropriate intrauterine conditions or PARs and may be difficult to manage with lifestyle interventions in adulthood. Our proposed model suggests that improving maternal and fetal health will allow humans to cope better with current postnatal nutritional conditions- conditions that we did not evolve to inhabit.

1.11.3 Pharmacological interventions- Statins

There is emerging evidence that hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor (Statin) treatment improves lipoprotein profile towards more physiological levels and restores endothelial function.³⁴⁴ Landmark clinical trials with pravastatin (WOSCOPS) and simvastatin (4S) demonstrate that these statins markedly decrease serum cholesterol levels and reduce the incidence of myocardial infarction and also cardiovascular mortality in primary and secondary prevention in individuals.³⁴⁵⁻³⁴⁶ Additionally, several other trials such as AFCAPS/TEXCAPS and LIPID report that other statins also had similar beneficial effects.³⁴⁷⁻³⁴⁸ In these studies, Lovastatin reduced the risk for the first acute major coronary event in men and women with average total and low-density lipoprotein (LDL)-C levels and below-average high-density lipoprotein (HDL)-C levels. While it is crucial to achieve lipid goals, it is also important to realize that half of all acute MI occur in patients with normal lipid levels.³⁴⁹ Emerging work in the literature cues to the beneficial effects of statins in the prevention of myocardial infarction independent to their effect of reducing serum cholesterol levels.³⁴⁹

Other pleiotropic effects of statins other than lipid lowering are speculated. A non randomized cohort of patients with CVD demonstrates that statins decrease plasma levels of CRP.³⁵⁰ Other effects includes improvement of endothelial function, reduction of pro-inflammatory events such as a decrease in monocyte adhesion and infiltration, and increases plaque stability.³⁵¹⁻³⁵² A number of studies described other properties of statin drugs that possibly contributed to their pleiotropism and interaction with the ubiquitin–proteasome system.³⁵³⁻³⁵⁵ Statins are shown to reduce atherogenic plaques *in vitro*³⁵⁶, scavenges free radicals and inhibits lipid peroxidation.³⁵⁷⁻³⁵⁸ Wiegman and co-workers demonstrated earlier that long-term pravastatin therapy induced a significant regression of carotid atherosclerosis in children with family history.³⁵⁹ In addition, statin therapy had promising short-term efficacy and reassuring safety in terms of changes in hepatic and muscle enzymes in children.³⁵⁶ Although statin therapy is not yet the recommended therapeutic approach for children with familial hypercholesterolemia, it might prove a reasonable treatment policy considering the limited window for cardiovascular prevention in this high-risk group of children. Inhibitors of cholesterol absorption had also been tested in children with familial hypercholesterolemia with a significant reduction in the levels of LDL cholesterol.³⁴⁴

First evidence for potential pharmacological modulation of systemic EPC levels by atheroprotective drugs came from studies using statins. Statins are shown to increase the number and the functional activity of EPCs *in vitro*, in mice, and in patients with stable CAD.³⁵⁹⁻³⁶¹ The increase in EPC numbers was associated with increased bone marrow-derived cells after balloon injury and accelerated endothelial regeneration.³⁶²⁻³⁶³ Although statins are shown to increase the number of stem cells within the bone marrow, the mechanism for enhancing EPC numbers and function may additionally include an increase in proliferation, mobilization, and prevention of EPC senescence and apoptosis.³⁶¹⁻³⁶⁴ The molecular signalling pathways for such an effect have not been identified so far. However, several studies indicate that the activation of the PI3K/Akt pathway, which has first been shown to be activated in mature endothelial cells by statins,³⁶⁵⁻³⁶⁶ may also play an important role in statin-induced increase in EPC levels.^{361, 364}

1.11.4 Therapeutic potential of endothelial progenitor cells

It is now well established that endothelial dysfunction underlies all of the major CVD process. Pathologic conditions such as hyperlipidemia, hyperglycemia, and hypertension impair the ability of the vascular endothelium to produce vasodilatory and anti-adhesion moieties.³⁶⁶ This increases the production of vasoconstrictor, pro-adhesion, and pro-thrombotic molecules, leading to elevated vascular tone, enhanced cell adhesion, proliferation of media smooth muscle cells, and propensity toward thrombosis.³⁶⁶

Studies have suggested that EPCs originating in the bone marrow play a significant role in endogenous neovascularization of ischemic tissues³⁶⁷ and re-endothelization of injured vessels.³⁶⁸⁻³⁷¹ In addition, EPC mobilization and proliferation is reported to contribute to the salutary effects of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors and estrogen.³⁷¹⁻³⁷² EPC transplantation has been shown to induce new vessel formation in ischemic myocardium and hind limb³⁷³ and to accelerate re-endothelialization of injured vessels and prosthetic vascular grafts in humans and in various animal models.³⁷⁴ This demonstrates their therapeutic potential as a cell-based strategy for rescue and repair of ischemic tissues and injured blood vessels. Furthermore, EPCs are amenable to genetic manipulation, underscoring their usefulness as vectors for local delivery of therapeutic genes.³⁷³ However, despite the excitement regarding the possible clinical use of EPC, upcoming studies have shown that age and other risk factors for CVD reduce the availability of EPC and impairs their function to varying degrees,³⁷⁴ thus limiting their therapeutic usefulness in these patient populations. Furthermore, the relative scarcity of EPCs and their finite proliferative potential limit the ability to expand these cells in sufficient numbers for some therapeutic applications.³⁷⁵⁻³⁷⁶

1.12 Summary

In summary, epidemiological observations and biomedical evidence discussed so far (sections 1.1-1.5) have focussed on the possible role of early life influences in altering later disease risk i.e. the “developmental origins of health and disease” (DOHaD) paradigm. These observations have led to the recognition of concepts towards our understanding of human disease biology in specific CVD and metabolic syndrome. It is suggested in these reports (sections 1.5.2, 1.5.3 & 1.6) that the DOHaD phenomenon can be considered as a subset of the broader processes of developmental plasticity by which organisms adapt to their environment during their life course. Mismatch between the anticipated and the actual mature environment exposes the organism to risk of adverse consequences—the greater the mismatch, the greater the risk. For humans, prediction is inaccurate for many individuals because of changes in the postnatal environment toward energy-dense nutrition and low energy expenditure, contributing to the epidemic of CVD and metabolic syndrome (section 1.7).

There has been increased attention on the possible association between altered maternal nutrition and cardiovascular or metabolic disease in the offspring. Namely, alterations in fetal nutrition (either under- or over-nutrition) may result during critical periods when offspring are most vulnerable to developmental adaptations that permanently change the structure, physiology and metabolism of the offspring, thereby predisposing individuals to metabolic and cardiovascular diseases in adult life. Today the most common maternal dietary imbalance in populations is an excessive intake of dietary fat. There is growing body of evidence that significant health problems for women of reproductive age result from being overweight or obese due to overeating. Upcoming studies to some extent have shown that maternal overnutrition retards placental and fetal growth, and increases fetal and neonatal mortality in animal models. As described in the earlier paragraphs, results of recent epidemiological studies indicate that almost 65% of the adult population in the U.S. is overweight [defined as a body mass index (BMI) $> 25 \text{ kg/m}^2$], while 31% of the adult population is obese (defined as BMI $> 30 \text{ kg/m}^2$). Many overweight and obese women unknowingly enter pregnancy and

continue overeating during gestation. These women usually gain more weight during the first pregnancy and accumulate more fat during subsequent pregnancies. Maternal obesity or overnutrition before or during pregnancy may result in fetal growth restriction and increased risk of neonatal metabolic syndrome and cardiovascular risk factors (sections 1.8-1.9).

Previously, studies have demonstrated abnormalities in plasma lipids, vascular fatty acids, and evidence for reduced endothelium-dependent relaxation in adult offspring of rodent models fed a lard-rich diet during pregnancy, suckling or lactation. However neither the design of these studies carried out nor the fat intake mimic the typical high-fat Western diet and human situation. Although the epidemiological associations are strong, a mechanistic understanding is essential in order to demonstrate that these associations are causal and in what way can be identified for the potential of intervention. Mechanistically and experimentally, there is an increasing body of knowledge showing that manipulation of the environment in the period extending from conception to infancy can be associated with permanent changes in physiology and/or structure. In turn, many of these changes are associated with permanent alterations in gene expression regulated by epigenetic factors such as DNA methylation and histone methylation/acetylation. Moreover, to date no one has determined the role of early pharmacological intervention in mothers and its effects on offspring in terms of cardiovascular control using the animal model. In this thesis, it is proposed that until more insights on the pathogenic mechanisms associated with high fat high cholesterol during pregnancy have been gained, an intense understanding into the influence on early CVD risk in offspring (in their adult life) from such high fat high cholesterol mothers is difficult; let alone the interventions and which critical window when is best suited (section 1.8).

The proposed model will change perspectives on how to intervene in the “life-style” disease epidemic. It shows that lifestyle interventions alone may be only partially effective in those who are most affected by inappropriate PAR and may be difficult to manage with life-style interventions in adulthood. The model suggests that improving maternal and fetal health will allow humans to cope better with current postnatal nutritional conditions—conditions that we

did not evolve to inhabit. If high fat environment is the core process underlying such programming in today's world, then priority should be given to understanding what changes occur, when they occur, the key mechanisms involved, and whether there are windows of opportunity during which the effects can be reversed (section 1.11). The whole discussion presented here and the proposed model are summarised in Figure 1.9.

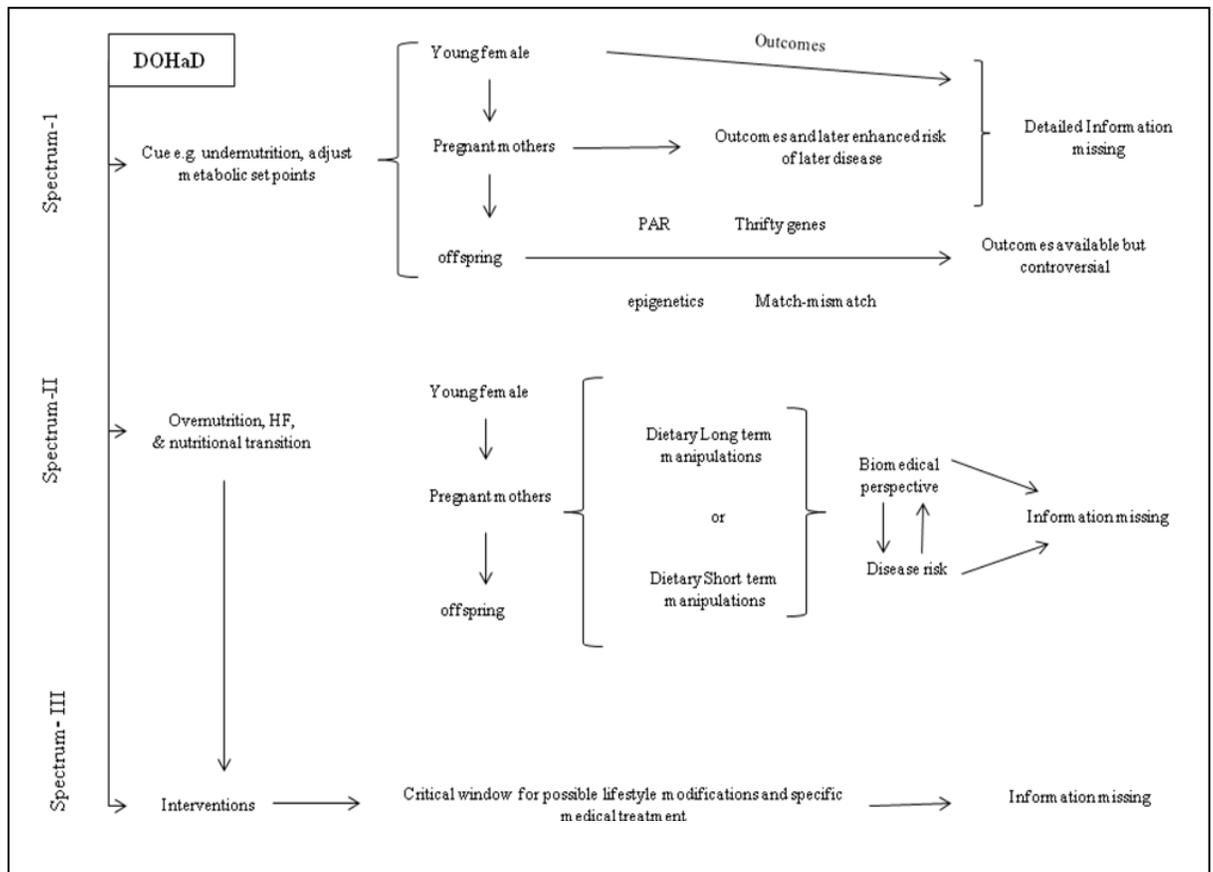


Figure-1.9: A model that demonstrates the effect DOHaD paradigm representing three spectrums. Spectrum-I demonstrates the undernutrition limb of the DOHaD, where considerable yet controversial amount of information is available. Spectrum- II characterizes the high fat (HF) overnutrition environment and its effect on short and long term adaptations of the offspring and then the adult in clinically contracting the disease (CVD and metabolic syndrome). This information with regard to biomedical perspective and disease risk is either still scarce or missing. Spectrum- III represents the arm of interventions and which critical window is the best opportunity to combat this epidemic of CVD and metabolic syndrome. Again, the information is missing in the published literature so far.

In populations of the world that are undergoing the nutritional and epidemiologic transition to Western style of diet, sedentary behaviour, obesity and chronic diseases, the ominous pattern that Baker *et al* identify- the lower birth weight followed by excess weight gain in childhood- is both common and liable to persist for the foreseeable future. It is therefore imperative that along with vigorous efforts to optimise childhood growth, researches and policymakers identify, quantify and evaluate strategies to modify prenatal and perinatal determinants of adverse adult health outcomes.

1.13 Hypotheses

Based on the discussions and spectrums presented in section 1.12 and Figure 1.9 respectively, the main hypotheses of this thesis are;

1. Does long-term prenatal and postnatal high fat nutritional environment induce metabolic and cardiovascular disease in offspring later in its adult life?
2. Does early pharmacological intervention in mothers consuming a high fat diet exert beneficial effects in preventing metabolic and cardiovascular disease in the adult life of the offspring?

1.14 Aims

1.14.1 General Aims

The thesis consists of small animal (rodents) based laboratory investigations. The primary focus of this work is to investigate the long term effects maternal high fat overnutrition may exert in their adult offspring both in prenatal and postnatal environments. The thesis also seeks to explore the effects of high fat rich in cholesterol nutrition in dams. The underlying mechanisms by which these effects play a role on biochemical and cardiovascular patho-

physiology including liver, kidneys, blood and bone marrow derived endothelial progenitor cells are studied. Paying particular attention to a possible critical window for lipid lowering pharmacological intervention in mothers, the thesis also aims to address the effect of short and long term maternal treatment on biochemical and vascular physiology of their offspring in preventing metabolic and cardiovascular disease in offspring later in adult life.

1.14.2 Specific Aims

1. To investigate whether long-term maternal high-fat (HF) feeding during pregnancy and lactation predisposes offspring to hypertension, raised plasma lipids and fatty liver in mice.
2. To investigate whether there are additive effects of feeding the offspring a HF diet from weaning to adulthood.
3. To study whether lipid lowering pharmacological intervention with statin in late pregnancy in HF fed dams reduces cardiovascular and metabolic risk factors in their offspring.
4. To investigate whether there are any sex differences in the offspring from these statin-treated HF-fed dams in their cardiovascular and metabolic responses to post-weaning HF feeding.
5. To study whether statin administration to female mice from the time they were weaned until weaning of their offspring reduces cardiovascular risk factors in these offspring, even if offspring are fed a high fat diet
6. To study the expression of EPC in bone marrow cultures from the female offspring.

In addition this study will also assess, and investigate the biochemical variants involved, by various biochemical and molecular biological techniques. The plan of investigations and laboratory techniques are therefore discussed in Chapter-2.

*Just as the highest tower needs a deep foundation,
so too the highest thinking is based on going deep within.*

CHAPTER 2

General Methods

2.1 Animal Model

Animal models provide a direct approach to determining possible mechanisms involved in disease processes. They are advantageous in that they allow the study of the interaction between physiological and genetic mechanisms during the animal's lifespan. Furthermore they also allow the study of intergenerational effects, for example, induced vascular defects have been shown to be transferred from the mother to the next generation in rats.³⁷⁷ Rodent models are widely used, and many experiments examining the DOHaD phenomenon have used these models. Rodents are advantageous in that they have a short gestation period and a relatively large number of offspring in which to study any effects. Furthermore, the rodent genomes have been widely studied and the genome sequences for mice and rats have recently been completed. However comparisons to humans must be made carefully as we have a much longer gestation period and fewer offspring. What is more, the brains of mice and rats are not fully developed at birth but continue to develop during the first few postnatal weeks, unlike humans whose brains are more fully developed at birth. Hence any effort to compare these studies and to apply them clinically must take into account interspecies differences in the stages of brain development occurring before and after birth. For example, the rat is an altricial species, born with the thyroid- hypothalamic- pituitary axis not yet fully matured. The sheep, in contrast, is a precocial animal born with the development of the thyroid-pituitary axis nearly complete at birth³⁷⁸.

The human at birth is somewhat intermediate between the rodent and sheep in that it has a relatively immature central nervous system but a more fully developed thyroid hormone axis. The importance of such differences in developmental timescales is illustrated by the fact that the majority of experimental studies in the rodent have analyzed the effect of thyroid hormone deprivation in the early neonatal period which corresponds to the late intrauterine phase of development in sheep and humans. Thus, whereas there is evidence of a role for thyroid hormone in brain maturation during the intrauterine period in human³⁷⁹⁻³⁸¹ and sheep,³⁸²⁻³⁸³ this is not yet clearly established in the rodent. Thyroid hormone appears to regulate processes associated with terminal brain differentiation such as dendritic and axonal growth, synaptogenesis, neuronal migration, and myelination. It is for these reasons that sheep models are also of great use for studying DOHaD. Sheep have a longer gestation period (5 months), more similar to humans and also give birth to one or two offspring.

However, this thesis is concerned with the process of rodent development and how it can be affected by maternal and environmental conditions. The processes and mechanisms under investigation in this work are manifested at the level of the 'organism', either before or after *in utero* development. Ethical constraints on experimentation in humans and the absence of suitable alternatives (e.g. computer models) for studying the DOHaD phenomenon, mean that rodent models are vital in the study of developmental physiology and investigation of the mammalian developmental process. Specific to this thesis objectives, I shall be collecting various samples at different stages of development and taking an integrated approach by combining *in vivo* systems physiology with cell/tissue culture and conventional biochemical analyses in order to maximise the amount of information obtained from an individual animal.

I chose rodents as the appropriate animal model for this research program because;

- The overwhelming bulk of our knowledge to date on reproductive and developmental biology in mammals is based on the rodent. Their developmental profile is well established and the periods in which different organs develop are known

- Rodents offer the best “functionally” characterised mammalian model system. In particular the rodent maternal diet model has been firmly established for analysis of mechanisms involved in fetal programming at the laboratory where I have carried out the research work.
- The size of these animals makes them ideal for physiological manipulations.
- They have a high reproductive capacity. They produce many fetuses/ offspring per dam thus reducing the need for a large cohort of mothers per experimental group.
- They have comparatively short generation time and lifespan.
- And they are easy to care for and to handle and are small and inexpensive

I used C57BL/6J mice, purchased from Charles River Labs UK, for my experiments in this thesis. C57BL/6 is the most widely used inbred strain. It is commonly used as a general purpose strain and as a background strain for the generation of congenics carrying either spontaneous or induced mutations. Although this strain is refractory to many tumours, it has a permissive background for maximal expression of many mutations. C57BL/6J mice are used in a wide variety of research areas including cardiovascular biology, developmental biology, diabetes and obesity, genetics, immunology, neurobiology, and sensorineural research. C57BL/6 mice breed well and are long-lived. Other characteristics include;³⁸⁴⁻³⁸⁵

- 1) A high susceptibility to diet-induced obesity, type-2 diabetes, and atherosclerosis.
- 2) A high incidence of microphthalmia and other associated eye abnormalities.
- 3) Resistance to audiogenic seizures
- 4) Low bone density
- 5) Hereditary hydrocephalus (early reports indicate 1 - 4 %)
- 6) Hair loss associated with overgrooming
- 7) Preference for alcohol and morphine
- 8) Late-onset hearing loss
- 9) Increased incidence of hydrocephalus and malocclusion.

The first characteristic on this list is critical to this thesis. C57BL/6J mice when fed a HF diet develop obesity, hyperglycemia, and hyperinsulinemia. For example, feeding these mice an atherogenic diet (1.25% cholesterol, 0.5% cholic acid and 15% fat) for 14 weeks led to development of atherosclerotic lesions in the aorta ranging from 4500 to 8000 μm^2 in cross-sections.³⁸⁴⁻³⁸⁷

2.2 The High Fat High Cholesterol Diet Model

All animal procedures carried out in this study are in accordance with the regulations of the British Home Office Animals (Scientific Procedures) Act, 1986. The protocols of the experiments are described below;

2.2.1 Animal husbandry and experimental diet

Virgin female C57BL/6N mice (supplied by Charles River Labs UK) at 4 weeks of age and weighing approximately 8 to 10 gm were fed *ad libitum*, before mating and through to pregnancy and lactation, either a control diet of standard laboratory chow (C) or a high fat-high cholesterol (HF) experimental diet supplemented 18% wt/wt with animal lard with additional vitamins, protein, choline and minerals to correct for the dilution (Rat and Mouse Diet no.3, Special Diet Services, Witham, Essex, UK)³⁸⁸ as shown in Table-2.1.

Table-2.1: Composition of diets

Components (wt/wt)	Standard Laboratory Chow Diet (C)	High-Fat High Cholesterol Diet (HF)
Protein	21.2	20
Fat	5.3	45
Carbohydrate	49.2	35
Fibre	4.65	24.3
Ash	6.0	0
Moisture, Minerals & Vitamins	10.1	8.2

Maternal weight and food intake were measured twice a week. The females were time mated with in-house males and the day of vaginal plug detection was defined as day 0 of pregnancy. After confirmation of pregnancy, dams were housed individually in standard mouse cages containing wood shavings as bedding and were given experimental diet each day throughout pregnancy, with free access to water. This amount of the diet consumed had been shown to be approximately 10 grams above pregnant dam's average daily food intake and could therefore be considered *ad libitum*. Prior to any new food being placed in the cages, all uneaten food was removed and weighed. All mice were kept in the same room with constant temperature maintained at 25°C and a 12:12-h light-dark cycle.

The dams were allowed to deliver naturally at term (approximately 21 days). After birth, pups were weighed and litter size recorded. Two days postpartum (to avoid rejection) pups were sexed and to standardise litters, culled to eight by cervical dislocation, leaving four male and four female offspring where possible. The offspring were weaned from their mothers at 21 days of age and separated into male and female cages when each mouse was given an

individual identification number. After weaning offspring of HF and C dams were fed either HF or C diets themselves, as shown in Figure-2.1.

The adult offspring born to HF dams are referred as HF/HF and HF/C according to the post weaning diet. Similarly adult offspring born to C dams are referred to as C/HF and C/C according to post-weaning fed diet. Food intake and animal weights were recorded from 1 week of age (to avoid maternal rejection of the pups) until animals were fully grown. At the time of the sampling, animals were euthanized by isoflurane inhalation and cervical dislocation. This was at an average age of 36 days depending on the studies, as described later in the thesis. Blood was collected by cardiac puncture and liver tissue was dissected and transferred to 10% neutral buffered formaldehyde and stored for further histological analyses. Other maternal and offspring samples including heart, lung, kidneys, adrenals, pancreas and adipose tissues were dissected, weighed, snap frozen in liquid nitrogen and stored at -80°C for further analysis.

2.2.2 Assessment of offspring blood pressure by tail cuff method

Blood pressure (BP) was measured by tail-cuff plethysmography using an IITC blood pressure monitor (model BP 2000 Blood Pressure Analysis System, Visitech Systems; Apex, NC). This was based on a method of Krege *et al.*³⁸⁹ and the manufacturer's instructions. For 5–7 days, mice were acclimatized to restraint and tail-cuff inflation. The restraint platform was maintained at 33–34°C. In order to achieve vasodilatation of the tail artery, mice were allowed to acclimatise in their cages in a room maintained at 28°C for two hours and then settled into a Perspex tube, (Figure 2.2a). To minimise the stress incurred, the tube was covered by a paper towel and then a suitably sized cuff was fitted over the tail. The cuff was inflated to 200 mmHg and deflated over a period of 10 seconds. Systolic blood pressure was then calculated (Figure 2.2b).

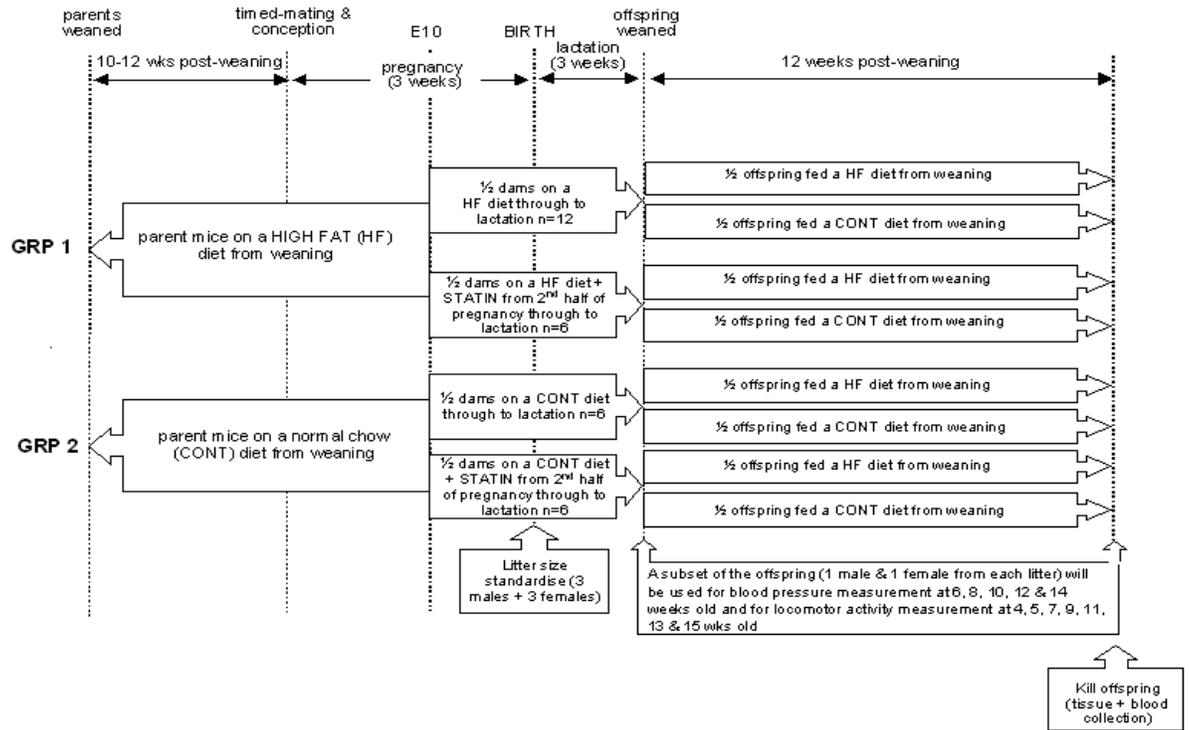


Figure-2.1: Flow diagram of the experimental protocol (please note that for practical reasons the experiments are staggered). Symbols in the parenthesis indicate breeding animals drawn from the stock colony as explained in the text.

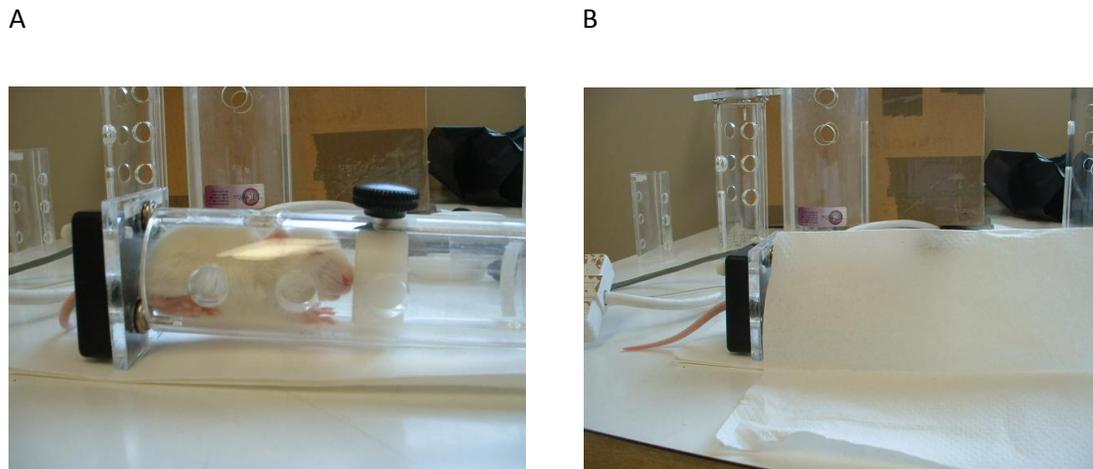


Figure- 2.2: (a) 28 day old mice inside Perspex tube with tail cuff around tail. (b) Perspex tube is covered in paper towel to make mice feel more secure and calm during blood pressure measurement.

For each session, the mouse was placed in a plastic restraint tube with its tail passing through the optical sensor and compression cuff and taped to the platform. A tail-cuff occluder was placed proximal on the mouse's tail. Pressure was then released at a rate of 6 mmHg per second and systolic blood pressure determined during deflation by use of IITC acquisition software (Figure 2.2c). On inflation, the occluder stopped blood flow through the tail, and on deflation the return of blood flow was detected by the sensor.

An initial series of inflation-deflation cycles was used to set amplifier and instrument levels. Blood pressure measurement was repeated five times for each animal with a total recording time per mouse of approximately 15 minutes. To control for the reproducibility of the method, measurements were taken until 3 similar readings were obtained for each animal. The experimenter was blinded to the groups to reduced intra-observer error. Day to day measurements produced a coefficient of variation equivalent to 7.5 %. The same subgroup of mice was measured at each age point.

In my preliminary experiments, I passed the tail through a cuff (13 mm long, with a 9-mm diameter) and immobilized it by adhesive tape in a V-shaped block between a light source above and a photoresistor below the tail. Evaluated photoelectrically, blood flow in the tails produced oscillating waveforms that were digitally sampled 200 times per second per channel. The waveforms, displayed in real time on a monitor, were computer analyzed before and during a programmable routine of cuff inflation and deflation. Programmable functions available by drop-down menu included:

1. The number of waveforms analyzed to identify the amplitude and heart rate before each cuff inflation,

2. Number of preliminary unrecorded measurements

3. Number of recorded measurements per session.

C

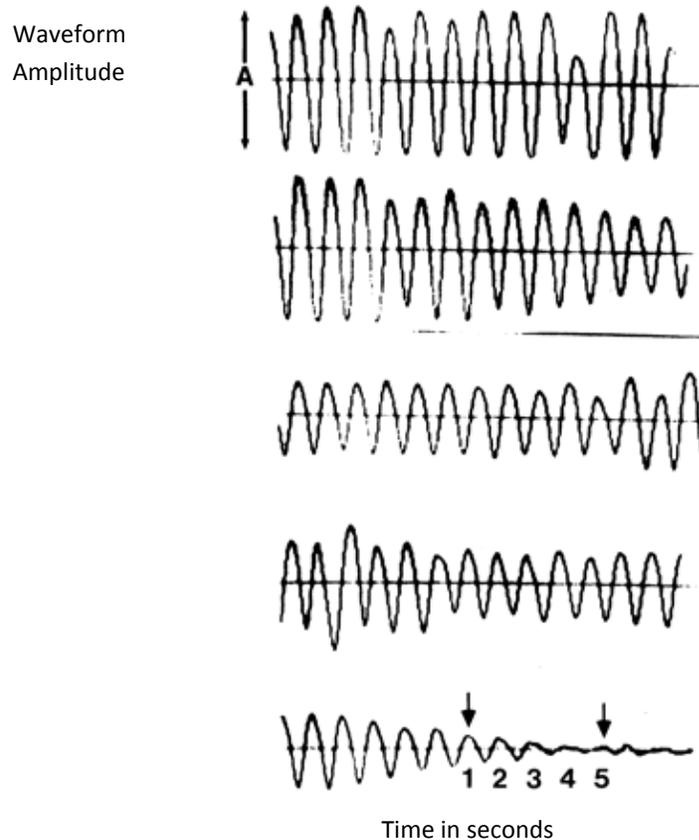


Figure 2.2c: Consecutive reading of representative flow waveforms shown during blood pressure determination. The system first determines full waveform amplitude (A, top) and then seeks waveforms having amplitudes less than 20% of A (numbered in the bottom waveform). Note that the waveform present at the second arrow is indistinguishable from baseline noise; therefore, the cuff pressure at this point (when five consecutive waveforms are less than 20% of A) accurately represents blood pressure

The software assigned BP values from a further set of programmable parameters. Tail-cuff BP was defined as the cuff inflation pressure at which the waveform amplitude fell below a programmable percentage, p , of its original amplitude for a specified number, n , of waveform

cycles. Adjustment of these parameters allowed me to determine systolic blood pressures without interference from background noise. In Figure 2.2 C, I show a representative waveform and decay and illustrate here a BP determination when p is 20% and n is 5—the values chosen for my preliminary study. If the system was unable (before a cuff pressure of >200 mm Hg is reached) to identify a waveform decay (usually because of excessive movement of the mouse), the computer recorded "systolic time-out" for the measurement. Care was taken to make sure that systolic time-out did not happen routinely. Owing to the stressful nature of the restraint tube, the tube was covered to minimize the stress and time was allowed for the rats to acclimatise before any measurements were made.

2.2.3 Open-field activity testing protocol

The open field test is now one of the most popular procedures in animal psychology.³⁸⁹ Voluntary locomotor activity was assessed in the offspring at predetermined times during the peripubertal period and in adulthood. This was done using a behavioural testing apparatus consisting of transecting photon beams. Behaviour was measured in terms of distance travelled, stereotypic movement, ambulatory time and time spent resting. Different versions are available, differing in shape of the environment (circular, square or rectangular), lighting (lighting from above with a bulb above the open field or lighting from underneath with a bulb placed under a transparent floor, sometimes using red light and presence of objects within the arena such as platforms, columns, tunnels.³⁹⁰ The procedure generally involves forced confrontation of a rodent with the situation. The animal is placed in the centre or close to the walls of the apparatus and the following behavioural items are recorded for a period ranging from 2 to 20 min (usually 5 min): horizontal locomotion (number of crossings of the lines marked on the floor), frequency of rearing or leaning (sometimes termed vertical activity) and grooming (protracted washing of the coat). In such a situation, rodents spontaneously prefer the periphery of the apparatus to activity in the central parts of the open field. Indeed, mice and rats walk close to the walls, behaviour called thigmotaxis. Increase of time spent in the central part as well as of the ratio central/total locomotion or decrease of the latency to enter

the central part is an indication of anxiolysis. Some authors use a procedure in which the animals are allowed free access to the open field, from a familiar cage.³⁹¹

Behaviour in the open field measurement technique allowed me to measure the different variables such as distance, time taken to cover specific distance and other episodes of activity. The computerised software gave these activities a mathematical number at predetermined times. I must thus emphasize there is a possibility of misinterpretation of data related to effects of some treatments on the sensory characteristics of the animals. Behaviour of mice in the open field depends mainly on the tactile sensory factors.

I used the standard protocol.³⁹² I placed the mice individually into brightly lit automated activity cages equipped with rows of infrared photocells interfaced with computer (San Diego Instruments, San Diego, CA) as described previously.³⁹²⁻³⁹³ I used a 100 X 100-cm arena with 45-cm- high walls constructed of four pieces of grey Trovicel plastic. I defined a minimum displacement of 2.5 cm to constitute a change in location, a minimum angle of 30⁰ to constitute a turn, and a 3-cm-wide wall zone. After a 1-min adaptation period, open field activity was recorded for 3-min. Within a measurement day, I assigned randomly the order of the mice. At the start of each trial, a mouse was grasped by the tail and placed in the centre of the arena. The arena floor was wiped with a moist sponge (1mM acetic acid) between trials and allowed to dry before the next trial, as is standard protocol in open-field testing. Recorded beam breaks were used to calculate active times, path lengths, rearing times and rearing events. An example is presented here to show the behavioural open- field measurements in two mice on two different diets over 3 minutes. The measurements are single runs (Table 2.2).

Table-2.2:

Variables	C/C mice	HF/HF mice
Distance (mm)	662.45	440.37
Time stereotypic	0.54	0.42
Stereotypic counts	732	522
Average Velocity	29.25	27.85
Jump Counts	9	4
Ambulatory counts	442	235
Episodes	35	19
Time Resting	1:36	2:01

2.3 Blood Measurements

2.3.1 Lipid profile

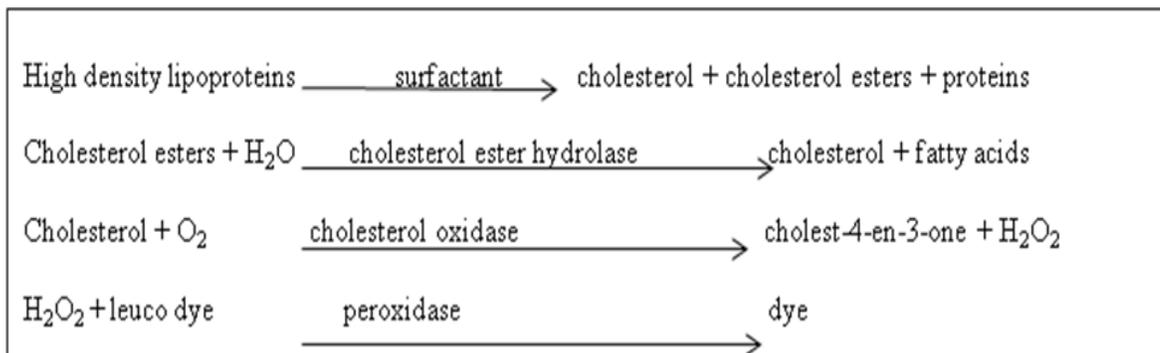
I drew blood samples by direct heart puncture after terminally anaesthetizing the animal with isoflourane. I followed the previously reported study³⁹⁴ for measuring serum cholesterol, LDL-cholesterol and HDL-cholesterol with commercially available kits (Vitros Products, NY, USA) using enzymatic methods and measured by reflectance spectrophotometry.

a) HDL cholesterol analysis

I used the VITROS Magnetic HDL-Cholesterol Reagent and VITROS CHOL Slides that quantitatively measure HDL cholesterol (HDLc) concentrations in serum and plasma. I prepared the samples for analysis of HDL cholesterol with VITROS Magnetic HDL-Cholesterol Reagent before measuring by the VITROS CHOL Slide.

The principle of this assay is that the reagent separates HDL by precipitating LDL and VLDL using dextran sulfate (MW 50,000) and magnesium chloride.³⁹⁵ The reagent also contains iron particles that are coated with polymer. Once the drop of pretreated sample is deposited on the slide, iron particles in the reagent then enhance the capture of the non-HDL lipoproteins when a magnetic field is applied on the slide. HDL cholesterol undergoes reactions to produce hydrogen peroxide. Hydrogen peroxide reacts with a leuco dye to produce a blue dye complex. After a fixed incubation period of 5 minutes at 37⁰C, the amount of colorimetric light reflected from the dye at a detection wavelength level of 670nm, gives the amount of HDL cholesterol present in the sample.

In summary, the reaction sequence at a sample drop of 10µL is described as follows;



I then turned my attention to the untreated sample, diluted with an equal volume of VITROS 7% BSA Diluent and pipetted 500 µL of serum into the sample tubes. The reagent was swirled and 100µL of it was added. I immediately vortexed the mixture for 5 to 10 seconds, placed the sample tube in the test tube rack and incubated for minimum of one minute. Following the incubation, the sample tube was left for three minutes to check the tube for a clear supernatant as shown in Figures 2.3a & b.

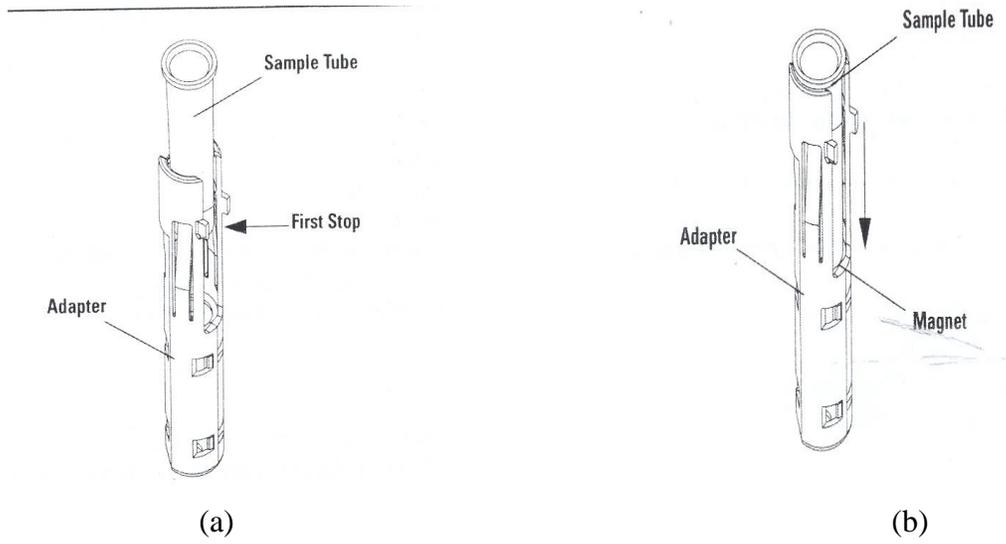


Figure 2.3a & b: Sample Tube at first stop and sample tube on magnet. (derived from VITROS Products operating manual).

The lower limit of the reportable (dynamic) range is 3.0 mg/dL (0.08 mmol/L). The plot in Figure 2.3c shows a comparison of this method analyzed on the VITROS system with CDC Dextran Sulfate/ Enzymatic reference method,³⁹⁶ using pretreated serum specimens.

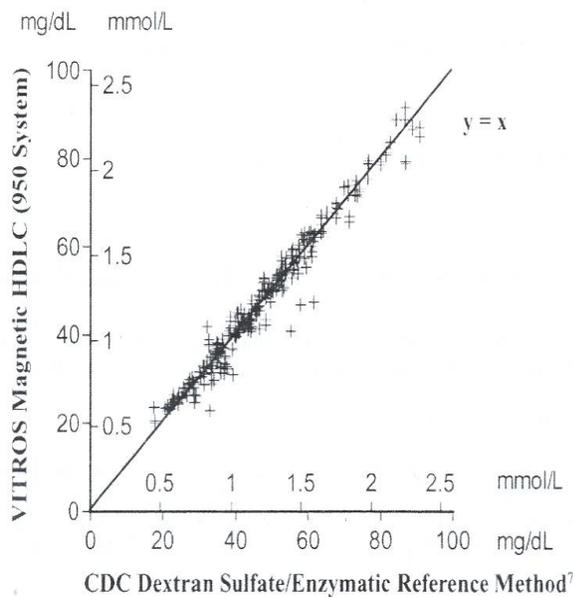
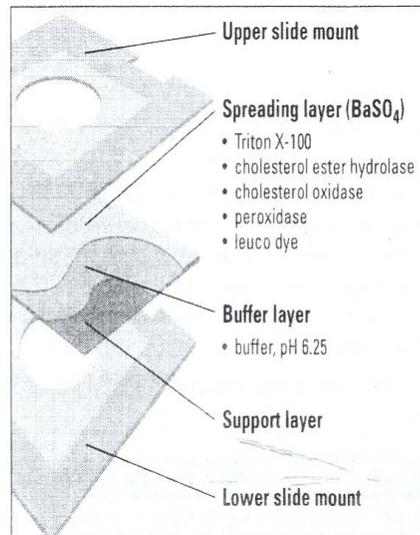


Figure 2.3c: The plot shows a comparison of the VITROS Magnetic HDL- Cholesterol Method analysed on the VITROS system with CDC Dextran sulphate/ enzymatic reference method. Cholesterol in duplicate dextran

sulfate-Mg²⁺ supemates of 48 subjects, as assayed by the Lipid Research Clinic assay with Liebermann-Burchard reagent (x) and the enzymic assay (y) as described. The *dotted line* indicates the line of perfect agreement, $y = x$. The *solid line* illustrates the relationship between the two cholesterol assays by linear regression: slope 0.958, y-intercept 9.2 mg/L, and $r = 0.988$. [Ref=395]

b) Cholesterol measurements

The measurement of cholesterol is based on an enzymatic method.³⁹⁷ I deposited a drop of sample on the VITROS CHOL Slide (dry, multilayered, analytical element coated on a polyester support). The Triton X-100 (TX100) surfactant on the slide aided in dissociating the cholesterol which was then acted on by cholesterol ester hydrolase. Free cholesterol was then oxidized in the presence of cholesterol oxidase to form cholestenone and hydrogen peroxide. Finally I added the leuco dye that oxidized hydrogen peroxide in the presence of peroxidase to generate coloured dye (Figure 2.3d). The density of dye formed is proportional to the cholesterol concentration present in the sample and is measured by reflectance spectrophotometry at a wavelength of $540\pi\text{m}$ at 37°C . The sample drop volume is usually $5.5\mu\text{L}$.



(d)

Figure 2.3d: Slide diagram used for measuring cholesterol. As shown the slide components are upper slide mount, spreading, buffer and support along with lower slide mount (Derived from VITROS Products operating manual).

(e)

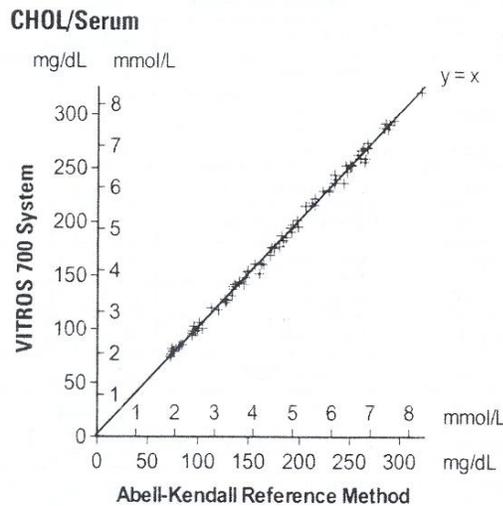


Figure 2.3e: The plot shows the results of a comparison of serum specimen analysed on the VITROS system (y) with those analysed using the Abell-Kendell reference method (x). (derived from VITROS Products operating manual). The *dotted line* indicates the line of perfect agreement, $y = x$. The *solid line* illustrates the relationship between the two cholesterol assays by linear regression: slope 0.958, y-intercept 9.2 mg/L, and $r = 0.988$.

The lower limit of the reportable dynamic range is 50mg/dL (1.29 mmol/L; 0.50g/L). The plot in Figure 2.3e shows the results of a comparison of serum specimens analyzed on the VITROS system with those analyzed using the Abell-Kendell reference method³⁹⁸.

c) Triglyceride measurement

The analysis for triglycerides is based on an enzymatic method as described by Spayd *et al*³⁹⁹. I deposited a drop of sample on the VITROS TRIG Slide (dry, multilayered, analytical element coated on a polyester support). The Triton X-100 (TX100) surfactant on the slide aided in dissociating the triglycerides from lipoprotein complexes present in the sample. The triglyceride molecules were then hydrolyzed by lipase to yield glycerol and fatty acids. Glycerol diffused to the reagent layer, where it was phosphorylated by glycerol kinase in the presence of adenosine triphosphate (ATP). In the presence of L- α -glycerol-phosphate oxidase, L- α -glycerophosphate was then oxidized to dihydroxyacetone phosphate and hydrogen peroxide. The final reaction involved the oxidation of a leuco dye by hydrogen peroxide,

catalyzed by peroxidase. The density of the dye formed is proportional to the triglyceride concentration present in the sample and is measured by reflectance spectrophotometry at a wavelength of 540nm at 37°C (Figure-2.3f).

The volume of the sample drop is usually $5.5\mu\text{L}$ and the reaction sequence is:

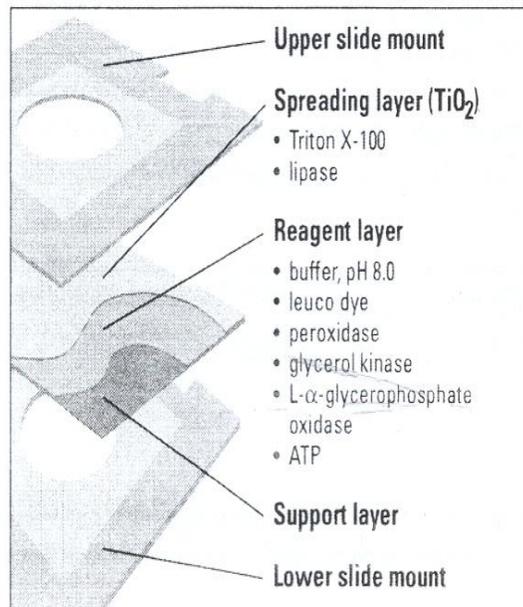
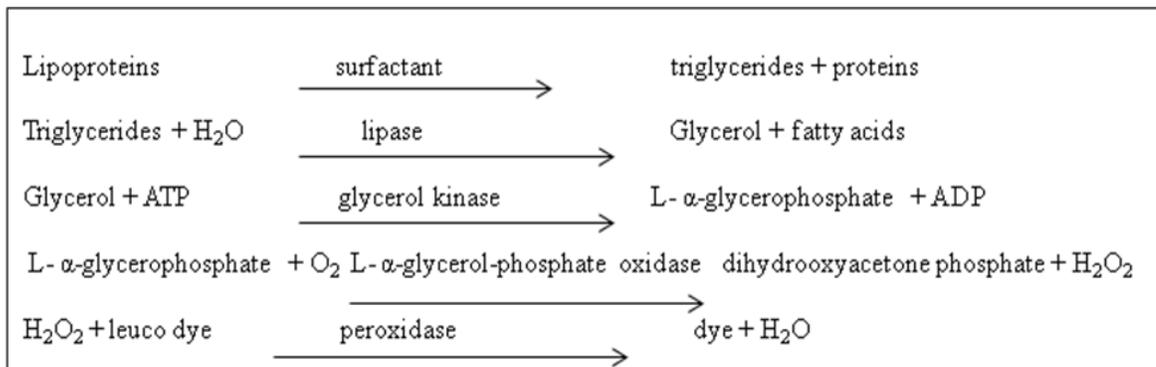


Figure 2.3f: Slide diagram used for measuring Triglycerides. As shown the slide components are upper slide mount, spreading, Reagent and support along with lower slide mount. (derived from VITROS Products operating manual).

2.3.2 C-reactive protein

I measured the CRP using a quantitative sensitive double-antibody sandwich immunoassay enzymatic ELISA using rabbit antihuman CRP and peroxidase conjugated rabbit anti-mouse CRP. In this assay, monoclonal anti-CRP antibody conjugated to horseradish peroxidase (HRP) serves as a signal generator. The assay is linear up to 5mg/l and logarithmic thereafter⁴⁰⁰. I pre-coated the microtiter plate provided in the kit with the monoclonal antibody specific for CRP. I added samples to the appropriate microtiter plate wells and incubated them according to the manufacturer's instructions. If present CRP would bind and become immobilised by the antibody pre-coated on the wells. I thoroughly washed the microtiter plate wells to remove unbound CRP and other components of sample. In order to quantify the amount of CRP present in the sample, I added a standardised preparation of horseradish peroxidase (HRP)-conjugated antibody specific for CRP to each well to sandwich the CRP immobilised during the second incubation. I again thoroughly washed the wells to remove all unbound HRP-conjugated antibodies and added TMB (3,3',5,5' tetramethyl-benzidine) substrate solution to each well with a short incubation period. Only those wells that contain CRP and enzyme conjugated antibody exhibit a change in colour. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the colour change was measured with spectrophotometry at a wavelength of $450\text{nm} \pm 2\text{nm}$. In order to determine the correct concentration of CRP in the samples, the assayed standard kit was used to produce standards.

2.4 Tissue Histology

I performed the microscopic examination on the livers of all animals to investigate the effects of the experimental diets used in my work. I dissected the livers, fixed for 24h in 10% neutral buffered formalin, processed and embedded in paraffin. From fixed liver tissues embedded in paraffin 5-10 micron sections were cut and mounted on glass slides. After deparaffinization, sections were stained with hematoxylin and eosin. Microscopic examination was performed on

stained liver sections from representative animals in each group. This is in accordance with *Ehrlich's* protocol⁴⁰¹ already established in our laboratory. For further details of Ehrlich's Method and preparation of solutions please refer to appendix-i.

2.5 Endothelial Progenitor Cells

2.5.1 Culture and staining

The use of endothelial progenitor cells (EPCs) for angiogenic therapies or as biomarkers to assess cardiovascular disease risk and progression relies on their identification. However, there is neither a uniform definition of these cells nor a method without limitations. At present, bone marrow (BM) is the most common source of cells used for clinical therapeutic strategy⁴⁰².

To date, experimental approaches to produce EPCs mainly involve culture of mononuclear cells (MNCs) on fibronectin in the presence of growth factors in order to bring about differentiation of MNCs into EPCs or mature endothelial cells. The majority of the cultured MNC population remains committed to being leukocyte or lymphocyte lineage and only a very small percentage can be identified as EPCs^{325, 402-403}. Here I adopted and refined a more efficient technique for culturing EPCs from autologous BM. For reagents and materials used to culture and stain BM, please refer to appendix-ii.

I collected the bone marrow from mice femurs into tubes containing buffered saline (HANKS). I carefully layered 6ml of diluted sample over 3ml lymphoprep in a 15ml centrifuge tube, centrifuged at 800 x g for 20 minutes at room temperature (approximately 20⁰C). While the samples were spun, I coated the wells (Lab-Tek TM II Chamber slide) with the D-MEM/F-12, Dulbecco's modified Eagle medium (400 microlitres (μL)). The plates were left in an incubator

until required for cells (incubation time 30 min for 1hr). I also added a small vial containing the same medium (~ 5mls) in the incubator.

Once the centrifugation was over, I carefully took out mononuclear cells in the interphase and transferred it to another 15ml centrifuge tube. I then mixed the cells (interphase) with 10ml saline buffer and centrifuged at 200 g for 5min at room temperature (25°C) with brakes. This step was repeated twice more. The supernatant was removed and the cells suspended in the preincubated plates. This time, I placed the plates in the incubator for another hour. After an hour, I gently aspirated 200µl out of each well, added 200-300 µl of fresh medium to each well, and placed the slide back to the incubator for further 48-96 hrs. After > 48 hrs, I examined the slide for live cells.

2.5.2 Counting of the cells:

The cells were counted with a haemocytometer, after mixing 10µl of cell solution with 0.4% trypan blue of stock solution under the microscope. I used the following formula for counting:

$$\text{Count of cells} \times 20,000 = \text{number of cells/ml.}$$

2.5.3 Detection of EPCs with double staining

To 500 µl of incubated wells, I added 20-25 ul of acetylated low density lipoprotein labelled with 1,1'-deoctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil-Ac-LDL) stain to culture medium from the stock solution of 10µg/L and gently mixed it once or twice. I then incubated this time for 4hrs at 37⁰C. After 4hrs, the chamber slides were observed under the microscope with green filter for red staining (RITC). Following this step, I removed the media from the wells, washed them with PBS X3 gently, fixed cells in 500µl/well of formaldehyde (3.7%) in PBS on ice for 30 min. I incubated them with UEA (10 µl of UEA in 990ul of PBS (stock 1mg/ml) and added 200µl/ well for 1hr at 37⁰C in incubator. This time the incubation

was without CO₂ and in the dark. Washings were performed for 3 times each 5 minutes in the dark. The slides were mounted on citiflour and examined under UV purple by inverting coverslip on a drop of 90% glycerol and 10% PBS onto a microscope slide.

2.5.4 Phase contrast and fluorescence imaging

Phase contrast and fluorescence images were collected using a Zeiss Axiovert 2 inverted microscope with a 5x CP-ACHROMAT/0.12 NA objective. Images were acquired using a SPOT RT colour camera (Diagnostic Instruments, Sterling Heights, MI) with the manufacturer's software. Composite images were assembled in Adobe Photoshop version 8.0.

2.6 Statistical Analysis

All of the data are expressed as means +/- SEMs. In the female offspring, effect estimates are from a mixed model analysis that considers all of the time points through the study, controlling for the set of dam-pup relationships.

All data was tested for normality using a Kolmogorov-Smirnov test. Subsequently statistical significance of differences between biochemical and biophysical parameters was determined by using 1-way ANOVA followed by the Tukey-Kramer comparisons test. A $P \leq 0.05$ was considered to be statistically significant. All of the statistical analysis was calculated with SPSS 14.0 (SPSS Inc). The initial ANOVA model incorporated sex as an independent variable and, where this showed significance, sexes were analyzed separately. Risk estimates for HF diet when compared with C diet towards developing CVD and metabolic syndrome were obtained with the use of logistic regression analyses.

2.6.1 Power calculations

The statistical power of a null hypothesis (H_o) significance test is the probability that the test will reject H_o when a research hypothesis (H_a) is true⁴⁰⁴⁻⁴⁰⁶. Knowledge of effect size is particularly important for statistical power analysis. Power calculations are performed when setting up an experiment to determine the sample size required in order to test for a significant difference of given magnitude. Thus they are performed to maximise the chance of finding a significant result if indeed it exists. The power calculation is based around two variables, the power ($1-\beta$, typically 80-90%) and the standardised difference (δ/σ , where σ is the standard deviation of the mean and δ the difference between the control and study group considered *a priori* to be meaningful). These numbers are then applied to the nomogram (Figure 2.4) to determine the n number required to demonstrate a given change at suitable power and significance (α). However, as the nomogram shows, calculating the power requires approximating both the expected magnitude of change and the standard deviation around the mean. This can be dealt with either by calculating the power from a preliminary study or the analysis of the first numbers through the study, or alternatively the power may be calculated based on the results of a similar study.

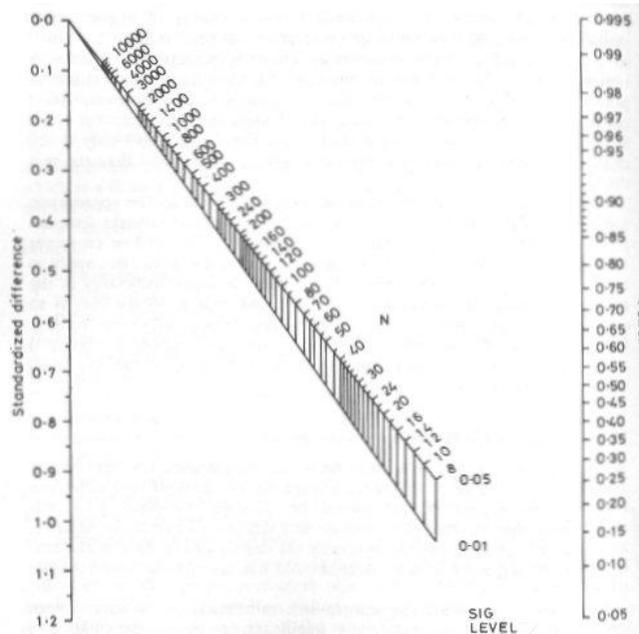


Figure 2.4: Nomogram for calculating sample size or power. From Altman, *Practical Statistics for Medical Research*. Chapman and Hall (Ref 403)

There are many kinds of effect size measures available (e.g., Pearson's r , Cohen's d , Hedges's g), but most of these fall into one of two major types, namely the r family and the d family⁴⁰⁴. The r family shows the strength of relationship between two variables while the d family shows the size of difference between two variables. As a benchmark for research planning and evaluation, Cohen in 1988⁴⁰⁶ proposed 'conventional' values for small, medium, and large effects: $r = .10, .30, \text{ and } .50$ and $d = .20, .50, \text{ and } .80$, respectively (in the way that p values of .05, .01, and .001 are conventional points, although these conventional values of effect size have been criticized; e.g., by Rosenthal *et al.*, 2000⁴⁰⁷). Bonferroni corrections are employed to reduce Type I errors (i.e., rejecting H_o when H_o is true) when multiple tests or comparisons are conducted. Two kinds of Bonferroni procedures are commonly used. One is the standard Bonferroni procedure, where a modified significant criterion (α/k where k is the number of statistical tests conducted on given data) is used. The other is the sequential Bonferroni procedure, which was introduced by Holm⁴⁰⁸ in 1979. A serious problem associated with the standard Bonferroni procedure is a substantial reduction in the statistical power of rejecting an incorrect H_o in each test. The sequential Bonferroni procedure also incurs reduction in power, but to a lesser extent (which is the reason I used the sequential procedure)

The number of mice used in this study was based on my previous preliminary work to assess the power of the study. I conducted the blood pressure measurements in mice with different treatment groups i.e. control (fed on normal laboratory Chow; C); High Fat (HF) and High fat with treatment (Statin; HF+S). The initial results obtained from this work showed systolic blood pressure on average of C = 110 ± 0.16 , HF = 140 ± 0.04 , and HF + statin = 120 ± 0.06 ; $p < 0.05$. A statistical simulation of probable effects brought about by different treatments suggested a minimum n size of 8 mice per group was required to detect 90% power for detecting differences in blood pressure at the 5% level of statistical significance). However, no power calculations were performed on work presented on EPC studies in the offspring. Due to the novelty of the EPC work in the thesis, it is felt that no previous studies are appropriate to

base a power calculation on, and as a result anything gained from such a calculation would be meaningless.

2.6.2 Maternal and offspring data

All values are expressed as mean \pm SEM. Differences between the means of each group were calculated using one-way ANOVA with Bonferroni *post hoc* test to correct for type-1 statistical error.

2.6.3 Systolic blood pressure

All values are given as systolic blood pressure (mmHg) and are expressed as mean \pm SEM. Means were then taken for each group and differences analysed using one-way ANOVA with Bonferroni *post hoc* test to correct for type-1 statistical error.

2.6.4 Offspring weight gain

All values are given as weight in grams and are expressed as mean \pm SEM. Differences between the groups were analysed for each age using mixed analysis, which takes into account the variation both within and between each litter thus minimising any litter effects. Differences between the growth curves of the mean weights of the groups were analysed using time and group as the two factors.

2.6.5 Post-mortem organ weights

All values are given as either weight in grams or organ to body weight ratio and are expressed as mean \pm SEM. Differences between the groups were analysed for each organ, both as a weight and as a ratio to body weight using a mixed analysis, taking age, weight and diet as variable parameters.

2.6.6 Immunohistochemistry

Values are given as percentage of area stained for each protein and expressed as mean \pm SEM for each group. Differences between the means of each group were calculated using one-way ANOVA.

The following sections described in chapters 3 to chapter-6 will now describe the results of the experimental work carried out in this thesis.

My mother is my root; my foundation. She planted the seed in me that I base my life on.

She always advises me

“A bend in the road is not the end of the road....Unless I fail to make the turn”

CHAPTER 3

Effects on Offspring of Long-term Maternal High Fat Feeding from Weaning through Pregnancy and Lactation

3.1 Introduction

Coincident with the rise in obesity internationally, the number of women who enter pregnancy obese has reached an all-time high. Based on the prevalence of obesity among women of reproductive age, it appears that at least one third of all pregnant women in the United States are obese. Data from the Centres for Disease Control and Prevention collected in 2000 showed that 50% of African American women, 40% of Hispanic women and 30% of white women were obese.⁴⁰⁹⁻⁴¹⁰ Many studies of thin, undernourished women have shown that maternal nutrition at conception influences the metabolic response to pregnancy and fetal growth and its development.⁴¹¹⁻⁴¹² Hence a transition from an environment where food is poor/ adequate to one where diet is high in fat and carbohydrates, especially during pregnancy, is associated with higher prevalence of the metabolic syndrome, typified by type-2 diabetes, hypertension, hypercholesterolemia and obesity in their offspring.^{15,207,413-416} Such a 'nutritional transition' to a Western diet may have deleterious consequences on the health of future generations by affecting early development and influencing susceptibility to disease in later life.

Today, there is considerable evidence that undernourished women gain less weight, have smaller increases in basal metabolism, and give birth to smaller babies.^{89,91,94-100,193,195,210} But the number of studies of pregnancy outcomes among women who enter pregnancy with excessive amounts of fat stores is more limited. As the prevalence of obesity increases, it is becoming evident that these women are susceptible to an increased risk of metabolic complications and poor pregnancy outcomes.^{211,416-418} Fetal anomalies as well as deviations in

fetal growth rates are more common among obese compared with normal-weight women, suggesting that maternal adiposity affects development during both the embryonic period as well as later in gestation. The intrauterine effects on fetal growth and development may also affect postnatal development of the child, particularly if fetal growth rates are abnormal. Large-for-gestational age infants are at increased risk for childhood obesity, which can lead to insulin resistance, diabetes, and hypertension later in life.⁴¹⁹ Small-for-gestational age infants born to obese women are susceptible to CVD and diabetes later in life, particularly if their postnatal catch-up growth produces truncal obesity but the child remains short.

Two factors can influence fetal growth and development—genetics and the maternal environment.⁴²⁰ Although genetics influences intrauterine growth rates and development of fetal anomalies, it is unlikely that the increased prevalence of fetal complications among obese mothers is due to genetics alone. It is more likely that the environment of obese mothers interacts with genetic factors to induce changes in fetal growth and development. The fetus “perceives” the maternal environment through signals transmitted by the placenta, such as nutrient transfer, blood hormone or oxygen levels. The fetus uses this information in two ways: (a) to make immediate survival choices and (b) to make longer-term adjustments to maximize its advantage postnatally.⁴²¹ For example, if blood oxygen levels drop, fetal growth rates slow to conserve oxygen. On the other hand, if the fetus experiences a chronically high blood glucose level, it might anticipate being born into a carbohydrate-rich environment and prepare for that situation by synthesizing and secreting more insulin.

Environmental signals to the fetus from obese mothers that influence fetal development include adjustments in the placental transfer of nutrients (e.g., glucose, fatty acids, amino acids), hormones (e.g., insulin, leptin, adiponectin), and, possibly, inflammatory markers. When these metabolic parameters reach abnormal levels, metabolic complications such as gestational diabetes, pregnancy-induced hypertension, and preeclampsia are diagnosed. Although it is well known that diet influences the development of diabetes and hypertension in

nonpregnant adults, the role diet plays among obese pregnant women and their offspring physiology has received little attention.

On the other hand, non-alcoholic fatty liver disease (NAFLD), a common cause of chronic liver disease occurs commonly with metabolic syndrome (central obesity, type-2 diabetes, dyslipidemia and CVD). NAFLD is the subject of substantial research interest, since its incidence in adults and children is rising rapidly along with obesity and type-2 diabetes.⁴²²⁻⁴²⁵ NAFLD is linked to excessive triglyceride (TG) accumulation and encompasses a broad spectrum of liver disease ranging from simple fatty liver (steatosis), to non-alcoholic steatohepatitis (NASH) with fibrosis that may progress to liver cirrhosis and portal hypertension.⁴²⁶⁻⁴²⁷ Recent estimates of prevalence in the USA are 20%-30% for hepatic steatosis, 3.5%-5% for NASH, and up to 80% of people with type-2 diabetes may have some form of NAFLD.⁴²⁷⁻⁴²⁸

It is unlikely that such a high prevalence of NAFLD can be explained by obesity alone, since not all obese individuals develop NAFLD, and not all individuals with simple steatosis progress to NASH. In fact, the pathogenic mechanisms involved in the disease progression from simple steatosis to NASH are still unrevealed. One candidate factor for disease progression is maternal obesity, since one third of women of child bearing age in the US are currently obese.⁴²⁹ Maternal obesity at conception has been shown to alter gestational metabolism and affects placental, embryonic and fetal growth and development.⁴³⁰ I hypothesize that maternal HF feeding can influence the development of hepatic steatosis in adulthood mice.

Developmental induction of cardiovascular and metabolic risk factors has been observed in rodent models in which the pregnant dam is exposed to nutrient restriction and the offspring fed normal chow diet.^{15,232} However, there are fewer studies that examined

adaptive/maladaptive mechanisms during fetal development when pregnant dams are exposed to overnutrition. Several studies, including ours, have shown in rats that a maternal diet rich in fat during pregnancy results in features of the metabolic syndrome such as obesity, sedentary behaviour and vascular dysfunction in the offspring.^{212-213,246-248} However, these studies were confined to short-term modifications in the maternal diet during pregnancy and/or lactation periods alone. Altering the maternal diet during critical periods of gestation^{216,249} or throughout gestation and/or the suckling period^{217,250} results in a varying degree of phenotypic outcomes associated with the metabolic syndrome, suggesting the importance of the timing and duration of the nutritional insult.

In humans, consumption of HF diet just during pregnancy or suckling is not common, but there is increasing concern about the effects of obesogenic diets in children on the health of their future offspring. Hence, the aim of this study is to;

1. Investigate in our animal model, the consequences for their offspring of feeding dams a HF diet from the time that they were weaned, through their pregnancy and lactation until their pups are weaned.
2. Determine whether there are additive effects of feeding these offspring themselves a HF diet from weaning to adulthood.

3.2 Methods

Methodology has been discussed earlier in detail in Chapter-2. Here I summarise the salient points for this specific research work.

1. Four week old virgin Female C57BL/6 mice were randomly allocated to either a control diet of standard laboratory chow (C) or a HF experimental diet. At 10 weeks old the females were time-mated with C57BL/6 males and after confirmation of mating were individually housed

2. After birth, pups were weighed and litter size was reduced to 8 pups and, when possible, to equal numbers of males and females. Following weaning (21 days post partum) offspring from the HF and C dams were fed either HF or C diets. I referred to the offspring born to HF dams as HF-HF and HF-C according to their post-weaning diet. Similarly offspring born to C dams were referred to as C-HF and C-C according to post-weaning diet. The experimental protocol is explained in Figure-3.1.
3. Food intake and body weights were monitored weekly until they reached adulthood. 36 weeks old adult offspring were killed by cervical dislocation and the samples collected (blood, fat depots, and livers)
4. Cumulative fat depot weights for each animal were compared to total body weights and body fat as a percentage of total body weight was calculated.

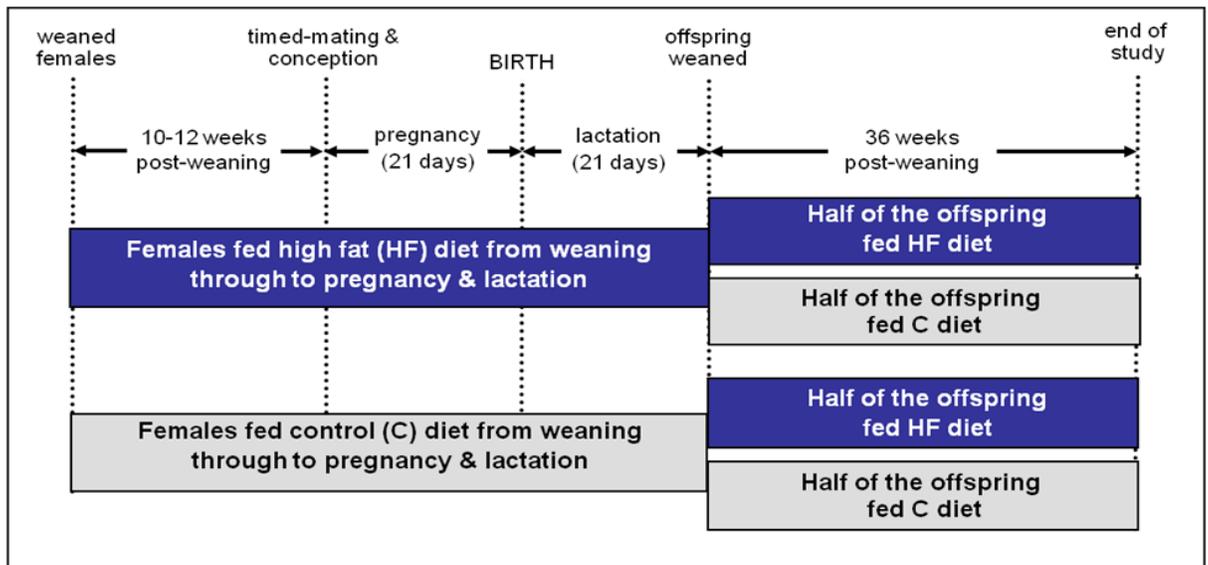


Figure-3.1: Flow diagram of the experimental protocol. Symbols in the parenthesis indicate breeding animals drawn from the stock colony as explained in the text.

5. BP was measured at 13, 18, 23, 27, 30 and 36 weeks post weaning.
6. C-reactive protein (CRP) and total cholesterol were measured in the serum.

7. Fixed liver sections were stained with hematoxylin and eosin (H&E) for visual assessment of steatosis and cellular infiltrate. Alternate sections were stained with Oil-Red-O to visualise lipid accumulation.
8. The Kleiner scoring system⁴³¹ was used to assess the severity of non-alcoholic fatty liver disease (NAFLD). The NASH Clinical Research network Committee⁴³¹ agreed that only H& E and Masson' trichrome stains would be sufficient to perform the evaluation. An activity score (AF) was generated by adding the individual score for necroinflammatory features; Steatosis (<5% = 0, 5-33% = 1, 33-66% = 2, >66% = 3); Ballooning (none = 0, few = 1, prominent = 2); and Lobular inflammation (none = 0, <2 foci = 1, 2-4 foci = 2, >4 foci = 3). A score of <3 correlates with mild NAFLD, a score of 3-4 correlates with moderate NAFLD and a score of 5 or more correlates with NASH. The average (mode) score for each histological characteristic in each group was used.
9. The results were statistically analysed and value of $P < 0.05$ was set as significant.

3.3 Results

3.3.1 Maternal parameters

The body weights of the HF dams were significantly greater at day 7 and day 16 of gestation and also at birth and at weaning (HF study group vs. controls at each stage, $p < 0.05$). Although average daily food intake was significantly reduced in the HF dams during pregnancy [average daily food intake (g) days 0-20, 13.6 ± 0.99 for HF vs. 18.9 ± 0.94 for C dams $P < 0.005$], the higher gross energy value of the HF diet meant that gross energy intake of the HF dams were similar to the C dams [average daily gross energy intake (kJ) 38.9 ± 4.85 for HF vs. 41.7 ± 8.42 , for C dams; $P = 0.436$].

During lactation, gross energy intake in the HF dams was significantly higher compared with C dams [average daily gross energy intake (kJ) 47.3 ± 8.9 for HF vs. 28.8 ± 3.5 for C dams, P

< 0.0001] despite a significant reduction in their average daily food intake [average daily intake (g) during suckling, days 22-42, 15.2 ± 1.26 for HF vs. 19.9 ± 0.69 , for C dams $P < 0.005$]. Overall HF mice were significantly heavier, hypertensive and hypercholesterolemic than C during pregnancy and at weaning of their offspring as shown below (Table-3.1)

Table-3.1: Statistical analyses of biochemical and biophysical parameters in dams.

Variables	HF			C			ANOVA P Value
	Pre-pregnancy	Pregnancy	Weaning	Pre-pregnancy	Pregnancy	Weaning	
Weight (gm)	54.3±2.0	67.4±2.4	57.4±3.4	31.2±2.8	41.2±1.8	30.7±1.3	<0.001
systolic blood pressure (mmHg)	135.2± 2.6	139.4 ± 1.8	136.2±1.4	110.5 ± 1.7	112.7 ± 2.0	108.7±2.4	<0.001
LDL cholesterol (mmol/L)	2.1±0.2	4.1±0.4	3.5±0.1	0.8±0.02	1.1±0.04	0.2±0.03	<0.001
cholesterol (mmol/L)	4.08±0.4	6.3±0.5	6.1±0.2	1.99±0.2	2.3 ±0.2	2.3±0.1	<0.001

Values mean ± SEM ($n = 8$ for all groups). Statistical test is the ANOVA result for all groups. Statistical result displayed in each cell is for the dietary group comparison with the others group using the Tukey-Kramer test. The significance of $P < 0.001$, is for all the columns between HF vs. C groups at pre-pregnancy, pregnancy and weaning.

3.3.2 Offspring

3.3.2.1 Food intake

I first examined the effects of maternal HF diet during pregnancy on daily energy intake in the offspring. As shown in Figure- 3.2, I observed a gender specific effect in offspring from HF mothers on their food intake. As shown in Figure-3.2 (a), at weaning no difference was observed in male offspring either fed HF or C diets. At 15 weeks of age, there was no difference in food intake but by 18 weeks till 27 week, HF/HF male offspring were prone to significantly increased food intake than C/C ($p<0.001$). In case of female offspring from HF mothers either fed on C or HF diets (Figure 3.2 (b)), at weaning no difference was observed in the food intake as like males. However, with age feeding a HF diet resulted in the reduction of total energy intake as compared to C/C female from 15 weeks ($p<0.001$).

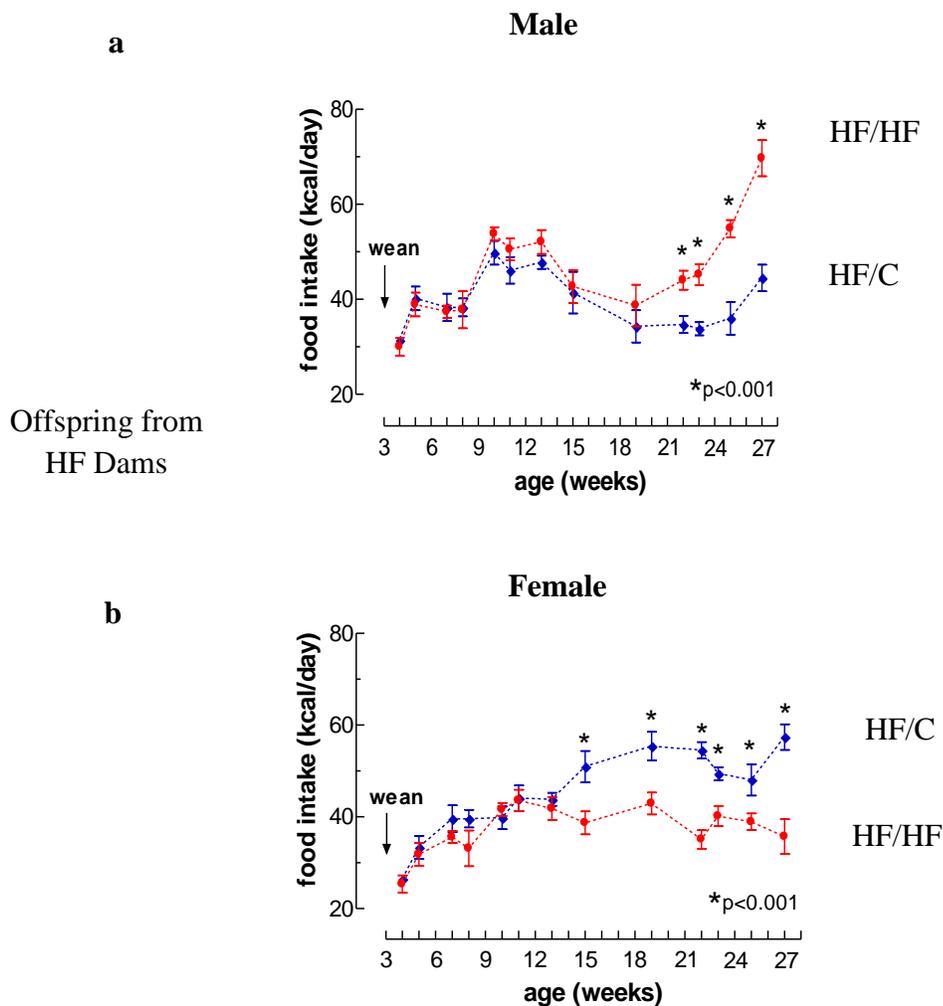


Figure-3.2: Maternal high fat diet during pregnancy has a gender specific effect on daily energy intake in the offspring

3.3.2.2 Body weight and adiposity

In female offspring the HF-C offspring were lighter than the HF-HF and C-HF animals but heavier compared with the C-C animals (Figure 3.3a). Corresponding increases in total body fat were observed in these groups compared with the C-C females (Fig 3.3c). HF-HF and C-HF male offspring were heavier at 36 weeks post weaning than HF-C and C-C males (Fig. 3.3b). These changes were also reflected in their total body fat (as % body weight), where the HF-HF and C-HF males had greater fat depots compared with the C-C and HF-C groups (Figure 3.3d). However, the combined effect of the maternal and postweaning diets (i.e. HF/HF) in both sexes had a greater effect ($p < 0.001$) on fat accumulation.

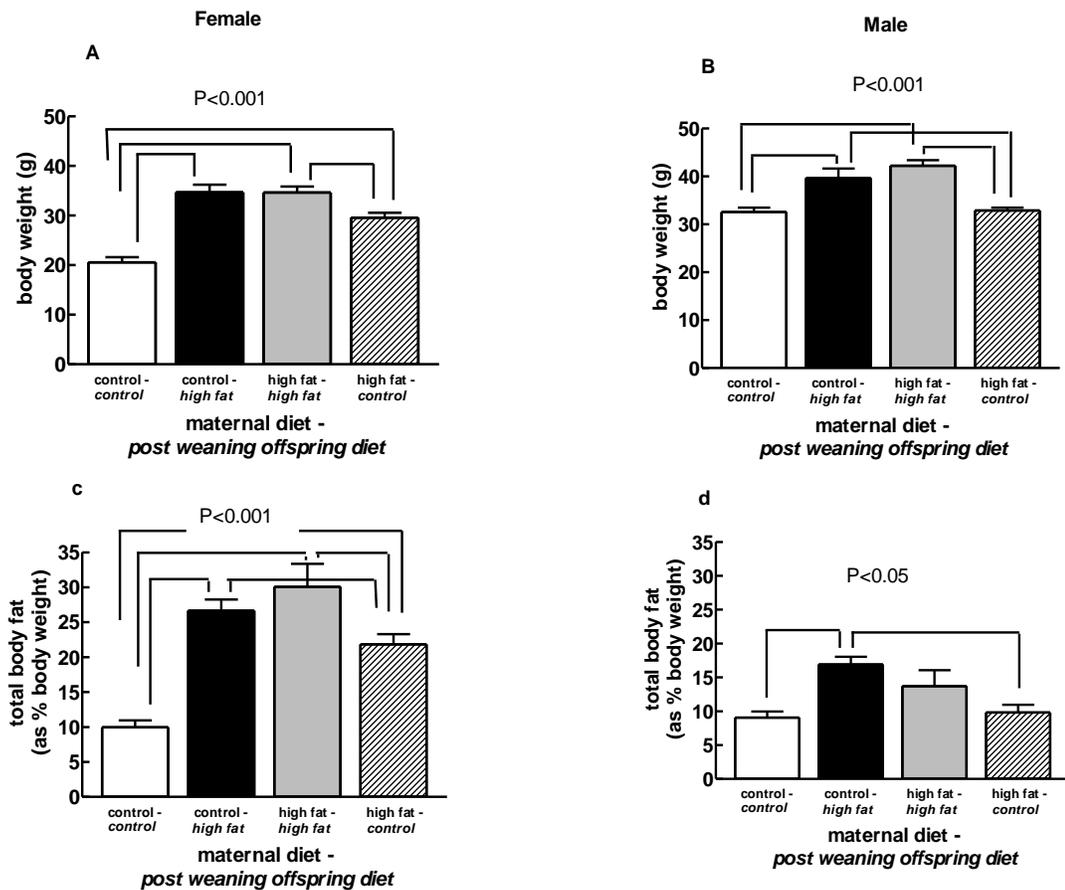


Figure- 3.3: Comparison of body weight (a, b) and total body fat (c, d) in male (left panels) and female (right panels) offspring from control fed mothers that were then fed a chow diet (C-C) or a high fat diet (C-HF) and from high fat fed mothers that were then fed a high fat diet (HF-HF) or a chow diet (HF-C). Values are means \pm SEM ($n = 8-10$ /group). Bars with different letters are significantly different ($p < 0.05$) from each other (by the Tukey-Kramer comparisons test).

3.3.2.3 Blood pressure

Systolic blood pressure was elevated in the HF-HF, HF-C and C-HF male and female offspring at 36 weeks post weaning compared to C-C offspring (Figures 3.4a & b). The HF-C males had the highest blood pressure and this was significantly greater than the HF-HF and C-HF groups. In the females, the HF-HF and HF-C groups had significantly elevated blood pressure levels compared with the C-HF offspring.

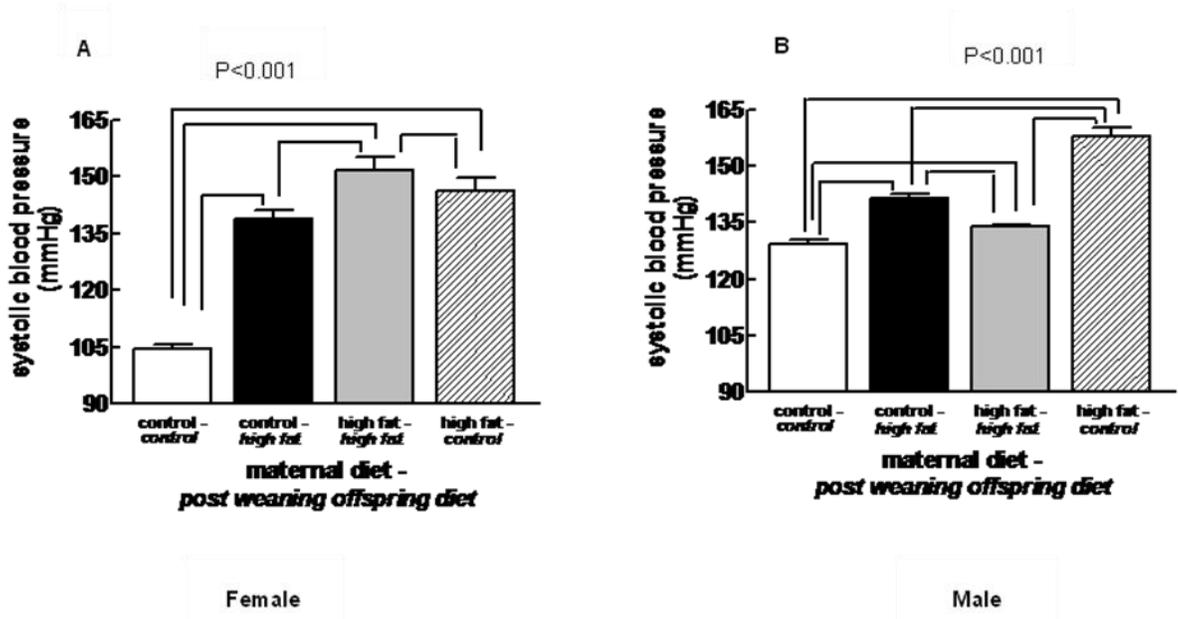


Figure 3.4: Comparison of systolic blood pressure (a, b), in male (left panels) and female (right panels) offspring from control fed mothers that were then fed a chow diet (C-C) or a high fat diet (C-HF) and from high fat fed mothers that were then fed a high fat diet (HF-HF) or a chow diet (HF-C). Values are means \pm SEM ($n = 8-10$ /group). Bars with different letters are significantly different ($p < 0.05$) from each other (by Duncan's Multiple Range Test). Bars with different letters are significantly different ($p < 0.05$) from each other (by the Tukey-Kramer comparisons test).

3.3.2.4 C-reactive protein and cholesterol levels

In females, total cholesterol and CRP level was elevated in the HF-HF and C-HF animals compared to the C-C offspring (Figures 3.5a & c, respectively). Total cholesterol in the HF-C group was also elevated but was not significantly different from C-C or from the C-HF and HF-HF groups. Total cholesterol was elevated in the HF-HF, HF-C and C-HF males at 36

weeks post weaning compared with the C-C males (Figures 3.5b). Interestingly, no difference was observed in CRP levels among these four treatment groups (Figure 3.5d).

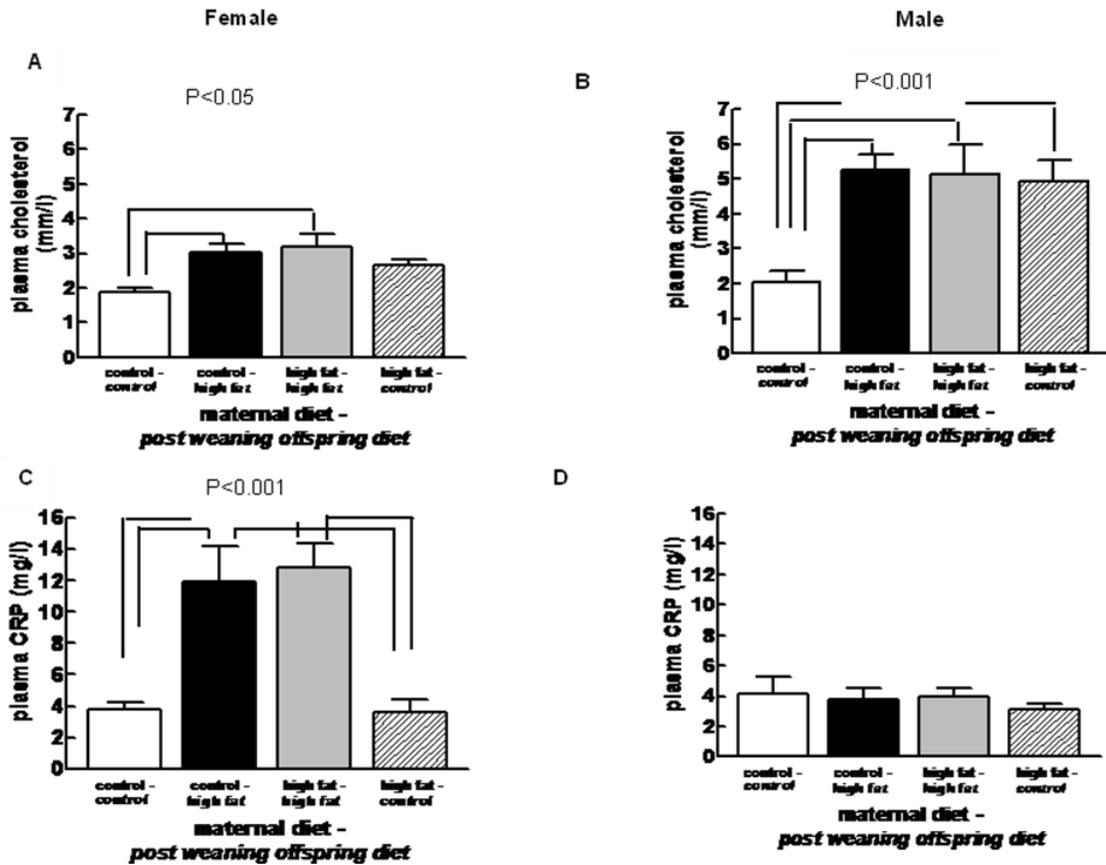


Figure 3.5: Comparison of total cholesterol (a, b) and C-reactive protein (CRP) levels (c, d) in male (left panels) and female (right panels) offspring from control fed mothers that were then fed a chow diet (C-C) or a high fat diet (C-HF) and from high fat fed mothers that were then fed a high fat diet (HF-HF) or a chow diet (HF-C). Values are means \pm SEM ($n = 8-10$ /group). Bars with different letters are significantly different ($p < 0.05$) from each other (by Duncan's Multiple Range Test). Bars with different letters are significantly different ($p < 0.05$) from each other (by the Tukey-Kramer comparisons test).

3.3.2.5 Development of fatty liver in adult offspring

I assessed liver morphology in 36 week old female offspring (Figure 3.6). No lipid accumulation and a normal hepatic architecture were observed in livers from C/C group. In livers from HF/C some lipid vacuoles were observed. C/HF offspring livers showed mild to moderate steatosis. However, histological examination of the liver from HF/HF demonstrated several lipid vacuoles of various sizes within hepatocytes, mononuclear cell infiltration, indicating an evidence of severe inflammation.

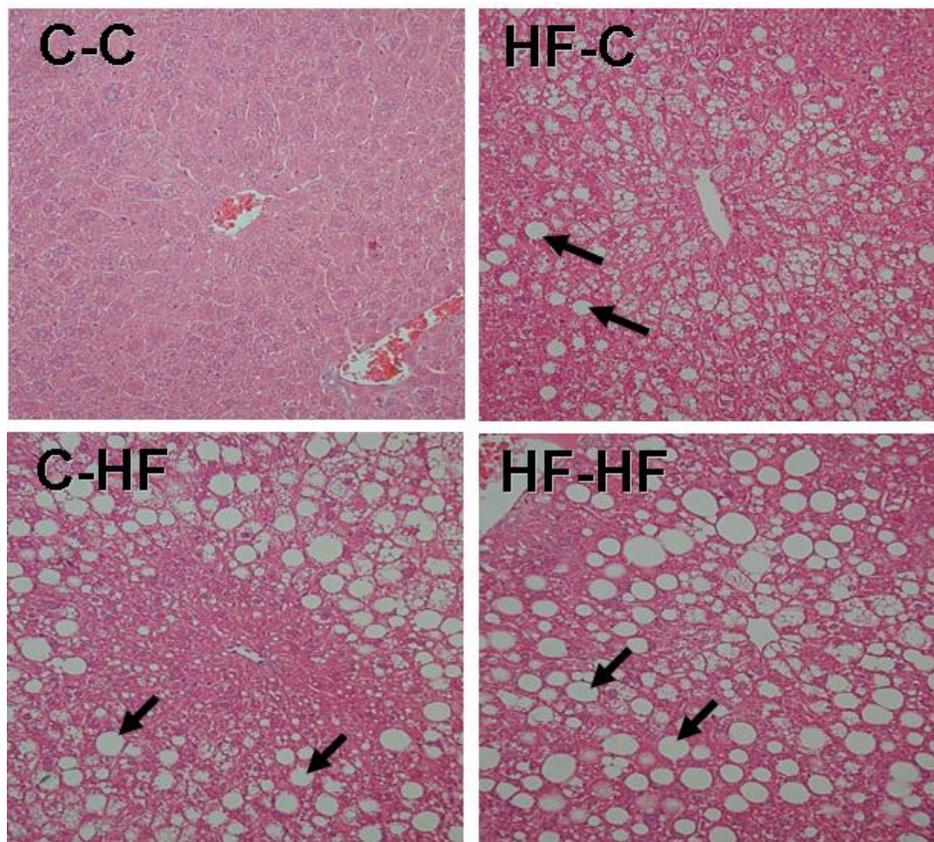


Figure 3.6: Liver histology in female offspring from control fed mothers that were then fed a chow diet (C-C) or a high fat diet (C-HF) and from high fat fed mothers that were then fed a high fat diet (HF-HF) or a chow diet (HF-C). C-C offspring had normal liver structure. However, lipid vacuoles of various sizes could be observed within hepatocytes of the C-HF, HF-HF and HF-C offspring. Staining with hematoxylin and eosin; magnification x20; bar scale = 40 μ m.

The severity of the NAFLD in the offspring livers assessed using the Kleiner scoring system, demonstrated that C/C offspring received no scores, the HF/C and C/HF offspring achieved a score of 4, indicative in human histopathology Kleiner scoring as NAFLD (Table 3.2). In contrast, the HF/HF liver generated a necro-inflammatory score of 7, compatible with a diagnosis of NASH. These findings demonstrate that exposure to a post-weaning HF diet causes hepatic steatosis and that this effect is markedly exaggerated when the offspring have also been exposed to a HF diet in the developmental environment leading to development of NASH in the offspring.

Table-3.2: Assessment of NAFLD severity in offspring’s liver using the Kleiner scoring system.

Groups	Steatosis	Ballooning	Lobular Inflammation	Activity Score	Indication
C/C	0	0	0	0	Normal
C/HF	2	1	1	4	NAFL
HF/C	2	1	1	4	NAFL
HF/HF	3	2	2	7	NASH

For each group (n=5) the mean score for each characteristic is given.

3.4 Discussion

Nutritional status during critical periods of early life has important influences on development. Modification of the quality and/or quantity of maternal nutrition during pregnancy has been shown to have consequences on the later health of the offspring, changing their responses to environmental challenges and thus their predisposition to disease.⁴³²⁻⁴³³ In the present study, the consequences to the offspring of a long-term maternal HF dietary regimen, starting from when the prospective mothers were themselves weaned until weaning of their offspring is studied. This contrasts with earlier animal experiments, which focussed on the consequences

for the offspring of maternal HF feeding during only gestation and/or lactation periods.^{218,254, 434} The long-term HF feeding results in changes in the dam's physiology, including increased body weight and raised circulating total and LDL cholesterol levels compared with C-fed group, similar to what we have observed in our previous work.⁴³⁵ These changes in the HF dams may provide 'cues' used by the developing offspring in altering phenotype in anticipation of their postnatal environment. The long-term dietary regimen used in the present study may better represent the situation in human populations, following socioeconomic transitions where consumption of HF food occurs very early in life and continues in women through pregnancy and lactation. The present study demonstrates that offspring of such mothers are predisposed to becoming fat, hypercholesterolemic and hypertensive in adulthood, thus perpetuating the cycle of chronic disease.

Our laboratory previously reported that prenatal and early postnatal exposure to a maternal diet rich in animal fat led to the development of characteristics similar to the human metabolic syndrome in adult rats, even when they were reared on a balanced diet.²⁴⁶⁻²⁴⁸ The present study clearly shows that long-term consumption of a HF diet by the female dams predisposes their offspring to obesity, hypercholesterolemia, hypertension and fatty liver in adult life, at least when they are also fed a HF diet. Greater adiposity is also seen in females, even when they are fed a C diet post-weaning, suggesting that predisposition to obesity has been induced during development. This is not however seen in the HF-C male offspring.

In males the elevation of SBP is less pronounced in the HF-HF group than in HF-C or C-HF groups. This supports the partially beneficial cardiovascular effect of reducing the dietary mismatch in the HF-HF offspring reported in our earlier study.²⁴⁷ In that study the dams were only fed the HF diet during pregnancy and weaning and endothelial dysfunction rather than elevated SBP was observed to be attenuated when compared with C fed offspring. These data are broadly in support of the predictive adaptive response concept²⁴⁷. However, as outlined in the original exposition of the concept⁹⁰ such an effect only operates within a range of

postnatal environments, beyond which the risk of pathophysiology is increased. The postnatal HF used in the present study is likely to take the offspring into such a range, one which from an evolutionary point of view is novel, possibly explaining the pathophysiological effects observed in them.

I also observed a substantial number of lipid vacuoles within hepatocytes of the HF-HF, HF-C and C-HF but not the C-C offspring. Previous studies in rats have demonstrated that offspring of fat-fed dams show profound metabolic defects such as increased liver weight and liver triglyceride content.^{218, 254} It has been reported in humans that there is a strong association between hypercholesterolemia, NAFLD and increased risk of CVD⁴³⁶ but it is unclear whether certain diets are more likely to produce these effects than others. NAFLD and atherosclerosis are both produced in apo-E lipoprotein deficient mice, suggesting pathophysiological effects on both the liver and aorta of cholesterol-enriched diets.⁴³⁷⁻⁴³⁸ The accumulation of lipids in hepatocytes suggests possible interference with mitochondrial and microsomal function leading to disruption in lipoprotein transport and fatty acid accumulation vs. metabolism.²⁴⁸ The accumulation of lipids in hepatocytes in the HF-C offspring suggests that susceptibility to development of fatty liver can be induced during early development.

This study demonstrates that offspring of dams fed a HF diet, which were also fed a HF diet post-weaning (HF/HF), are predisposed to develop steatosis, a phenotype similar to humans; progressive liver condition that may result in cirrhosis and end stage liver failure. This was also true for offspring from C mothers that were only fed a HF diet post-weaning and exhibited steatosis with evidence of inflammation. Therefore exposure to a HF diet during development and post-weaning is worse than HF exposure post-weaning alone.

My further observations from these animals show that a maternal HF diet can lead to development of NAFLD later in life, even if a control diet has been adopted in the adult

environment, evidence that changes occurring during development are persistent, and affect disease out-come in later life. Importantly, analysis of the HF/HF offspring's livers, revealed a progressive form of steatosis, which was (again) severe than their C/HF counterparts. This collectively demonstrates that HF exposure in early development exacerbates the effect of a HF diet in later life, leading to progressive fatty liver disease and highlighting the critical nature of maternal nutrition.

Reports have suggested that a stimulus in addition to baseline steatosis is required for NAFLD disease progression to occur.⁴³⁹⁻⁴⁴⁰ Based on the findings from this present study, it is investigated whether a HF maternal diet may constitute this stimulus and lead to increased susceptibility to NASH development in adult offspring. At this point I am unable to define a mechanism that leads to this developmental priming as this was not the focus of my aims here. However it is plausible to speculate that this suboptimal nutrition during the developmental period may alter the epigenetic profile of key metabolic genes, subsequently leading to persistent modulations in gene transcription, thus increasing the risk. But chronic inflammation is central to lipid homeostasis and is maternally inherited; therefore providing a good candidate vector for the inheritance of developmentally primed contributor to atherosclerosis and CVD.²⁷⁹ An important marker of inflammation is an elevation in CRP, an acute-phase reactant secreted by hepatocytes in response to pro-inflammatory cytokines such as interleukin (IL)-6.⁴⁴⁰⁻⁴⁴¹ In large epidemiological studies, CRP has been shown to be a strong, independent predictor of CVD risk in both men and women.⁴⁴²⁻⁴⁴³ The mechanism by which inflammation increases CVD risk is not known, but during periods of acute inflammation lipid metabolism is altered, giving a proatherogenic profile.⁴⁴⁴

Emerging reports are suggesting that alterations in mitochondrial form and function could constitute a central element for this pathophysiology^{438,445-446} and is likely to lead to increased generation of ROS.⁴⁴⁷⁻⁴⁴⁸ This has previously been shown to initiate lipid peroxidation and trigger the release of inflammatory cytokines during late pregnancy when stored lipids are

needed during the postabsorptive state, to minimize protein catabolism, and to preserve glucose and amino acids for the fetus. The breakdown of fat stores in adipose tissue is enhanced, increasing plasma free fatty acid (FFA) and glycerol levels⁴⁴⁸⁻⁴⁵⁰ (Figure-3.7). In case of maternal hyperlipidemia, triglyceride production by the liver increases thus representing the enhanced levels of lipolytic products in circulation. Although triglycerides do not cross the placenta, they represent a “floating energy deposit” that is easily accessible by the actions of lipoprotein lipase and other lipases releasing ketogenic byproducts that cross the placenta and provide a source of ROS for the fetus, thus increasing the element of inflammation⁴⁵⁰. These elevated expression profiles then persist into early adulthood⁴⁴⁹⁻⁴⁵⁰. Therefore, offspring that have received a HF diet in development and are also exposed to a HF diet in adulthood (HF/HF offspring) shuttle their cytosolic fatty acids towards lipogenesis resulting in severe steatosis and a metabolic syndrome like phenotype. Conversely, this hypothesis also explains why the offspring exposed to a HF diet in the adult life do not exhibit such a severe phenotype; since their mitochondrial function may be maintained and their lipogenesis pathways are not developmentally primed.

Moreover, HF-HF and C-HF female offspring in this study demonstrate elevated CRP levels. Interestingly, such increase in CRP levels are not observed in the male offspring. This suggests that the effect of HF exposure on CRP levels is sex specific. The increase in CRP levels found only in female offspring on HF diet may be a consequence of increased sensitivity to the HF diet brought about by circulating female sex hormones⁴⁴²⁻⁴⁴³ or due to the fact that the HF diet itself may result in high CRP in females. This observation is supported by human studies where CRP levels tend to be higher in women than in men.^{441-442, 444,451}

This study shows for the first time that the maternal HF feeding developmentally and biochemically “primes” metabolic pathways associated with NAFLD disease onset and progression. Specifically, in the case of the HF/HF offspring exposed to HF in both

developmental and adult environments, these “primed” pathways are induced to a greater effect resulting in a florid NAFLD phenotype.

In conclusion, our data provides evidence that exposure to a maternal HF diet primes an increased susceptibility to hepatic steatosis and inflammation in adult offspring. Specifically, if previously “primed” offspring (due to increased maternal fat intake) are subsequently fed a HF diet, this results in severe disease similar to progressive human metabolic syndrome and NAFLD. These data emphasise the importance of the consumption of a balanced diet during pregnancy and lactation and the consequences for incidence of chronic disease if an unhealthy diet is consumed across generations.

Here, I reasoned that it is crucial to understand the mechanisms contributing to disease progression from exposure to a HF diet in the developmental period. This is important in developing strategies to prevent the maternal influences on the offspring’s susceptibility towards the metabolic syndrome and NAFLD later in its life. To explore such possibilities will be the focus of the chapter-4.

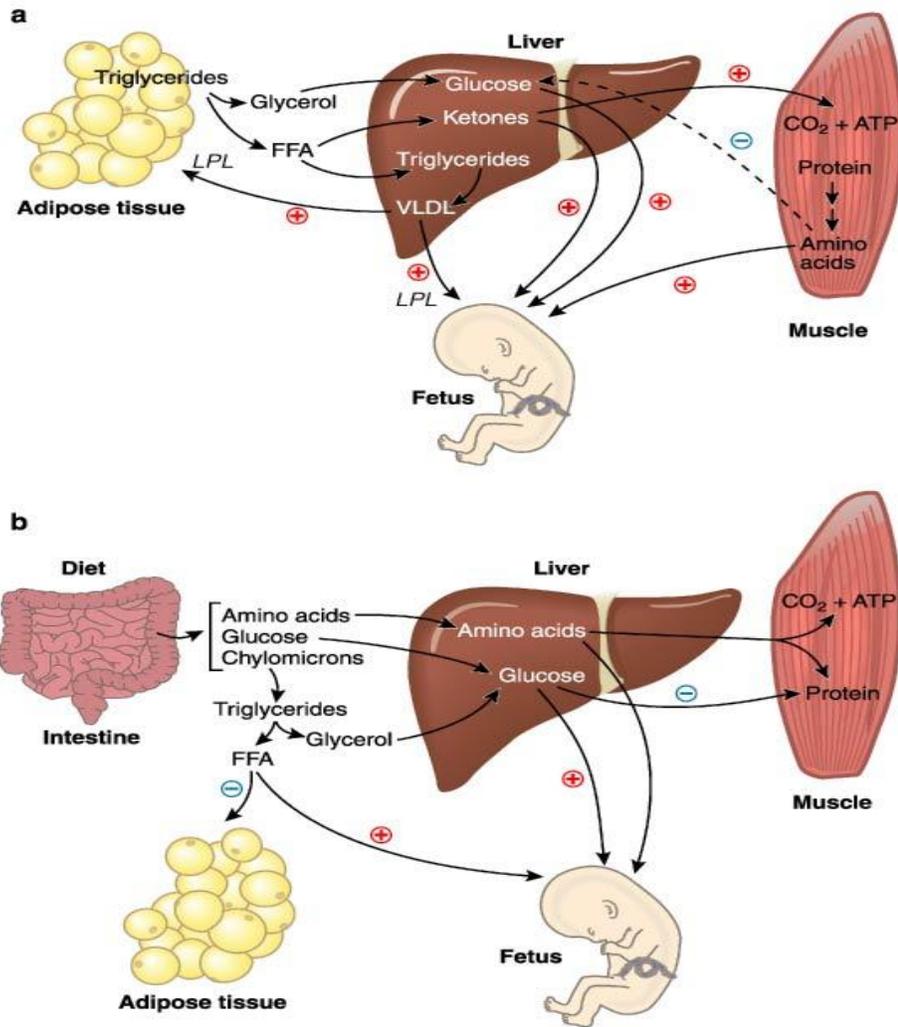


Figure 3.7: Schematic representation of the effect of pregnancy on carbohydrate, lipid, and amino acids metabolism. Arrows with (+) markings are those pathways enhanced during late pregnancy; arrows with (-) markings are those reduced. In general, these metabolic shifts are more enhanced in obese than in normal-weight women. *Part A* shows the response in a postabsorptive state; *part B* depicts a postprandial state. FFA, free fatty acids; VLDL, very-low-density lipoproteins; LPL, lipoprotein lipase. From Herrera with permission ⁴⁴⁹

My father always mentored me. I remembered him advising me once

“Son! Never lose three things in life; 1. Hope 2. Words 3. Honesty”

I have never lost this advice in my life.

CHAPTER-4

Effects on Offspring of Statin Treatment in Hypercholesterolemic Mice from Pregnancy to Lactation

4.1 Introduction

Five key messages emerge from the results described earlier in Chapter-3: 1) Long-term HF feeding results in changes in the dam's physiology, including increased body weight and raised circulating total and LDL cholesterol levels compared with C-fed group 2) Long-term consumption of a HF diet by the dams predisposes their offspring to obesity, hypercholesterolemia, hypertension and fatty liver in adult life, when these offspring are also fed a HF diet. 3) There is greater propensity towards adiposity in offspring from HF dams, even when they were fed a C diet post-weaning thus suggesting that predisposition to obesity had been induced during development; 4) Feeding a HF diet during pregnancy and/or lactation induces gender-specific BP and CRP effects in the offspring and 5) A maternal HF diet can lead to development of NAFLD in offspring later in life, even if a control diet has been adopted in the adult environment. Taken together, these findings extend our knowledge of the pathophysiology of maternal HF diet and its effects on offspring. This provides further evidence that changes occurring during development are persistent, and effect disease outcome in later life.

In recent years there has been increasing focus on optimizing women's nutrition and lifestyle in the periconceptual period, as this is a key time for fetal development.⁴⁵² For example, clinical trials have shown that folic acid supplementation during the periconceptual period reduces the risk of neural tube defects.⁴⁵³ Therefore promoting

good health and nutrition before pregnancy may be at least as important as during pregnancy. Recently the National Institute for Health and Clinical Excellence (NICE) has reinforced this focus on the periconceptional period. Its guidance on nutrition of mothers and children⁴⁵⁴ not only advises improving the nutrition of pregnant women but also includes recommendations for those who may become pregnant. Indeed a review commissioned by NICE focused exclusively on interventions to improve the health of women during the periconceptional period⁴⁵⁵ and the health of their babies. Arguably the best known international recommendation so far for women who plan a pregnancy is to take 400 µg of folic acid a day in supplements to prevent neural tube defects⁴⁵⁶⁻⁴⁶³. Until recently women planning a pregnancy in the United Kingdom are encouraged to limit alcohol consumption, to eat a healthy balanced diet, take exercise, and avoid smoking.⁴⁶⁴⁻⁴⁶⁷ Despite the information available for women planning a pregnancy, data are limited on how closely the recommendations are followed. Recently, Southampton Women's Survey and others⁴⁶⁸⁻⁴⁷⁴ reported that only a small proportion of women planning a pregnancy follow the recommendations for nutrition and lifestyle. This proportion is even less for the population as a whole as many pregnancies are unplanned.

So improved nutrition and lifestyles are becoming as important for women of childbearing age as women planning a pregnancy; we now face a bigger challenge than just the noncompliance of pregnant women adhering to healthy lifestyle. Today in a culture where food is more abundant and high in fat and carbohydrates, there exists a higher prevalence of metabolic syndrome especially before and during pregnancy and this may impact the health of future generations. A pattern of fat distribution, development of type-2 diabetes and obesity in young women are becoming more prevalent not only in developed countries (e.g. USA, UK) but also in developing countries (e.g. Mexico, India).⁴⁷⁴⁻⁴⁷⁶ Reports have suggested that higher waist circumference⁴⁷⁷ and higher waist to hip ratio⁴⁷⁸⁻⁴⁷⁹ are both associated with type-2 diabetes and CVD. This suggests that impaired fetal growth could lead to increased metabolic syndrome in a given population. This is especially true in the case of females where events in their fetal lives could lead to persisting changes in the body's structure and metabolic function.

In humans, the influence of a HF diet is unlikely to commence during pregnancy. Early interventions may be effective, either in the form of low fat diets (as shown in studies where the introduction of a low saturated fat diet in infancy and maintaining it during the first decade of life is associated with a reduction in serum cholesterol concentration and enhanced endothelial function in children.^{254,481} Lipid-lowering interventions such as HMG-Co-A reductase inhibition using statins are widely used to control hypercholesterolemia; is proven to reduce risk of CVD.⁴⁸²⁻⁴⁸⁷ Because maintaining the population on low fat diet all the time is neither possible no ethical, statins may be a good choice as an early intervention.

The existing data thus raise the important question of whether lipid-lowering interventions during pregnancy in mothers already consuming a HF diet could provide long-lasting benefits to their offspring. Palinski *et al*²⁵⁴ demonstrated a reduction of atherosclerosis in offspring of rabbits treated with cholestyramine or vitamin E, as well as those receiving combined treatments. Prevailing practice advocates that interruption of total cholesterol synthesis during first trimester is potentially hazardous to the growing embryo. The cholesterol-lowering statin drugs are therefore clinically contraindicated in pregnancy, and initial animal studies have shown that they are potentially teratogenic.⁴⁸⁸⁻⁴⁸⁹ Although a recent study reported no evidence of an increase in congenital anomalies in humans compared within the general population after maternal exposure to simvastatin or lovastatin,⁴⁹⁰ these statins are still highly lipophilic and can result in embryo-placental concentrations similar to those in maternal plasma. Pravastatin, on the other hand, is the most hydrophilic statin and has not been reported to induce abnormal pregnancy outcomes, even in animals.⁴⁹⁰⁻⁴⁹¹

We now know that pravastatin is an HMG-CoA reductase inhibitor that lowers plasma cholesterol levels by inhibiting de novo cholesterol synthesis, increasing the receptor-

mediated catabolism of low density lipoprotein (LDL).⁴⁹² Pravastatin produces consistent dose-dependent reductions in both total and low density lipoprotein (LDL)-cholesterol levels in patients with primary hypercholesterolaemia.⁴⁹² Reductions in LDL-cholesterol levels reported are 18% (10 mg/day), 23% (20 mg/day) and 31% (40 mg/day) after 12 weeks (discussed in reference 493). Favourable changes in other parameters such as decrease in total triglyceride and consistent increase in high density lipoprotein (HDL) are generally not dose dependent⁴⁹⁴⁻⁴⁹⁵. Combination therapy with other antihyperlipidaemic agents such as cholestyramine further enhances the efficacy of pravastatin in patients with severe dyslipidaemia.⁴⁹²⁻⁴⁹³ Available data suggest that pravastatin is effective in CVD and in patients with hypercholesterolaemia secondary to diabetes mellitus or renal disease.⁴⁹²

The effect of pravastatin on cardiovascular events related to elevated plasma cholesterol levels is under investigation in several large scale disease regression and primary and secondary prevention trials. Regression studies such as PLAC I, PLAC II, and REGRESS⁴⁹⁵⁻⁴⁹⁷ showed that pravastatin slows progression of atherosclerosis and lowers the incidence of coronary events in patients with mild to moderately severe hypercholesterolaemia and known coronary heart disease. Large scale primary (WOSCOPS and PROSPER) and secondary prevention studies (as summarised by Rajpathak *et al*,⁴⁹⁸) moreover demonstrate that pravastatin has beneficial effects on coronary morbidity and mortality. In WOSCOPS, all-cause mortality was reduced by 22%, Pravastatin was generally well tolerated by most patients.⁴⁹⁸ As with other HMG-CoA reductase inhibitors, myopathy occurs rarely (< 0.1% of patients treated with pravastatin): approximately 1 to 2% of patients may present with raised serum levels of hepatic transaminases. Thus, with its favourable effects on cardiovascular morbidity/mortality and total mortality, pravastatin is considered a first-line agent in patients with elevated cholesterol levels, multiple risk factors or coronary heart disease who are at high risk of cardiovascular morbidity.⁴⁹⁸⁻⁵⁰⁰

Pravastatin is also the most rigorously tested drug in the “first generation” of statins due to its pleiotropic properties. Despite, the identification of CVD and metabolic syndrome risk factors (e.g. hypertension, hyperlipidemia, smoking, and diabetes) by the Framingham Heart Study, these traditional factors showed a very low specificity.⁵⁰¹⁻⁵⁰³ Therefore, the American Heart Association (AHA) suggested that inflammatory soluble mediators such as acute phase reactant CRP, (shown to play a central role in all phases of atherosclerosis), might be useful indicator of cardiovascular risk⁵⁰⁴⁻⁵⁰⁸. Basic research and clinical studies have shown that pravastatin not only lowers LDL cholesterol levels but also inhibits atherosclerotic inflammatory processes. As I mentioned earlier that pravastatin is a hydrophilic compound (different from fluvastatin or simvastatin that are lipophilic), and does not get metabolized by the cytochrome P450 complex.⁵⁰⁹ Probably due to this property, treatment with pravastatin induces different effects even in vascular cells. Wiesbauer and coworkers showed that among six different statins, only pravastatin did not decrease PAI-I production in human endothelial and smooth muscle cells.⁵¹⁰ Furthermore, pravastatin increased E-selectin and vascular cell adhesion molecule (VCAM)-1-induced expression on vascular endothelial cells stimulated with TNF- α or LPS.⁵¹¹ This suggests that pravastatin could be considered a very promising pleiotropic agent against inflammatory processes as well. Figure 4.1 summaries this as follows.

Due to its beneficial lipid lowering effects, reducing CVD risks and pleiotropic effects inducing molecular intracellular mechanisms in both vascular and immune cells statin (pravastatin) therapy should be considered as a promising approach to give cardiovascular protection in future generations. Therefore the aim of this chapter is to test the hypothesis that statin therapy (pravastatin) during pregnancy and lactation in hypercholesterolemic mothers prevents adverse phenotype in their offspring. Once again C57BL/6 hypercholesterolemic mouse model was used, giving the HF diet from the time the dams were weaned, then throughout pregnancy and lactation

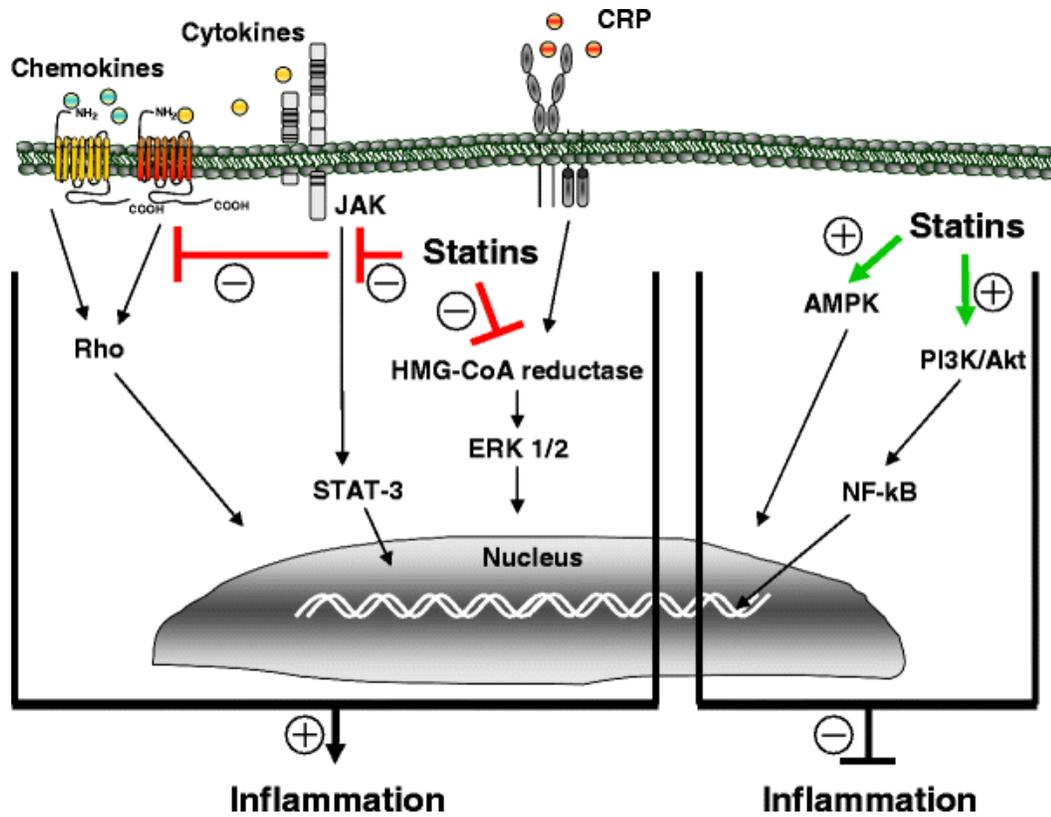


Figure-4.1: “Activatory” and “inhibitory” intracellular signaling pathways regulate pravastatin-induced pleiotropic activities. Pravastatin diminishes proinflammatory effects and promote anti-inflammatory activities through the direct inhibition/activation of chemokine-, cytokine-, and acute phase reactant-induced intracellular signaling pathways in several cell types (such as leukocytes, vascular cells, adipocytes, and hepatocytes). In particular, the inhibition of ERK 1/2, rho, JAK/STAT3, or mevalonate pathways is crucial to reduce inflammation. On the other hand, statins directly activate AMPK and PI3K/Akt/NFkB pathways, thus, increasing cell survival, endothelial, and neuronal protection. (Adapted from references⁵⁰⁹⁻⁵¹⁰)

4.2 Methods

The protocol for conducting the experiments is shown in Figure-4.2.

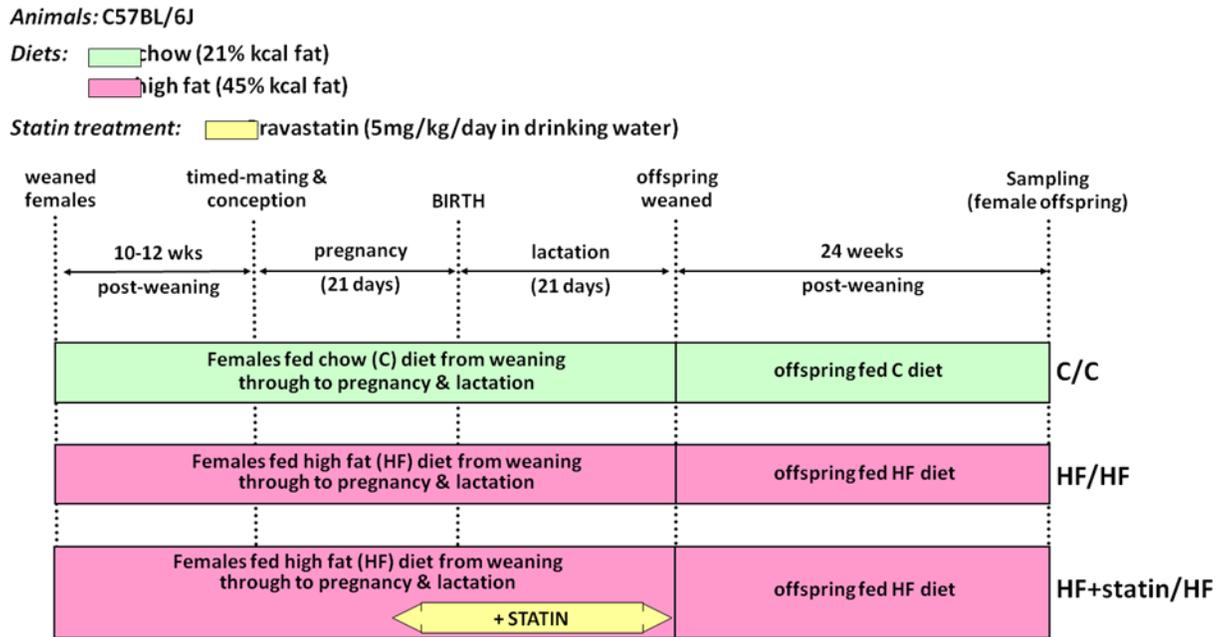


Figure-4.2: Flow diagram of the experimental protocol.

The main point of this experimental protocol was the administration in their drinking water of a water-soluble Pravastatin (Sigma UK; 5mg/kg/day), to half of the pregnant females on HF diet from the second half of the pregnancy and throughout lactation. Later in this chapter I explain the results of my preliminary experiments for pravastatin dose optimisation. This let me decide the appropriate standardized dosages of Pravastatin to be given to HF pregnant dams in this protocol.

The pregnant dams were allowed to give birth, pups were weighed and litter size was standardised to 8 pups with equal numbers of males and females if possible. From weaning (3 weeks post partum) offspring from the C dams were fed the same C diet as their mothers. The offspring of HF and HF-S dams were fed *ad libitum* the HF diet to generate the HF/HF and HF-S/HF groups. Body weights of the offspring (from 1 week of age to avoid maternal rejection of the pups) and food intake (from weaning) were monitored this time until 27 weeks of age. Systolic blood pressure, biochemical markers (total, LDL and HDL cholesterol) and

physical activity was measured at 13, 18, 23 and 27 weeks. All the data were expressed as mean +/- SEM. A p<0.05 was considered to be statistically significant.

4.2.1 Metabolism and optimization of pravastatin in C57BL/6 Mice- Preliminary experiments

Before I discuss my preliminary experiments, I would like to mention the mechanism of action and functional properties of pravastatin as below;

a) Mechanism

As I mentioned earlier, pravastatin catalyses the conversion of HMG-CoA to mevalonate, the rate-limiting step in de novo cholesterol synthesis. Competitive inhibition of this enzyme by the statins decreases hepatocyte cholesterol synthesis (Figure-4.3).

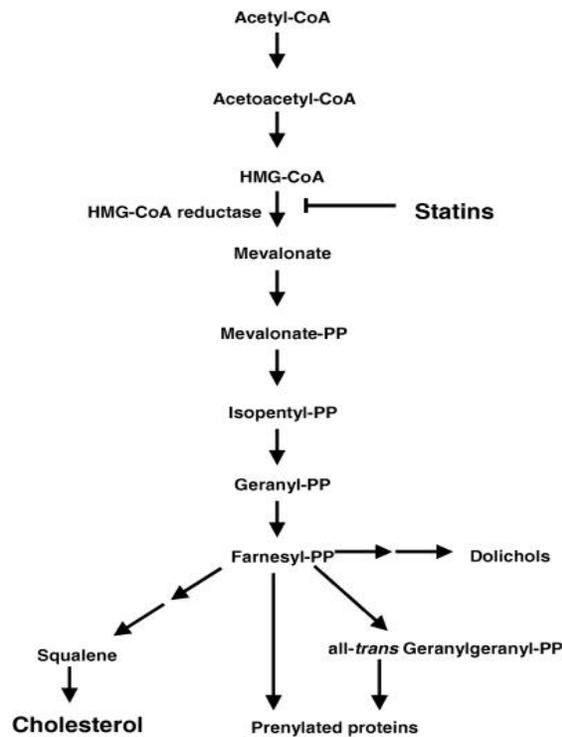


Figure-4.3: The mammalian mevalonate pathway; PP, pyrophosphate. Adopted from Corsisni *et al*⁵¹²

The associated reduction in intracellular cholesterol concentration induces LDL-receptor expression on the hepatocyte cell surface, which results in increased extraction of LDL-C from the blood and decreased circulating LDL-C concentrations.⁵¹²⁻⁵¹³ Pravastatin also has beneficial effects on other lipid parameters, including increases in high-density lipoprotein cholesterol (HDL-C) concentration and decreases in triglyceride concentration.⁵¹⁴ Secondary mechanisms by which pravastatin may reduce levels of atherogenic lipoproteins include inhibition of hepatic synthesis of apolipoprotein B100 and a reduction in the synthesis and secretion of triglyceride-rich lipoproteins.⁵¹⁴⁻⁵¹⁵ In addition, pravastatin may exert beneficial cardiovascular effects independent of their lipid-modifying properties.⁵¹⁶ These pleiotropic properties may be explained by inhibition of synthesis of nonsteroidal isoprenoid compounds, which are also produced from mevalonic acid (Figure-4.3),⁵¹⁷ and include improvement of endothelial cell function, modification of inflammatory responses, and reduction of smooth muscle cell proliferation and cholesterol accumulation.^{516,518}

b) Chemistry and functional properties

Pravastatin is a fungal-derived inhibitor of HMG-CoA reductase, and is not a fully synthetic compound as like other statins.⁴⁹⁴ The chemical structures of the different statins are shown for comparison in Figure-4.4.

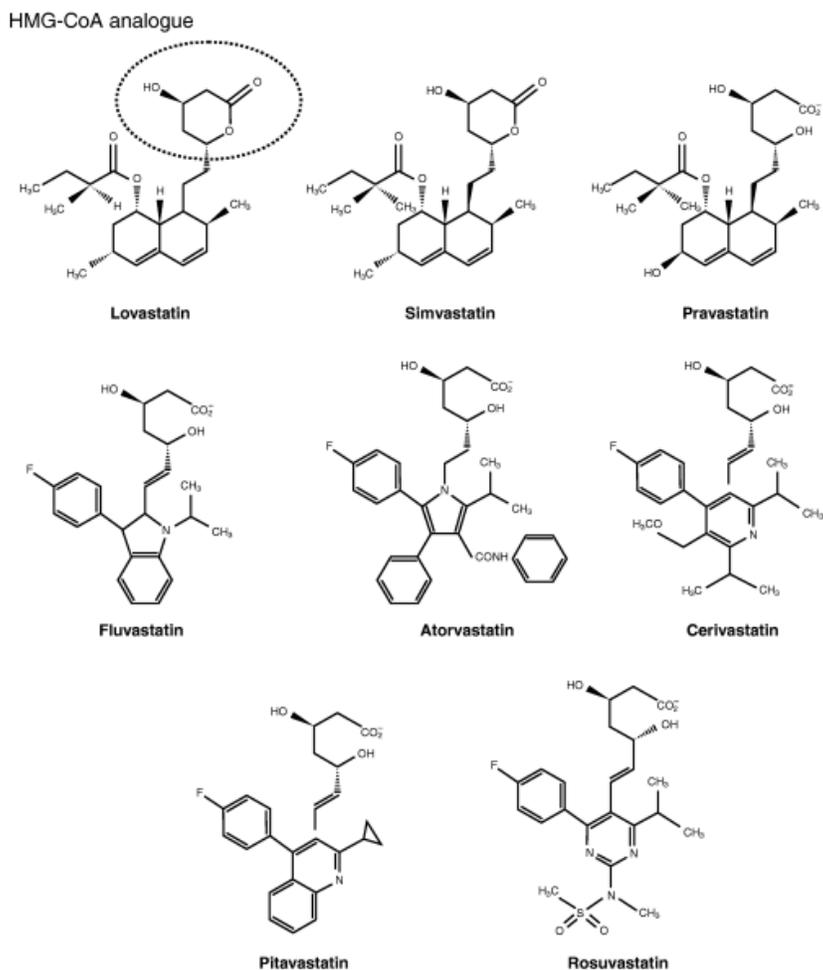


Figure-4.4: Chemical structures of the statins⁵¹²

These structures can be broadly divided into three parts⁵¹⁹: an analogue of the target enzyme substrate, HMG-CoA; a complex hydrophobic ring structure that is covalently linked to the substrate analogue and is involved in binding of the statin to the reductase enzyme; side groups on the rings that define the solubility properties of the drugs and therefore many of their pharmacokinetic properties. Atorvastatin, fluvastatin, lovastatin and simvastatin are relatively lipophilic compounds, while pravastatin and rosuvastatin are more hydrophilic as a result of a polar hydroxyl group and methane sulphonamide group, respectively. For a detailed comparison of pharmacokinetic properties of the statins, please refer to Appendix-iii

Pravastatin, unlike other statins does not bind fully to plasma proteins and as a result systemic exposure to unbound, pharmacologically active drug is relatively high.⁵²⁰ Thus widespread tissue distribution is prevented by the hydrophilic nature of the drug.⁵²¹ Pravastatin is relatively hepatoselective with respect to inhibition of HMG-CoA reductase, an important property given that the majority of endogenous cholesterol production occurs in the liver. The mechanisms contributing to this hepatoselective effect are governed by the solubility profile of the statin. For lipophilic statins, passive diffusion through the hepatocyte cell membrane is primarily responsible for efficient first-pass uptake, while for hydrophilic statins extensive carrier-mediated uptake is the major mechanism.⁵²¹⁻⁵²² This contributes to the fact that hydrophilic statins exhibit greater hepatoselectivity. Indeed, the lack of influence of pravastatin on smooth muscle cell proliferation is likely to be due to low penetration of the cells by the drug.⁵²³

c. Safety of pravastatin for use in experiments

Current utilization studies that ascertain the most commonly used drugs in pregnancy have suggested the teratogenic potential of statins mainly in the first trimester.⁵²⁴⁻⁵²⁵ Thus the selection and timing of administration of statin to the female dams in my experiments was a major challenge. Consideration of the differences between the statins (as described above) helped me to provide a rational basis for the safe and effective use of pravastatin in my experiments. On the basis of the current knowledge about pravastatin (salient features; that include hydrophilic nature, higher and rapid peak plasma concentration within 4hrs, easy systemic bioavailability, more liver protection, low penetration into smooth muscle cells and increased effectiveness in lowering cholesterol and LDL without too much affecting HDL levels), I used this statin in the second half of the pregnancy through to lactation.

d. Determination of pravastatin levels in samples using liquid chromatography

20 Female C57 mice (Charles River Labs UK) maintained on a HF experimental diet were randomly assigned to either pure water available *ad libitum* or water with soluble pravastatin added (100µg/ ml made up solution; Sigma UK) This group is called HF-S. This study is the first to use hydrophilic statins in the drinking water rather than given via intraperitoneal (i.p.) injection. This is to avoid unnecessary stress to the animals, especially during pregnancy that could affect the findings of the study. As there was no prior study available in terms of dosing regimen in drinking water, the calculation for the dosing regimen was based on previous studies where a single 0.5 gm/kg dose administered to C57BL/6 mice caused an elevation in both plasma cholesterol and triglycerides 2 h post-dosing and a subsequent return of both lipids to normal plasma concentrations after approximately 120 h.⁵²⁶ On the basis of this, it was calculated that an average 25 gm mouse would require 5mg/kg/day of pravastatin dissolved solution for effective results. Water with or without pravastatin was available to mice the whole day, as it was established that these agents did not need to be specifically dosed prior to sleep when HMGR (3-hydroxy-3-methylglutaryl Coenzyme A reductase) attained its maximum biological activity for maximum effects.⁵²⁶

From Sigma- Aldrich UK, Pravastatin sodium salt that came in the form of white powder with the solubility of H₂O > 10mg/mL was purchased. The linear formula of this product is C₂₃H₃₅O₇NA⁵²⁷⁻⁵²⁸. This powder was dissolved in an appropriate volume of deionized sterilized water and working solution of statin and all standard solutions were stored in a -10 °C freezer to minimize the interconversion of statins. Samples were collected, separated for plasma and serum and stored at -20C before being transferred to Wickham Laboratories in Hampshire UK (www.wickhamlabs.co.uk) for liquid chromatography. Though I did not directly carry out the analysis, the principles of the mass spectrometry and liquid chromatography are mentioned in Appendix-iv.

Time scheduled chromatography performed on the standard solution of statin containing 10ng/ml pravastatin identified, in at least 50% of the samples, the presence of analytes over a period of 7 days (Figure 4.5).

Chromatogram for 7 Day Statin Serum 1 Metabolites

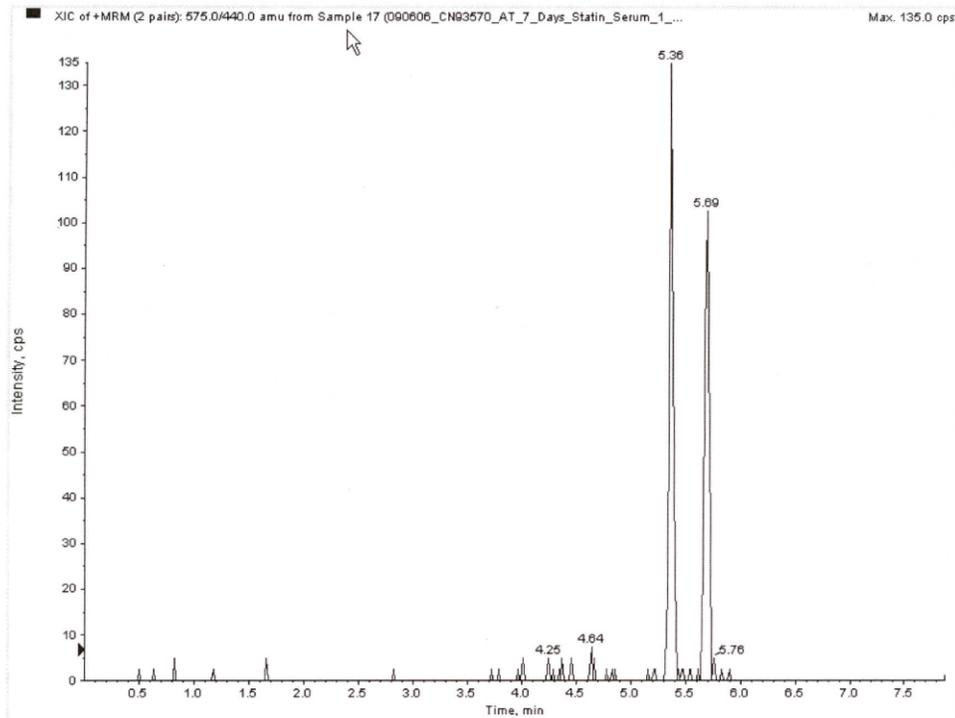


Figure-4.5: Time scheduled chromatogram of a standard solution of statin.

Extracted ion chromatograms were constructed from the FT-MS data, and peak area was determined with LCQuan software (Thermo Electron). Ion chromatograms were reconstructed for the peptide charge state identified by SEQUEST. Differences in statin levels over time were determined by analyzing peak areas using a repeated-measures 2-way ANOVA as within-subject factors based on natural log-transformed values⁵²⁸. The following Table-4.1 demonstrates the results in control sera and plasma as well in treated animals. Table 4.1a shows non-detectable peaks however, Table 4.1b demonstrates the peak levels within sera and plasma.

Results for Early Statin Samples

ND – No peak detected

<1 – less than lowest standard

Control Sera and Plasmas (no statin given)

	Sample	Conc. (ng/ml)
a	Control 1 Serum 25-04-06	ND
	Control 2 Serum High Lipid 25-04-06	ND
	Control 1 Plasma 26-04-06	ND
	Control 1 Plasma High Lipid 26-04-06	ND

Control Plasmas Spiked with 10 ng/ml Statin

	Sample	Conc. (ng/ml)
b	QC10 Serum	6.9
	QC10 Serum High Lipid	9.1
	QC10 Plasma	9.7
	QC10 Plasma High Lipid	10.6

Moreover the bottom Table-4.1 (c) demonstrates concentrations (ng/ml) in adult at 1 and 8 days of statin treatment and in weaned and adolescent mice at 15 days treatment (n=5/sample group)

c Statin Plasma Samples

Sample	Conc. (ng/ml)	Metabolite 1 (Peak Area)	Metabolite 2 (Peak Area)
Adult (mean+/-sem) – 1 day of statin treatment	0.8+/-0.2	77.7+/-10.4	172.3+/-46.6
Adult – 8 days of statin treatment	2.3+/-0.42	236.9+/-38.2	213.3+/-30.7
Weaned young – 5 days treatment	27.8+/-8.2	1633+/-419	3397+/-1693
Adolescent – 15 days treatment	5.7+/-1.3	475+/-109	1294+/-239

Table-4.1 (d) demonstrates the concentration of pravastatin (ug/ml) in the aqueous phase over a storage period of 90 days by chromatographic examination in 100µg/ml of stock solution.

d

Dosing Solutions (100µg/ml made up)

Sample	Dilution	Conc. (µg/ml)
CMC Fresh	200	79.2
Stored Solution 1	200	99.0
Stored Solution 2	200	89.9

4.3 Results**4.3.1 Statin treatment in hypercholesterolemic dams ameliorates their blood pressure and alters total cholesterol, LDL cholesterol and HDL cholesterol Profiles**

Prior to becoming pregnant, 14-week old HF females (fed the HF diet for 10 weeks) showed significantly raised total cholesterol (4.08 ± 0.4 vs. 1.99 ± 0.2 mmol/l, $p < 0.001$) and LDL cholesterol (2.1 ± 0.2 v. 0.8 ± 0.02 mmol/l, $p < 0.001$) relative to their C counterparts. HF dams were heavier than C during mid pregnancy (body weight 67.4 ± 2.4 v. 41.2 ± 1.8 g, $p < 0.001$) and at the time of weaning their offspring (57.4 ± 3.4 v. 30.7 ± 1.3 g, $p < 0.001$). At weaning of their offspring, HF dams were more hypertensive with raised total cholesterol and LDL cholesterol compared with C dams (Table 4.2). Statin treatment in HF dams reduced systolic BP, total cholesterol and LDL cholesterol levels at weaning of their offspring. Additionally, administering statin to HF dams increased HDL cholesterol concentrations compared to the untreated HF mothers.

Table-4.2: Statistical analyses of biochemical and biophysical parameters in dams at weaning, with comparisons among the groups. Values mean \pm SEM ($n = 8$ for all groups). Statistical test on the right hand column is the ANOVA result for all groups. Statistical result displayed in each cell is for the dietary group comparison with the other groups such as HF vs. HF-S; HF vs. C and C vs. HF-S using the Tukey-Kramer test. ** $P < 0.01$, *** $P < 0.001$.

Variables	Dietary Groups				ANOVA P Value
	HF	HF		C	
		vs HF-S	vs C	vs HF-S	
total cholesterol (mmol/l)	6.02 \pm 0.20	3.45 \pm 0.24***	2.29 \pm 0.12***	***	<0.001
systolic blood pressure (mmHg)	136.2 \pm 1.4	123.6 \pm 1.1***	108.7 \pm 2.4***	***	<0.001
LDL cholesterol (mmol/L)	3.50 \pm 0.09	1.13 \pm 0.09***	0.21 \pm 0.03***	***	<0.001
HDL cholesterol (mmol/L)	1.12 \pm 0.17	2.60 \pm 0.05***	1.78 \pm 0.10**	***	<0.001

4.3.2 Statin treatment in hypercholesterolemic dams has beneficial effects on offspring blood pressure, total cholesterol profiles and locomotor activity

HF/HF offspring were of similar weight as the C/C offspring one week postpartum (Figure 4.6a and Table 4.2). However they became heavier at weaning (3 weeks of age) and at 13 to 27 weeks of age compared to the C/C offspring. The HF-S/HF offspring showed a smaller increase in body weight gain compared with the HF/HF offspring. Systolic blood pressure (SBP) was lower at 13 to 27 weeks in HF-S/HF compared to HF/HF offspring (Figure 4.6b and Table 4.3).

As expected, SBP for the C/C group was lower at all time points examined. We also found that SBP in the HF-fed offspring at 27 weeks of age was much more elevated compared with their HF-fed mothers (151.6 ± 3.6 v 136.2 ± 1.4 mmHg, respectively, $P < 0.01$). Offspring from HF-S mothers were significantly more active at 13 to 27 weeks of age than HF/HF offspring, although not as much as the C/C animals (Figure 4.6c and Table 4.3). Total serum and LDL cholesterol concentrations for offspring on HF or C diets followed a similar pattern to dams on HF or C, respectively and previous exposure of their dams to pravastatin resulted in significantly lower total or LDL cholesterol levels, similar to its effect in the dams themselves (Figure 4.6d and 4.6e respectively, and Table 4.3). The elevated levels of total cholesterol observed in HF/HF offspring at 27 weeks were similar to levels found in the HF dams. It is interesting to note that total serum and LDL cholesterol concentrations in HF-S/HF offspring become progressively closer together over time to levels found in the HF/HF group. The HDL cholesterol concentrations for offspring on HF or C diets also showed a similar pattern to dams on HF or C respectively and previous exposure of their dams to pravastatin resulted in significantly higher HDL cholesterol concentration, similar to its effect in the dams themselves (Figure 4.6f and Table 4.3).

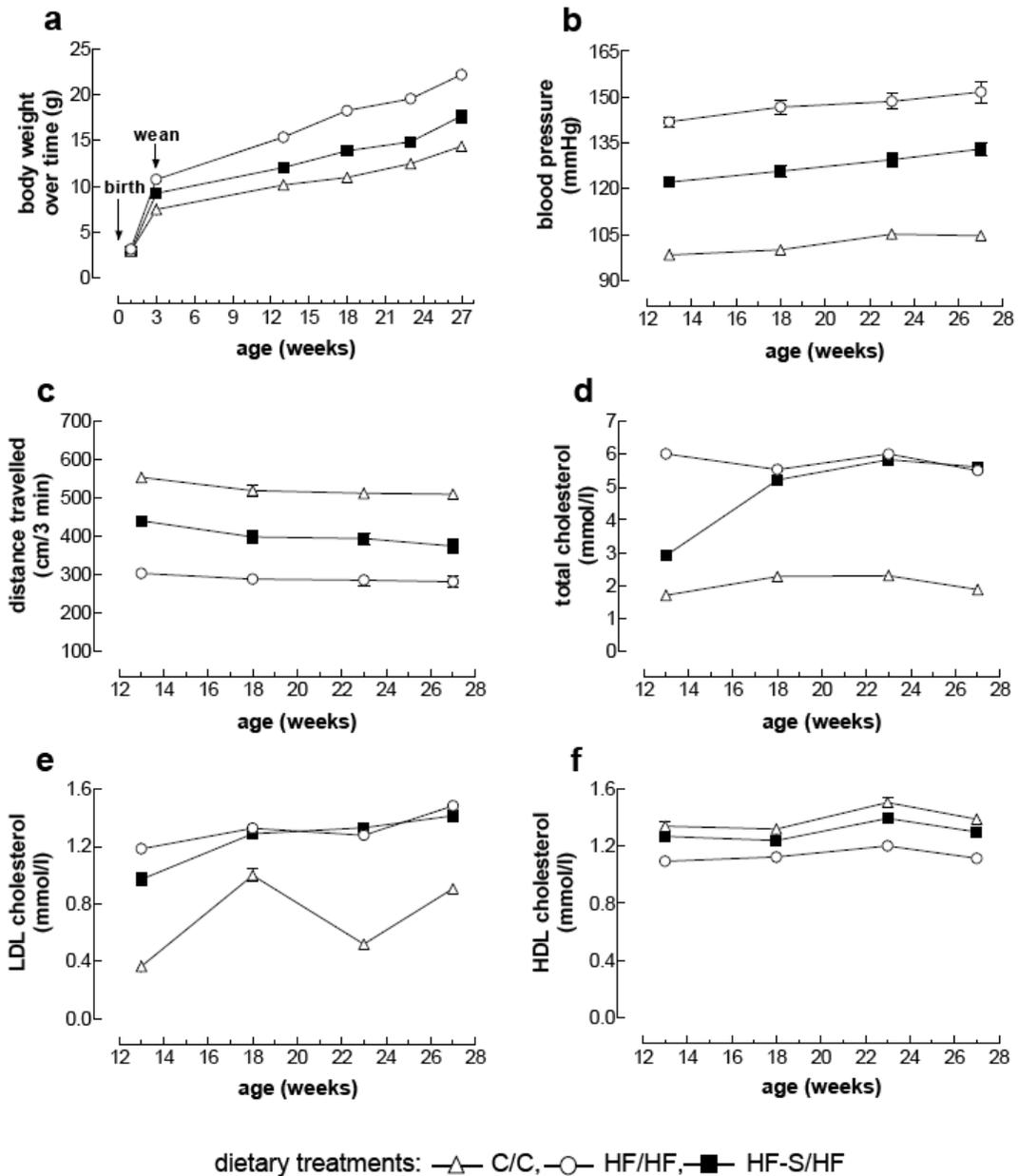


Figure-4.6: Statin treatment in hypercholesterolemic mothers during late pregnancy and lactation has beneficial effects on the cholesterol profile, blood pressure and locomotor behaviour in their offspring. (a) Body weight gain, (b) systolic blood pressure (c) locomotor activity, (d) serum cholesterol, (e) serum LDL and (f) serum HDL in offspring from mothers on standard chow (C), high fat-high cholesterol (HF) diet or HF diet and treated with statin during pregnancy and lactation (HF-S). Weaned offspring were then fed either HF or C diets to adulthood, thus generating the dam/offspring dietary groups: HF/HF, HF-S/HF and C/C (n = 8 per group). See Table 2 for result of statistical analysis.

Table-4.3: Statistical analyses of biochemical and biophysical parameters in offspring.

Variables	Age (weeks)	Dietary Groups				ANOVA P Value
		HF/HF	HF-/HF		C/C	
			vs. HF-S/HF	vs. C/C	vs. HF-S/HF	
body weight (g)	1	3.2±0.1	2.9±0.2 ns	3.3±0.2 ns	ns	ns
	3	10.8±0.2	9.3±0.2***	7.5±0.2***	***	<0.001
	13	15.4±0.2	12.1±0.2***	10.2±0.4***	***	<0.001
	18	18.3±0.4	13.9±0.6***	11.0±0.5***	***	<0.001
	23	19.5±0.5	14.9±0.4***	12.5±0.4***	***	<0.001
	27	22.2±0.4	17.7±0.7***	14.4±0.5***	***	<0.001
systolic blood pressure (mmHg)	13	141.9±1.7	122.2±1.4***	98.4±1.1***	***	<0.001
	18	146.7±2.3	125.7±1.9***	99.9±0.6***	***	<0.001
	23	148.6±2.6	129.5±2.2***	105.2±1.3***	***	<0.001
	27	151.6±3.6	132.9±1.9***	104.7±1.0***	***	<0.001
total cholesterol (mmol/l)	13	6.0±0.2	3.0±0.3***	1.8±0.4***	**	<0.001
	18	5.3±0.3	5.2±0.3 ns	2.3±0.3***	***	<0.001
	23	6.0±0.4	5.9±0.2 ns	2.3±0.3***	***	<0.001
	27	5.5±0.2	5.6±0.2 ns	1.9±0.3***	***	<0.001
HDL cholesterol (mmol/l)	13	1.1±0.2	1.3±0.2 ns	1.3±0.2 ns	ns	ns
	18	1.1±0.2	1.2±0.2 ns	1.3±0.2 ns	ns	ns

	23	1.2±0.2	1.4±0.2 ns	1.5±0.2 ns	ns	ns
	27	1.1±0.2	1.3±0.2 ns	1.4±0.3 ns	ns	ns
LDL cholesterol (mmol/l)	13	1.2±0.2	1.0±0.1 ns	0.4±0.0***	**	<0.001
	18	1.3±0.1	1.3±0.4 ns	1.0±0.1 ns	ns	ns
	23	1.3±0.2	1.3±0.2 ns	0.5±0.0**	**	<0.01
	27	1.5±0.2	1.4±0.2 ns	0.9±0.0*	*	<0.05
locomotor activity (cm/3min)	13	302.8±5.1	440.5±7.7***	554.3±7.2***	***	<0.001
	18	287.8±6.5	398.5±17.4***	519.6±14.7***	***	<0.001
	23	285.0±13.3	393.9±14.8***	512.7±11.8***	***	<0.001
	27	281.4±15.4	373.9±18.4***	510.3±6.7***	***	<0.001

Values mean ± SEM ($n = 8$ for all groups). Statistical test on the right hand column is the ANOVA result for all groups. Statistical result displayed in each cell is for the dietary group comparison with the other groups using the Tukey-Kramer test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant.

4.4 Discussion

The present study demonstrates that treating pregnant animals that are hypercholesterolemic and hypertensive with pravastatin not only improves their health but can also have long-lasting beneficial effects on their offspring in terms of blood pressure and activity, and induces a transient reduction of cholesterol in their offspring even if they consume a similar high fat diet. Further, the present findings provide the first indication that cholesterol-lowering, or other effects of statins, benefits postnatal blood pressure.

Several studies, including ours, have shown that a maternal diet rich in fat and cholesterol during pregnancy can induce obesity, vascular dysfunction, impaired skeletal muscle

development, sedentary behaviour and gender-specific hypertension in the offspring.^{213,217,246,247,250,253,254} However these studies have been confined to short term modifications in the maternal diet such as during pregnancy and/or lactation periods only. In the present study, future mothers were given a HF diet very early in life to produce an effect on offspring health. Thus the experimental approach is more relevant to the human condition.

Altering the maternal diet during critical periods of gestation^{216,249} or throughout gestation and/or the suckling period^{247,250} results in a varying degree of phenotypic outcomes, suggesting the importance of the timing and duration of the nutritional insult. This is emphasized by one of the novel findings using this animal model, namely that SBP in the HF/HF offspring was much greater than their HF-fed mothers despite having similar cholesterol levels (Table-4.4). Such effects are fundamental to the concept of the developmental origins of disease¹⁵ and also indicate that intervention in early life may be particularly important in reducing later risk of disease in the face of lifestyle factors such as a HF diet.

Table-4.4: HF Dams vs. HF/HF offspring at 27 wk

Variables	HF (dams)	HF/HF (Female offspring)	t-test
cholesterol conc. (mmol/l)	6.02±0.2	5.5±0.2	p=ns
Systolic blood pressure (mmHg)	136.2±1.36	151.625±3.59	p<0.01

The significance of maternal hypercholesterolemia in DOHaD remains to be investigated. We believe that the presence of severe developmental physiological defects in offspring (reported here and in Chapter-3; e.g. HF/HF) and with inborn errors of high cholesterol synthesis could

result from the distinct processes involved in cholesterol transfer across the placenta. It has been long assumed that most, if not all, cholesterol required for fetal growth is synthesized de novo by the fetus itself, thus making it autonomous from maternal or placental cholesterol supply. However, several lines of evidence have cast doubt on this notion.⁵²⁸⁻⁵³¹ For example, fetuses that lack the ability to synthesize cholesterol, such as those with the Smith–Lemli–Opitz syndrome, are nevertheless born with low levels of tissue and plasma cholesterol, indicating that they have acquired maternal cholesterol in utero.⁵³² Similarly, studies demonstrate a strong correlation between the size and number of atherosclerotic lesions in human fetal arteries and maternal cholesterol levels.^{278,292} Moreover, maternal hypercholesterolemia also modifies early predictors of CVD in the offspring, thus corroborating the concept of DOHaD in humans.⁵³³ Considering that progression of atherosclerosis in adults takes time, these striking results support the assumption of a strong maternal impact on the comparatively short period of fetal development.

These results have sparked strong interest in delineating the mechanism of maternal-to-fetal cholesterol transfer, because identifying possible mechanisms might offer an insight into the statin intervention adopted in this study. Maternal cholesterol entering the fetal circulation traverses the syncytiotrophoblast layer and the endothelium of the fetoplacental circulation. *In vitro* studies on human syncytiotrophoblast membranes and on human primary trophoblasts⁵³⁴⁻⁵³⁶ demonstrated the presence and functional role of LDL receptor and HDL receptor scavenger receptors in cholesterol uptake and subsequent transport across the syncytiotrophoblast layer. Recent work has further suggested that endothelial cells (ECs) of the fetoplacental vasculature display a high and tightly regulated capacity for cholesterol release.⁵³⁷ In brief, the sequential steps in transplacental transfer of lipoprotein-derived cholesterol at the end of gestation are demonstrated in Figure-4.7

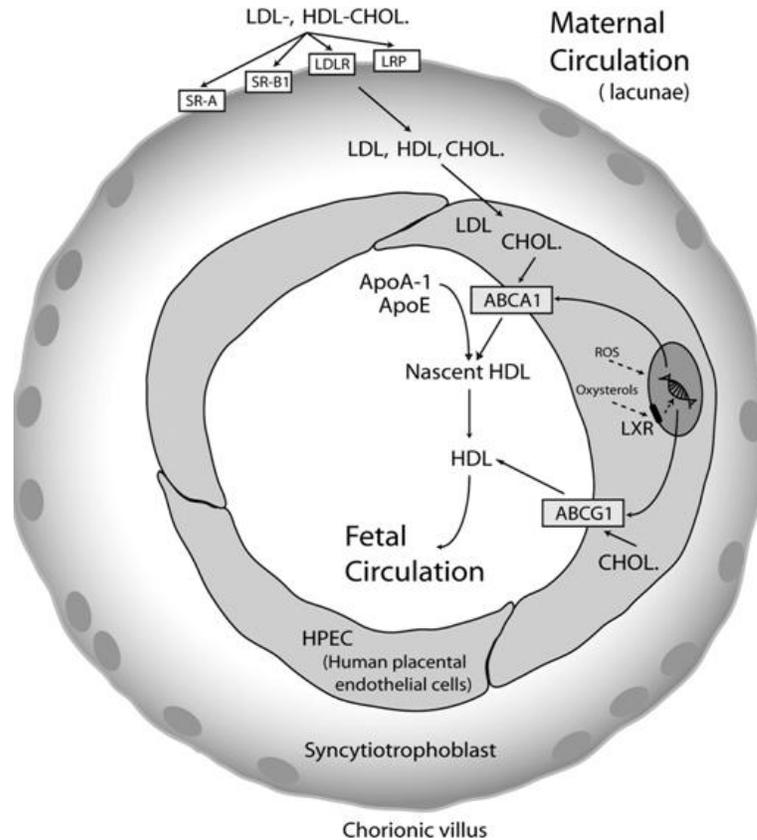


Figure-4.7: Overall maternal–fetal transfer likely encompasses an "uptake/influx" component for maternal lipoproteins and/or their cholesterol into the syncytiotrophoblast (STB), transport of lipoprotein-derived cholesterol (C) to the basal side of the STB, subsequent release into the villous core for passage through the extracellular matrix (ECM), uptake into ECs, and, finally, an efflux component by which cholesterol is released from ECs into the fetal circulation. To date, only the uptake and degradation of LDL and HDL in cultured trophoblasts have been described. However, other lipoproteins and their respective receptors have not been analyzed, and the subsequent intraplacental transport steps are still uncharacterized. (Adapted with permission⁵³⁵)

It is plausible that statin may indirectly affect growth and development of the fetus by influencing maternal-fetal cholesterol transfer across the placenta, and prevent changes in liver, kidney and vascular function in the fetus. This may also up-regulate EC activity by enhancing eNOS, increase NO bioavailability and decrease oxidative stress.⁵³⁷ Statin can therefore blunt the deleterious effects of placental insufficiency by preventing the alteration of these pathways associated with such inadequate utero-placental function.

It is also possible that effects of statin on offspring might be related to excretion of statin during lactation and this is supported by recent studies.^{492,493,538} In this respect, characteristics of statins vary. Atorvastatin, simvastatin, lovastatin, fluvastatin, cerivastatin and pitavastatin are lipophilic in nature and are metabolized by cytochrome P(450). They are more likely to be excreted in substantial amount in the milk. However, this is not the case with pravastatin that is relatively hydrophilic and not significantly metabolized by cytochrome P(450) enzymes. Hence its concentrations in milk should usually be negligible.⁵³⁹⁻⁵⁴⁰ Though the HMG-CoA reductase inhibitors are not generally recommended for nursing mothers, a previous study conducted in humans that pravastatin excretion in milk is negligible.⁵⁴¹ There may therefore be an argument for testing statin treatment in early postnatal life in hypercholesterolemic mothers, initially in animal models.

We also observed that offspring from hypercholesterolemic dams were less active, providing another aspect of the model that mimics the early origins of the ‘couch-potato’ syndrome in humans⁵⁴². Although this has been previously observed when dams were undernourished during pregnancy⁵⁴³⁻⁵⁴⁴ the present study is the first to show that a maternal HF diet during pregnancy can also result in sedentary behaviour in the offspring. Moreover statin treatment of the dams ameliorates this effect. The mechanisms underlying these effects are not known. Although inadequate cholesterol provision to the developing fetus is deleterious to development of the central nervous system⁵⁴⁵ the effects of a hypercholesterolemic condition during pregnancy have not been reported.

We recognise that prolonged exposure to the HF diet not only led to hypercholesterolemia and hypertension prior to and during pregnancy, but can also result in the development of obesity. This would almost certainly be associated with insulin resistance, increased inflammation and concomitant immune responses, which also affect developmental origins of cardiovascular disease.^{532,538} It is therefore not possible at this time to attribute unequivocally the changes in offspring blood pressure and activity level to maternal hypercholesterolemia. It also remains to be determined whether the protective effects of statin treatment are due to cholesterol-

lowering during pregnancy or to a lesser degrees of obesity, hypertension, or insulin resistance in mothers, i.e. whether statins prevent pathogenic effects by improving maternal health or whether they interfere with *in utero* mechanisms. It is more likely that the protective effect is due to cholesterol lowering. We however cannot discount the possibility that there may be other pleiotropic effects of the drug (e.g. on endothelial cells).

Although it has been suggested that maternal hypercholesterolemia enhances atherosclerosis in offspring, and is inhibited by antioxidant or lipid-lowering intervention during pregnancy,^{253,278, 287,546} it is difficult to establish that this is also true for the effect of statin on postnatal BP or activity levels. To resolve these questions, it would be necessary to compare the effect of statin to that of other hypocholesterolemic drugs, and to utilize an experimental design that minimizes differences in body weight and other parameters prior to pregnancy. This would be beyond the scope of the present work but should be considered a limitation of this work. It is also possible that the programming effect of statin occurs during early postnatal period since we continued giving it in the dam's drinking water during the lactation period. Studies have shown excretion of statin into the milk in rat dams that were treated postpartum with the statin atorvastatin.²²⁴ Statin could at this point reverse the programming effect of the maternal hypercholesterolemia similar to the effect of leptin administration in early postnatal life to offspring from undernourished mothers in preventing obesity, vascular dysfunction, sedentary behaviour and gender-specific hypertension in the offspring.^{287,547} More recently, it is debated about the pleiotropic actions of statin that includes increased NO bioavailability and reduced inflammation and oxidative damage.⁵⁴⁸ In this context, in a model of endothelial dysfunction, independent of dyslipidemia [rats fed a control (18% casein) or protein-restricted (9% casein) diet throughout pregnancy], statin treatment on vascular function was assessed. At weaning, a subset of the protein-restricted group was given atorvastatin (10 mg/kg per day) in the drinking water. The authors demonstrated that atorvastatin restored endothelial function and CRP to control levels in the females ($P < 0.05$) but its effects were gender specific and dependent on the vascular bed.⁵⁴⁹

Statins in general are still regarded as contraindicated during pregnancy, mainly due to previous reports of their teratogenic effects. Despite widespread use of statins however, and in many instances their inadvertent use during pregnancy, there is little evidence of their adverse effects in humans. A reconsideration of the use of statin in high risk situations therefore seems to be indicated, but this will remain controversial.

To summarise, the findings indicate that 1) Statin administration to HF pregnant dams during second half of pregnancy is safe and does not indicate any adverse effects on dams or their offspring; 2) Statin administration to HF pregnant dams not only improves their cardiovascular and metabolic health but it also gives some post-weaning protection to their offspring even when they are fed a HF diet 3) This might allow time for other intervention strategies to be put in place before the effects of a poor post-weaning diet set in, and 4) The improvement in offspring phenotype following statin treatment of their dams seen in our study further signifies the importance of mother's health during pregnancy as a major contributing factor to the rapidly developing cardiovascular epidemic.^{287,546}

The significance of this new evidence from the current work suggests that maternal hypercholesterolemia has a detrimental effect on the next generation. This is in line with interest by US National Cholesterol Education Program (NCEP) for drug therapy in children aged >10years of age whose LDL cholesterol remains elevated after dietary therapy, though this has not been tested fully.⁵⁴⁸ Based on this background, it would now be interesting to examine whether early postnatal statin treatment to offspring from hypercholesterolemic mothers would show the same protective effects against a post weaning HF diet, both in themselves and in their offspring. This will be the focus of the next chapter.

*For me when GOD solves my problems, I have faith in HIS abilities;
when God doesn't solve my problems, I believe HE has faith in my abilities*

CHAPTER-5

Effects of Long-term Statin Treatment in Hypercholesterolemic Pregnant Mice on their Offspring Fed a High Fat Diet.

5.1 Introduction

As discussed earlier (Chapter-1), current literature supports the concept that maternal under-nutrition of calories, proteins and a number of micronutrients has a profound effect on the fetus and increases its risk of metabolic and CVD disease.⁵⁵⁰⁻⁵⁵⁵ But what is more important today is the notion of HF overnutrition-mediated teratogenesis, predominantly based on animal studies, such as described by Freinkel and colleagues.⁵⁵⁶ This explains the wide-ranging fetal effects of maternal hyperglycemia and related metabolic abnormalities. Pettit and colleagues made very interesting observations in Pima Indians. Using prospective serial databases from a Pima Indian community, they assessed the contribution of genetics and the intrauterine environment to the risk of obesity and metabolic syndrome in the offspring.⁵⁵⁷⁻⁵⁵⁸ These authors demonstrated that children of diabetic mothers had a higher risk of being obese. Subsequently, they showed that risk of diabetes in the child was many times higher if the mother had diabetes during pregnancy (intrauterine exposure) compared with the risk in the children whose mothers developed diabetes after pregnancy (genetic risk). These results suggest that intrauterine overnutrition is more important in intergenerational propagation of metabolic syndrome and eventually CVD compared with genetic factors. Therefore, overnutrition in young girls is beginning to become a major factor in the escalating epidemic of diabetes, obesity and hypercholesterolemia. Up to 70% of young diabetes could be ascribed to maternal diabetes and children of diabetic mothers have a higher risk of glucose intolerance at a young age.⁵⁵⁹⁻⁵⁶⁰

Previous studies have provided compelling evidence for close associations among obesity, essential hypertension, and metabolic disorders [discussed in Ref 559]. It is reported that these disorders could lead to end-stage renal disease (ESRD)⁵⁶¹⁻⁵⁶² associated with increased glomerular filtration rate, renal blood flow, glomerulomegaly and in extreme cases focal segmental glomerulosclerosis.⁵⁶² Though two main risk factors (hypertension and hyperglycemia) for ESRD are closely linked to excess body weight, this association in particular with metabolic syndrome still remains robust in some but not in all studies.⁵⁶³⁻⁵⁶⁶ Yet several lines of evidence now indirectly suggest that dyslipidemia may be an important factor in the development and progression of ESRD. Observational data and a recent meta-analysis point out that elevated triglycerides and low HDL are independent risk factors for the development or acceleration of ESRD and early atherosclerosis lesions and that the use of statin may slow their progression.⁵⁶⁷⁻⁵⁷⁴

In view of the current environment of increasing fat utilization, decreasing energy expenditure, adiposity and increasing body weights in young adults, we can assume that CVD (e.g. atherosclerosis) begins in early childhood,⁵⁷⁵ Also, it is reported that elevated serum levels of total and LDL-C are associated with fatty streaks and fibrous plaques in aorta in adolescents and young adults⁵⁷⁵⁻⁵⁷⁶ and reducing dietary saturated fat and cholesterol reduces blood total cholesterol and LDL-C.⁵⁷⁷ So diets reduced in total fat, saturated fat, and cholesterol are recommended for healthy children 2 years of age and older, with greater reductions recommended for children with elevated blood cholesterol and with a family history of premature coronary heart disease.⁵⁷⁵⁻⁵⁷⁸

Questions have been raised about the safety of such interventions, including possible growth retardation, nutritional inadequacy, and adverse psychosocial effects,⁵⁷⁹⁻⁵⁸⁰ and about the effects on blood lipids and cholesterol in children.⁵⁸¹ In 1987, the Dietary Intervention Study in Children (DISC), a multicentre, controlled, randomized trial, addressed these efficacy and safety questions in children aged 8-10 years with LDL-C levels greater than or equal to the

80th and less than the 98th percentiles for age and sex. The participants were randomized into an intervention group (n = 334) and a non-treatment group (n = 329). The trial reported that at 3 years, dietary total fat, saturated fat, and cholesterol levels decreased significantly in the intervention group (P < .001). Levels of LDL-C decreased in the intervention and non-intervention groups by 0.40 mmol/L (15.4 mg/dL) and 0.31 mmol/L (11.9 mg/dL), respectively. When variables adjusted for baseline level and sex, the mean difference between the groups was -0.08 mmol/L (-3.23 mg/dL) (95% confidence interval [CI], -0.15 to -0.01 mmol/L [-5.6 to -0.5 mg/dL]) (P = 0.02).⁵⁸²⁻⁵⁸³ Accordingly, early initiation of statin treatment in children might be advantageous to them, but unfortunately studies of such treatment have so far only addressed short-term tolerability and safety.⁵⁸⁴⁻⁵⁸⁸ These important findings reasons that once diagnosed, adult hypercholesterolemic patients are prescribed lifelong treatment with statin and that postponing statin treatment until adulthood could allow development of significant arterial lesions in young hypercholesterolemic patients.

The findings of preceding chapters in this thesis that a maternal diet rich in fat, either during pregnancy alone or long-term, induces obesity, vascular dysfunction, sedentary behaviour and gender-specific hypertension in the offspring.^{435,574} The situation is analogous to the description by Freinkel *et al*⁵⁵⁶ in a diabetic pregnancy. The results of the last chapter (Chapter-4) demonstrate that treating pregnant animals that were hypercholesterolemic and hypertensive with pravastatin during the second half of pregnancy and lactation shows evidence of improved dam's health and effects on total cholesterol, LDL and HDL cholesterol in offspring and lasting beneficial effects on blood pressure and activity if their offspring consumed a similar high fat diet.⁴³⁵ I also showed that maternal treatment with pravastatin is well tolerated and has no deleterious effects on their offspring.⁴³⁵

Now when the safety and effects of statin, given to dams consuming a high-fat, high-cholesterol diet in the second half of pregnancy and lactation, have been demonstrated in reducing cardiovascular risk factors in offspring (chapter-4), it is now hypothesized that statin

in mice from the time they are weaned until weaning of their offspring could modify their lipid/ lipoprotein profile to such an extent that effect could be sustained into adulthood of the offspring consuming HF diet.

Based on hypothesis, in this chapter I designed experiments 1) to evaluate the long-term administration of pravastatin in young mice fed a HF from the time they are weaned; 2) the overall safety and tolerability of long-term pravastatin treatment pre-pregnancy, pregnancy and lactation; 3) to assess the influence of such a regimen on CVD risk factors in their offspring, even if both dams and offspring are fed a HF diet and 4) finally whether there are any gender-linked differences in offspring of these dams in their cardiovascular responses.

5.2 Methods

The protocol for conducting experiments is explained in Figure-5.1

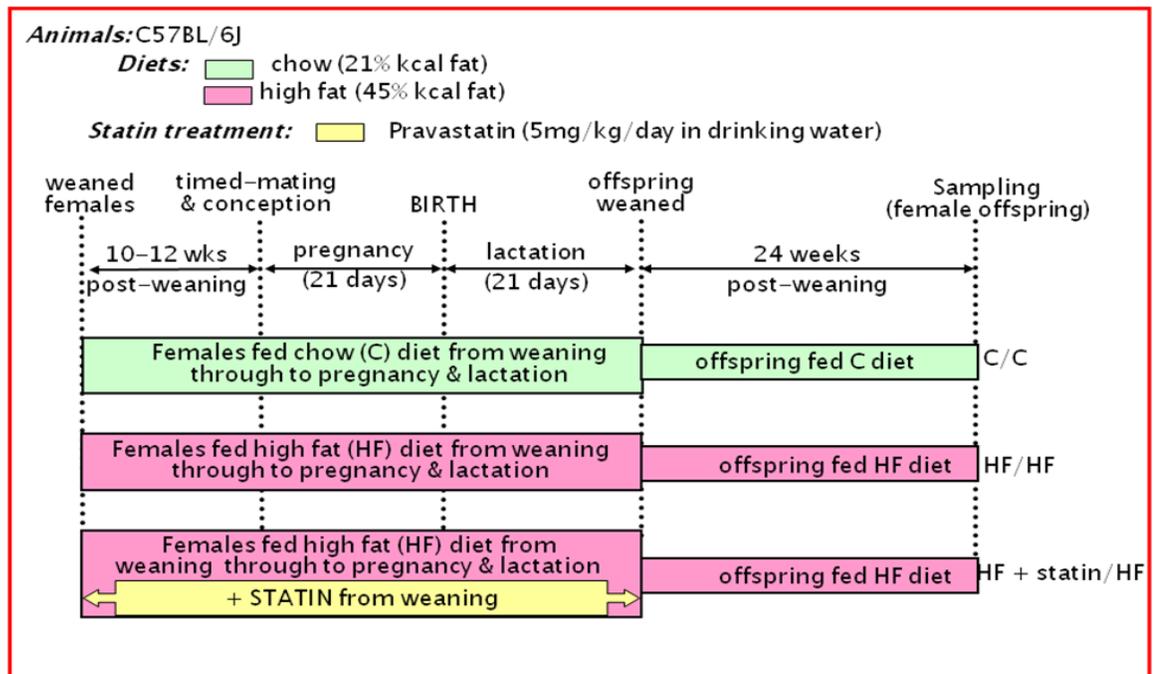


Figure-5.1: Flow diagram of the experimental protocol.

The main point of this experimental protocol was the administration of a water-soluble Pravastatin (Sigma UK; 5mg/kg/day), to females on HF diet from weaning through to pregnancy and lactation.

After birth, pups were weighed and litter size was reduced to 8 pups and, when possible, to equal numbers of males and females. From weaning (21 days post partum) offspring from the HF, C and HF+ S dams were fed HF, C and HF diets respectively. We refer to the offspring born to HF dams as HF-HF, born to C dams as C-C and born to HF+S and on HF diet as HF+S-HF according to their post-weaning diet (Figure-5.1). Body weights, SBP and biochemical markers (LDL-C and CRP) of the offspring were measured at 24 weeks. All the data were expressed as mean +/- SEM. A $p < 0.05$ was considered to be statistically significant.

5.2.1 Histological analyses of kidneys

Kidneys were removed at 24 weeks and immediately placed in ice-cold phosphate-buffered saline (PBS). A coronal cross-section containing the hilus was removed from the right kidney, fixed in neutral buffered formalin, and embedded in paraffin. Sections (5- μ m thick) of formalin-fixed, paraffin-embedded tissue were mounted on glass slides and stained with the hematoxylin and eosin, for general histological assessment. Frozen sections were used for Oil Red O staining to determine renal accumulation of neutral fats. All tissues were evaluated without my knowledge of the group from which they originated.

5.2.2 Other Blood and Urine Chemistries

Plasma glucose was measured using the glucose CII kit (Wako Chemicals USA, Inc., Richmond, VA). Plasma insulin was determined using a mouse insulin enzyme-linked immunosorbent assay kit (LINCO Research, Inc., St. Charles, MO). Urine albumin

concentration was determined by using a competitive enzyme-linked immunosorbent assay via the Albuwell M kit (Exocell, Philadelphia, PA). Urine creatinine concentration was determined by the Creatinine Companion kit (Exocell).

5.3 Results

5.3.1 Long-term statins administration to dams fed HF diet improves their body weights, systolic blood pressure and alters total cholesterol profile

The food and water intake was similar in female mice of all three groups throughout the study. However, body weight gain in C dams was lower than in HF dams at the weaning of their offspring. Predictably, the cholesterol-enriched HF diet increased cholesterol levels in HF dams. At mating, the total serum cholesterol levels were twofold higher in the HF group than the C group. At weaning, the cholesterol values in HF dams reached their highest levels, to three fold higher than in C dams. This was also reflected in the tail cuff SBP in the HF group when compared with C group. Long-term statin treatment in HF dams reduced SBP, total cholesterol and LDL cholesterol levels at weaning of their offspring (Table-5.1)

5.3.2 Long-term pravastatin treatment to dams fed a HF diet reduces body weight & improves systolic blood pressure in offspring on a post-weaning HF diet

In female offspring, a significant increase in weights was observed in HF diet fed offspring from mothers on similar diet (HF/HF) when compared to offspring fed laboratory chow from mothers on laboratory chow (C/C) ($P < 0.01$). Similarly, HF/HF male offspring were

Table-5.1: Body weight, total serum cholesterol, and tail cuff blood pressure evaluated in control (C), High Fat (HF) and High Fat + Statin from weaning (HF+S) female mice. Body weight gain was taken as difference between body weight at mating and body weight at weaning of their offspring (n=8/group)

Groups	Body weight (g)	Cholesterol mmol/L		Systolic Blood Pressure (mm Hg)		Plasma Glucose (mg/dL)		Plasma Insulin (ng/ mL)	
		1	2	1	2	1	2	1	2
C	34.2 ±3.0*	2.95 ± 0.2*	2.76 ± 0.3*	110.5 ±2.3*	114.3 ±2.4*	128.7± 12.3*	130.7± 14.3*	0.51 ±0.06*	0.49 ±0.04*
HF	52.3 ± 3.0	5.59 ± 0.3	6.31 ± 0.2	140.2 ± 3.2	137.6 ± 3.1	234.5± 16.7	233.5± 17.8	4.84 ± 0.30	5.34 ± 0.45
HF+S	45.2 ± 3.0‡	3.56 ±0.2‡	3.51 ±0.3‡	128.4 ±2.3‡	122.3 ± 3.0‡	130.6± 14.3‡	132.5± 15.4‡	0.50 ±0.10‡	0.53 ±0.08‡

1= at mating; 2= at weaning of their offspring. Statistical result displayed in each column is for the dietary group comparison with the other group using the Turkey-Kramer test.*P<0.01 and |P<0.001 for HF+S or C against HF. ‡P<0.001 for C against HF+S. The number of animals is 8 for all of the groups and the results are expressed as mean ± SEM

significantly heavier as compared to C/C ($P<0.001$). However, long-term pravastatin treatment to dams fed a HF diet reduced body weight in offspring on a post-weaning HF diet (HF+S/HF; $P<0.001$; Figure-5.2 a & b).

In female offspring, SBP was significantly lower at 24 weeks in HF+S/HF compared with HF/HF offspring ($p<0.001$). However, the differences were not so pronounced in male offspring although still significant ($p<0.05$). As expected, SBP for the C/C group was lower at the time point (24 weeks) examined (Figure-5.2 c& d).

5.3.3 Long-term pravastatin treatment to dams fed a HF diet reduces total cholesterol & effects CRP in offspring on a post-weaning HF diet

Total serum cholesterol concentrations for offspring on HF or C diets followed a similar pattern to dams on HF or C, respectively and long-term exposure of their dams to pravastatin resulted in significantly lower total cholesterol levels, similar to its effect in the dams themselves ($P<0.001$, Figure 5.3a and 5.3b respectively). Of particular note is that a similar effect was observed in males ($P<0.001$). In females, HF/HF exposure significantly increased CRP in contrast to C/C group. The effect was the same though the magnitude of difference was less in male offspring. Pravastatin administration in early life to dams fed a HF diet significantly abolished CRP mediated inflammation in their offspring on a post-weaning HF diet ($P<0.001$; Figure 5.3c and 5.3d respectively).

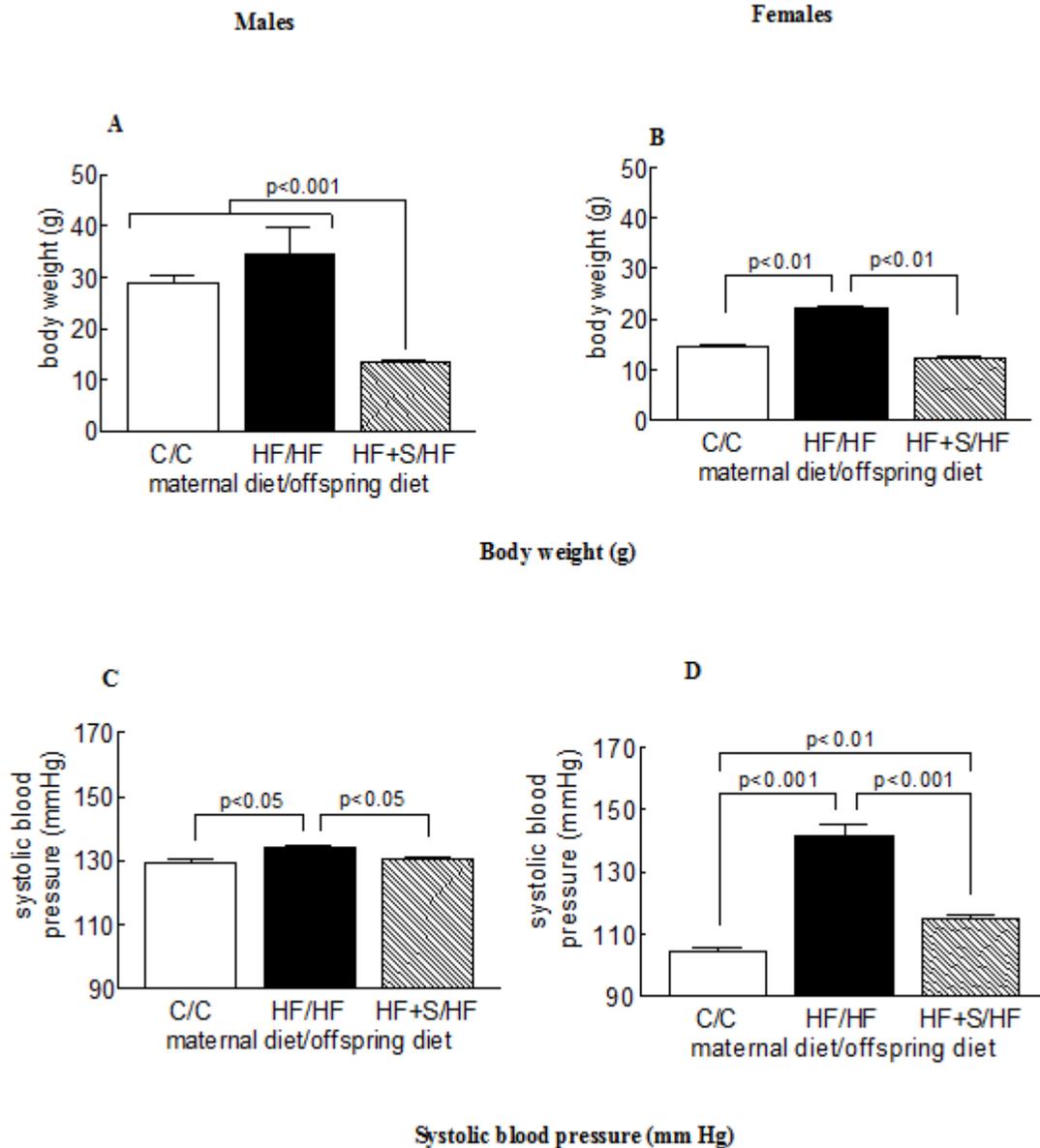


Figure-5.2: Long-term statin treatment in hypercholesterolemic mothers has beneficial effects on the body weights and blood pressure in their offspring. (a) Body weight gain males, (b) body weight gain in female (c) systolic blood pressure in male (d) systolic blood pressure in female offspring from mothers on standard chow (C), high fat-high cholesterol (HF) diet or HF diet and treated with statin during pregnancy and lactation (HF-S). Weaned offspring were then fed either HF or C diets to adulthood, thus generating the dam/offspring dietary groups: HF/HF, HF-S/HF and C/C (n = 8 per group).

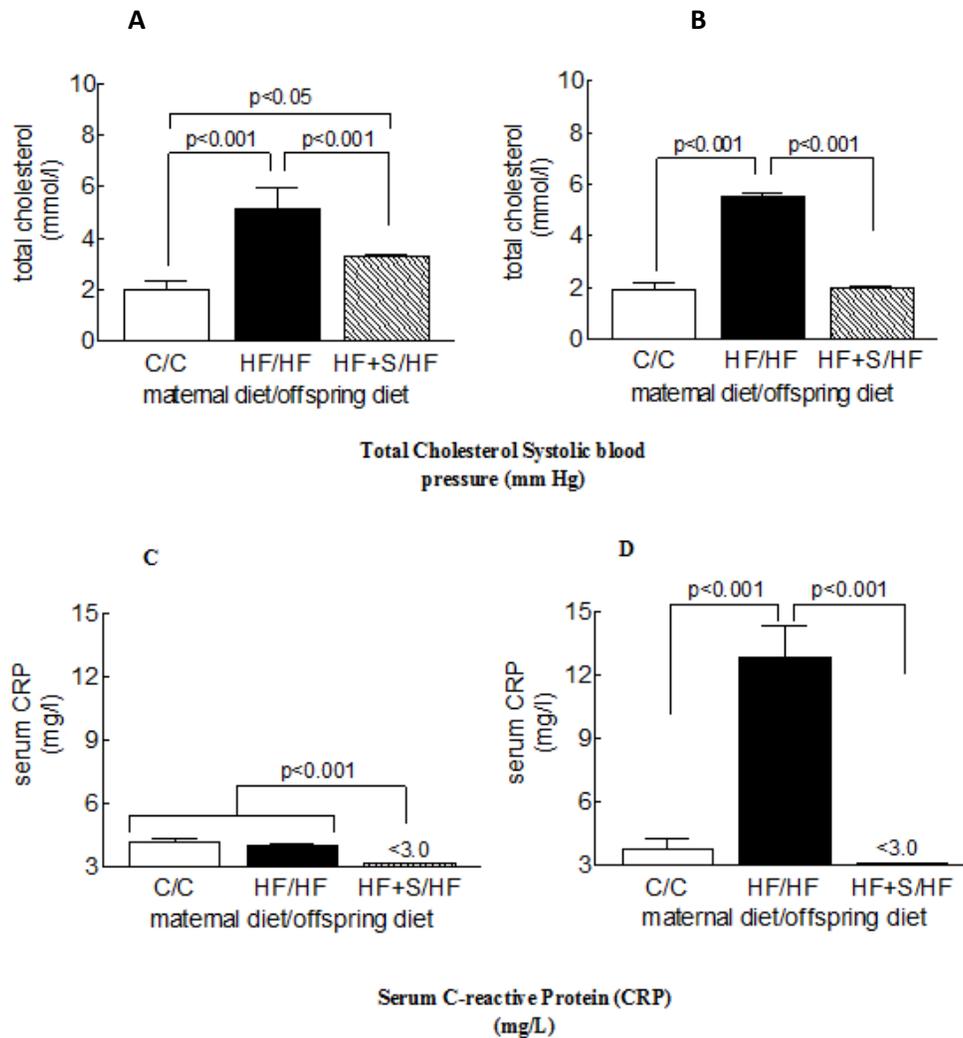


Figure-5.3: Long-term statin treatment in hypercholesterolemic mothers has beneficial effects on total cholesterol and CRP in their offspring. (a) Total cholesterol levels in males, (b) Total cholesterol levels in female (c) CRP levels in male (d) CRP levels in female offspring from mothers on standard chow (C), high fat-high cholesterol (HF) diet or HF diet and treated with statin during pregnancy and lactation (HF-S). Weaned offspring were then fed either HF or C diets to adulthood, thus generating the dam/offspring dietary groups: HF/HF, HF-S/HF and C/C (n = 8 per group).

5.3.4 Long-term pravastatin treatment to dams fed a HF diet reduces plasma glucose and insulin in offspring on a post-weaning HF diet

Plasma glucose and insulin levels were significantly higher in HF/HF than in those with C/C ($p < 0.001$). However, these diet-induced changes were not observed in HF+S/HF. In fact, HF+S/HF exhibited no diet-induced hyperglycemia and hyperinsulinemia. The diet-induced changes in plasma free fatty acid levels were not statistically significant in either of the group (Table-5.2)

Table 5.2: Comparison of variables in three groups.

	C/C	HF/HF	HF+S/HF
Plasma glucose (mg/dl)	131.5 \pm 14.3	215.6 \pm 15.6	139.6 \pm 13.3
Plasma Insulin (ng/ml)	0.42 \pm 0.07	5.48 \pm 0.40	.48 \pm 0.09
Plasma free fatty acid (mM)	0.69 \pm 0.06	0.88 \pm 0.08	0.71 \pm 0.1

Results are expressed as mean \pm SE. Each group includes 8 animals.

5.3.5 Long-term pravastatin treatment to dams fed a HF diet reduces renal triglycerides and total cholesterol and lipid accumulation in offspring on a post-weaning HF diet

Kidney weights were similar between the HF/HF and C/C offspring. HF/HF showed lower values than C/C group when kidney weights were expressed relative to body weight ($P < 0.01$). However, when kidney weights were expressed relative to body weight, HF+S/ HF showed higher values than HF/HF offspring ($P < 0.01$). To study whether HF caused an increase in renal lipid accumulation I performed oil red O staining in kidney sections, which demonstrated the accumulation of lipids in the glomerular and tubulointerstitial cells of HF/HF. There were

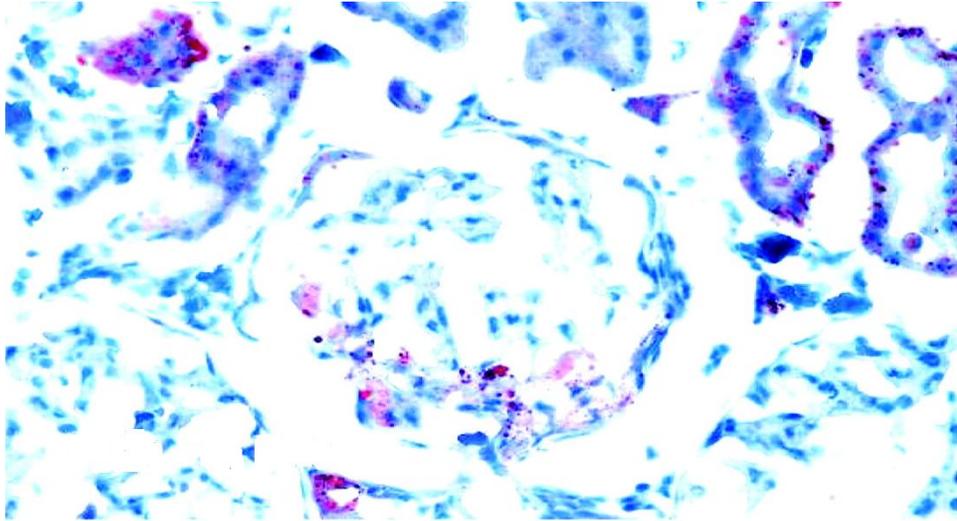
significant increases in renal triglycerides and cholesterol content in HF/HF, which cholesterol with the increased oil red o staining (Figure 5.4a). However, HF+S/HF mice had no increases in renal triglycerides and cholesterol contents (Figure 5.4b). No attempt was made to quantify the cell types using a definitive method.

5.4 Discussion

The purpose of this chapter was to investigate the role of long-term statin administration in a HF-induced metabolic syndrome mouse model. I examined 1) whether statin treatment was efficacious earlier in the life course; 2) whether lipid lowering in HF fed dams prevented future CVD/ metabolic syndrome in their offspring in which the response to lifestyle could often be inadequate; 3) whether cholesterol lowering in dams or other effects of statins benefit postnatal kidney specific changes.

Once again characteristics of metabolic syndrome such as obesity, hypercholesterolemia, hypertension and hyperinsulinemia in HF dams as well as in HF/HF offspring are observed and thus confirmed that maternal nutrition with a HF diet induces developmental disturbances in their offspring. The results also demonstrate that treating dams fed a HF diet with pravastatin after weaning not only improved their health but also showed long-term beneficial effects on offspring BP, cholesterol profile, body weight and CRP levels even if their offspring consumed a similar HF diet. This effect is seen in males and females although more pronounced in females. The latter is an interesting finding that I did not observe in my previous study where statin therapy was initiated in the 2nd half of pregnancy (Chapter-4). Furthermore, the present findings provide the first indication that renal lipid accumulation could mediate the kidney disorder associated with HF feeding in dams, however, cholesterol lowering in these mice on HF diet after their weaning prevents such lipid accumulation in HF+S/HF offspring.

(a)



(b)

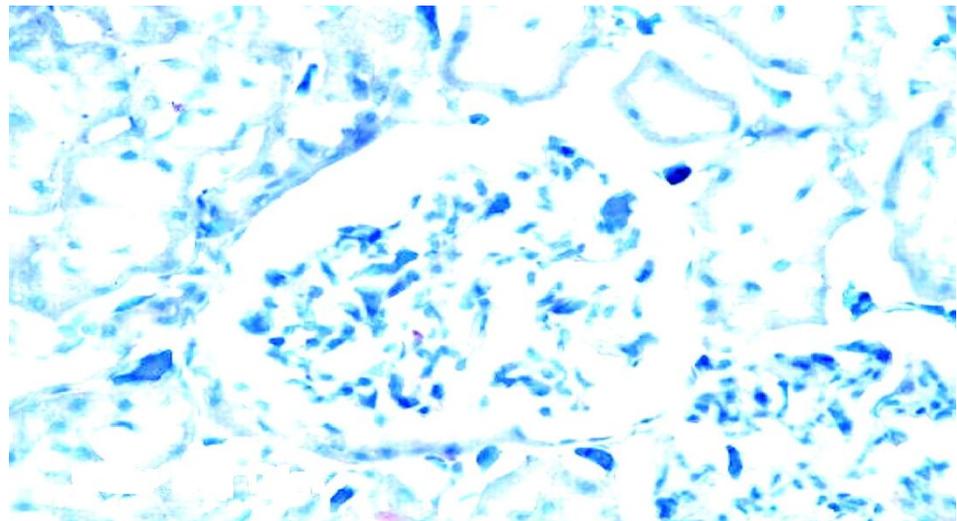


Figure 5.4: Representative photomicrographs of Oil Red O stained renal sections from (a) HF/HF and (b) HF+S/HF mice. The sections were counterstained with hematoxylin and eosin. Lipid droplets appeared as red spots that revealed the accumulation of neutral lipids in the glomerular and tubulointerstitial cells and showed an increase in HF/HF compared with HF+S/HF (n=8)

It is very clear now that the early life environment has a significant role in the aetiology of later CVD disease.⁵⁸⁹⁻⁵⁹⁰ Treating future mothers on HF diet with pravastatin very early in life (post weaning) to study the effect on their offspring health is more representative experimental approach of the human situation. Though, giving pregnant HF dams pravastatin induces healthy changes in them and their offspring when put on similar HF diet, the approach in Chapter-4 was confined to short-term administration of pravastatin to pregnant HF dams, during pregnancy and lactation only. This may explain why the effects of such treatment were only transient in their offspring for 10-12 weeks post weaning. Such short term effects are not observed the present work.

Only a few studies have been conducted to date evaluated statin therapy in a young age group (children and adolescents). Stein was the first to show a 40% reduction of LDL-C in hypercholesterolemic and obese children treated with statin. But this study was not controlled and involved only a small group of boys⁵⁹¹. Ducobu and colleagues in a small (n=32) and uncontrolled study with simvastatin reported a 37% LDL-C reduction and excellent tolerability.⁵⁹² Later, three other statin studies in children or adolescents were reported. In the first study,⁵⁹³ 72 hypercholesterolemic children (66% girls), aged 10 to 16 years, were randomized to placebo or pravastatin (5, 10, or 20 mg/ day). After 12 weeks, LDL-C levels were reduced by 23%, 24%, and 33% in the groups receiving pravastatin at 5, 10, and 20 mg/day, respectively. The second study⁵⁹⁴ was an uncontrolled study in which boys were randomized to lovastatin at 10, 20, 30, or 40 mg/day for 12 weeks. LDL-C levels were reduced by 21% to 36%. In the last study⁵⁹⁵, 132 boys, aged 10 and 17 years were randomized to either lovastatin or placebo. Lovastatin was started at 10 mg/dL, and the dosage was doubled every 8 weeks to a maximum of 40 mg/d. Mean LDL-C levels decreased significantly relative to placebo in all treatment groups. In these studies, short-term safety and tolerability were observed to be excellent with no serious adverse events (in terms of myopathy, drug-related events or clinically meaningful elevations in hepatic transaminases (ALT and AST) and creatine phosphokinase). These results are comparable to those observed in the present work namely that cholesterol and SBP in HF+S remained within normal limits at all times (at

mating and weaning; Table 5.1) relative to their HF fed counterparts. This is a very novel finding. I believe that such effects are fundamental to the DOHaD concept and indicate that intervention in early life may be particularly important in reducing later risk of disease in the face of lifestyle factors such as a HF Western cafeteria diet.

Another original finding is, that in HF/HF mice, there is increased Oil Red O staining in the kidney sections and higher renal content of cholesterol. Thus confirming that HF diet in mothers cause histological, biochemical and functional changes in the kidneys of their offspring fed a similar diet (i.e. HF/HF). These changes include accumulation of neutral lipids in glomerular and tubulointerstitial cells (Figure-5.4a). These changes are also associated with marked hyperinsulinemia, hypertension and hypercholesterolemia. This is not the case in HF+S/HF kidney sections (Figure 5.4b) where quite normal histological morphology is demonstrated. It is observed that long-term statin administration in HF dams ameliorates these effects in their offspring even when fed HF diet postnatally.

Although I did not design these studies to elucidate the specific mechanisms by which a HF diet caused structural changes in the kidney, there are several hemodynamic and hormonal alterations in obese dams fed a HF diet that might contribute to the renal changes in their offspring. For example, I observed that a HF diet caused increased SBP, glomerular hyperfiltration, and increased renal fat deposits and that these hemodynamic alterations were sustained for up to 24 wk in HF/HF. The combination of increased arterial pressure and renal vasodilation likely caused marked glomerular hypertension, that stretched the mesangium and initiates a complex cascade of biochemical and histologic changes.⁵⁹⁶ Similarly, using isolated glomeruli, Cortes *et al.*⁵⁹⁷ reported that an acute increase in glomerular pressure could stretch the mesangium and stimulate collagen production.

In addition to the hemodynamic alterations, there are also hormonal changes that could contribute to renal remodeling in obesity. One possibility includes hyperinsulinemia that occurred in this model. Evidence that elevated insulin levels could contribute to glomerular structural changes is derived primarily from *in vitro* studies of mesangial cell cultures.⁵⁹⁸⁻⁵⁹⁹ Moreover, the effects of insulin on angiotensin II seemed to be additive⁵⁹⁸. However, these effects had been observed *in vitro* using very high concentrations of angiotensin II (usually 10^6 M or higher) and insulin. *In vivo* studies with animals and humans suggest that blockade of angiotensin II reduces renal function associated with severe renal disease, but angiotensin II blockade also decreases arterial and glomerular hydrostatic pressures. However, it is yet not clear whether angiotensin II blockade prevents or ameliorates changes in renal structure and function mainly occurring early in obesity, even before severe renal injury. Hence, the role of increased angiotensin II levels and hyperinsulinemia in mediating the renal changes associated with a HF diet and obesity yet remains to be elucidated.

These observations may partly explain the structural changes observed in glomeruli after a HF diet. Yet the contribution of long-term statin treatment in HF dams and its effect on glomerular hyperfiltration to the production of biochemical and structural changes in the renal cortex of their offspring (HF+S/HF) still remains unclear. There is increasing evidence that demonstrates the role of lipid metabolism in renal disease. It has been reported that inhibition of cholesterol synthesis by statins and of triglyceride synthesis by peroxisome proliferator activated receptor- α agonists (fibrates) protects against diabetic and non-diabetic renal disease.⁶⁰⁰⁻⁶⁰¹ A recent meta-analysis of several small scale interventional studies in diabetic and non-diabetic human subjects with glomerulosclerosis and proteinuria indicates that long term treatment with statin and/or fibrates significantly prevents the decline in glomerular filtration rate.⁶⁰²

The importance and direct effects of lipids *per se* in causing kidney cell injury is mediated by pro-inflammatory cytokines that includes interleukins, TNF- α and CRP.⁶⁰³ The findings of this study demonstrate an increase in total cholesterol and result in CRP upregulation towards

glomerular injury. This association has also been demonstrated in cell culture studies.⁶⁰⁴⁻⁶⁰⁵ Studies in renal mesangial and tubular cells grown in culture have shown that incubation, of these cells with LDL or vLDL, results in up-regulation of the growth factors, including transforming growth factor- β and platelet derived growth factor; ECM proteins; adhesion molecules, including monocyte chemoattractant protein-1, intercellular adhesion molecule 1, and vascular cell adhesion molecule-1.⁶⁰⁴⁻⁶⁰⁶ My previous findings in chapter-3 suggested that hepatocytes lipid accumulation associated with increased lipid synthesis is involved in NAFLD seen in the HF animal model. Whether a similar alteration in renal lipid metabolism and accumulation of lipids mediates the kidney disease associated with obesity-initiated metabolic syndrome, is yet to be established.

In summary, the present evidence links pathological effects in offspring with impaired fetal environment modulated by maternal HF diet. These changes are more pronounced in the female offspring unlike the non-gender effects observed in chapter-4. HF/HF exposure increases inflammation (CRP) and results in renal fat deposits, early components of functional changes and structural changes in the kidney (ESRD). Pravastatin administration in early life to dams fed a HF diet protected their offspring from risk of later cardiovascular pathophysiology even if they consumed a HF diet. Furthermore, pravastatin administration to dams before and during pregnancy produced no deleterious effects on their offspring. Because the phenotypic changes observed in the HF+S-HF offspring appears to be permanent in nature, the question of an extension of the clinical use of statin arises, yet caution.

Therefore, after defining these important links, it is speculated that these changes may possibly relate to the effects on endothelial dysfunction in a manner dependent or independent of cholesterol levels, inflammation or even hepatic and renal lipid accumulation. Because endothelial dysfunction is a pathological precursor for early hypertension and CVD, it would be interesting to study the ability of HF and pravastatin in modulating vascular responses in

offspring. One way of investigating this is to study the impact of maternal HF diet on endothelial progenitor cells (EPCs) in offspring. This will be the focus of the next chapter.

*Life has taught me a succession of lessons
which I think must be lived to be understood*

CHAPTER-6

Effects of Maternal High Fat Diet and Statin Treatment on Bone Marrow Derived Mononuclear Cells in Offspring fed a Similar Diet.

6.1 Introduction

The results presented so far in the thesis lead to the next question of whether HF diet consumption in pregnancy plays any role in vascular response. This is characterised by endothelial dysfunction in their offspring; thus increasing the risk of early CVD in their adult life.

Endothelial dysfunction is a common phenomenon in CVD, occurs in the metabolic syndrome and is known to be a primary event in the aetiology of atherosclerosis.⁶⁰⁷⁻⁶⁰⁸ Experimental and clinical evidence suggest that diabetes and hyperlipidemia are related to endothelial cell dysfunction, peripheral blood and bone marrow stem cell dysfunction, and excessive vasoconstriction.^{360,609-614} In addition, the extent of diabetes- and hyperlipidemia-related impaired potency of bone marrow mononuclear cells can affect effective vascular angiogenesis.^{247,615} Endothelial dysfunction is also a common phenotype in a number of rodent models of DOHaD that include both maternal HF and total maternal nutrient restriction models.⁶¹⁶⁻⁶¹⁸

Recent work has raised the possibility that bone marrow derived endothelial progenitor cells (EPCs; mononuclear in phenotype) may help restore normal vascular function after injury or in disease.⁶¹⁹⁻⁶²⁰ Despite much that is known about EPCs (see Chapter-1), there are some important points that warrant discussing before focusing on the specific study question in this chapter.

It is reported that functional integrity of the endothelial morphology is essential to protect against the initiation of atherogenesis.^{76,280,621} Marked changes in endothelial morphology include a loss of orientation of endothelial cells in the direction of the blood flow, decreased overlap between adjacent endothelial cells, and an accelerated turnover of endothelial cells in response to hyperlipidemia, hypertension and inflammatory stress.⁶²² It has been proposed that this kind of disruption of vascular homeostasis predisposes the vessel wall to vasoconstriction, leukocyte adherence, platelet adhesion, thrombosis, vascular inflammation, and eventually atherosclerotic lesion formation.^{76,621-622}

Recent studies suggest that in response to these marked morphological changes in the surrounding mature endothelial cells, EPCs play a critical role in maintaining endothelial function in mature blood vessels by contributing to re-endothelialization and neovascularisation.⁶²³⁻⁶²⁴ It is therefore conceivable that the mobilization and differentiation of EPCs are important in this process of adult neovascularization^{623,625-628} and any impairment of this vasculogenic element of endothelial regeneration may account for the progression of endothelial dysfunction and CVD.⁶²⁸ Accumulating evidence also suggests that the number and migratory activity of circulating EPCs inversely correlate with circulating CRP^{349,361,442,629-630} one of the risk factors for CVD that interacts strongly with LDL cholesterol⁶³¹⁻⁶³².

Reports have proposed that CRP has a direct effect of inhibiting angiogenesis, promoting atherosclerotic processes and EC activation.^{308,309,633-637} In a synchronous fashion CRP downregulates eNOS and destabilizes eNOS mRNA transcription; decreases both basal and stimulated NO release;³⁰⁸ upregulates NFκ-B (a key nuclear factor that facilitates the transcription of numerous proatherosclerotic genes);⁶³⁸ mediate adhesion molecules and LDL uptake.⁶³³⁻⁶³⁴

These interrelations among LDL cholesterol, CRP and EPCs, suggest that CRP-related alteration in progenitor cell number and function in offspring may be induced by maternal HF consumption. Also it would be interesting to study, through net reduction effect on maternal dyslipidemic load, whether statin therapy in dams exerts any advantageous effect on EPC circulation in their offspring fed a similar HF diet. These hypotheses are supported by several experimental models and *in vivo* ischemic disease patients' studies.^{359,360,364,639-640} In these studies,^{359,360,364,639-640} the precise mechanisms remain unclear, yet it is shown that statin improves endothelial function by activating protein kinase Akt,⁶⁴⁰ mobilizing EPCs,³⁶⁰ reducing senescence, and increasing proliferation of EPC.³⁶³

A major area of DOHaD research also concerns the fate of progenitor cells within the early embryogenesis. This involves modifications in vascular endothelial cell function and changes such as reduced numbers of nephrons, pancreatic beta cells or cardiomyocytes, enhanced development of the islets of Langerhans in fetuses of diabetic mothers and hypertrophy of the endocrine pancreas, induced in early life. These experimental data in laboratory animals are confirmed by epidemiological studies on infants of mothers suffering from diabetes or malnutrition during pregnancy. This provides a novel paradigm that disturbances in the maternal metabolism alter the nutrient supply from mother to fetus and induce structural and functional adaptations during fetal development which persist throughout life.^{15,65,86-94,110-111,129,134-137,152,157-158,170,176,178-182,193-197}

In this chapter, I propose to examine the question of how maternal HF diet consumption affects EPCs of their offspring also fed a HF diet. Therefore, the aims of the study are;.

Study-1:

1. To determine the effects of feeding a HF diet to dams during pregnancy and lactation on cardiovascular function, EPC expression and serum CRP levels in their offspring
2. To examine the effect of statin treatment of HF-fed dams during pregnancy and lactation on these cardiovascular variables (EPC expression and CRP) in their offspring

Study-II:

To study the effects of statin administration to HF-fed female mice (from the time they were weaned until weaning of their offspring) on expression of bone marrow derived EPC in offspring.

6.2 Methods

The method for isolating, culturing and staining of bone marrow derived mononuclear cells i.e. EPCs has already been described in chapter-2.

The experimental protocol for Study-I is described in Figure-6.1. As shown in protocol, water-soluble Pravastatin (Sigma UK; 5mg/kg/day) was given in their drinking water to half of the pregnant females on HF diet during the second half of pregnancy and throughout lactation. The female offspring of C, HF and HF-S dams were fed *ad libitum* with the C and HF diets after weaning to generate the C/C, C/HF, HF/HF, HF/C; HF-S/HF and HF-S/C groups respectively.

Body weights of the offspring (from 1 week of age to avoid maternal rejection of the pups) and food intake (from weaning) were monitored until 24 weeks of age. SBP, biochemical markers (total, LDL and HDL cholesterol) and CRP were measured at 24 weeks.

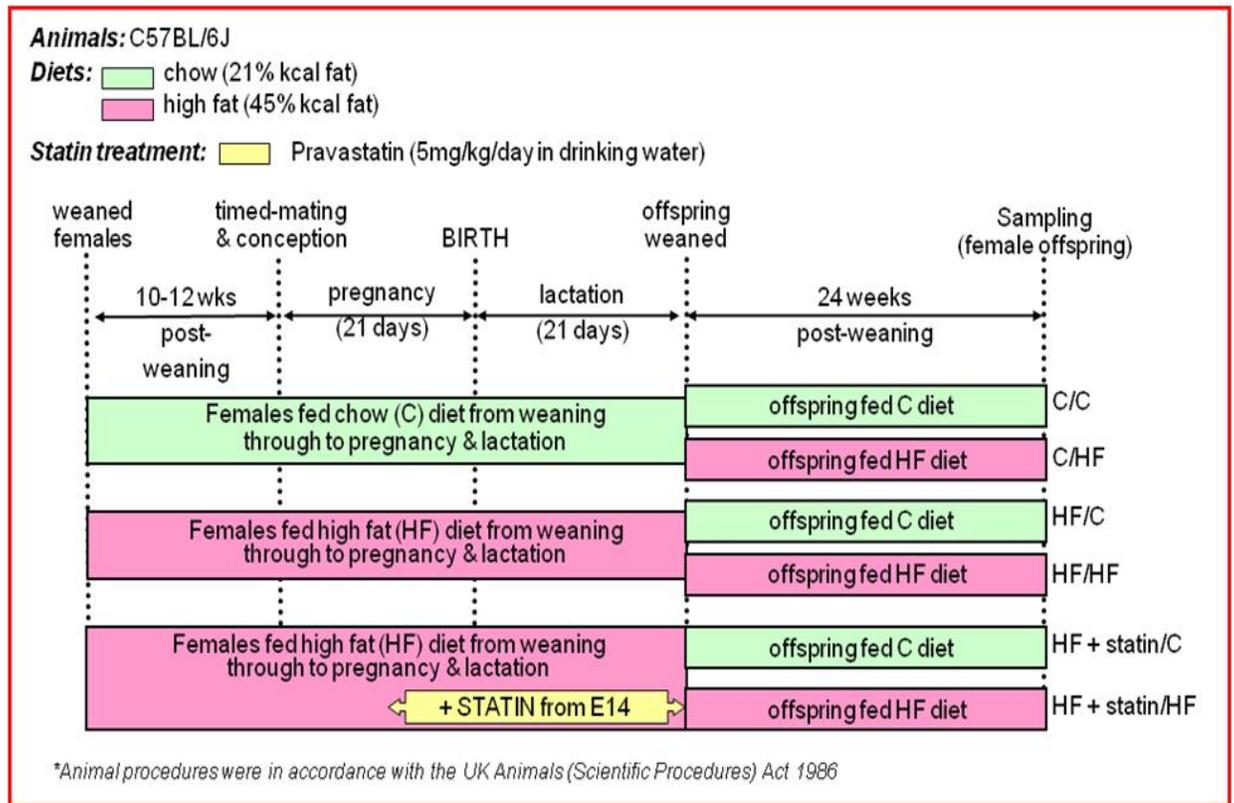


Figure 6.1: Experimental protocol for Study-I

Study-II:

This is an extension of work on the bone marrow derived EPC samples from the study described in Chapter-5. The EPCs were cultured and stained only in female offspring. The protocol for conducting experiments is already explained in Figure-5.1 and described as follows

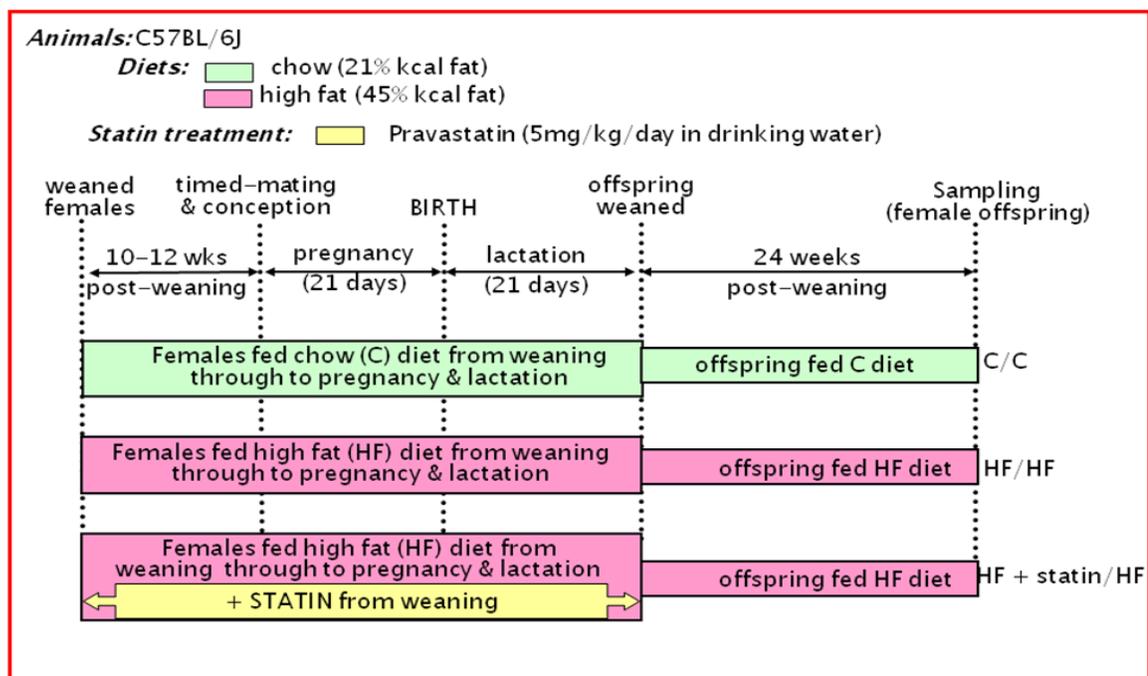


Figure-5.1: Flow diagram of the experimental protocol.

For both the studies, the data were expressed as mean \pm SEM and $p < 0.05$ was considered to be statistically significant.

6.3 Results

6.3.1 Study-I

6.3.1.1 Prenatal and postnatal HF diet consumption increase the risk of cardiovascular disorders in offspring

Female C/HF and HF/C and HF/HF offspring were heavier (Figure 6.2a), hypertensive (Figure 6.2b), with increased serum levels of LDL-cholesterol (Figure 6.2c) and CRP (Figure 6.2d) than C/C offspring at 24 weeks. However, the results demonstrated that total cholesterol levels

were significantly increased in HF/C and HF/HF not in C/HF female offspring than C/C at 24 weeks (Figure 6.2e). Short-term statin therapy (second half of pregnancy and lactation) in dams did not affect SBP (Figure 6.2b) but reduced the effect of prenatal and postnatal HF diet on bodyweight and LDL-cholesterol. Interestingly, pravastatin therapy reduced the levels of CRP to negligible in HF+S/C and HF+S/HF offspring.

6.3.1.2 Prenatal and Postnatal HF diet consumption attenuates bone marrow derived circulating endothelial progenitor cells in offspring

HF diet (prenatal or postnatal) had significantly reduced percentage of positively stained mononuclear cell and decreased number of double stained colonies with inhibited expression of acetylated low-density lipoprotein (Figure 6.3). Pravastatin treatment to these hypercholesterolemic dams significantly improved and increased the number of EPC observed in the culture. Representative photomicrographs of EPC colonies stained for endothelial markers Dil-Ac-LDL (red) and lectin (green) are shown in Figure 6.4)

6.3.1.3 Correlation of hypercholesterolaemia to EPC number

The number of EPCs was inversely correlated with total cholesterol (Figure 4A) and LDL-cholesterol (Figure 4B) levels, whereas no correlation between the number of EPCs and HDL-cholesterol levels ($r=0.237$, $P>0.05$) was observed. By multivariate analysis, total cholesterol (standard coefficient= -0.530 , $P<0.001$) and LDL cholesterol (standard coefficient= -0.417 , $P<0.01$) levels remained independent predictors of lower EPC numbers.

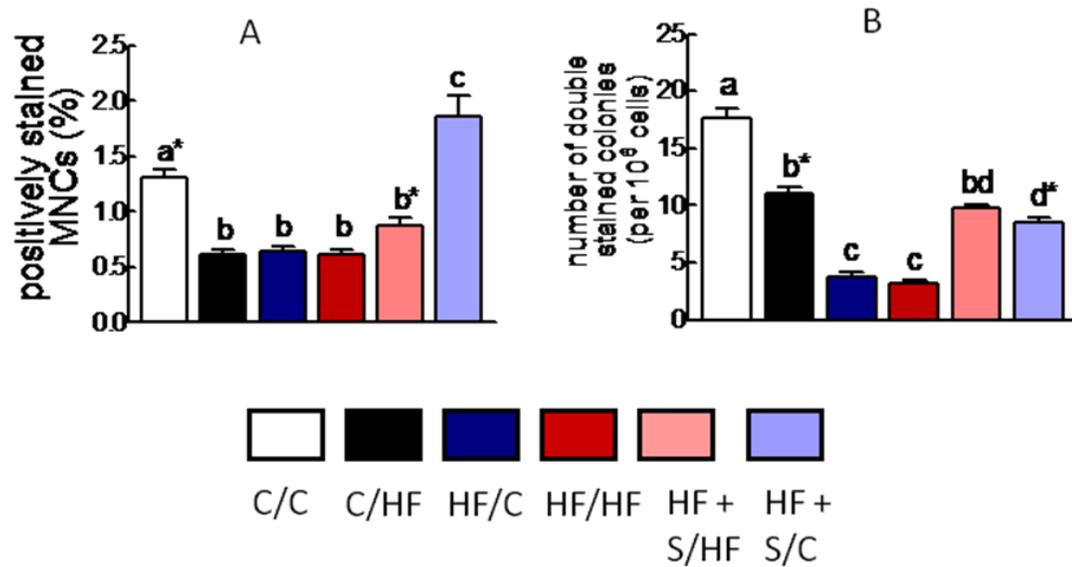


Figure 6.3: Pravastatin treatment in hypercholesterolemic mothers during late pregnancy and lactation has beneficial effects on (a) the positively stained mononuclear cells (MNCs) and (b) the number of double stained colonies in offspring from mothers high fat-high cholesterol (HF) diet. Different letters indicate $p < 0.001$ except between bars with different letters and asterisks (*) where $p < 0.05$, showing the level of significance.

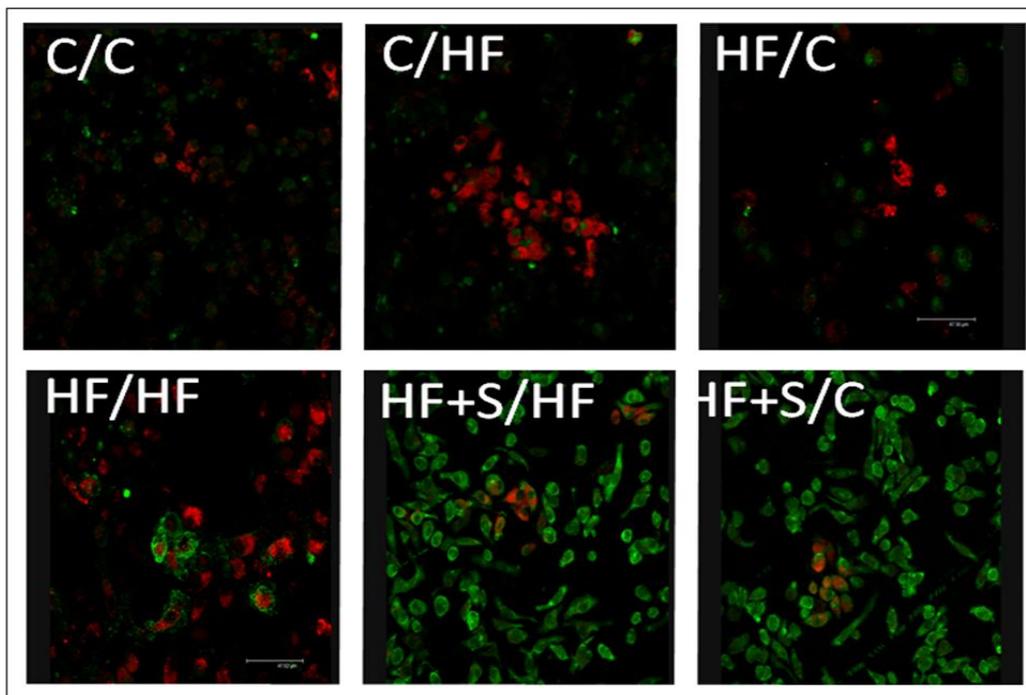


Figure- 6.4: Expression of endothelial markers on EPCs. Representative photomicrographs of EPC colonies stained for endothelial markers Dil-Ac-LDL (red) and lectin (green). EPC colonies demonstrate reduced staining in HF/HF vs C/C. Statin treatment to HF-fed dams abolished these effects in their offspring.

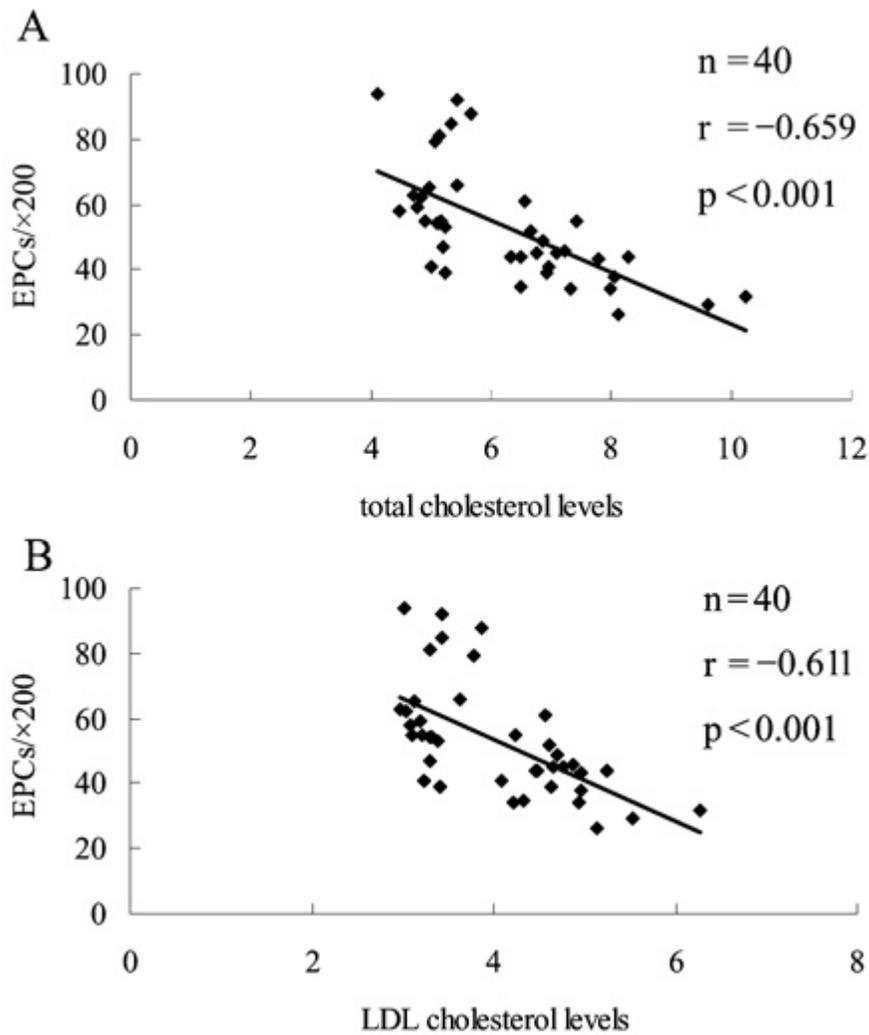


Figure-6.5 Correlation between the number of EPCs from HF/HF offspring with hypercholesterolaemia and total cholesterol (A) and LDL-cholesterol (B) levels

6.3.2 Study-II:

Long-term pravastatin treatment to dams fed a HF diet improves EPCs number and colonies in offspring on a post-weaning HF diet

Once again, HF/HF exposure significantly inhibits EPC numbers and colonies, therefore affecting key components of angiogenesis and endothelial repair in these offspring. Long term

pravastatin treatment in early life to dams fed a HF diet protects their offspring in a post-weaning HF nutritional environment from such effects of EPC (Figure 6.5).

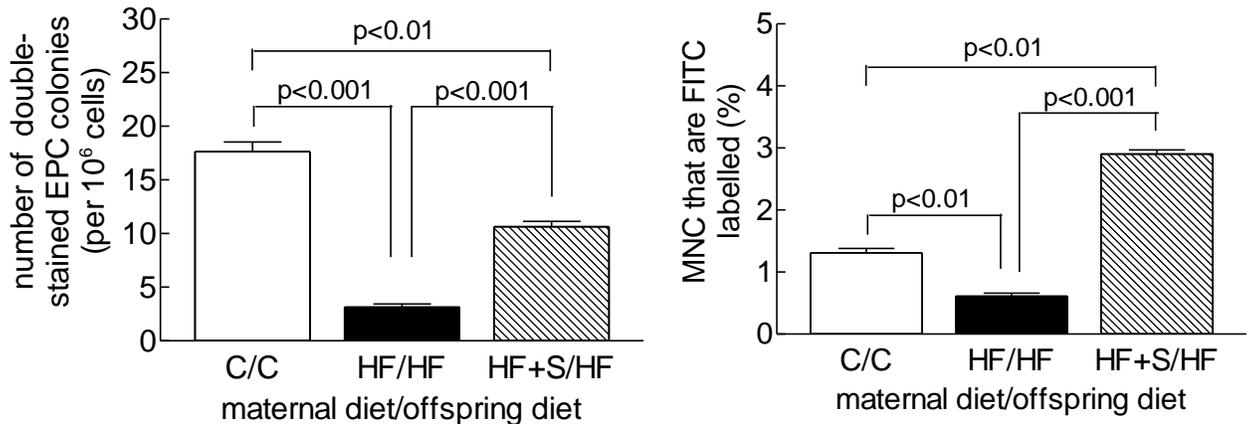


Figure -6.6: Long-term statin treatment in hypercholesterolemic mothers increased circulating endothelial progenitor cells (a) number of stained EPC colonies (b) percentage of mononuclear cells that are FITC labelled. Weaned offspring were then fed either HF or C diets to adulthood, thus generating the dam/offspring dietary groups: HF/HF, HF-S/HF and C/C (n = 8 per group).

6.4 Discussion

The present study investigates whether long-term maternal HF diet has an impact on the expression of bone marrow derived mononuclear cells (double stained endothelial progenitors,

EPCs) in their offspring even if they were fed a HF or C diet in adulthood i.e. to study the role of prenatal and postnatal diet on EPCs. This work also studies the effect of pravastatin {both short-term (second half of pregnancy and lactation) and long-term (soon after weaning through to pregnancy and lactation)} treatment in dams on HF diet on the number of EPC colonies in their offspring even when these offspring are fed the HF diet.

The results demonstrate that (1) EPC numbers and expression (Figure-6.4) in female offspring exposed prenatally or postnatally (C/HF, HF/HF and HF/C) to the HF diet are significantly decreased; (2) treating dams with pravastatin (in both protocols) proves beneficial for improving EPC colony forming units in their offspring irrespective of their postnatal diet; (3) number of EPC is inversely correlated with total cholesterol and LDL-cholesterol levels (4) LDL-cholesterol is a predictor of EPC expression and (5) maternal hypercholesterolemia increases CRP levels in plasma and inhibits number of EPC colonies expressed in adult offspring (Figure 6.7). Though the mechanism behind this pathophysiology still remains elusive, improvement of EPC numbers observed in HF+S/HF offspring is probably related to the blockade of CRP induced excessive inflammation and the improvement in cholesterol profile. Previous studies have reported the role of statin in improving angiogenesis^{359,639-640} but not directly in the context of DOHaD phenomenon. This is a very important and novel finding and has not been reported before.

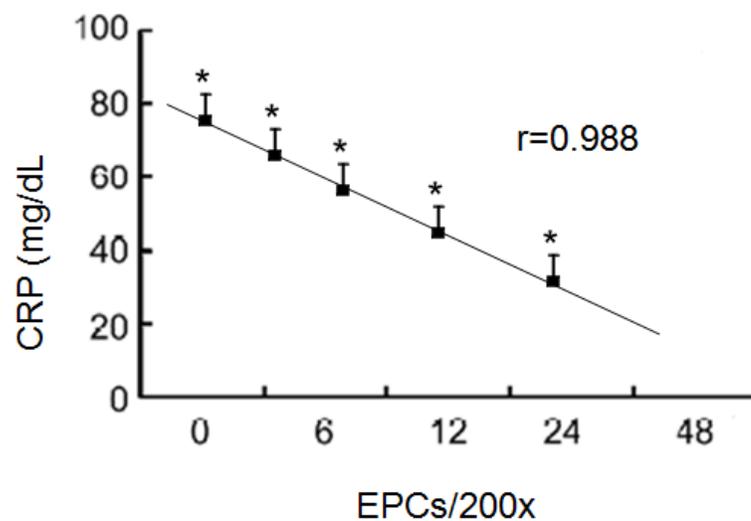


Figure-6.7: The plot shows the linear regression results in HF/HF offspring. The *solid line* illustrates that increase in plasma CRP levels in offspring plasma correlates with decrease in number of bone marrow derived EPC colonies expression in adult offspring. Asterisks (*) where $p < 0.05$, shows the level of significance.

Studies and laboratory evidence have identified that EPCs participate in postnatal neovascularization and reendothelialization.^{318,360,620,623,626,362,642-645} However, our present findings have documented that hypercholesterolaemia can decrease EPC number and activity. Given the well-established role of EPCs in neovascularisation and re-endothelialization, our findings may establish a novel pathophysiological mechanism of hypercholesterolaemia: namely, hypercholesterol not only impairs endothelial cells directly, but also affects EPC number and function at the same time. Thus hypercholesterolaemia may influence the endothelial repair process and disturb the balance between the magnitude of injury and the capacity for repair, which leads to endothelial dysfunction, and promote the early progression of coronary artery disease in adult offspring.

On the other hand statins have been developed as lipid-lowering drugs, and are well established to reduce morbidity and mortality from CAD.⁶⁴⁶⁻⁶⁴⁷ Besides lipid lowering, primary and secondary prevention trials and laboratory investigations established, that statin possessed favourable effects independent of cholesterol reduction.⁶⁴⁶⁻⁶⁴⁸ In particular, statins have recently been reported to promote EPC proliferation, migration, and cell survival *in vitro*.^{359-361,364} A recently performed clinical study demonstrated an increase in EPC number with enhanced migratory activity by statin treatment in patients with stable CAD.³⁶⁰ Results of this work, together with the findings of other investigators suggested a novel mechanism of statins' action in augmentation and promotion of EPC functional activity.

More recently, two groups have documented in animals and human subjects that EPCs contribute up to 25% of endothelial cells in newly formed vessels.^{314,370} Thus, increasing the number of circulating EPCs by transplantation of hematopoietic stem cells or by injection of *in vitro*-differentiated EPCs has been shown to improve neovascularization of ischemic hindlimbs^{312,620} accelerate blood flow in diabetic mice⁶¹¹, and improve cardiac function⁶⁴³. More importantly, reports suggest that patients with CAD reveal reduced levels and functional impairment of EPCs, that correlate with risk factors for CAD.⁶⁴⁸⁻⁶⁴⁹ Therefore, the stimulation

of mobilization and/or differentiation of EPCs by statin may provide a useful novel therapeutic tool to improve postnatal neovascularisation and reendothelialization in patients with CAD.

There are several possible scenarios by which hypercholesterolaemia could have this effect. First, this might be due to increased apoptosis of premature progenitor cells, as CD34-positive EPCs have been shown to be very sensitive to apoptosis induction. Moreover, ox-LDL is known to induce apoptotic cell death. Secondly, hypercholesterolaemia may interfere with the signalling pathways regulating EPC differentiation or mobilization. Thirdly, the continuous endothelial damage or dysfunction may lead to an eventual depletion or exhaustion of a presumed finite supply of EPCs. The mechanistic effects of statins on EPC in such settings may well be via increased regional perfusion of blood flow through increased endothelial NO production.⁶⁵⁰⁻⁶⁵¹ Or statin may induces EPC differentiation by reducing the CRP mediated inflammation via the PI 3-kinase/Akt (PI3K/Akt) pathway.³⁶¹⁻³⁶².

Evidence demonstrated by this study supports the notion that decrease in number of EPCs in HF/HF is heavily influenced by the maternal diet and thus contribute to the defect in postnatal vascular response i.e. EPCs mediated mobilization and endothelial function. It would have been advantageous to conduct fluorescence-activated cell sorting (FACS) for cultured mononuclear cells to evaluate whether these cells progressed to an EPC-like phenotype rather than relying on the double staining alone to identify them.

In conclusion, the ability of maternal hypercholesterolemia to reduce EPC numbers and differentiation may represent an important mechanism in the developmental origins of CVD. Studies of this design are likely to be valuable to our understanding for the design of intervention studies in human populations.

*When my inner landscape is full of beautiful and spiritual thoughts,
everything then I do is an absolute pleasure.*

CHAPTER-7

General Discussion

Overall this thesis provides evidence that offspring's prenatal environment is responsible for a cascade of pathogenic events (i.e. cardiovascular and metabolic disorders) later in life. Current literature so far lacks details of how we humans live in our environments and how the combination of genetics and developmental constraints affects our lives. Do our new emerging environments i.e. HF and over-nutrition, which we ourselves have created, answer the question of developmental origins of CVD/ metabolic syndrome in the fetus? Especially it is yet to be established whether maternal HF actually translates into fetal hypercholesterolemia and causes early CVD in its adult life. This aim led to the series of investigations in the thesis.

Animal models have unequivocally shown that adverse prenatal nutrition, such as maternal diet rich in fat during pregnancy, enhances susceptibility of the offspring to metabolic syndrome and other features of the human CVD in adulthood.^{247,249,250,253,254,287,652} However, previous experimental studies were confined to short-term modifications of the maternal diet during pregnancy and/or lactation periods, a situation uncommon in humans. Also in humans, the offspring may consume a HF diet, which may take them beyond the range to which their development adapts them to respond healthily. But it still remains unknown to what extent this contributes to CVD in humans. In this regard, the thesis is the first work investigating whether the effects of long-term consumption of a HF diet by the mother predisposes her offspring to developing a CVD/ metabolic syndrome-like phenotype in adult life.

In C57/BL6 mice, the effects on offspring of feeding their mothers a HF or C diet from weaning through pregnancy and lactation were studied. Additive phenotypic effects of feeding these offspring a similar HF diet from weaning to adulthood (dam–offspring dietary group HF-HF) were investigated. This group was compared with offspring from HF-fed dams fed a C diet from weaning to adulthood (HF-C) and offspring from C-fed mothers fed the C or HF diet (C-C and HF-C, respectively). HF-HF, HF-C and C-HF adult female offspring were observed to be heavier, fatter, and had raised serum cholesterol and BP compared with C-C female offspring. A similar trend was observed in male offspring except for the HF-C group which was not heavier or fatter than male C-C offspring. Liver histology demonstrated lipid vacuoles within hepatocytes in the HF-HF, HF-C and C-HF but not the CC offspring. CRP was observed to be elevated in female (C-HF and HF-HF) but not in male offspring. Elevated BP in the HF-C and C-HF groups was attenuated in the HF-HF group in males but not in females. The findings indicate that adverse effects on the offspring are induced during development and are not necessarily or completely reversed by either consumption of a postnatal C diet or a HF diet. The results from this study provide an experimental basis for investigating consequences of dietary transitions relevant to humans, where the woman’s diet both before and during pregnancy and lactation may be a contributing factor to the development of metabolic and CVD in her children (Appendix- v).

The role of pharmacological intervention using statins in late pregnancy to alleviate the detrimental effects of maternal HF diet on the health of the dams and their offspring was examined. This time pregnant C57/BL6 mice on HF diet were given pravastatin in the drinking water (5 mg/kg of body weight per day) in the second half of pregnancy and during lactation to lower cholesterol and improve post-weaning maternal BP. Weaned offspring were then fed HF diet until adulthood (generating dam/offspring dietary groups HF/HF and HF+S/HF). These groups were compared with offspring from C mothers, fed C diet as well to adulthood (C/C). Compared with HF/HF, HF+S/HF showed significantly reduced total cholesterol concentrations and reduced SBP. The HF+S/HF offspring were significantly lighter, less hypertensive, and more active compared with the HF/HF group. Total cholesterol and LDL concentrations were significantly lower, and HDL concentrations were increased in HF+S/HF

offspring, compared with the HF/HF. The findings from this work indicate that statin administration to HF-fed pregnant dams not only improves their cardiovascular and metabolic health but also gives postweaning protection to their offspring. This might allow time for other intervention strategies to be put in place to protect the offspring, which are also likely to consume a poor post-weaning diet. This new evidence proposes that maternal hypercholesterolemia has a detrimental effect on the next generation and may necessitate a reconsideration of present recommendations against the use of interventions during pregnancy (Appendix vi).

Moreover, the effects of long-term pravastatin administration in the drinking water to HF-fed female mice from the time they were weaned until weaning of their offspring were investigated. Weaned offspring were fed the HF diet until adulthood, generating dam/offspring dietary groups HF/HF and HF+S/HF. These groups were compared with offspring from C dams fed on C diet post-weaning to adulthood (C/C). HF+S dams demonstrated significantly reduced total cholesterol and SBP vs. HF dams. The HF+S/HF offspring were significantly lighter, with lower systolic blood pressure and serum cholesterol concentrations vs. HF/HF ($P < 0.001$). HF/HF offspring also had elevated CRP but it was reduced in the HF+S/HF animals to levels found in the C/C group. I conclude that long-term pravastatin administration to dams not only protects them from the deleterious effects of a HF diet but completely protects their offspring from cardiovascular and metabolic risk factors in later life, even if these offspring consume a HF diet (Appendix-vii).

These findings led to the study reported in chapter-6, where it was investigated whether any or all of the cardiovascular defects in female dams exposed to a long-term HF diet could have an impact on the expression of bone marrow derived mononuclear cells (double stained endothelial progenitors; EPCs) in their offspring if they were fed a HF or C diet in adulthood. It was also investigated whether pravastatin treatment in dams either short-term or long-term

affect the EPC in offspring. Two study protocols were adopted with short term and long-term administration of pravastatin to dams on HF diet. The results suggest that maternal hypercholesterolemia increases CRP and inhibits EPC whereas pravastatin treatment to these hypercholesterolemic dams improves EPC numbers and reduces CRP in their adult offspring (Appendices viii & ix).

Although the cardiovascular hazards of a raised intake of dietary fat in adults are well recognised, it is yet not known whether a maternal diet containing a disproportionately HF content through the female life span (pre-pregnancy, pregnancy and lactation) is a threat to health and wellbeing of the offspring, even into adulthood. The evidence presented here suggests in animal model that this may be the case, though the mechanisms underlying this phenomenon are unknown. Therefore, in this final chapter I bring together a critical analysis of my approach, discuss at length the plausible mechanisms and review the possible limitations.

7.1 HF diet & DOHaD-why it is important in cardiovascular medicine?

A paradigm shift in how we perceive the early origins of many of the chronic diseases of adult life caused clinicians and scientists to focus on CVD at its earliest stages. Hence for those of us whose work centres on paediatric and adult cardiovascular medicine, this is an exciting subject to explore. It has of course been appreciated for many years that events before and soon after birth have important implications throughout an individual's lifespan. But the impetus for many of the recent scientific advances in cardiovascular and metabolic medicine comes from the fact that current levels of nutrition in Western countries remain unbalanced and rich in HF with associated high birth weight, type-2 diabetes, CVD and many other components of metabolic syndrome in adult life.

Today, central to our understanding of predisposition to health and disease is the concept that a pregnant woman programs her fetus for the metabolic world in which she is living and in which it is assumed her offspring will also live. This explains the massive epidemic of CVD and obesity currently underway in many developing countries and in populations making a rapid transition to a Westernized lifestyle.^{126,653} Today a pregnant woman lives a sedentary life, characterized by low levels of exercise, a high plane of HF intake and the adaptations in metabolism resulting in obesity, diabetes and a host of related conditions.^{90;94,654} This process is relevant to the millions of people currently making rapid transitions from thrift to plenty, and explains why such individuals are so particularly prone to the early development of diseases when compared with those whose ancestral line has made the transition over many generations.

More recently, it has become evident that birth weights in the upper part of the range, in most cases as a result of exposure to fetal hyperglycemia from poorly controlled maternal diabetes, may also be an antecedent of metabolic syndrome and CVD.^{123,655} Thus, these most important diseases of adult life have a U-shaped association with birth weight, with increased rates in both the lower and upper parts of the range, each reflecting different causal pathways.⁶⁵⁶ In numerical terms, the epidemic of obesity, diabetes and related conditions currently underway across the world represents the greatest health challenge ever experienced by humans.⁶⁵⁷

This points our focus on the relationship between serum cholesterol levels and the risk of CVD⁴⁻⁵ in the abundance of HF diet available in the 21st century world. Indeed, experimental, clinical, and epidemiologic data capped by dramatic interventional results with statins and controlled diet had established hypercholesterolemia as a major causative factor in CVD; clinically manifested. It is equally clear that from the very beginning the HF induced hypercholesterolemic related phenotypic changes had a strong inflammatory component, characterized by ox-LDL generated in response to pro-oxidative changes in the CVD as a plausible candidate. Hence both phenomena could be intertwined. The nature of the persistent

changes in the fetus responsible for such increased adverse outcome and the mechanisms by which maternal hypercholesterolemia induce these changes requires to be investigated. This prompted me to choose this very thesis for research.

7.2 Was the experimental model the best to use in this study?

When designed within the context of evidence obtained from human populations, animal models are able to test specific hypotheses whilst overcoming the major limitations of epidemiology study designs. Animal models have been absolutely essential in demonstrating the biological plausibility of the associations observed in human populations, providing proof of principle to the theory of DOHaD. Use of a C57BL/6 mouse model in this thesis gave control over confounding factors, and allows the measurement of invasive end points and the characterisation of downstream events across the full life span and into subsequent generations. Its physical and biochemical phenotype greatly resembled human disease. It can thus be anticipated that this model will be helpful in providing new answers to a number of unresolved questions in this field. The homogeneous genetic background, rapid breeding, short growth phase, and short life-span of this mouse model accelerate the acquisition of knowledge that may take longer time to accumulate in the human disease situation. The use of C57BL6 has exhibited circulating lipid profiles end points similar to those of human subjects.

Literature demonstrates consistent evidence of moderate hyperglycemia, hyperinsulinemia and hypercholesterolemia in response to HF feeding.¹² These outcomes are associated with increased number and size of adipocytes,¹³⁻¹⁴ decreased skeletal muscle insulin sensitivity¹⁵ and hepatic steatosis¹⁶ all key components of the metabolic syndrome. It was therefore possible to induce a metabolic phenotype in my experimental model similar to that observed in the human subject, despite not exhibiting progression to an atherosclerotic state. Furthermore, the model permits a detailed study on body composition and tissue characteristics at the histological, histochemical, and molecular levels—information that cannot be obtained in

humans for ethical and technical reasons. Finally, the mouse will serve as a useful null hypothesis for future studies (discussed in chapter-8) on the impact of genes in a global or tissue-specific manner for physiological studies in the background of DOHaD.

When comparing the outcomes of interventions such as statins during development in this model and when extrapolating conclusions to man, it is important to remember that the timing and trajectory of developmental processes differs between species. A particular limitation of the mouse model is that they are generally altricial species and are therefore relatively immature at birth in comparison with man and large animal models (e.g. sheep and pig). Mice are born with a poorly developed central nervous system and autocrine system and the development of organs implicated in the induction of disease (kidney and liver) continues into postnatal life. The periods of vulnerability of the developing systems therefore differ between species and therefore interventions directed at the same stage of gestation cannot be considered comparable. For example, nephrogenesis is complete by 32-34 weeks of gestation in man and by 130 days of gestation in sheep. In contrast, nephrogenesis in the mice continues into the postnatal period. It is therefore important that the mechanisms of impact of interventions during early life are discussed in the context of their timing in relation to developmental processes rather than stage of gestation per se (discussed in chapter-2).

The purified HF dietary pattern observed with my experimental diet more closely reflects that of the 'non prudent' diet of human subjects⁶⁵⁸⁻⁶⁶⁰ and avoids the very high intakes of specific and potentially biological active fatty acids {i.e. n-6 fatty acid, linoleic acid (LA), n-3 fatty acids, linolenic acid (LNA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)} that are observed in other models of HF feeding. It may be therefore be a useful tool for modelling the effects of non-prudent diets in human subjects. We now know through reported epidemiological and clinical studies that the n-6 fatty acid, LA, n-3 fatty acids, LNA, EPA and DHA collectively protect against CAD. Such fatty acids regulates LDL-C metabolism by downregulating LDL-C production and enhancing its clearance in the blood. Further, these are critical factors determining the hyperlipemic effects of other dietary fat components, such as

saturated and trans-fatty acids, as well as cholesterol [discussed in Ref-660]. The distinct functions of these two families make the balance between dietary n-6 and n-3 fatty acids an important consideration influencing cardiovascular health⁶⁶¹.

7.3 Do the findings answer predictive adaptive response theory?

The findings of the thesis demonstrate that a maternal HF diet alters plasma lipid levels, SBP and cause alterations in the activity levels in the offspring. The novel observation not only emphasizes the development of characteristics similar to the human metabolic syndrome in adult mice, the programming effects of maternal diet, but further provides a plausible explanation with regards to the developmental programming of CVD.

We now know that fetus constantly ‘interprets’ the environment created by the maternal milieu. Some fetal responses are homeostatic or immediately adaptive; others reflect developmental disruption but might have echoes throughout life (e.g. reduced nephron numbers). Still others have little immediate adaptive value, but confer advantage by establishing metabolic physiology appropriate for the postnatal environment predicted to exist (Figure 7.1)

Gluckman & Hanson⁹⁵ explain in this figure the nature of the PAR that is determined by the predicted and actual postnatal environment. As explained earlier, the fetus sets a range of homeostatic settings appropriate for postnatal life according to the information it receives *in utero*. The postnatal range associated with health is narrowed if the fetal environment has been unbalanced simply because the plastic responses made during development may later act to limit the range of postnatal adaptive responses. The findings of the thesis support this concept in male offspring where the elevation of BP was less pronounced in the HF-HF group than in HF-C or C-HF groups. This supports the partially beneficial cardiovascular effect of reducing

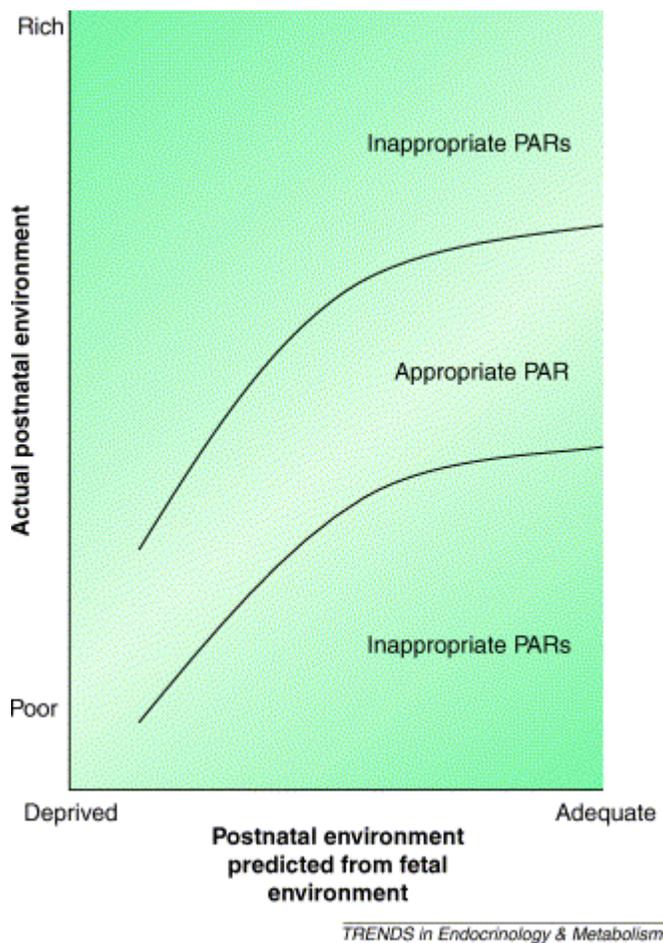


Figure-7.1: Adopted from Gluckman & Hanson with permission.⁹⁵

the dietary mismatch in the HF/HF offspring. Khan *et al*²⁴⁷ reported the same, but in his study (1) the dams were only fed the HF diet during pregnancy and weaning, and (2) endothelial dysfunction rather than elevated BP was observed to be attenuated when compared with the C fed offspring. These data are broadly in support of the PAR concept.^{247-248,251} However, as outlined in the original exposition of the concept such an effect only operates within a range of postnatal environments, beyond which the risk of pathophysiology is increased. The upper limit of the postnatal nutritional environment tolerated without risk of CVD depends on the fetal prediction and this in turn depends on maternal physiology. As certain features of the

metabolic syndrome are acquired by mice offspring whose mothers consume a HF diet either *in utero* and/or during suckling, this study demonstrates a broad window of developmental susceptibility to dietary imbalance of a kind prevalent both among populations of developed countries and developing countries undergoing rapid economic transition. The data suggest that PAR occurs *in utero* to protect against a subsequent "dietary challenge" in the postnatal period.

In addition, the results demonstrated that offspring of both sexes at weaning from HF dams and on HF diet (HF/HF) were heavier than those from C dams and on C diet (C/C). This greater weight was associated with hypercholesterolemia and hyperinsulinemia. Moreover, the relative accumulation of fat in hepatocytes reflects an early sign of the adverse effects of diets rich in saturated fatty acids.⁶⁶¹ Another example has been recently illustrated in Sprague-Dawley rats, with regards to hypertension, where it was not prevented in offspring raised on similar HF diet to their mothers.^{247-248, 251} In agreement with the present study female offspring in this thesis are the most affected, which may indicate a similarity of underlying mechanism between these studies.^{247-248, 251}

7.4 Does the sex of the offspring need to be considered?

The thesis demonstrates subtle offspring gender differences in food intake, BP and CRP levels. Sex differences are now well established and reports have suggested female sex hormones,^{247,250,662-663} (estradiol exposure and somatosensory stimuli) partly associated with the sex difference.⁶⁶²⁻⁶⁶⁴ However, the cardiovascular protective role of oestrogen discussed in literature still remains controversial. Peng *et al*⁶⁶⁵ reported that young female spontaneously hypertensive rats (SHR) are relatively well protected from NaCl-sensitive hypertension, but depletion of both endogenous and dietary estrogens greatly exacerbates NaCl-sensitive hypertension. The study tested the hypothesis that oestrogen also protected late middle-aged

female SHR from NaCl-sensitive hypertension and that this effect was mediated by an oestrogen-related effect on hypothalamic norepinephrine release.

So far, no data has investigated the oestrogen response towards hypertension in females on HF diet. In the thesis hypertension and increased CRP levels in females were observed to be positively correlated with hypercholesterolemia. This relation could indirectly be deduced for the effect of HF diet on female hormones in downregulating their protective effect on CVD; though the mechanism requires further studies (Figure 7.2).

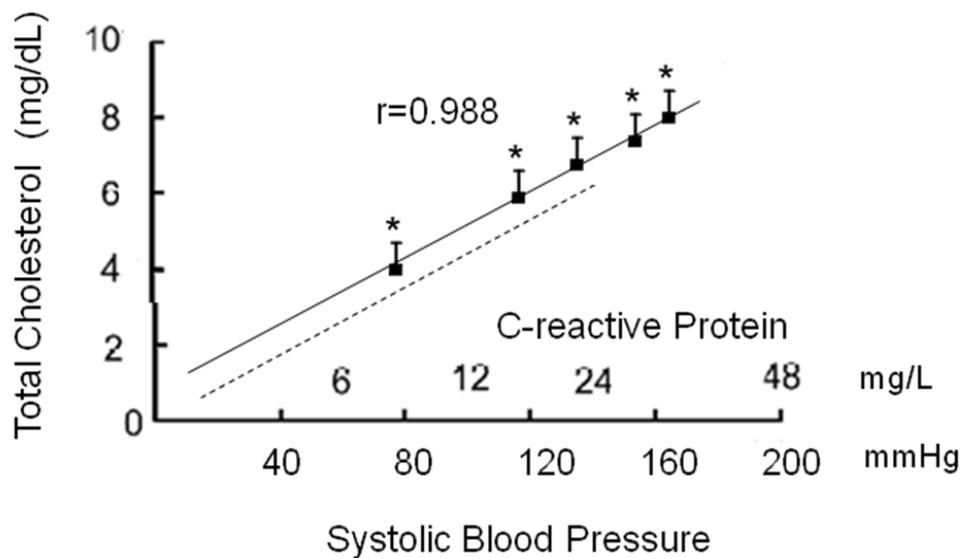


Figure 7.2: The plot shows the linear regression analysis in female HF/HF offspring. The *solid line* illustrates the level dependent increase in total cholesterol to increase in systolic blood pressure in 28 weeks old offspring. Similarly the *dotted line* demonstrates the rises in cholesterol levels positively correlate to increase in C-reactive protein values in same offspring (n=8). *p<0.001

Emerging studies have reported that HF offspring birth weight correlates with⁶⁶⁵ both maternal weight gain and a high intake of HF⁶⁶⁶ and is suggested to reflect abnormal fetal oestrogenic environment.⁶⁶⁷ The daughters of women who suffered from pregnancy-induced hypertension (pre-eclampsia/eclampsia) have a 5-fold reduction with lower circulating oestrogen and PUFA levels.⁶⁶⁸⁻⁶⁶⁹ These data, if extrapolated to humans, may explain the link among diet and CVD.

7.5 Was tail-cuff method appropriate for BP measurement?

The suitability of any research methodology is largely dependent on the investigative objective. Thus, a particular technique for measuring BP may be well suited for one type of study but less useful for another. Accordingly, the advantages and disadvantages of various BP measurement methods with the goal of providing specific answers depend on the study objective. For example, if the primary objective is to determine whether a drug protects against atherosclerosis or cardiovascular damage independent of any effects on BP, then the investigator should use a monitoring technique that provides a comprehensive measure of the total BP load on the vasculature. For this kind of study objective, techniques that provide only sporadic measurements of BP would be less useful or even potentially misleading no matter how accurate those measurements might be.

Techniques for measuring BP in experimental animals can be divided into indirect method (tail cuff) and direct method (radiotelemetry). Most methods for measuring BP can be applied in a range of animals, although certain technical modifications may be required depending on the species undergoing study. In most cases, the choice of method should be driven by the investigative objective rather than the species of animal being studied. It should be emphasized that regardless of the method used for measuring BP, systemic anaesthesia should be avoided whenever feasible because of the well-documented effects of anaesthetics on cardiovascular function.⁶⁷⁰ It has long been recognized that commonly used anaesthetics can affect multiple

aspects of circulatory system and that integrative cardiovascular responses often differ greatly in anaesthetized versus conscious animals.⁶⁷⁰

In animals, the most commonly used indirect method for monitoring BP is the cuff technique in which BP is measured in a tail or limb by determining the cuff pressure at which changes in blood flow occur during occlusion or release of the cuff. A variety of methods have been used for sensing the point at which some type of change in blood flow occurs during manipulation of cuff occlusion pressure, including, but not limited to, photoelectric sensors, oscillometric sensors, doppler sensors, chamber volume sensors and acoustic sensors. Several improvements in sensor technology have occurred, but regardless of the type of sensor used, all of these methods share certain advantages and disadvantages that should be carefully considered when deciding whether to use an indirect technique in a particular study.

Tail cuff method has served a valuable role in experimental hypertension research for many years and continues to be useful in certain kinds of study designs. This method used in the thesis shares some of the same advantages and disadvantages in blood pressure measurement. Tail cuff method has 4 main advantages^{389,670,671}: (1) It is noninvasive and does not require surgery; (2) it can be used to obtain repeated measurements of SBP in conscious animals during studies of short or long duration; (3) It requires less expensive equipment than radiotelemetry and can also be less expensive to operate; and (4) it can be used to screen for systolic hypertension or substantial differences in SBP among large numbers of animals. Thus, I preferred this method to noninvasively detect or confirm the presence of frank systolic hypertension, substantial differences in SBP between groups and substantial changes in SBP over time, particularly when dealing with large numbers of mice.

Although tail cuff method is clearly suitable for measuring BP for the thesis protocol, it has 3 main disadvantages' (1) it only measures BP in a very small sample of cardiac cycles and hence is incapable of assessing the average level of BP throughout the day and night over the course of a study. This problem limits the value of this method, regardless of how accurate this method is thought to be in measuring SBP during an individual cardiac cycle. Therefore to overcome this problem the BP measurements in this thesis were performed during only a very brief portion of the day. Although cuff devices for ambulatory BP monitoring are increasingly being used to indirectly measure BP over many cardiac cycles in humans, such approaches have not proven to be practical for measuring BP in animals. (2) Despite the noninvasive nature of indirect methods and well-intended efforts by investigators to train and acclimatize animals to undergo the procedures, these methods impose significant stress though less than telemetry that disturbs multiple aspects of the cardiovascular system. The notion that one can truly acclimatize rodents to indirect tail-cuff procedures and effectively minimize the impact of the procedural stress on cardiovascular and endocrine function is doubtful.⁶⁷²⁻⁶⁷⁴ Tail-cuff measurements of BP in rodents impose substantial amounts of thermal and restraint stress that are known to affect BP, heart rate, and stress hormones.⁶⁷²⁻⁶⁷⁵ In fact, acute restraint has been shown to lead to acute BP increases and activation of vascular wall mitogen-activated protein (MAP) kinases comparable to those observed after acute infusions of angiotensin II or phenylephrine.⁶⁷⁶ Moreover, the assumption that different experimental groups within a given study would be expected to demonstrate similar quantitative responses to restraint stress may also not be valid.⁶⁷⁷⁻⁶⁷⁹ Thermal stress is caused by increased body temperature required to dilate the tail artery and allow sufficient blood flow into the tail. When the animals are confined to the restraining cages, substantial increases in body temperature occur even without intentional warming of the animal.⁶⁷⁵ Tail-cuff measurements are also commonly performed during the day, which disrupts rodent sleep cycles. Therefore I trained/ conditioned the animals for 5 to 14 days before commencing tail-cuff measurements, yet some investigators have demonstrated that even 10 days of conditioning can fail to prevent the large changes in BP and heart rate induced by restraint stress.⁶⁷² (3) Several studies have been published purporting to validate cuff methods based on correlations between indirect cuff measurements of BP and direct measurements of BP simultaneously or subsequently obtained with arterial catheters.^{388,675,680-684} Most such validation studies have relied on simple correlation/regression

analyses that can be misleading and obscure large individual differences or even systematic differences between measurement methods.⁶⁸⁵ When more appropriate analytical techniques have been used, such as agreement analysis, BP measurements obtained by indirect methods have shown poor agreement with BP measurements simultaneously obtained by direct methods.^{684,686} In some cases, tail-cuff measurements of systolic BP have appeared suspect because they have shown large differences with direct measurements of systolic pressure⁶⁸⁴ or because they have shown minimal differences with direct measurements of mean arterial pressures.³⁸⁹

As noted by Reddy *et al*, another major limitation of most tail-cuff methods is that they are not well suited to measuring diastolic pressure.⁶⁸⁴ Diastolic BPs measured with a custom-made instrument incorporating a pulsed Doppler tail-flow sensor have been reported to show good agreement with directly measured diastolic pressures⁶⁸⁴; however, this method requires the use of anaesthesia, which has obvious drawbacks in cardiovascular research. Based on a validation protocol for evaluating automated sphygmomanometers that was developed by the Association for the Advancement of Medical Instrumentation, Jamieson *et al* compared systolic and diastolic BP results obtained with a commonly used tail-cuff instrument to those obtained by direct arterial recordings.⁶⁸⁷ For both systolic and diastolic pressure measurements, the disagreement between the indirect and direct methods exceeded clinically acceptable standards, and 74% of diastolic pressures showed disagreements >5 mm Hg.⁶⁸⁷ Finally, even if an indirect method can be shown to provide an accurate measurement of BP during a particular moment in time, this does not validate the method for assessing an animal's true average BP or for detecting the extent to which BP exceeds certain thresholds during the course of a study.

Given the major limitations of indirect BP measurement methods and particularly their inability to determine true average BP or the frequency of large BP fluctuations, these techniques are not recommended for studies intended to quantify the relationship between BP

and other variables (eg, vascular damage, atherosclerosis, etc). This was not the case in my experiments.

7.6 Maternal hypercholesterolemia and obesity programs disease risk

The findings of the thesis provide evidence of metabolic imprinting of the progeny born to dams fed a long-term HF diet, that are overtly obese, hypercholesterolemic and hypertensive. The results also suggest that some of the effects of the HF diet in such offspring are adaptive in nature (discussed in section 7.3). This is in agreement with published literature.^{118,688-693} Long-term HF diet induced hypercholesterolemia and hypertension prenatal and during pregnancy in dams and led to postnatal hypertension even the offspring was fed C diet. Lowering maternal cholesterol with statin reduced offspring cholesterol, LDL- cholesterol, CRP and hypertension. Studies have suggested that maternal treatment with a statin during pregnancy reduce atherogenic programming in mice.^{254,288,435} The first indication that *in utero* exposure to cholesterol may program adult CVD came from the observation that maternal hypercholesterolemia, even if temporary and limited to pregnancy only, is associated with a marked increase in fatty streaks in the aorta of premature human fetuses.²⁷⁸ The same study also showed that fetal cholesterol levels at the end of the second trimester were very high, even in fetuses of normocholesterolemic mothers, and declined linearly until term. This is consistent with the well-documented fact that in the absence of inherited genetic defects of lipid metabolism such as familial hypercholesterolemia, cholesterol levels of term-born children are very low, even if their mothers are hypercholesterolemic. It is therefore conceivable that fetal lesions might regress towards full term. Instead, the Fate of Early Lesions in Children (FELIC) study, a morphometric assessment of aortic atherosclerosis in normocholesterolemic children who died before the age of 14, demonstrated that maternal hypercholesterolemia was associated with greater atherogenesis throughout childhood⁵⁴⁷. This observation could not be explained by conventional risk factors in mothers or children and suggested programming by maternal hypercholesterolemia.

7.7 HF diet plays a role in developmental programming of hepatic and renal metabolism

An understanding of the mechanisms contributing to disease progression from hepatic steatosis to NAFLD is crucial in developing strategies to prevent chronic irreversible liver disease. Exposure to a HF diet in the developmental period could be an important factor that influences the offspring's susceptibility to the development of NAFLD later in life (see Chapter-3). The work suggested that 27 week old offspring of dams fed a HF diet that were also fed a HF diet post-weaning (HF/HF), were predisposed to develop hepatic steatosis, a phenotype similar to humans. This also demonstrates (1) exposure to a HF diet during development and post-weaning is worse than HF exposure post-weaning alone and (2) maternal HF diet can lead to development of hepatic steatosis later in life, even if a C diet has been adopted in the adult environment. Further studies are required to investigate the underlying mechanisms that may lead to this developmental priming. However it is plausible to speculate that this HF nutrition during the developmental period may alter the epigenetic profile in key metabolic genes, subsequently leading to persistent modulations in gene transcription, and increases the risk of developing hepatic disorder in adulthood.¹³⁶ Our data indicates that exposure to a maternal HF diet primes an increased susceptibility to hepatic steatosis and inflammation in adult offspring.

These findings highlight a public health problem which coincides with the obesity and metabolic syndrome.⁶⁹⁴⁻⁶⁹⁷ Reports of paediatric fatty liver disease and steatohepatitis in obese children have been increasing⁶⁹⁸⁻⁷⁰⁰ and include cases of cirrhosis⁷⁰¹⁻⁷⁰⁴ requiring liver transplantation.⁷⁰⁵ It is alarming to note that the prevalence of fatty liver in obese children in China, Italy, Japan, and the US stands between 10% and 77%.⁷⁰⁶⁻⁷¹⁰ According to the 'double-hit' hypothesis,⁴³⁹⁻⁴⁴⁰ HF diet leads to hepatocellular lipid accumulation as the 'first hit', followed by a 'second hit' in which proinflammatory mediators and reactive oxygen species

(ROS) induced inflammation, hepatocellular injury, and fibrosis.⁷¹¹ While this is a useful conceptual framework with knowledge expanding on the role of macrophages, immune tolerance and lipid homeostasis (Figure-7.3⁷¹¹), our understanding of the cellular and molecular mechanisms with regard to DOHaD phenomenon in defining NAFLD and thereby guiding therapeutic approaches remains insufficient.

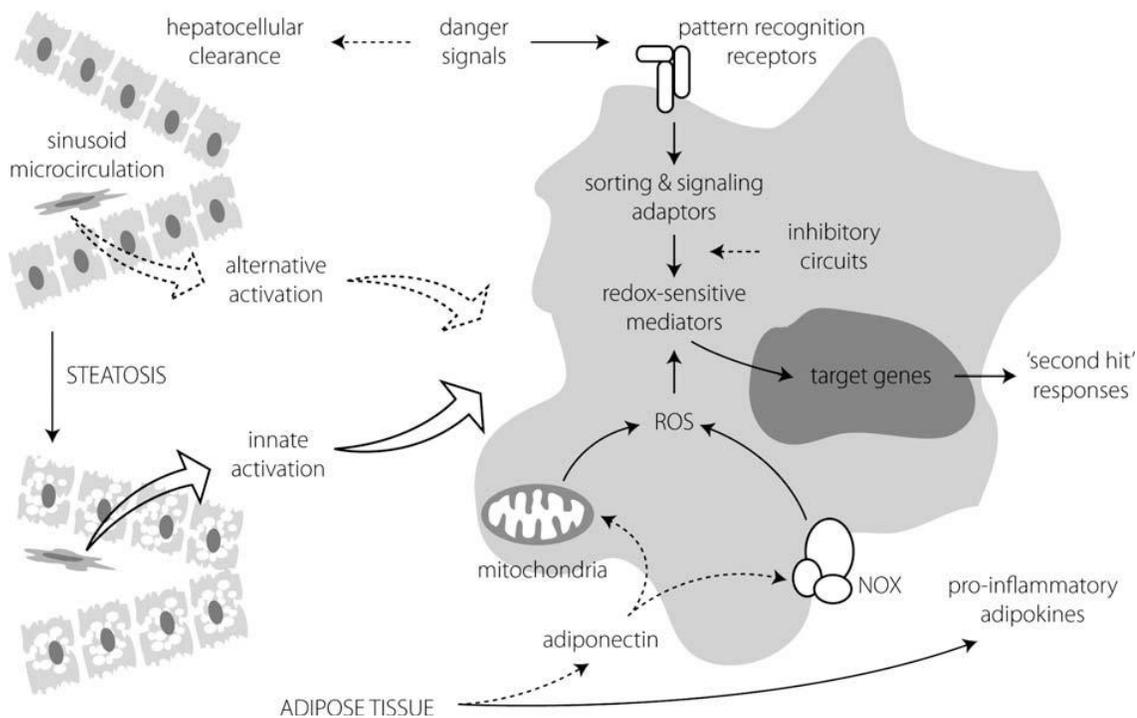


Figure-7.3: Scheme for dysfunctional activation of Kupffer cells in NAFLD. Pattern recognition receptors of Kupffer cells such as TLR4 may be increasingly exposed to exogenous and endogenous danger signals (e.g., LPS, excess fatty acids, modified lipoproteins) via the portal circulation, enhanced by lack of hepatocellular clearance. Pattern recognition pathways may intensify due to altered sorting and signalling, impaired inhibitory circuits, or amplification of redox-sensitive signalling loops. Adipokine imbalance may contribute to these events including low adiponectin levels that fail to suppress intracellular ROS generation. Fat-laden hepatocytes may compromise sinusoid microcirculation leading to entrapment of inflammatory cells. Finally, steatosis may shift away Kupffer cells from alternative activation. Solid lines, pro-inflammatory effects; dotted lines, anti-inflammatory mechanisms. Malfunction at one or more steps may promote ‘second hit’ responses, while cellular targeting of these checkpoints has the potential for identifying novel treatment strategies in NAFLD.⁷¹¹

Moreover the findings in chapter-5 suggested that HF diet, fed for as little as 24 weeks in offspring from HF dams, could cause an increase in renal lipid accumulation in the glomerular

and tubulointerstitial cells (Figure 5.5a). It is noteworthy that relation to infer causality between the maternal HF diet and risk for chronic kidney disease or microalbuminuria or glomerular filtration rates in offspring were not performed here. Yet the results of the thesis have important clinical and public health implications with regard to metabolic syndrome in the West.⁷¹²

7.8 CRP and HF diet consumption

Growing literature has drawn attention at CRP being an important predictor of subsequent risk of CVD, type-2 diabetes,⁷¹³ metabolic syndrome⁷¹⁴ and mortality.⁷¹⁵ Considerable insights into the dynamics of disease in developmental origins of CVD/ metabolic disorder pathophysiology could be gained by investigating CRP levels in dams and their offspring. The present results demonstrate in HF dams at 52 weeks of age (1) reduction of serum CRP levels and (2) reduced number of bone marrow derived EPC. Sex specific response to CRP is observed i.e. in HF/HF female offspring CRP levels are raised and in HF/HF males CRP levels remain normal. Spearman's correlation coefficients between CRP and variables such as total cholesterol, LDL and HDL show significant positive correlation ($P < 0.0001$) between CRP and total cholesterol, LDL and a significant negative correlation with HDL ($P < 0.0001$; Table 7.1).

Table 7.1: Spearman rank correlations between CRP and different variables in female offspring.

Variable	CRP	P
Total cholesterol	0.19	<0.0001
LDL	0.29	<0.0001
HDL	-0.13	<0.0001

It is speculated that raised CRP in offspring may lead to a more systemic inflammatory reaction encompassing a wide range of organ systems, including hepatic production of CRP and accumulation of fat in the hepatocytes.⁷¹⁶⁻⁷¹⁸ However, little is known about this underlying mechanism and future studies are required to further test this hypothesis.

7.9 Bone Marrow derived EPCs and increase CRP levels in offspring

Given the importance of bone marrow derived EPC to postnatal vascular endothelial biology, the current work demonstrate that HF prenatal and postnatal diet and raised CRP exert direct effects to inhibit EPC expression and numbers in the circulation. Accumulating evidence suggests that circulating CRP is one of the significant predictors of atherogenesis and vascular death in CVD pathology.^{302,307,308, 309,348,442,350,719-724} CRP down-regulates eNOS transcription and destabilizes eNOS mRNA, which decreases both basal and stimulated NO release.³⁰⁸ In addition, CRP stimulates endothelin release, upregulate adhesion molecules and stimulate monocyte chemotactic protein. It facilitate macrophage LDL uptake,³⁵⁰ EC apoptosis and inhibit angiogenesis with upregulation of NF- κ B, a key nuclear factor for transcription of numerous proatherosclerotic genes.⁷²⁵⁻⁷²⁶ It is hypothesized that the effects of maternal HF diet observed on offspring EPCs in the present work may involve this mechanistic interaction.

7.10 Effects of maternal statin treatment on offspring phenotype

The present work demonstrates that statin treatment of hypercholesterolemic dams during pregnancy and lactation reduces obesity, hypertension and sedentary behaviour in their offspring when fed on a similar diet. This effect is more permanent when the dams are given long-term statin from the time they were weaned. Moreover, the novel effects of statin on

EPC numbers and on CRP levels in adult offspring in preventing the risk of later CVD is observed. It is plausible that statin specifically alters the expression of enhanced NO secretion by ECs and suppresses NF- κ B activity, outcomes that may have favoured the developing offspring. Statins in dams appear to have a generalized antihypertensive effect by attenuating endothelial dysfunction and increasing NO production, inhibiting thrombin-induced endothelin synthesis, and interfering with leukocyte-EC interactions.^{508-510,516,522,526,537,592-594} The results also suggest that part of the beneficial cardiovascular effects observed after treatment with statins may be due to the favourable effect on EPCs. The present study has clinical implications regarding the usefulness of statin therapy in repairing vascular injury by circulating EPCs. Our findings revealed a new mechanism of statin therapy in vascular disorders involving circulating vascular progenitor cells. Determination of the relationship between EPC and timing of any stem cell intervention may contribute to establishment of vascular repair and atherosclerosis as new targets of treatment.

A limitation to the present study is that the *in vivo* effects of CRP and statins on EPC number and vasculogenic function are not determined; however, such studies are the future work to elucidate such mechanisms. The present data add to the growing body of evidence that proposes the pleiotropic vasculoprotective effects of statins and their ability to increase EPC population and counteracting the deleterious effects of CRP. To summarise the possible mechanisms by which cholesterol lowering in dams by statins affects the offspring phenotype and protect it from future CVD is illustrated in Figure 7.4.

7.11 Conclusions

Considering that a typical Western diet is rich in dietary fat content, especially saturated fats, this thesis investigated the role of HF feeding in the concept of developmental origins of CVD

and metabolic syndrome. These studies in an animal model indicate that maternal HF consumption during the life course can induce features of metabolic syndrome/ CVD including

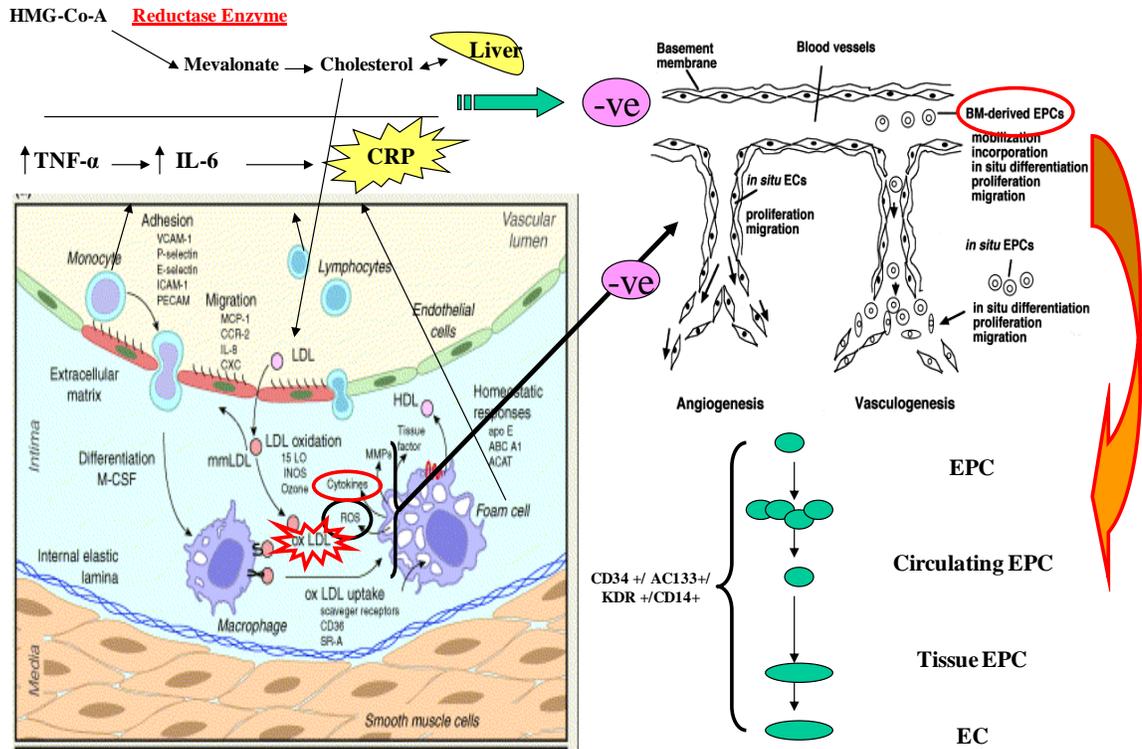


Figure 7.4: Hypercholesterolaemia plays a crucial role in the development of atherosclerotic diseases in general and CVD in particular. Statin, converts HMG-CoA to mevalonate. Inhibition of HMG-CoA reductase by statins decreases intracellular cholesterol biosynthesis, which then leads to transcriptionally upregulated production of microsomal HMG-CoA reductase and cell surface LDL receptors. This resets intracellular cholesterol homeostasis in extrahepatic tissues. The liver is the target organ for the statins, since it is the major site of cholesterol biosynthesis, lipoprotein production and LDL catabolism, inhibits CRP metabolism and exerts a healthy effect on the bone marrow derived endothelial progenitor cells in the circulation. The beneficial effects of statin in offspring from HF dams on long term treatment may depend in part upon the degree to which statin act on extrahepatic tissues. (Adapted from references^{508-510,516,522,526,537,592-594})

obesity, hypercholesterolemia, hypertension, increased inflammation and decreased bone marrow derived EPC numbers in offspring in their own adult life. The data, however, indicate the involvement of gender-associated mechanisms behind programming of dyslipidemia. The study design allowed investigating whether programming effects of the maternal diet could be

reversed by interventions in dams or with postnatal diet. The offspring obtained from HF mothers continued on C diet postweaning (HF/C) failed to show a phenotypic benefit as compared to C/C offspring. However, use of statin in dams reversed the programming effects of the maternal HF feeding no matter what diet offspring had postnatally. Feeding a HF maternal diet caused a reduction in bone marrow derived endothelial progenitor cells, whereas given statins to mother positively affected EPC numbers and expression in adult offspring. Overall, it is observed that the relative risk of developing CVD and metabolic syndrome in dams on HF diet intake increased significantly (RR 1.47; 95% CI 1.11–1.96; $p < 0.01$) than their counterparts on C diet. This risk significantly reduced if dams on HF diet were given statins (RR 1.63; 95% CI 1.07–2.49; $p < 0.02$). The novel observations in the offspring obtained from mothers fed HF diet, not only emphasize the programming effects of maternal diet, but can further provide plausible mechanisms behind the developmental programming of CVD in the context of over-nutritional HF environment, presently encountered by developed and developing populations.

On the basis of the presented work and the other large body of animal studies so far quoted in the thesis, it is clear that maternal HF/ hypercholesterolemic environment could exert a programming effect on the fetus and increase its risk of metabolic syndrome and CVD. The situation is analogous to the description by Norbert Freinkel in a diabetic pregnancy discussed earlier in chapter-3. Therefore on the basis of the results, I would like to propose the term “Hypercholesterolemic-mediated Teratogenesis” for my animal model based work to describe these phenomena observed in my thesis. This is illustrated in Figure 7.5.

7.12 Implications of this thesis to current population situation

The findings of the thesis that HF diet mediates hypercholesterolemia in young female mammals may indicate a similar phenomenon in humans, a possible significant factor in the escalating epidemic of CVD/ metabolic syndrome. In a female, such a situation exposes her

fetus to multiple adverse programming influences, resulting in a complex phenotype including exaggerated tendency to develop adiposity, obesity, hypertension, hypercholesterolemia and hyperinsulinemia at a young age.

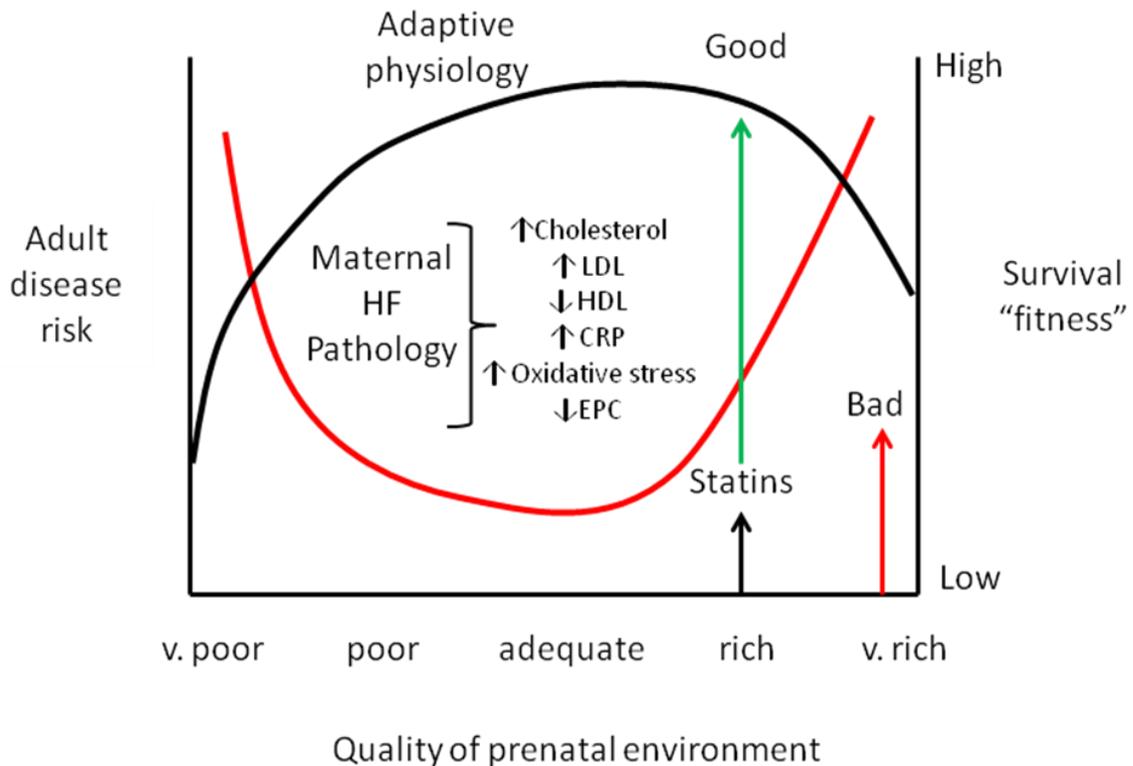


Figure 7.5: The hypothesized “*Hypercholesterolemic-mediated Teratogenesis*” model originating from this thesis. This model demonstrates on one extreme (right side) the risk of adult disease and on the other extreme (left side) range of postnatal physiological settings for survival fitness. At the x-axis (bottom) five different quality of prenatal environments from very poor to very rich are presented. The red line curve represents the environment that the fetus anticipates postnatally judged from the nutritional and related signals it receives from the mother through the placenta. Provided that the postnatal environment matches the range for which the fetus has set its postnatal physiology by the processes of predictive adaptive responses, the disease risk is low. However, when it lies outside that range then the postnatal environment turns bad (indicated by the red arrow) and the disease risk increases. The fetus perceives the more restricted environment and predicts a poorer postnatal survival. But maternal high-fat dietary stress worsens the situation. Overnutritional high fat environment prenatally and postnatally shifts the postnatal survival associated with increased disease risk further to the upper limit (represented with curved black line). Maternal high fat diet causes hypercholesterolemic mediated teratogenesis with deranged lipid profile, increased oxidative stress and decreased activity of endothelial progenitor cells in the circulation of the offspring. This even further potentiates the CVD risk with early manifestation of cardiovascular disease/ metabolic syndrome in its own adult life. From an adequate nutritional environment, the offspring then lies outside the band of rich and very rich environment that can be detrimental to its survival fitness. However, administration of statin to mothers on high fat diet, even at rich nutritional upper limit, can provide a good safeguard from the disease process and maintains the survival fitness even if increasing affluence raises the richness of the mature postnatal environment.

Fueled by urbanization and the advent of the global economy, these changes in eating patterns are the most rapid and dramatic in the course of human history. Radical dietary shifts in many developed and developing nations are supplanting traditional patterns of eating with Western diet high in animal products and refined carbohydrates and low in whole grains, fruits, and vegetables. For example, in China, consumption of animal products increased by nearly 40% between 1989 and 1997⁷²⁷ and HF food sales more than doubled between 1999 and 2005.⁷²⁷ The term "Coca-colonization," a reference to the ubiquitous presence of Coca-Cola, Pepsi, and McDonald's, describes a world that is moving toward a common diet, one accompanied by the more sedentary lifestyles associated with increased risk of chronic disease.⁷²⁸⁻⁷²⁹

According to the World Health Organization's global database, countries like China, India and Pakistan has a preschool childhood obesity prevalence of about 1%.⁷³⁰ Childhood obesity increases the risk of obesity in adulthood and parental obesity interacts quite strongly to alter this risk. There are several other interactive factors as well contributing to the increased prevalence of obesity in childhood. Societies like China, India and Pakistan, which are rapidly urbanising, demonstrate increases in energy intake, dramatic increases in fat intake along with increased levels of sedentarianism. Food balance data from the Food and Agriculture Organization (FAO) show that the change in energy intake in Asian countries has been small, but there have been large changes in consumption of animal products, sugars and fats.⁷³¹ The net effect has been a marked shift in the diet with energy from fat (both animal and vegetable) increasing each year.⁷³¹ Data from India show that higher-income groups consumed a diet with 32% of the energy from fat while the lower-income groups consumed only 17% energy from fat.⁷³²⁻⁷³⁵

Therefore, nutritional history of current populations becomes an important determinant of their health. There is a growing recognition of the importance of environmental factors acting on

the genotype throughout the life cycle of an individual to progressively modify its phenotype. The periconceptual and intrauterine period seem to be the most crucial, when a small change

in environment could have a large effect on the phenotype. . The slogan of the UN Expert Meeting held in April 2008, “Woman's Health is a Nation's Wealth” reflects this philosophy.⁷³⁶

The basic principle of prevention of cardiovascular/metabolic syndrome events consists of the control of classical risk factors with specific interventions. This clinical approach has led to an improvement in prognosis, although still far below expectations. In other words, the mere control of hypertension, hypercholesterolaemia, or obesity, although definitely beneficial, leaves a residual risk, which is still greater than that reduced by treatment. Thus, it is necessary to identify windows of time in the lifecycle when the susceptibility of the genome to such an influence is very high for different treatment targets, which could more successfully impact on CVD. Any preventive intervention will therefore have to start *in utero*, and improving the health of young girls will be a very important aspect of such an approach.

Today, we have to recognize that it may be still too early to test this hypothesis in the clinical setting, yet the animal model in the present work has established cause-effect relationships for some maternal factors and led to the identification of specific questions in a HF maternal environment. Further mechanistic studies, both in animals and in humans, are needed in order to achieve a better understanding of the role of maternal hf diet in the pathophysiology of CVD, metabolic syndrome and atherosclerosis. Further methods that allow a better characterization of the vascular milieu in humans are necessary. Only a more in-depth knowledge of these important pathophysiological mechanisms can allow identifying and

testing a therapeutic strategy aimed at targetting the early developmental origins of cardiovascular disease in the populations.

The story of the Eagle always fascinates me. It has the longest life span of 70 years but to reach it takes hard decisions in life. Many a times, in order to survive the next step even in life one has to also start a change process. Only then one can take advantage of the present!

CHAPTER-8

Future Work

In the thesis, we have learnt about the effects of longterm maternal HF diet and statin treatment on the offspring predisposition to early cardiovascular and metabolic disease. However, much of our understanding of the disorder is far from complete. Future research originating from present work could focus to translate unexplored involved signalling pathways in answering the central question- how such maternal HF diets affect the longterm cardiovascular health of offspring. I therefore, propose some mechanistic studies (currently ongoing or to be initiated) along the following lines:

8.1. Effects of maternal HF diet on Genes Involved in Offspring Cardiac Development

Throughout fetal development, the main nutritional source for the fetus comes from the mother. As a result, the quality of the maternal diet affects growth and development of the fetus during this period,^{88,89,112,135,158,737} though the mechanisms involved are poorly understood. Nevertheless, experimental animal models whereby the maternal diet is manipulated have provided insight into the links between *in-utero* environments and susceptibility to disease in later life. Rodent offspring from dams that were fed a protein-restricted diet during pregnancy later developed hypertension in adulthood.^{377,433} However, developmental programming of CVD in adulthood is not limited to maternal dietary restriction alone. Ours and other groups have shown that feeding dams a HF diet during pregnancy and/or lactation can also lead to the offspring developing vascular dysfunction and

hypertension^{247,248,251,435} and that changes in the maternal diet during pregnancy are linked with an increase in cardiomyocytes and fetal left ventricular mass.⁷³⁸ Cardiomyocytes go

through terminal differentiation at or after birth and this is characterised by the transition from hyperplastic growth to hypertrophic growth.⁷³⁹⁻⁷⁴¹ Many genes are reported to be involved in cell cycle progression and are associated with cardiac hypertrophy.⁷⁴² It remains however to be determined whether the hypercholesterolemic condition during pregnancy leads to any long term changes in these genes in the adult offspring heart; and whether the cholesterol-lowering effect of pravastatin given to pregnant dams consuming a HF diet influence the expression of these genes. In these settings particular pathways are of prime importance to explore.

8.1.1 Cyclin dependent pathways

Particular molecules involved in various stages of the cell cycle determine the progression of the normal cell cycle to assist with cell replication. Cyclins and cyclin dependent kinases (CDK) are a group of catalytic kinases that play a critical role in formation, activation and inactivation of the progression of cell from a quiescent state, G1 phase into the cycle.⁷⁴³ Cyclin G1, one of the G1 phase cyclins, is up-regulated in nutrient restricted fetal heart and has been found to be involved with cardiac hypertrophy in adults. This gene is shown to encourage entry into the cell cycle therefore increasing cell numbers. Protein synthesis is initiated by cyclin G1 to cause cardiac hypertrophy, rather than DNA synthesis through entry into the cell cycle in an adult heart.⁷⁴³ It seems that changes in this gene expression may be either a cardio-protective response due to a limited nutrient supply, a response to myocyte stretch from increased systemic vascular resistance or possibly a response to a changed endocrine situation. It may be interesting to find out how this gene behaves in a hypercholesterolemic state of mother and adult offspring as cyclin G1 is expressed in the only phase of the cell cycle that can be influenced by external stimuli; the G1 phase.⁷⁴⁴ It is also advantageous to investigate the consequences of lowering maternal cholesterol with pravastatin on these HF-fed dams in

late gestation and look at the expression level of cyclin G1 gene involved in cell cycle progression (Appendix-x Figures a & b).

8.1.2 Brain natriuretic peptide

Amongst the molecules which are considered vital in ventricular dysfunction, brain natriuretic peptide (BNP) is an important marker of cardiac function. It is actually an indicator of increased intra-cardiac pressure not taking into account whether the raised pressure is caused by LV systolic dysfunction, LV hypertrophy or valve disease.⁷⁴⁵ However, BNP is secreted to a lesser degree in fetal life compared to adult life.⁷⁴⁶ BNP is also thought to be a marker along with a modulator of cardiac hypertrophy⁷⁴⁶⁻⁷⁴⁷ with a significantly increased expression of BNP in cardiac hypertrophy and cardiac stress. However, it remains to be determined whether BNP levels are affected by maternal HF diet and whether lowering maternal cholesterol with pravastatin on these HF-fed dams in late gestation plays a role in ventricular remodelling (BNP expression) in the offspring left ventricle (Appendix-x Figure-c).

8.1.3 Small proline-rich repeat protein 1A

Small proline-rich repeat protein 1A (SPRR 1A) is known to be a stress inducible cardio-protective protein in cardiomyocytes responding to either ischemic or biochemical stress.⁷⁴⁸ Histological results discovered that SPRR1A induction after mechanical stress from pressure overload was restricted to myocytes that surrounded necrotic lesions. In post-infarcted rat hearts, a similar expression pattern was found. *In vitro* and *in vivo* over-expression of SPRR1A protected cardiomyocytes from ischemic injury.⁷⁴⁹ Therefore SPRR1A has a cardio-protective effect against ischemic stress. A different role for SPRR1A may be its association with actin cytoskeleton. It has been reported that ectopic expression of SPRR1A may prevent disruption of myofibrils and accumulation of nuclear actin after ischemic stress.⁷⁵⁰ It is further suggested that the cytoprotective action of SPRR1A may be due to mechanical stabilisation of myofilament/Z disc structure in cardiomyocytes, therefore preventing cells from permanent

damage. However, the role of SPRR1A as a marker of cardiac stress in this experimental model still remains to be investigated. Moreover, it warrants investigations whether treating maternal cholesterol with pravastatin has any effect on the SPRR 1A expression in the offspring left ventricle (Appendix-x Figure-d).

8.2 Effects of maternal HF on offspring bone structures

Epidemiological studies suggest that skeletal growth is programmed during intrauterine and early postnatal life. It may be interesting to find out in this model, how hypercholesterolemic state of mother plays a role in the development of optimal peak bone mass, in fetus. Using the model, femur samples were taken at 30 weeks of age and bone structure, adiposity and strength analysed. Sample sizes were four to six for each sex and each diet group. The results demonstrated that offspring from HF-fed dams showed increased adiposity in the femur in comparison to offspring from C-fed dams. Female offspring from HF dams exhibited altered trabecular structure indicative of *in utero* programming. This concludes that maternal HF diet during pregnancy increases bone marrow adiposity and alters bone structure in their offspring (For further readings see Appendix-xi).

8.3 Mechanistic Studies

8.3.1 Placental role in transplacental cholesterol transport

The CVD programming effect of maternal hypercholesterolemia and the observation of strikingly high fetal cholesterol levels at the end of 2nd trimester raise interest in maternal–fetal cholesterol transport. It is speculated that maternal cholesterol might cross the placental barrier such as in Smith Lemli Opitz syndrome where survival of fetus depends on maternal cholesterol.⁷⁵¹ Identification of active transport mechanisms on the maternal side of the placenta in rabbits⁷⁵² and hamsters⁷⁵³ has provided evidence for maternal–fetal cholesterol

transport. Transplacental cholesterol transport mechanism mediated by endothelial cells lining of the placental villi into the lumen of fetal microvessels has recently been elucidated.⁷⁵⁴ Results indicate that regulation of these mechanisms by increased fetal demand for cholesterol, differs from that induced by maternal hypercholesterolemia.⁷⁵⁵ Increased cholesterol transport, at least during parts of gestation, may therefore constitute one mechanism by which maternal hypercholesterolemia affects fetal programming and hence requires further investigations.

8.3.2 Maternal hypercholesterolemia and oxidative stress

It is reported that hypercholesterolemia is associated with enhanced lipid peroxidation levels in fetal plasma and aortic lesions.^{278,279,288,293,295} The first indication that increased oxidative stress plays a role in CVD programming is provided by a study in premature human fetuses that showed intracranial arteries with higher activities of antioxidant enzymes developed less atherosclerosis in response to maternal hypercholesterolemia than extracranial arteries.²⁷⁸ Given that, OxLDL enhances adult atherogenesis by a number of mechanisms, including interference with many oxidation-sensitive nuclear signalling pathways, it is plausible that oxidative stress is an important element in the developmental programming induced by maternal HF nutrition. Hence the mechanism whereby maternal HF diet intake plays a role in inducing oxidative stress mediated phenotypic changes in the vasculature of the offspring requires further research in preventing atherogenic programming.

8.3.2 Maternal HF diet and Epigenetic changes in the development

Epigenetic changes associated with increased disease susceptibility such as obesity, type-2 diabetes and cardiovascular dysfunction are emerging as critical components of enduring effects induced by early life experiences. The available data are now beginning to provide a molecular basis for involvement of oxidative stress in modifying biological properties of DNA methylation and other epigenetic effects.⁷⁵⁶ It is reported that *in utero* conditions result in

specific epigenetic change.⁷⁵⁷⁻⁷⁵⁸ Developing organisms seem to have a wide window of susceptibility to epigenetic changes but the periconceptual period is particularly important towards sensitivity to suboptimal nutrition during this developmental stage in which widespread reprogramming of the epigenome occurs.^{15,87,88,91-93,95,136-138,142} It is speculated that HF over-nutrition in pregnancy and/or postnatal could also lead to metabolic dysfunction in major organs such as kidneys and liver later in life.^{150,208,211,215,222-226} These epigenetic changes within suboptimal nutrition can have long-lasting effects on epigenomic methylation patterns in specific gene promoters. Moreover, the study of epigenetics will further guide us in understanding the hormonal regulation of enzymes controlling acetylation and methylation and sex differences across the epigenome. Therefore, the animal model used in this thesis will be ideal to demonstrate (1) adverse effects of impaired early-life maternal HF nutrition associated epigenetic changes in offspring and (2) the mechanisms/ impact of early interventions such as statins in mothers on HF diet for prevention or reversal of such epigenetic consequences during intrauterine development.

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I don't work for a job, I work for a cause!

Heart surgery and research for me is not a living but "the love of my life"

Appendices

Appendix i: Ehrlich's tissue histology protocol

1. *Haematoxylin*

Haematoxylin	16 g
Industrial methylated spirit	240 ml
Potassium or ammonium alum	48 g
Distilled water	240 ml
Glycerol	240 ml
Glacial acetic acid	24 ml

Haematoxylin is dissolved in the alcohol with the aid of gentle heat (56°C oven or water bath). The alum is then dissolved in the distilled water by using heat (hot plate) and whilst warm glycerol is added. Allow to cool. Alcoholic haematoxylin solution is added in small volumes to the alum glycerol solution, mixing well. Acetic acid is added and mixed. The container is plugged with cotton wool and allowed to oxidise by exposure to light; this will take at least 6 weeks but ripening may be allowed to continue indefinitely beyond the period. Filter prior to use. Immediate oxidation may be achieved by adding 0.1 g of sodium iodate / 100 ml of solution. Then mixing and allowing standing for at least 1 hour before use.

2. *Eosin*

Eosin Yellowish	5 g
Calcium chloride	5g
Tap water	500 ml

Added eosin and calcium chloride to the water, mixed thoroughly and filtered; the stain is ready for use immediately.

3. *1% Acid Alcohol*

Alcohol (IMS)	700 ml
Distilled water	300 ml
Concentrated hydrochloric acid	10 ml

Alcohol is added to the water and mixed thoroughly, then hydrochloric acid is added and mixed again

4. *Staining Method*

1. Dewax sections in xylene, hydrate through graded alcohols to water
2. Stain in Ehrlich's Haematoxylin for 20 minutes
3. Wash in running tap water for 5 minutes
4. Differentiate in 1% acid alcohol for 5 seconds
5. "Blue" in running tap water for 5 minutes
6. Stain in eosin for 5 minutes
7. Rinse briefly in water
8. Dehydrate through alcohols, clear in xylene and mount in DPX

5. *Results*

Nuclei—Blue/ Black

Cytoplasm—varying shades of pink

Muscle fibres—deep pink red

Red blood cells, eosinophils—orange red

Fibrin--- deep red

Appendix ii: Reagents and materials used to culture and stain bone marrow derived mononuclear cells

1. Fresh mononuclear cells derived from mice
2. D-MEM/F-12, Dulbecco's modified eagle medium. Nutrient Mixture F-12 (Ham) 1:1 with glutamine + pyridoxine HCl without herpes buffer (Does not matter if it is with phenol red or not) [Invitrogen Corporation GIBCO]. The constitution is in 500 ml of bottle add antibiotics (5mls), L-glutamine (5mls) and 5mls of fetal bovine serum (FBS). Turn upside down and save it in 2-80C. ▲Critical: Once prepared can stay in the fridge.
3. HANKS Buffered saline without phenol red.
4. Lymphoprep™ (cat no: 1031966) is a ready made sterile and endotoxin tested solution for the isolation of pure lymphocyte suspensions. The solution contains sodium diatrizoate (9.1% w/v) and polysaccharide (5.7% w/v) [AXIS-SHIELD PoC AS Norway] ▲Critical: Lymphoprep™ should be stored at room temperature.
5. Phosphate buffer solution (PBS) from Sigma
6. Lab-Tek™ II Chamber slide™ system (cat.no 154534) NUNC, UK.
7. Haemocytometer
8. Dil-Ac-LDL catalogue No: BT-902, concentration: 200 ug/L; Lot No: 9020506; Absorbance ratio= 5.6 ▲Critical: Dil-Ac-LDL is stable 3 months when kept sterile at 4oC. Never Freeze.
9. Lectin from Ulex europaeus (Sigma, Cat. No L9006) FITC conjugate, lyophilised powder, potency of <5 ug/mL agglutination activity. Storage temp. -20oC.
10. Citiflour
11. Kroeings wax (Pfaltz & Bauer, Wterbury, CT 06708. Cat No C25974)

Appendix-iii: Pharmacokinetic properties of different statins (modified from references^{70, 113-114})

	Atorvastatin	Cerivastatin	Fluvastatin	Lovastatin	Pravastatin	Simvastatin	Rosuvastatin	Pitavastatin
Optimal timing of dosing	Anytime of day	Evening	Bedtime	With meals morning and evening	Any time of day	Evening	Anytime of day	na
Bioavailability (%)	12	60	24	5	18	5	20	Nearly 80
Solubility	Lipophilic	lipophilic	lipophilic	lipophilic	hydrophilic	lipophilic	hydrophilic	lipophilic
Effect of food	Bioavailability	No effect	Bioavailability	Bioavailability	Bioavailability	No effect	No effect	na
Protein binding (%)	98	>99	>98	>95	Nearly 50	95-98	90	96
Active metabolites	√	√	x	√	x	√	minor	minor
Elimination of half life (h)	14	2.5	1.2	3	1.8	2	19	11
CYP450 metabolism & isoenzyme	√3A4	√3A4,2C8	√2C9	√3A4	X	√3A4	limited	limited
Renal excretion	<5	30	6	10	20	13	10	na

Appendix iv:

Mass spectrometry

Mass spectrometry is performed using a Quattro LCTM tandem quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an ESI source. The capillary voltage is 4.0 kV, and the voltages of extractor and RF lens are 3.0 and 0.8 V, respectively. The entrance and exit energies of the collision cell are set at 15.0 V. The cone voltage is operated at an optimal value for each analyte in positive-ion mode. Nitrogen is used as the drying and nebulizing gas at flow rates of 500 and 70 l/h, respectively. The source and desolvation temperatures are optimized under LC–MS–MS operation, and were kept at 100 and 350 °C, respectively.

During method development, individual standard solutions are infused through a syringe pump (Harvard Apparatus, Holliston, MA, USA) at a flow-rate of 10 µl/min into the mass analyzer. Following the selection of precursor ions by the first quadrupole mass analyzer, collision-induced dissociation (CID) is carried out using 2.0×10^{-3} mbar UHP argon (Praxair Products, Peterborough, ON, Canada) in the hexapole collision cell. Product ion mass spectra are obtained at a series of collision energies so as to characterize each compound's fragmentation behavior. When LC is used for analyte separation prior to tandem spectrometry, the mass spectrometer is operated in selected reaction monitoring (SRM) mode with 1.3 of low mass resolution (LM Res) and high mass resolution (HM Res) on both of the first and second analyzers. A dwell time of 200 ms per ion pair is used, and the inter-channel delay was 10 ms.

Liquid chromatography

Analytes are separated using a Waters 2695 liquid chromatograph (Waters, Milford, MA, USA) with a Genesis C18 column (2.1×50 mm, 3 µm) (Jones Chromatography, Hengoed, UK). The mobile phase solvents are acetonitrile and water, respectively, containing 2 mM methylamine with 0.1% acetate acid. The mobile phase gradient is started at 60% of A, which is increased linearly to 100% within 3 min and held for 2 min. The flow-rate of the mobile phase is 0.2 ml/min and the injection volume was 20 µl.

Appendix v:

Long-term maternal high-fat feeding from weaning through pregnancy and lactation predisposes offspring to hypertension, raised plasma lipids and fatty liver in mice

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In rodents, adverse prenatal nutrition, such as a maternal diet rich in fat during pregnancy, enhances susceptibility of the offspring to hypertension, type 2 diabetes and other features of the human metabolic syndrome in adulthood. However, previous experimental studies were confined to short-term modifications of the maternal diet during pregnancy and/or lactation periods, a situation uncommon in humans. Moreover in humans, the offspring may also consume a high-fat diet, which may take them beyond the range to which their development has adapted them to respond healthily. We examined in C57 mice the effects on offspring of feeding their mothers a high-fat (HF) or standard chow (C) diet from weaning through pregnancy and lactation, and whether there are additive phenotypic effects of feeding the offspring an HF diet from weaning to adulthood (dam–offspring dietary group HF-HF). This group was compared with offspring from HF-fed dams fed a C diet from weaning to adulthood (HF-C) and offspring from C-fed mothers fed the C or HF diet (C-C and HF-C, respectively). HF-HF, HF-C and C-HF adult female offspring were heavier, fatter, and had raised serum cholesterol and blood pressure compared with C-C female offspring. We observed a similar trend in male offspring except for the HF-C group which was not heavier or fatter than male C-C offspring. Histology showed lipid vacuoles within hepatocytes in the HF-HF, HF-C and C-HF but not the CC offspring. Serum C-reactive protein was elevated in female (C-HF and HF-HF) but not in male offspring. Elevated blood pressure in the HF-C and C-HF groups was attenuated in the HF-HF group in males but not in females. These findings indicate that long-term consumption of an HF diet by the mother predisposes her offspring to developing a metabolic syndrome-like phenotype in adult life, although cardiovascular effects of an HF diet are related to sex specificity in the HF-HF group.

Hypertension: Pregnancy: Diet: Obesity: Metabolic syndrome

In humans it has been well documented that transition from an environment where food is poor or adequate to one where diet is high in fat and carbohydrates is associated with higher prevalence of the metabolic syndrome, typified by type 2 diabetes, hypertension, hypercholesterolaemia and obesity^(1–6). It has been suggested that such a 'nutritional transition' to a Western diet may have deleterious consequences on the health of future generations by affecting early development, influencing susceptibility to disease in later life. We recently proposed that the extent of such susceptibility depends on the degree of mismatch between the developmental and later environments, for example, in nutritional content⁽⁷⁾.

Developmental induction of cardiovascular and metabolic risk factors has been observed in rodent models in which the pregnant dam is exposed to nutrient restriction and the offspring fed a normal chow diet^(8–10). However, there are fewer studies that have examined adaptive and maladaptive

mechanisms during fetal development when pregnant dams are exposed to overnutrition. The issue is particularly important in the light of the increasing consumption of refined foods with a high glycaemic index and fat content among men and women now consuming 30% more saturated fats than the recommended daily intake⁽¹¹⁾. Several studies, including ours, have shown in rats that a maternal diet rich in fat during pregnancy results in features of the metabolic syndrome such as obesity, sedentary behaviour and vascular dysfunction in the offspring^(12–15). However, these studies were confined to short-term modifications in the maternal diet during pregnancy and/or lactation periods alone. Altering the maternal diet during critical periods of gestation^(16,17) or throughout gestation and/or the suckling period^(18,19) results in a varying degree of phenotypic outcomes associated with the metabolic syndrome, suggesting the importance of the timing and duration of the nutritional insult. In humans, con-

Abbreviations: BP, blood pressure; C, chow; CRP, C-reactive protein; HF, high-fat.

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sumption of a high-fat diet just during pregnancy or suckling is not common, but there is increasing concern about the effects of obesogenic diets in children on the health of their future offspring. We therefore extended our animal model to investigate the consequences for their offspring of feeding dams a high-fat (HF) diet from the time that they were weaned, through their pregnancy and lactation until their pups are weaned. In addition we determined whether there are additive effects of feeding these offspring themselves an HF diet from weaning to adulthood.

Methods

Experimental protocol

All animal procedures were in accordance with the UK Animals (Scientific Procedures) Act 1986. Female C57BL/6 mice (Charles River Laboratories, Margate, Kent, UK) were maintained under a 12h light–dark cycle at constant temperature ($25 \pm 2^\circ\text{C}$) with food and water available *ad libitum*. At age 4 weeks they were randomly allocated to either a control diet of standard laboratory chow (C; 5.3% fat (maize oil), 21.2% protein and 49.2% carbohydrate; Special Diet Services, Witham, Essex, UK) or an HF experimental diet supplemented 18% (w/w) with animal lard with additional vitamins and minerals, protein and choline to correct for the dilution (final composition in g% (w/w): lard, 17.8; casein, 26.5; choline chloride, 0.3; L-cysteine, 0.4; rice starch, 28.3; cellulose, 6.1; soya oil, 4.3; sucrose, 10.4; minerals, 4.3; vitamins, 1.2; Special Diet Services diet no. 824053, Witham, Essex, UK). This HF diet is as used in previous studies⁽²⁰⁾. At age 10 weeks, the females were time-mated and, after

confirmation of mating (i.e. presence of vaginal plug), were individually housed under a 12h light–dark cycle at constant temperature with water available *ad libitum*.

After birth, pups were weighed and litter size was reduced to eight pups and, when possible, to equal numbers of males and females. From weaning (21 d post-partum) offspring from the HF and C dams were fed either HF or C diets. We refer to the offspring born to HF dams as HF-HF and HF-C according to their post-weaning diet. Similarly, offspring born to C dams are referred to as C-HF and C-C according to their post-weaning diet. Food intake and body weights were monitored weekly until the offspring reached adulthood. Adult offspring were killed at age 36 weeks by cervical dislocation. Blood was collected by cardiac puncture and fat depots (i.e. gonadal, retroperitoneal, interscapular, inguinal and peri-renal) were dissected and weighed. Cumulative fat depot weights for each animal were compared with total body weights and body fat as a percentage of total body weight was calculated. The livers were also dissected, fixed in 10% neutral buffered formaldehyde and stored for further histological analyses.

Blood pressure measurements

Systolic arterial blood pressure (BP) was measured by tail-cuff plethysmography, as described previously by Krege *et al.*⁽²¹⁾ who showed that BP taken by this method was highly correlated with intra-arterial BP measured by telemetry in unrestrained, unanesthetised animals⁽²¹⁾. We conducted the measurements in a heated room ($27\text{--}28^\circ\text{C}$) in order to get optimal BP readings at the same time during the day. All animals were made accustomed to the procedure for 7 d before

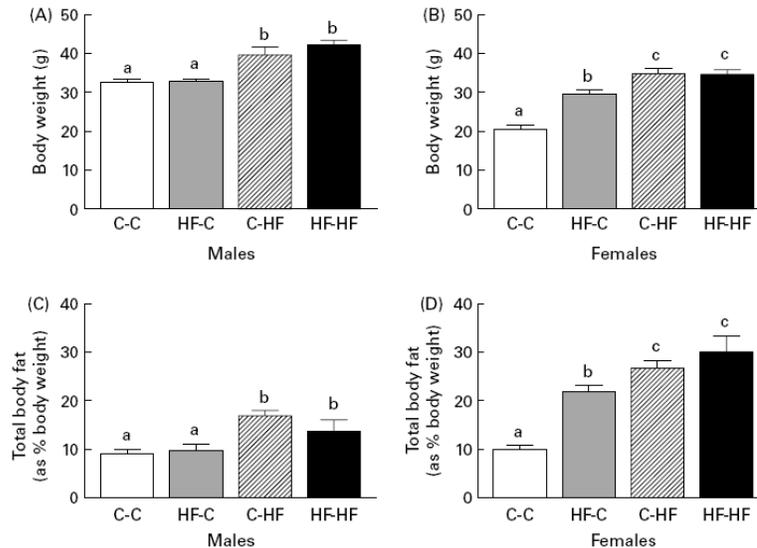


Fig. 1. Comparison of body weight (A, B) and total body fat (C, D) in male (A, C) and female (B, D) offspring from control-fed mothers that were then fed a chow diet (C-C) or a high-fat diet (C-HF) and from high-fat-fed mothers that were then fed a high-fat diet (HF-HF) or a chow diet (HF-C). Values are means ($n = 8\text{--}10$ per group), with standard errors represented by vertical bars. ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.05$; Tukey–Kramer comparisons test).

each BP measurement session. At least five readings were taken from each animal per session and averaged to get a single session value. BP was measured at 13, 18, 23, 27, 30 and 36 weeks post-weaning. At each time point, we took the average BP values from eight offspring of each sex picked randomly from each of the eight litters in each treatment group.

Measurement of plasma C-reactive protein and cholesterol

C-reactive protein (CRP) in the serum was measured using a sensitive double-antibody sandwich immunoassay enzymic ELISA rabbit anti-human CRP and peroxidase-conjugated rabbit anti-mouse CRP (VITROS CRP Slides; Vitros Products, Rochester, NY, USA)⁽²²⁾. Monoclonal anti-CRP antibody conjugated to horseradish peroxidase served as the signal generator. The assay was linear up to 5 mg/l and logarithmic thereafter. The inter-assay CV were less than 10% across the range of measured results. Serum total cholesterol was measured with commercially available kits (Vitros Products)

using enzymic methods and measured by reflectance spectrophotometry as previously reported^(15,18).

Histology

Fixed liver tissues were embedded in paraffin and 5–10 μ m sections were cut and mounted on glass slides. Deparaffinised, fixed sections were stained with haematoxylin and eosin. Microscopic examination was performed on stained liver sections from representative animals in each group.

Data analysis

The biochemical and biophysical parameters in dams were analysed using one-way ANOVA followed by the Tukey–Kramer comparisons test. All data are expressed as mean values with their standard errors. A *P* value less than 0.05 was considered to be statistically significant. All statistical analysis was calculated with SPSS 14.0 (SPSS, Inc., Chicago, IL, USA).

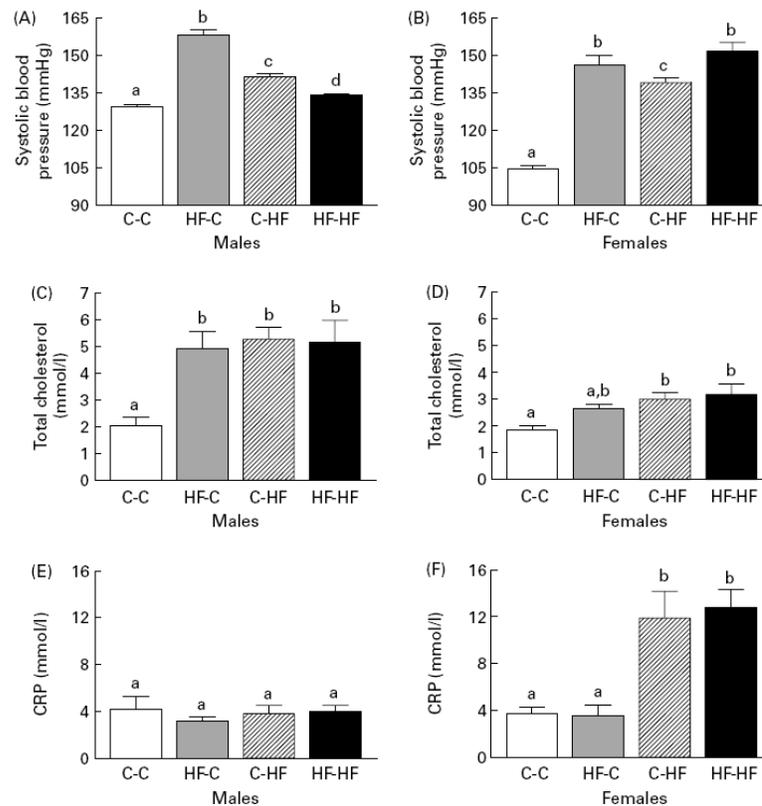


Fig. 2. Comparison of systolic blood pressure (A, B), total cholesterol (C, D) and C-reactive protein (CRP) levels (E, F) in male (A, C, E) and female (B, D, F) offspring from control-fed mothers that were then fed a chow diet (C-C) or a high-fat diet (C-HF) and from high-fat-fed mothers that were then fed a high-fat diet (HF-HF) or a chow diet (HF-C). Values are means (n 8–10 per group), with standard errors represented by vertical bars. ^{a,b,c} Mean values with unlike letters were significantly different (P < 0.05; Tukey–Kramer comparisons test).

Results

Body weight and adiposity

We did not find any significant difference in food intake among the various treatment groups during the experimental period (data not shown). HF-HF and C-HF male offspring were heavier at 36 weeks post-weaning than HF-C and C-C males (Fig. 1 (A)). These changes were also reflected in their total body fat (as percentage body weight), where the HF-HF and C-HF males had greater fat depots compared with the C-C and HF-C groups (Fig. 1 (C)). In female offspring the HF-C offspring were lighter than the HF-HF and C-HF animals but heavier compared with the C-C animals (Fig. 1 (B)). Corresponding increases in total body fat were observed in these groups compared with the C-C females (Fig. 1 (D)).

Blood pressure

Systolic BP was elevated in the HF-HF, HF-C and C-HF male and female offspring at 36 weeks post-weaning compared with C-C offspring (Fig. 2 (A) and (B)). The HF-C males had the highest BP and this was significantly greater than the HF-HF and C-HF groups. In the females, the HF-HF and HF-C groups had significantly elevated BP levels compared with the C-HF offspring.

C-reactive protein and cholesterol levels

Total cholesterol was elevated in the HF-HF, HF-C and C-HF males at 36 weeks post-weaning compared with the C-C males (Fig. 2 (C)). Interestingly, no difference was observed in CRP levels among these four treatment groups (Fig. 2 (E)).

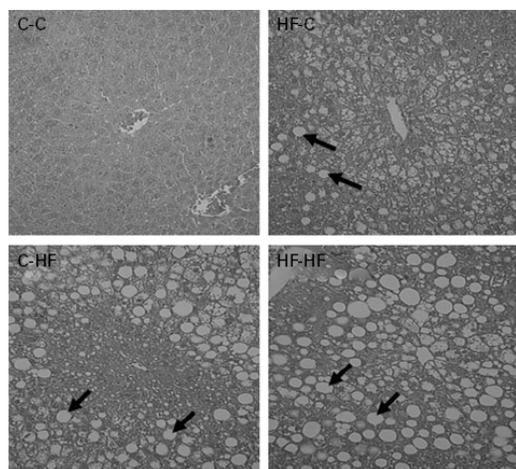


Fig. 3. Liver histology in female offspring from control-fed mothers that were then fed a chow diet (C-C) or a high-fat diet (C-HF) and from high-fat-fed mothers that were then fed a high-fat diet (HF-HF) or a chow diet (HF-C). C-C offspring had normal liver structure. However, lipid vacuoles (—) of various sizes could be observed within hepatocytes of the C-HF, HF-HF and HF-C offspring. Staining with haematoxylin and eosin; magnification $\times 20$; bar scale = 40 μm .

In females, total cholesterol and CRP level were elevated in the HF-HF and C-HF animals compared with the C-C offspring (Fig. 2 (D) and Fig. 2 (F), respectively). Total cholesterol in the HF-C group was also elevated but was not significantly different from the C-C or from the C-HF and HF-HF groups.

Development of fatty liver in adult offspring

Histological examination of the liver showed that the C-C offspring had normal liver structure (Fig. 3). However, we observed lipid vacuoles of various sizes within hepatocytes of the C-HF, HF-HF and HF-C offspring. Moreover, mononuclear cell infiltration, pyknotic nuclei and the rupturing of the endothelium of some central veins were observed in the livers in these groups of offspring compared with C-C offspring (data not shown).

Discussion

Nutritional status during critical periods of early life has important influences on development, and modification of the quality and/or quantity of maternal nutrition during pregnancy has been shown to have consequences on the later health of the offspring, changing their responses to environmental challenges and thus their predisposition to disease^(23,24). In the present study we examined in a mouse model the consequences to the offspring of a long-term maternal HF dietary regimen, starting from when the prospective mothers were themselves weaned until weaning of their offspring. This contrasts with earlier animal experiments, which focused on the consequences for the offspring of maternal HF feeding during only gestation and/or lactation periods^(12,13,15). The long-term HF feeding results in changes in the dam's physiology, including increased body weight and raised circulating total and LDL-cholesterol levels compared with the C-fed group, as observed in our previous study⁽²⁵⁾. These changes in the HF dams may provide 'cues' used by the developing offspring in altering phenotype in anticipation of their postnatal environment. The long-term dietary regimen used in the present study may better represent the situation in human populations, following socio-economic transitions where consumption of high-fat food occurs very early in life and continues in women through pregnancy and lactation. The present study demonstrates that offspring of such mothers are predisposed to becoming fat, hypercholesterolaemic and hypertensive in adulthood, thus perpetuating the cycle of chronic disease.

We previously reported that prenatal and early postnatal exposure to a maternal diet rich in animal fat leads to the development of characteristics similar to the human metabolic syndrome in adult rats, even when they are reared on a balanced diet^(13,14). The present study clearly shows that long-term consumption of an HF diet by the female dams predisposes their offspring to obesity, hypercholesterolaemia, hypertension and fatty liver in adult life, at least when they were also fed an HF diet. Greater adiposity was also seen in females, even when they were fed a C diet post-weaning, suggesting that predisposition to obesity had been induced during development. This was not, however, seen in the HF-C male offspring.

In males the elevation of BP was less pronounced in the HF-HF group than in the HF-C or C-HF groups. This supports the partially beneficial cardiovascular effect of reducing the dietary mismatch in the HF-HF offspring reported in our earlier study⁽¹³⁾ although in that study the dams were only fed the HF diet during pregnancy and weaning, and endothelial dysfunction rather than elevated BP was attenuated compared with the C-fed offspring. These data are broadly in support of the predictive adaptive response concept⁽¹³⁾. However, as outlined in the original exposition of the concept⁽³⁾, such an effect only operates within a range of postnatal environments, beyond which the risk of pathophysiology is increased. The postnatal HF used in the present study is likely to take the offspring into such a range, one which from an evolutionary point of view is novel, possibly explaining the pathophysiological effects observed in them.

We observed a substantial number of lipid vacuoles within hepatocytes of the HF-HF, HF-C and C-HF but not the C-C offspring. Previous studies in rats have demonstrated that offspring of fat-fed dams have profound metabolic defects such as increased liver weight and liver TAG content^(26,27). It has been reported in humans that there is a strong association between hypercholesterolaemia, steatohepatitis (non-alcoholic fatty liver) and increased risk of CVD⁽²⁸⁾ but it is unclear whether certain diets are more likely to produce these effects than others. Steatohepatitis and atherosclerosis are both produced in apo-E lipoprotein-deficient mice, suggesting a pathophysiological effects on both the liver and aorta of cholesterol-enriched diets^(29,30). The accumulation of lipids in hepatocytes suggests possible interference with mitochondrial and microsomal function leading to disruption in lipoprotein transport and fatty acid accumulation v. metabolism⁽¹⁴⁾. The accumulation of lipids in hepatocytes in the HF-C offspring suggests that susceptibility to development of fatty liver can be induced during early development.

Chronic inflammation is a major contributor to atherosclerosis and CVD⁽³¹⁾. An important marker of inflammation is an elevation in CRP, an acute-phase reactant secreted by hepatocytes in response to pro-inflammatory cytokines such as IL-6⁽³²⁾. In several large epidemiological studies, CRP has been shown to be a strong, independent predictor of CVD risk in both men and women^(33,34). The mechanism by which inflammation increases CVD risk is not known, but during periods of acute inflammation lipid metabolism is altered, giving a proatherogenic profile⁽³⁵⁾. This is what we observed in our HF-HF and C-HF female offspring, which had elevated CRP levels. Interestingly, we did not find such increases in the male offspring, suggesting that the effect of HF exposure on CRP levels is sex specific. The increase in CRP levels found only in female offspring on the HF diet may be a consequence of increased sensitivity to the HF diet brought about by circulating female sex hormones^(34,36) or due to the fact that the HF diet itself may result in high CRP in females. This observation is supported by human studies where CRP levels tend to be higher in women than in men^(33,34,37–39).

Perspectives

The current findings indicate that long-term consumption of an HF diet by the mother predisposes her offspring to obesity,

hypercholesterolaemia, hypertension and development of fatty liver in adult life. This adverse effect on the offspring is induced during development and is not necessarily or completely reversed by either consumption of a postnatal C diet or indeed an HF diet. The results from the present study provide an experimental basis for investigating consequences of dietary transitions relevant to humans, where the woman's diet both before and during pregnancy and lactation may be a contributing factor to the development of metabolic and cardiovascular disease in her children.

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Statin Treatment in Hypercholesterolemic Pregnant Mice Reduces Cardiovascular Risk Factors in Their Offspring

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Abstract—Increasing evidence suggests that hypercholesterolemia during pregnancy initiates pathogenic events in the fetus leading to increased risk of cardiovascular disease in the adult offspring. In this study we examined in mice whether pharmacological intervention using statins in late pregnancy could alleviate the detrimental effects of a high-fat, high-cholesterol (45% fat) maternal diet on the health of the dams and their offspring. Pregnant C57 mice on high-fat, high-cholesterol diet were given the 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor pravastatin in the drinking water (5 mg/kg of body weight per day) in the second half of pregnancy and during lactation to lower cholesterol and improve postweaning maternal blood pressure. Weaned offspring were then fed the high-fat, high-cholesterol diet until adulthood (generating dam/offspring dietary groups high-fat, high-cholesterol/high-fat, high-cholesterol and high-fat, high-cholesterol plus pravastatin during the second half of pregnancy and lactation/high-fat, high-cholesterol). These groups were compared with offspring from mothers fed standard chow (control), which were then fed control diet to adulthood (control/control). Compared with high-fat, high-cholesterol, high-fat, high-cholesterol plus pravastatin during second half of pregnancy and lactation dams showed significantly reduced total cholesterol concentrations and reduced systolic blood pressure. The high-fat, high-cholesterol plus pravastatin during second half of pregnancy and lactation/high-fat, high-cholesterol offspring were significantly lighter, less hypertensive, and more active compared with the high-fat, high-cholesterol/high-fat, high-cholesterol group. Total serum and low-density lipoprotein cholesterol concentrations were significantly lower, and high-density lipoprotein cholesterol concentrations were raised in high-fat, high-cholesterol plus pravastatin during the second half of pregnancy and lactation/high-fat, high-cholesterol offspring, compared with the high-fat, high-cholesterol/high-fat, high-cholesterol group. The control/control offspring showed the lowest blood pressure and cholesterol levels. These findings indicate that the cholesterol-lowering effect of statins in pregnant dams consuming a high-fat, high-cholesterol diet leads to reduced cardiovascular risk factors in offspring that are sustained into adulthood. (*Hypertension*. 2008;51:1-6.)

Key Words: statins ■ diet ■ hypercholesterolemia ■ hypertension ■ pregnancy

Increasing evidence suggests that hypercholesterolemia during pregnancy initiates pathogenic events in the fetus and, thus, increases the risk of cardiovascular disease in the offspring.^{1,2} Atherosclerosis progresses faster in offspring of hypercholesterolemic mothers than those of mothers with normal cholesterol levels.³ In animals, we and others have shown that feeding a high-fat, high-cholesterol diet (HF) during pregnancy and/or lactation induces obesity, vascular dysfunction, impaired skeletal muscle development, sedentary behavior, and gender-specific hypertension in the offspring.^{4–10} Thus, early interventions may be very effective as shown in studies where the introduction of a low saturated fat diet in infancy and maintaining it during the first decade of life is associated with a reduction in serum cholesterol concentration and enhanced endothelial function in children.¹¹

In humans, the influence of an HF diet is unlikely to commence during pregnancy. The existing data thus raise the important question of whether lipid-lowering interventions during pregnancy in mothers already consuming an HF diet could provide long-lasting benefits to their offspring. Palinski et al¹² demonstrated a reduction of atherosclerosis in offspring of rabbits treated with cholestyramine or vitamin E, as well as those receiving combined treatments. Prevailing practice advocates that interruption of total cholesterol synthesis during the first trimester is potentially hazardous to the growing embryo. The cholesterol-lowering “statin” drugs are, therefore, clinically contraindicated in pregnancy, and initial animal studies have shown that they are potentially teratogenic.^{13,14} Although a recent study reported no evidence of an increase in congenital anomalies in humans compared within the general population after maternal exposure to simvastatin

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or lovastatin,¹⁴ these statins are still highly lipophilic and can result in embryo-placental concentrations similar to those in maternal plasma. Pravastatin, on the other hand, is the most hydrophilic statin and has not been reported to induce abnormal pregnancy outcomes, even in animals.¹⁵ We, therefore, chose to study the consequences of lowering maternal cholesterol with pravastatin treatment in late pregnancy and lactation with the view of testing the hypothesis that this could also reduce cardiovascular risk factors in the adult offspring. We used the C57BL/6 hypercholesterolemic mouse model, giving the HF diet from the time the dams were weaned, then throughout pregnancy and lactation. We measured blood pressure, body weight, and physical activity in female offspring, because a previous study revealed more pronounced effects on blood pressure in the female offspring of lard-fed pregnant rats compared with males.⁷ In addition, we compared plasma cholesterol concentrations in the dams with that of their offspring.

Methods

Experimental Protocol

All of the animal procedures were in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Female C57 mice (Charles River Laboratories United Kingdom) were maintained under a 12-hour light-dark cycle at constant temperature ($25\pm 2^\circ\text{C}$) with food and water available ad libitum. At 4 weeks old, the females were randomly allocated to either a control diet of standard laboratory chow (C; 5.3% fat [corn oil], 21.2% protein, 49.2% carbohydrate; Special Diet Services United Kingdom) or an HF experimental diet supplemented 18% weight/weight with animal lard with additional vitamins and minerals, protein, and choline to correct for the dilution [final composition in percentage of grams [weight/weight]: lard 17.8; casein 26.5; choline chloride 0.3; L-cystine 0.4; rice starch 28.3; cellulose 6.1; soya oil 4.3; sucrose 10.4; minerals 4.3; vitamins 1.2; Special Diet Services diet E24053]. This HF diet has been used in previous studies.¹⁶ At 10 weeks old, the females were time mated and after confirmation of mating (ie, presence of vaginal plug), were individually housed. From the second half of the pregnancy and throughout lactation, half of the pregnant females on the HF diet were given a water-soluble 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor (pravastatin, Sigma United Kingdom) in their drinking water (HF-S). Pravastatin was dissolved at a concentration that gave a daily dose of 5 mg/kg per day, based on the daily water consumption of pregnant and lactating mice, which we had determined in a preliminary study. The pregnant dams were allowed to give birth, pups were weighed, and litter size was standardized to 8 pups with equal numbers of males and females if possible. From weaning (3 weeks postpartum), offspring from the C dams were fed the same C diet as their mothers. The offspring of HF and HF-S dams were fed ad libitum with the HF diet to generate the HF/HF and HF-S/HF groups. Body weights of the offspring (from 1 week of age to avoid maternal rejection of the pups) and food intake (from weaning) were monitored until 27 weeks of age. We took the average body weights and food intake from each of the 8 litters in each treatment group.

Blood Pressure Measurements

Systolic arterial pressure was measured by tail cuff plethysmography, as described previously by Kreege et al.¹⁷ Measurements were conducted in a heated room ($\approx 34^\circ\text{C}$) to get optimal blood pressure (BP) readings and were conducted at the same time during the day (afternoon). All of the animals were accustomed to the procedure for 7 days before each BP measurement session. At least 5 readings were taken from each animal per session with the highest and lowest readings discarded, and the remaining readings were averaged to get a single session value. BP was measured at 13, 18, 23, and 27 weeks

of age. At each time point, we took the average BP values from 8 female offspring picked randomly from each of the 8 litters in each treatment group.

Measurement of Locomotor Activity

Locomotor activity was measured by placing individual animals in automated activity cages equipped with infrared photocells interfaced with a computer, as described previously.¹⁸ Recorded beam breaks were used to automatically calculate the total distance traveled. Measurements were taken at 13, 18, 23 and 27 weeks of age. At each time point, we took the average measurements from 8 female offspring picked randomly from each of the 8 litters in each treatment group.

Measurement of Serum Lipid Profile

A blood sample was drawn by direct heart puncture after anesthetizing the animal with isoflurane and cervical dislocation. Blood samples were taken from a subgroup of females at the time of mating (14 weeks of age) and in dams after weaning their pups. Blood samples were also taken from offspring at 13, 18, 23, and 27 weeks of age. At each time point, we sampled 8 female offspring picked randomly from each of the 8 litters in each treatment group. Total cholesterol, low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol in the serum were measured with commercially available kits (Vitros Products) using enzymatic methods and measured by reflectance spectrophotometry, as reported previously.^{19,20}

Data Analysis

The biochemical and biophysical parameters in dams were analyzed using 1-way ANOVA followed by the Tukey-Kramer comparisons test. All of the data are expressed as means \pm SEMs. In the female offspring, effect estimates are from a mixed model analysis²¹ that considers all of the time points through the study, controlling for the set of dam-pup relationships. A $P < 0.05$ was considered to be statistically significant. All of the statistical analysis was calculated with SPSS 14.0 (SPSS Inc) (Disease and Stroke).

Results

Statin Treatment in Hypercholesterolemic Dams Ameliorates Their BP and Alters Total Cholesterol, LDL Cholesterol, and HDL Cholesterol Profiles

Before becoming pregnant, 14-week-old HF females (fed the HF diet for 10 weeks) showed significantly raised total cholesterol (4.08 ± 0.4 versus 1.99 ± 0.2 mmol/L; $P < 0.001$) and LDL cholesterol (2.1 ± 0.2 versus 0.8 ± 0.02 mmol/L; $P < 0.001$) relative to their C counterparts. HF dams were heavier than C dams during midpregnancy (body weight: 67.4 ± 2.4 versus 41.2 ± 1.8 g; $P < 0.001$) and at the time of weaning their offspring (57.4 ± 3.4 versus 30.7 ± 1.3 g; $P < 0.001$). At weaning of their offspring, HF dams were more hypertensive with raised total cholesterol and LDL cholesterol compared with C dams (Table 1). Statin treatment in HF dams reduced systolic BP, total cholesterol, and LDL cholesterol levels at weaning of their offspring. In addition, administering statin to HF dams increased HDL cholesterol concentrations compared with the untreated HF mothers.

Statin Treatment in Hypercholesterolemic Dams Has Beneficial Effects on Female Offspring BP, Lipid Profiles, and Locomotor Activity

HF/HF offspring were of similar weight as the C/C offspring 1 week postpartum (HF/HF, 3.2 ± 0.1 versus C/C, 2.9 ± 0.2).

Table 1. Statistical Analyses of Biochemical and Biophysical Parameters in Dams at Weaning

Variables	Dietary Groups, Mean \pm SEM			
	HF	HF-S	C	P
Total cholesterol, mmol/L	6.02 \pm 0.20	3.45 \pm 0.24†	2.29 \pm 0.12††	<0.001
Systolic BP, mm Hg	136.2 \pm 1.4	123.6 \pm 1.1†	109.7 \pm 2.4††	<0.001
LDL cholesterol, mmol/L	3.50 \pm 0.09	1.13 \pm 0.09†	0.21 \pm 0.03††	<0.001
HDL cholesterol, mmol/L	1.12 \pm 0.17	2.60 \pm 0.05†	1.73 \pm 0.10††	<0.001

Statistical test in the right column is the ANOVA result for all of the groups. Statistical result displayed in each column is for the dietary group comparison with the others group using the Tukey-Kramer test; n=8 for all of the groups.

†P<0.01 and ††P<0.001 for HF-S or C against HF.

‡P<0.001 for C against HF-S.

However, their overall body weight gain was significantly greater compared with the C/C offspring (Figure, part A and Table 2). The HF-S/HF offspring showed a smaller increase in body weight gain compared with the HF/HF offspring. Systolic BP was significantly lower at 13 to 27 weeks in HF-S/HF compared with HF/HF offspring (Figure, part B and Table 2). As expected, systolic BP for the C/C group was lower at all of the time points examined. We also found that systolic BP in the HF-fed female offspring at 27 weeks of age was much more elevated compared with their HF-fed mothers (151.6 \pm 3.6 versus 136.2 \pm 1.4 mm Hg, respectively; P <0.01). Offspring from HF-S mothers were significantly more active at 13 to 27 weeks of age than HF/HF offspring, although not as much as the C/C animals (Figure, part C and Table 2). Total serum and LDL cholesterol concentrations for offspring on HF or C diets

followed a similar pattern to dams on HF or C, respectively, and previous exposure of their dams to pravastatin resulted in significantly lower total and LDL cholesterol levels, similar to its effect in the dams themselves (Figure, part D and part E, respectively, and Table 2). The elevated levels of total cholesterol observed in HF/HF offspring at 27 weeks were similar to levels found in the HF dams. It is interesting to note that total serum and LDL cholesterol concentrations in HF-S/HF offspring become progressively closer together over time to levels found in the HF/HF group. The HDL cholesterol concentration for offspring on HF or C diets also showed a similar pattern to dams on HF or C, respectively, and previous exposure of their dams to pravastatin resulted in significantly higher HDL cholesterol concentration, similar to its effect in the dams themselves (Figure, part F and Table 2).

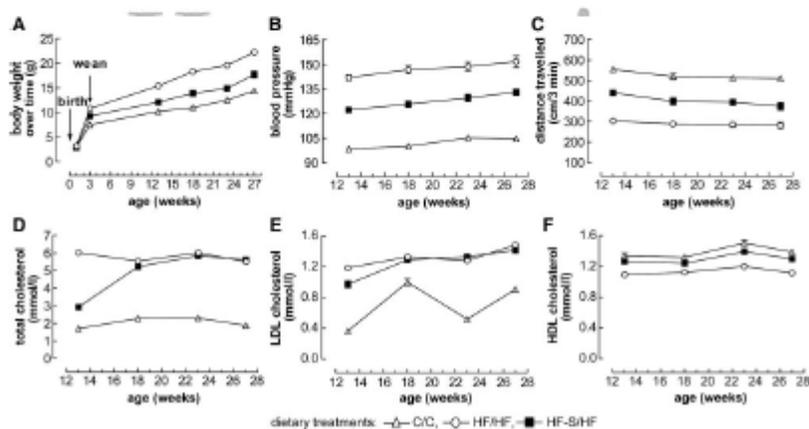


Figure 2. Statin treatment in hypercholesterolemic mothers during late pregnancy and lactation has beneficial effects on the cholesterol profile, blood pressure, and locomotor behavior in their female offspring. A, Body weight gain; B, systolic blood pressure; C, locomotor activity; D, serum cholesterol; E, serum LDL; and F, serum HDL in offspring from C mothers, HF mothers, or HF-S mothers. Weaned offspring were then fed either HF or C diets to adulthood, thus generating the dam/offspring dietary groups: HF/HF, HF-S/HF, and C/C (n=8 per group). See Table 2 for results of data analysis.

Table 2. Estimate of Mean Difference and 95% CI of Biochemical and Biophysical Parameters in Female Offspring

Variables	HF/HF vs HF-SHF			HF/HF vs C/C			HF-SHF vs C/C		
	Mean	95% CI	P	Mean	95% CI	P	Mean	95% CI	P
Body weight, g	2.9	2.2 to 3.6	<0.001	4.7	4.0 to 5.4	<0.001	1.8	1.1 to 2.6	<0.001
Systolic BP, mm Hg	20	15 to 25	<0.001	45	40 to 50	<0.001	26	21 to 31	<0.001
Locomotor activity, cm ² /min	112	85 to 140	<0.001	235	207 to 263	<0.001	122	95 to 150	<0.001
Total cholesterol, mmol/L	0.89	0.60 to 1.16	<0.001	3.68	3.39 to 3.96	<0.001	2.79	2.51 to 3.08	<0.001
LDL cholesterol, mmol/L	0.87	0.56 to 1.17	<0.001	3.73	3.43 to 4.04	<0.001	2.87	2.56 to 3.17	<0.001
HDL cholesterol, mmol/L	0.17	0.13 to 0.20	<0.001	0.26	0.22 to 0.29	<0.001	0.09	0.05 to 0.12	<0.001

Effect estimates ($n=8$ for all groups) are from a mixed-model analysis that considers all of the time points through the study, controlling for the set of dam-pup relationships.

Discussion

The present study demonstrates that treating pregnant animals that are hypercholesterolemic and hypertensive with pravastatin not only improves their health but can also have long-lasting beneficial effects on BP and activity and induces a transient reduction of cholesterol in their offspring even if they consume a similar high-fat diet. This work has underscored the importance of maternal nutrition even before pregnancy on metabolic and cardiovascular outcome of the future offspring. Furthermore, the present findings provide the first indication that cholesterol lowering, or other effects of statins, benefits postnatal BP.

Several studies, including ours, have shown that a maternal diet rich in fat and cholesterol during pregnancy can induce obesity, vascular dysfunction, impaired skeletal muscle development, sedentary behavior, and gender-specific hypertension in the offspring.⁴⁻²² However, these studies have been confined to short-term modifications in the maternal diet, such as during pregnancy and/or lactation periods only. In the present study, we gave future mothers an HF diet very early in life to produce an effect on offspring health. Thus, our experimental approach is more representative of the human condition.

Altering the maternal diet during critical periods of gestation^{22,23} or throughout gestation and/or the suckling period²⁴ results in a varying degree of phenotypic outcomes, suggesting the importance of the timing and duration of the nutritional insult. This is emphasized by one of the novel findings using our animal model, namely that BP in the HF/HF offspring was much greater than in their HF-fed mothers despite having lower cholesterol levels and the mother weighing more than their offspring at the time of BP measurement. Such effects are fundamental to the concept of the developmental origins of disease²⁴ and also indicate that intervention in early life may be particularly important in reducing later risk of disease in the face of lifestyle factors such as a high-fat diet.

It remains to be investigated what mechanisms may have changed the phenotype of the offspring through maternal pravastatin treatment. Statins are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase, which converts 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonate, an early rate-limiting step in cholesterol biosynthesis.²⁵ Moreover, previous studies have shown that there is intrauterine transfer of maternal cholesterol to the embryo, as well as the

fetus.^{26,27} Thus, statin treatment may indirectly affect patterns of growth and development of organs and tissues within the fetus by influencing maternal-fetal cholesterol transfer across the placenta and preventing changes in liver, kidney, and vascular function in the fetus because of the detrimental effects of the hypercholesterolemic condition. Aside from the lipid-lowering effects of statins, they have also been found to upregulate endothelial NO synthase, increase NO bioavailability, and decrease oxidative stress.²⁸ It is, therefore, possible that statins could blunt the deleterious effects of an imbalanced maternal diet by a range of mechanisms.

We also observed that offspring from hypercholesterolemic dams are less active, providing another aspect of the model that mimics the early origins of the "couch-potato" syndrome in humans.²⁹ Although this has been observed previously when dams were undernourished during pregnancy,^{29,30} the present study is the first to show that a maternal high-fat diet during pregnancy can also result in sedentary behavior in the offspring. Moreover, statin treatment of the dams ameliorates this effect. The mechanisms underlying these effects are not known. Although inadequate cholesterol provision to the developing fetus is deleterious to patterning and development of the central nervous system,³¹ the effects of hypercholesterolemic condition during pregnancy have not been reported.

We recognize that prolonged exposure to the HF diet can not only lead to hypercholesterolemia and hypertension before and during pregnancy but can also result in the development of obesity. This would almost certainly be associated with insulin resistance, increased inflammation, and concomitant immune responses, which also affect developmental programming of cardiovascular disease.^{32,33} It is, therefore, not possible at this time to attribute unequivocally the changes in offspring BP and activity level to maternal hypercholesterolemia. It also remains to be determined whether the protective effects of statin treatment are because of cholesterol lowering, per se, during pregnancy or because of the reduced obesity, hypertension, or insulin resistance in mothers in late pregnancy, ie, whether statins prevent pathogenic programming by improving maternal health or whether they interfere with in utero programming mechanisms. It is more likely that the protective effect is because of cholesterol lowering, because the statin that we have used, pravastatin, is hydrophilic and does not cross the placental barrier. How-

ever, we cannot discount the possibility that there may be other effects of the drug (eg, on endothelial cells).

It remains to be determined how the cholesterol-lowering effect of statin in the pregnant mothers affect postnatal BP or activity levels in the offspring. To resolve these questions, it would be necessary to compare the effect of statin with that of other hypocholesterolemic drugs and to use an experimental design that minimizes differences in body weight and other parameters induced by the diet before pregnancy. This was beyond the scope of the present study but should be considered as a limitation in interpreting the data. It is also possible that the beneficial effect of statin occurs during the early postnatal period, because we continued giving it in the dam's drinking water during the lactation period. Studies have shown excretion of statin into the milk in rat dams that were treated postpartum with the statin atorvastatin.²⁴ Statin could, at this point, reverse the programming effect of the maternal hypercholesterolemia similar to the effect of leptin administration in early postnatal life to offspring from undernourished mothers in preventing obesity, vascular dysfunction, sedentary behavior, and gender-specific hypertension in the offspring.^{25,26} It would, therefore, be interesting to examine whether statin treatment in early postnatal life of offspring from hypercholesterolemic mothers will also reduce the response to a postweaning HF diet to a similar degree to statin administration in late pregnancy alone. Further studies are also needed to comprehensively examine the effects of the HF diet and statin treatment on the dynamic nature of BP throughout the day and night in our experimental animals. Although our results indicate clear-cut differences in systolic BP among the various experimental groups, the tail-cuff method can only give a "snapshot" of the animal's BP at a particular time of day.

The statin class of drugs is still regarded as contraindicated during pregnancy, mainly because of previous reports of their teratogenic effects. Despite widespread use of statins, however, and many instances when they were inadvertently taken during pregnancy, there is little evidence of their adverse effects in humans. A reconsideration of the use of statins in high-risk mothers, therefore, seems to be indicated, but this remains controversial. Bearing this in mind, we, therefore, limited their use to the second half of pregnancy.

The present evidence linking an impaired fetal environment with later pathological effects in offspring supports the notion that maternal hypercholesterolemia during pregnancy should be included among the risk factors for disease in children. Moreover, the improvement in offspring phenotype after statin treatment seen in our study further indicates that poor health of the mother during pregnancy is a contributing factor to the rapidly developing cardiovascular disease epidemic.^{2,27} Primary prevention should aim to optimize body composition and diet in young women even before they reach childbearing age.

Perspectives

The present findings indicate that statin administration to HF-fed pregnant dams not only improves their cardiovascular and metabolic health but also gives some postweaning protection to their offspring. This might allow time for other

intervention strategies to be put in place to protect the offspring, which are also likely to consume a poor postweaning diet. This new evidence to suggest that maternal hypercholesterolemia has a detrimental effect on the next generation may necessitate a reconsideration of present recommendations against the use of statins during pregnancy.

Acknowledgments

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Sources of Funding

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Disclosures

None.

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Appendix vii:

Long-term statin treatment in hypercholesterolemic pregnant mice reduces cardiovascular and metabolic risk in their offspring also fed a high fat diet post-weaning.

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Introduction: We have shown that long-term maternal high-fat (HF) feeding during pregnancy and lactation predisposes offspring to hypertension, raised plasma lipids and fatty liver in mice¹. Recently, we have also demonstrated that pharmacological intervention with statin in late pregnancy in HF fed dams reduces cardiovascular (CV) and metabolic risk factors in their offspring². In this study, we examined the effects of long-term statin administration to HF-fed female mice on these risk factors when their offspring were also fed a HF diet. **Methods:** Pregnant C57 mice on HF diet (45% kcal fat) were given the 3hydroxy3methylglutaryl-coenzyme A reductase inhibitor pravastatin in their drinking water (5 mg/kg of body weight per day) from the time they were weaned until weaning of their offspring. Weaned offspring were then fed the HF diet until adulthood generating dam/offspring dietary groups HF/HF and HF+S/HF. These groups were compared with offspring from dams fed standard chow (C) which were fed chow diet post-weaning to adulthood (C/C). All data were expressed as mean \pm SEM. One way ANOVA followed by post-hoc test was used. Significance was assumed if the P value was <0.05. Animal procedures were in accordance with the UK Animals (Scientific Procedures) Act 1986. **Results:** HF+S dams showed significantly reduced total cholesterol concentrations and systolic blood pressure vs. HF dams (P<0.001). The HF+S/HF offspring were significantly lighter, with lower systolic blood pressure and serum cholesterol concentrations vs. HF/HF (P<0.001). HF/HF offspring also had elevated C-reactive protein (CRP) levels and these were reduced in the HF+S/HF animals to levels found in the C/C group (P<0.001). **Conclusions:** Long-term pravastatin administration to dams not only protects them from the deleterious effects of a HF diet but also protects their offspring from CV and metabolic risk factors in later life, even if these offspring consume a HF diet.

1. M.M Elahi et al., Br J Nutr., 10:1-6, 2009.
2. M.M.Elahi et al., Hypertension., 51:939-44, 2008

Poster presentation (P-9A-387) at the 6th World Congress on Developmental Origins of Health and Disease, 19-22 November 2009, Santiago, Chile. *J Develop Origins Health & Disease*, 2009; 1: 289.



Long-term statin treatment in hypercholesterolemic pregnant mice reduces cardiovascular and metabolic risk in their offspring also fed a high fat diet post-weaning.

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 Centre for Developmental Origins of Health & Disease & Wessex Cardiothoracic Centre, University of Southampton, UK.



- BACKGROUND -

- ◆ We have shown that long-term maternal high-fat (HF) feeding during pregnancy and lactation predisposes offspring to hypertension, raised plasma lipids and fatty liver in mice¹.
- ◆ Recently, we have also demonstrated that pharmacological intervention with statin in late pregnancy in HF fed dams reduces cardiovascular (CV) and metabolic risk factors in their offspring^{2,3}.
- ◆ We have now investigated whether statin administration to female mice from the time they were weaned until weaning of their offspring reduces CVD risk factors in these offspring, even if offspring are fed a high fat diet.

- AIMS -

1. To investigate the effects of long-term statin administration to HF-fed female mice on these risk factors when their offspring were also fed a HF diet.
2. To investigate whether there are any gender-linked differences in offspring of these dams in the cardiovascular responses.

- METHODS -

Animals and Experimental Diets



Body Weight & Blood Pressure

Body weight was measured on weekly basis. Systolic arterial pressure was measured by tail cuff plethysmography.

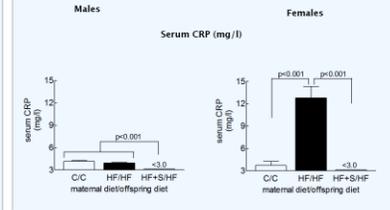
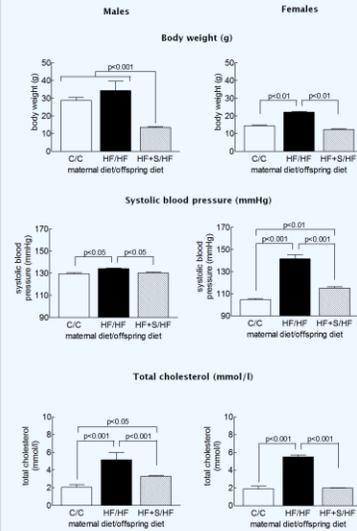
Plasma Lipid Profile & C-Reactive Protein

At 27 weeks post weaning, blood was collected by cardiac puncture under terminal anaesthesia. Lipid profile was measured by enzymatic methods using commercially available kits and reflectance spectrophotometry. C-reactive protein (CRP) was measured using a sensitive double-antibody sandwich immunoassay enzymatic ELISA.

Statistical Analysis

The statistical significance of differences between groups was determined by one way ANOVA followed by post-hoc test

- RESULTS -



- CONCLUSIONS -

- Our findings show that the HF/HF offspring were obese, had higher cholesterol concentrations, and were hypertensive compared with the C/C group. These changes were more pronounced in the female offspring.
- HF/HF exposure also increases CRP-mediated inflammation.
- Pravastatin administration in early life to dams fed a HF diet protects their offspring in a post-weaning HF nutritional environment from risk of later cardiovascular pathophysiology.
- We did not find any evidence that pravastatin administration to dams before and during pregnancy produced any deleterious effects on their offspring.

- REFERENCES -

1. M.M Elahi et al., *Br J Nutr.*, 10:1-6, 2009.
2. M.M.Elahi et al., *Hypertension.*, 51:939-44, 2008.
3. Elahi et al. 2007 *Early Human Dev* 83: 574.

- ACKNOWLEDGEMENTS -

MME and MAH are supported by BUPA/HOPE research fellowship and British Heart Foundation, respectively.

Appendix viii:

Statin treatment in female mice has sex specific effects on cardiovascular risk factors and circulating endothelial progenitor cells in their offspring.

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Objectives: Recently we reported that, in dams consuming a high-fat high-cholesterol diet, statin administration during the 2nd half of pregnancy and lactation reduces cardiovascular risk factors in their offspring. We have now examined the effects of statin administration to female mice from the time they were weaned until weaning of their offspring on cardiovascular risk factors in their offspring. **Materials and Methods:** Virgin C57BL/6 mice (n=8/group) were fed either a high fat-high cholesterol diet (HF; fat-45% kcal) or standard chow (C; fat-21% kcal) from weaning through their pregnancy and lactation. Each group was given the HMG-Co-A reductase inhibitor pravastatin (S) in the drinking water (5 mg/kg body weight per day) throughout this period to create HF+S and C+S dam groups. Offspring from each group were fed HF or C diets from weaning to adulthood, generating dam/offspring dietary groups HF+S/HF, HF+S/C, C+S/HF and C+S/C. Body weight, blood pressure, serum lipid profile were measured in offspring at 27 weeks of age, and bone marrow cells were cultured and expression of marrow-derived circulating endothelial progenitor cells (EPC) markers were assessed. **Results:** Compared with C+S/HF, HF+S/HF female offspring had lower total and low-density lipoprotein cholesterol concentrations, were less obese and hypertensive, and showed increased EPC expressing colony numbers (P<0.001). However, in male offspring the attenuation of EPCs and the increased cardiovascular risk factors brought about by prenatal or postnatal exposure to the HF diet was not blunted by prenatal statin therapy. **Conclusion:** Our results demonstrated that statin administration in early life to dams fed a HF diet produce gender specific effects, which can influence responses of the offspring to the pre- or post-weaning HF diet.

Poster Presentation at the Society for Gynaecologic Investigation 56th Annual Scientific Meeting, Reproductive Biology Session, 17-21 March, 2009. Reproductive Sciences, 2009; 16: 412.



Statin treatment in female mice has sex specific effects on cardiovascular risk factors and circulating endothelial progenitor cells in their offspring.
 Maqsood M. Elahi, Felino R. Cagampang, Dhea Mukhtar, Sunil K. Ohri, Mark A. Hanson
 Centre for Developmental Origins of Health & Disease & Wessex Cardiothoracic Centre, University of Southampton, UK.



- BACKGROUND -
 Risk of cardiovascular disease (CVD) may be established partly through adaptive responses to an adverse intrauterine environment^{1,2}, which can predispose the offspring to obesity, hypercholesterolemia and hypertension³. The components of such adaptive effects may involve endothelial progenitor cells (EPC) which play a role in CVD development^{4,5}. Statin treatment of hypercholesterolemic mouse dams during pregnancy and lactation reduces CV risk factors in their offspring⁶ and increases the number of EPC expressing colonies⁷. We have now investigated whether statin administration to female mice from the time they were weaned until weaning of their offspring reduces CVD risk factors in these offspring, even if both dams and offspring are fed a high fat diet.

- AIMS -
 1. To investigate the effects of statin administration to female mice from the time they were weaned until weaning of their offspring on cardiovascular risk factors in these offspring.
 2. To study the expression of EPC in bone marrow cultures from the female offspring.
 3. To investigate whether there are any gender-linked differences in offspring of these dams in the cardiovascular responses.

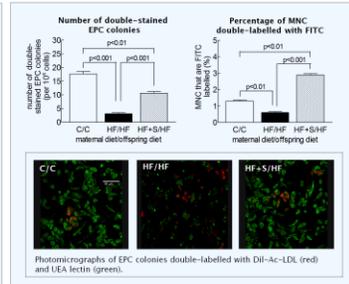
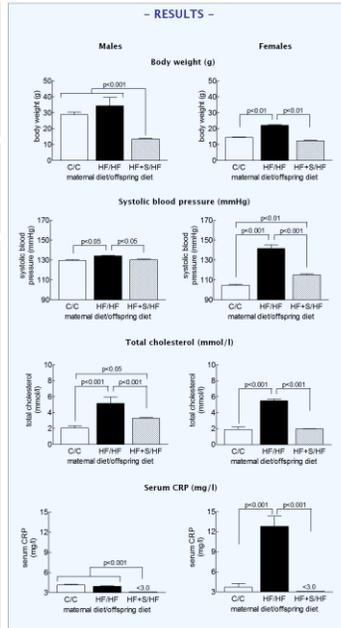
- METHODS -
Animals and Experimental Diets
 Animals (C57BL/6J) were divided into three groups: C/C (control), HF/HF (high fat diet), and HF+S/HF (high fat diet with statin). The experimental design is shown in the flowchart. All groups were weaned at 21 days of age and fed either a control diet (C/C) or a high fat diet (HF/HF or HF+S/HF) until weaning of their offspring at 21 days of age. The offspring were then divided into three groups: C/C, HF/HF, and HF+S/HF. All groups were fed a high fat diet until the end of the study at 28 weeks of age.

Body Weight & Blood Pressure
 Body weight was measured on weekly basis. Systolic arterial pressure was measured by tail cuff plethysmography.

Plasma Lipid Profile & C-Reactive Protein
 At 27 weeks post weaning, blood was collected by cardiac puncture under terminal anaesthesia. Lipid profile was measured by enzymatic methods using commercially available kits and reflectance spectrophotometry. C-reactive protein (CRP) was measured using a sensitive double-antibody sandwich immunoassay enzymatic ELISA.

Bone-Marrow Cell Isolation and Phenotyping
 Mononuclear cells (MNCs) were harvested from bone marrow, isolated and cultured in DMEM/F12 medium at a density of 10⁶ cells per well. To detect the phenotype for EPCs among the cultured MNCs, cells were incubated with TRITC-conjugated DiI-Ac-LDL and then fixed and labelled with FITC-conjugated lectin from *Ulex europaeus*. The double-labelled EPCs and their colonies were then counted.

Statistical Analysis
 The statistical significance of differences between groups was determined by one way ANOVA followed by post-hoc test.



- CONCLUSIONS -

- Our findings show that the HF/HF offspring were obese, had higher cholesterol concentrations, and were hypertensive compared with the C/C group. These changes were more pronounced in the female offspring.
- HF/HF exposure also increases CRP-mediated inflammation and inhibits EPC differentiation and survival in the offspring. It therefore affects key components of angiogenesis and endothelial repair in these offspring.
- Pravastatin administration in early life to dams fed a HF diet protects their offspring in a post-weaning HF nutritional environment from risk of later cardiovascular pathophysiology.
- We did not find any evidence that pravastatin administration to dams before and during pregnancy produced any deleterious effects on their offspring.

- REFERENCES -

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- ACKNOWLEDGEMENTS -
 MME and MAH are supported by BUPA/HOPE research fellowship and British Heart Foundation, respectively.

Appendix ix:

High Fat High Cholesterol Diet Consumption in Pregnancy attenuates Bone Marrow-derived Circulating Endothelial Progenitor Cells and Increases the Risk of Cardiovascular Disorders in the Offspring.

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Abstract:

Aims: We have previously shown that statin therapy in hypercholesterolemic dams during pregnancy and lactation alleviates the risk of adverse metabolic and cardiovascular disorders in their offspring. Here we examine C-reactive protein (CRP) levels and expression of endothelial progenitor cells (EPCs) in bone marrow cultures from these offspring. **Study Design & Subjects:** Virgin C57BL/6 mice strain were fed either a high fat diet (HF; fat-45% kcal) or standard chow (C; fat-21% kcal) from weaning through to pregnancy and lactation. Half of the pregnant mothers in each group were given the Pravastatin (5mg/kg/day) in their drinking water from the second half of pregnancy through to lactation. Female offspring from each group were then weaned onto either HF or C diets to adulthood. **Outcome Measures:** Body weight, blood pressure, plasma lipid profile and serum CRP were measured in offspring at 27 weeks, bone marrow cells were cultured and expression of EPC markers was assessed. **Results:** Compared to offspring from C dams female offspring from hypercholesterolemic dams had significantly ($P<0.001$) elevated CRP and fewer EPC expressing colonies in cultured bone marrow cells. Statin therapy in HF mothers resulted in their offspring having significantly lower CRP and increased EPC expressing colonies ($P<0.001$). **Conclusions:** These findings suggest that EPC expressing cells and CRP may be involved in the mechanisms whereby statin therapy to hypercholesterolemic dams during pregnancy and lactation reduces the risk of adverse metabolic and cardiovascular function in their offspring.

This work is supported by BUPA , HOPE Charity & BHF (UK)

Oral presentation at the 5th International Congress on Developmental Origins of Health & Disease Perth, Western Australia. 6-9 November 2007. Early Human Development, 2007; 83: 5-6, S74

Appendix x: Results

a. Heart weight in adult male and female offspring

The heart weights, expressed as % body weight, in the HF/HF females were heavier compared with C/C group ($p < 0.05$). Maternal pravastatin treatment during pregnancy and lactation resulted in reduction in heart weights in HF+S/HF female offspring ($p < 0.001$ respectively vs HF/HF) to levels similar to the C/C group. There was no effect of prenatal and post weaning exposure to the HF diet and of maternal statin treatment on heart weight male offspring.

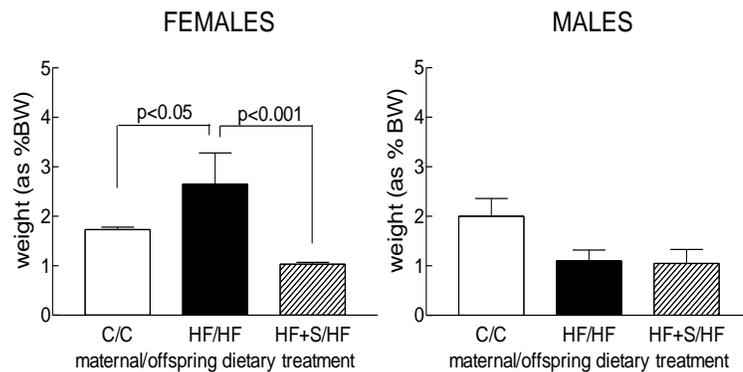


Figure-a: Heart weight, expressed as percent of body weight, in female and male adult offspring from HF-fed dams with or without pravastatin treatment during pregnancy and lactation (HF+S and HF, respectively) and fed post weaning a HF diet to adulthood (producing dam/offspring dietary groups HF+S/HF and HF/HF). The groups were compared to offspring from dams fed standard chow and fed post weaning the same chow diet (C/C). Values are expressed as mean \pm SEM.

b. Levels of mRNA expression for cyclin G1 in the adult offspring left ventricle

Left ventricular (LV) mRNA levels for cyclin G1 were lower in HF/HF offspring vs C/C groups ($p < 0.05$ for both sexes). Maternal statin treatment during pregnancy and lactation prevented the reduction in cyclin G1 mRNA levels in HF+S/HF offspring compared to those found in the HF/HF group.

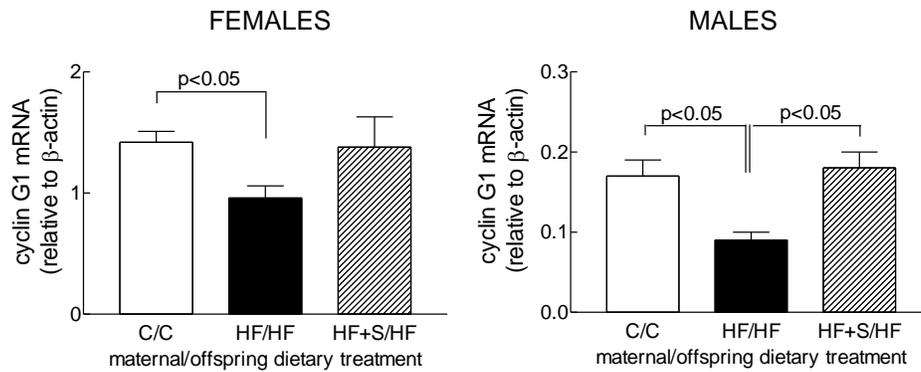


Figure b. Levels of mRNA expression for cyclin G1 in the left ventricles of female and male adult offspring from HF-fed dams with or without pravastatin treatment during pregnancy and lactation (HF+S and HF, respectively) and fed post weaning a HF diet to adulthood (producing dam/offspring dietary groups HF+S/HF and HF/HF). The groups were compared to offspring from dams fed standard chow and fed post weaning the same chow diet (C/C). Values are expressed as mean \pm SEM.

c. Levels mRNA expression for BNP in the adult offspring left ventricle

BNP mRNA levels in the LV (Figure 11) of female offspring remained unchanged following maternal and post weaning exposure to the HF diet, and following maternal statin treatment. In male offspring however, LV BNP levels were lower ($p < 0.05$) in the HF/HF group vs C/C. Maternal statin treatment did not significantly change BNP gene expression in the HF+S/HF offspring compared with HF/HF group.

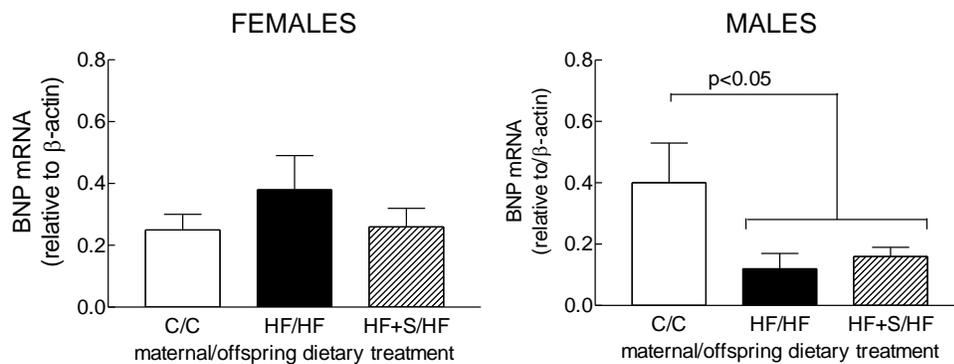


Figure c. Levels of expression for BNP in the left ventricles of female and male adult offspring from HF-fed dams with or without pravastatin treatment during pregnancy and lactation (HF+S and HF, respectively) and fed post weaning a HF diet to adulthood (producing dam/offspring dietary groups HF+S/HF and HF/HF). The groups were compared to offspring from dams fed standard chow and fed post weaning the same chow diet (C/C). Values are expressed as mean \pm SEM.

d. Levels mRNA expression for SPRR 1A in the adult female offspring left ventricle

SPRR 1A mRNA levels in the LV (Figure 12) were found to be similar in all groups.

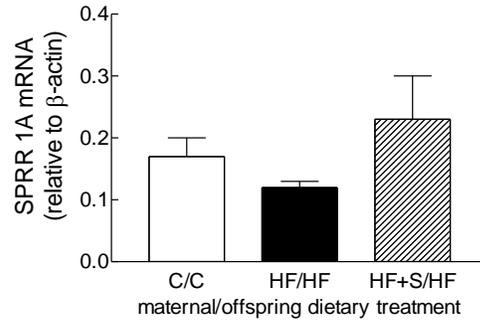


Figure d. Levels of expression for SPRR 1A in the left ventricles of female adult offspring from HF-fed dams with or without pravastatin treatment during pregnancy and lactation (HF+S and HF, respectively) and fed post weaning a HF diet to adulthood (producing dam/offspring dietary groups HF+S/HF and HF/HF). The groups were compared to offspring from dams fed standard chow and fed post weaning the same chow diet (C/C). Values are expressed as mean \pm SEM.

3.1 Appendix-xi:

Maternal high-fat diet: effects on offspring bone structure

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Abstract

Summary Peak bone mass is believed to partly be programmed in utero. Mouse dams and offspring were given a high-fat diet and offspring studied as adults. Female offspring from high-fat dams exhibited altered trabecular structure indicative of in utero programming. In utero nutrition has consequences in later life.

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Introduction Epidemiological studies suggest that skeletal growth is programmed during intrauterine and early postnatal life. We hypothesise that development of optimal peak bone mass has, in part, a foetal origin and investigated this using a mouse model of maternal dietary fat excess.

Methods Offspring from mouse dams fed either standard chow (C) or lifetime high-fat diet (HF) were maintained on a HF diet to adulthood. Femur samples were taken at 30 weeks of age and bone structure, adiposity and strength analysed. Sample sizes were four to six for each sex and each diet group.

Results Offspring from HF-fed dams showed increased adiposity in the femur in comparison to offspring from C-fed dams. Female offspring from HF dams exhibited altered trabecular structure indicative of in utero programming.

Conclusions A maternal HF diet during pregnancy increases bone marrow adiposity and alters bone structure in their offspring.

Keywords High fat · In utero · Micro-computed tomography · Programming · Structure

Introduction

Epidemiological data have led to the hypothesis that the risk of developing some chronic diseases, such as cardiovascular disease and type 2 diabetes, in adulthood is influenced not only by genetic and life style factors, such as diet and exercise, but also by environmental factors acting in early life. This is known as the developmental origins of health and disease hypothesis [1]. Maternal nutrition has been shown to be important in this hypothesis, with disease occurring when there is a mismatch between maternal and offspring diet. Developmental plasticity (the ability of an organism to alter development depending on

the particular environment) provides a conceptual explanation for this phenomenon [2]. Developmental plasticity requires stable regulation of gene expression, and this appears to be partly mediated by epigenetic processes such as DNA methylation and histone modification. Thus, both the genome and the epigenome influence the mature phenotype and determine not only sensitivity to later environmental factors but also the subsequent risk of disease. If the maternal environment is severe, such as inadequate maternal nutrition, then the foetus may make a defensive response such as a reduction in growth, which may be global or organ-specific. Alternatively, if the environment does not require an immediate defensive response, such as mild maternal nutritional stress, it may cause the developing foetus to make phenotypic changes that have a fitness advantage in later life by tuning its physiology to better match aspects of the predicted adult environment. Such modifications have been termed predictive adaptive responses [3].

Osteoporosis is a skeletal disorder characterised by low bone mass and micro-architectural deterioration, producing enhanced bone fragility and increasing susceptibility to fracture [4]. Peak bone mass attained at skeletal maturity is a major factor of fracture risk in later life. Current evidence suggests that peak bone mass is partly inherited; however, current genetic markers only partially explain the variance in an individual's bone mass or fracture risk [5]. Epidemiological studies indicate that impaired growth during foetal life, infancy and early childhood is associated with reduced bone mass in the adult [6–9]. Such associations suggest a possible role for maternal and foetal nutrition in determining peak bone mass and fracture risk in later life. The observation of altered skeletal structure as a consequence of undernutrition during a critical stage of early life, as exemplified by rickets, demonstrates that skeletal development can be disrupted by severe dietary change [10]. Cohort studies of female twins, including monozygous twins, showed significant intra-pair concordance between birth weight and bone mass. These studies suggest that variations in the intrauterine environment, even within the normal range, can influence skeletal development as well as birth weight, and they argue against an important role for fixed genetic variations in this respect [11]. Although we do not know, in detail, the molecular and cellular mechanisms underlying such effects, they are at least, in part, mediated through the changes in metabolic and endocrine homeostasis established during critical periods of development [12, 13].

Animal models for the developmental origins of osteoporosis have replicated the observations in humans and have provided crucial insight into factors that may mediate the association between unbalanced diet, e.g. producing dyslipidaemia, and osteoporosis. Bone marrow contains multipotential stromal cells commonly referred to as

mesenchymal stem cells (MSC) which can differentiate into fibroblastic, osteogenic, adipogenic and reticular cells [14–16]. Therefore, early environmental factors that regulate MSC differentiation are likely to be important candidate mechanisms for the developmental effects on skeletal growth. Parhami et al. [17] found that MSC obtained from mice placed on a high-fat diet demonstrated a significant decline in their ability to undergo osteoblastic differentiation compared to mice fed a normal chow diet. The inhibitory effect of an atherogenic diet was sustained over four cell cycles *in vitro*, suggesting a direct effect on the cells rather than an indirect effect mediated via altered bone marrow milieu or endocrine processes. The bone mineral content and density of mice fed a high-fat diet were also found to be reduced as measured by peripheral quantitative computer tomography [18].

In the last decade, there has been a marked increase in prevalence of obesity, metabolic syndrome, type 2 diabetes and cardiovascular disease. Emerging epidemiological evidence links osteoporosis with atherosclerosis and cardiovascular disease, independent of age, with evidence suggesting that dyslipidaemia may significantly contribute towards the progression of both diseases. Furthermore, cohort studies of aged men and postmenopausal women have found a statistically significant correlation between hypertriglyceridaemia and low bone mineral density [19, 20].

Developmentally induced risk of disease is readily observable in animal models using maternal nutrient restriction. Rodents exposed to maternal protein restriction only during development exhibit hypertension [21], impaired renal function [22] and cardiovascular disease [23] in adulthood despite being maintained on a balanced diet. In rats, maternal protein restriction has also been shown to affect skeletal development: Adult offspring from a protein-restricted group of dams were found to have reduced bone mineral content, area and altered growth plate morphology when compared to offspring from dams on normal protein diet [24]. Developmental induction of risk of cardiovascular and skeletal disorders is, however, not limited to effects of protein restriction. In recent years, a number of studies have shown that exposure to a maternal diet rich in saturated fats and cholesterol during development induces obesity, vascular dysfunction [25], impaired skeletal muscle development [26] and hypertension [27] in adult animals raised on standard chow.

Whilst the mechanisms involved in the developmental origins of disease are currently under investigation, the mechanisms involved have been conceptualised in terms of the deleterious consequences of mismatch between the postnatal environment predicted on the basis of cues during development and the environment actually experienced. The suggestion that some components of development constitute predictive adaptive responses (PAR) has received some support from studies of vascular function or body fatness [28–30].

Previously, using a rat model, we have shown that a low-protein maternal diet can alter bone structure and biochemistry in control-diet-fed offspring [31, 32]. The aims of this study, using a mouse model, were twofold: initially, to investigate the effects of a maternal and post-weaning high-fat diet on offspring skeletal development and, secondly, to determine whether there was evidence of a predictive adaptive response in relation to skeletal development as a consequence of maternal high-fat nutritional challenge.

Materials and methods

Animal and experimental design

All animal experimentation was performed under license from the Home Office in accordance with the Animals (Scientific Procedures) Act (1986). All mice were raised within the University of Southampton Biomedical Research Facility and were housed in appropriate environments in rooms maintained at $22\pm 2^{\circ}\text{C}$ with a 12:12-h light/dark cycle. At weaning, female C57BL/6 strain mice were allocated either a control (C) diet of standard chow [5.3% fat (corn oil), 21.2% protein, 49.2% carbohydrate; Special Diet Services, Witham, Essex, UK] or a high-fat (HF) diet supplemented 18% (w/w) with animal lard with additional vitamins and minerals, protein and choline [final composition in percentage of grams (w/w): lard 17.8; casein 26.5; choline chloride 0.3; L-cystine 0.4; rice starch 28.3; cellulose 6.1; soya oil 4.3; sucrose 10.4; minerals 4.3; vitamins 1.2; Special Diet Services). At 10 weeks of age, the females were mated and after confirmation of mating (presence of a vaginal plug) were individually housed and maintained on their corresponding diet. These C or HF diets were continued throughout pregnancy and lactation until the offspring had been weaned. The weaned offspring were then further subdivided to provide each parental dietary group with offspring that had been fed either the control (C/C group) or the high-fat diet (C/HF or HF/HF groups). This diet was continued up to the time of sampling at 30 weeks of age. Animals were killed by cervical dislocation.

Collection and preparation of bone specimens

Femora were dissected out, stripped of soft tissue using a scalpel and the right femur immediately frozen for later micro-computed tomography (CT) and three-point bend testing.

Anthropometric measurements

Following dissection, the left femur of each dietary group was measured (length and diameter) using digital callipers (Mitutoyo, Andover, Hampshire, UK).

Fixation and decalcification

The left femora were fixed in 4% formaldehyde in phosphate-buffered saline for 24 h. The fixed bones were then decalcified in a solution of 0.1 M TRIS and fresh 5% EDTA (pH 7.2) solution at 4°C . The 0.1 M TRIS and 5% EDTA solution was changed weekly until the bones were fully decalcified as proven by micro X-ray through the use of a Faxitron MX-20 (Faxitron X-Ray, Wheeling, IL, USA).

Processing and embedding

The decalcified bones were processed using a Shandon Citadel 2000 automated processor (Thermo Scientific, Basingstoke, Hampshire, UK). Bones were dehydrated through graded alcohols (50%, 90%, 100% and 100% ethanol), cleared in chloroform and bones were embedded in paraffin wax with a Blockmaster III (Raymond A. Lamb Ltd., Eastbourne, E. Sussex, UK). Seven-micrometre sections were cut from the blocks using a Microm HM330 microtome (Carl Zeiss Ltd, Welwyn Garden City, Hertfordshire, UK). Sections were mounted on Superfrost Plus slides in a waterbath and then dried for 4 h at 40°C in an incubator. Slides were wrapped in foil and stored at 4°C .

Alcian blue/Sirius red staining

Four to five sections of the left femur were selected for each sample. Sections were dewaxed in HistoClear twice for 7 min each then rehydrated in graded alcohol solutions (100%, 100%, 90% and 50%) for 2 min each. Sections were stained with Weigert's haematoxylin for 10 min, rinsed 10 min, dedifferentiated in acid alcohol solution and rinsed for a further 5 min. Sections were then stained in Alcian blue for 10 min, rinsed for 1 min, stained in 1% molybdophosphoric acid for 20 min and rinsed again for 1 min before being stained in Sirius red for 1 h. Sections were then rinsed for 1 min before being dehydrated through graded alcohols (50%, 90%, 100% and 100%) for 30 s each and cleared in HistoClear twice for 2 min. Sections were mounted with DPX and left to dry for 1 week.

Microscopy

Images from each section were captured using an Axio-CamHR camera mounted on a Zeiss Axiovert microscope and Axiovision software at a $1,300\times 1,300$ -pixel resolution. The images taken provide an overview of the trabecular structure of the proximal and distal end of the femur as well as higher magnification images of the growth plate at the proximal and distal end of the femur.

Adipocyte size and diameter

Greyscale images of the low-power magnification view of the distal end of the femur were taken and the white background of these images digitally removed. These images were then processed using Amira 4.1.2 package (Mercury Computer System Inc., Chelmsford, USA). The images were binarised so only areas originally containing adipocytes were visible. The software then calculated the number of individual spots and the mean area of these spots.

3D computed tomography

Right femora from 30-week-old offspring were scanned using an Xtek Benchtop 160Xi scanner (Xtek Systems Ltd., Tring, Hertfordshire, UK) equipped with a Hamamatsu C7943 X-ray flat panel sensor (Hamamatsu Photonics, Welwyn Garden City, Hertfordshire, UK). All scans were taken at 100 kV, 60 μ A using a molybdenum target with an exposure time of 534 ms and 4 \times digital gain. Image resolution was 14 μ m. Reconstructed volume images were analysed using VGStudio Max 1.2.1 software (Volume Graphics GmbH, Heidelberg, Germany). Using standards of known density, all the voxels which formed the structure were automatically assigned Hounsfield units.

Mechanical bone strength testing

All testing was performed on a Bose Electroforce 3200 electromagnetic test instrument (Bose Corporation, Eden Prairie, Minnesota, USA). The midshaft strength of the right femur was tested using a three-point bend test. Femora were placed anterior surface down on two supports equidistant from the ends and 8 mm apart. Femora were centrally loaded at a constant rate (6 mm/min) up to fracture. Load–displacement curves were used to calculate maximum load, maximum deflection, stiffness, energy and stress. Stiffness was calculated as the slope of the linear portion of the load–displacement curve. Energy was determined as the area under the curve. Stress was determined as the maximum load divided by the cross-sectional area as determined by computed tomography.

Statistics

ANOVA with Bonferroni post hoc statistical analysis was performed using the SPSS for Windows programme version 14 (SPSS UK, Woking, Surrey, UK). Data are presented as mean \pm standard deviation from $n=4-6$; significance was determined with a p level of 0.05 or lower. For the bone density graphs, data were compared every 100 Hounsfield units over the range of 2,000–7,500 units.

Results

Offspring mass

Mean offspring mass was measured after 30 weeks (Tables 1 and 2). In female offspring, a significant increase in mass was observed in HF-diet-fed offspring from both dams on a control diet (C/HF) or from dams on a high-fat diet (HF/HF; $p = 0.002$ and 0.0002 , respectively). Similarly, male offspring showed a significant increase in mass in HF-diet-fed offspring from both dams on a control diet (C/HF) and from dams on a high-fat diet (HF/HF; $p = 0.004$ and 0.02 , respectively).

Of particular note is that HF/HF female offspring gained significantly less weight when compared to C/HF female offspring. No such effect was observed in males.

Femur lengths

Femur lengths from C/C, C/HF and HF/HF female offspring were examined (Table 1). HF/HF female offspring showed significantly shorter femur length compared to C/C group ($p = 0.008$). In addition, the HF/HF offspring femora were significantly shorter than those from the C/HF group ($p = 0.008$).

Examination of femur lengths in male offspring from both the C/HF group and the HF/HF group showed they had significantly shorter femora ($p = 0.04$ and 0.03 , respectively) compared to the C/C group.

3D computed tomography analysis of femur

Female cohort

Following analysis at a scan resolution of 14 μ m, total bone volume of the femur from female groups indicated an increase in bone volume ($p = 0.03$) in the C/HF group only (Table 1). Examination of mean trabecular spacing showed a significant increase in the C/HF group ($p = 0.03$ compared to C/C controls and $p = 0.04$ compared to HF/HF group). Together with unaltered trabecular thickness, this alteration in bone parameter was also confirmed by a reduction in the bone volume to total volume ratio, although not to a statistically significant level ($p = 0.08$ compared to C/C group) and reduced number of trabeculae per millimetre, although, again, not to a statistically significant level ($p = 0.06$ versus C/C group and 0.08 versus HF/HF group). Interestingly, the HF/HF group showed increased structural model index (SMI) and increased fractal dimension (FD) values compared to C/C group and the C/HF group (SMI $p = 0.03$ versus C/HF and FD $p = 0.04$ versus C/C group). The increased SMI indicates a more rod-like structure [33], and the increased fractal dimension indicates a more complex surface structure [34].

Table 1 Bone structure and strength data from female femur

Factor	Diet group			p value
	C/C (mean ± SD) (n=5)	C/HF (mean ± SD) (n=5)	HF/HF (mean ± SD) (n=5)	
Bone structure data				
Body mass (g)	14.39 (1.27)	31.50 (1.99)	22.19 (1.11)	0.002a,c, 0.0002b
Femur length (mm)	15.04 (0.47)	15.14 (0.22)	13.88 (0.36)	0.008b,c
Bone volume (mm ³)	24.98 (2.31)	28.55 (1.82)	25.23 (2.26)	0.03a
Bone surface/bone volume ratio	15.23 (0.91)	15.12 (1.47)	15.22 (0.73)	
Bone volume/total volume ratio	0.56 (0.07)	0.49 (0.02)	0.55 (0.02)	
Trabecular thickness (mm)	0.132 (0.008)	0.133 (0.013)	0.132 (0.007)	
Trabecular spacing (mm)	0.105 (0.026)	0.139 (0.017)	0.106 (0.005)	0.03a, 0.04c
Number of trabeculae per millimetre	4.26 (0.36)	3.70 (0.40)	4.21 (0.13)	
Structural model index	0.07 (0.02)	0.06 (0.02)	0.09 (0.01)	0.03c
Fractal dimension	2.45 (0.04)	2.46 (0.03)	2.51 (0.04)	0.04b
Bone strength data				
Maximum load (N)	14.23 (1.51)	16.65 (0.83)	16.16 (1.57)	
Maximum displacement (mm)	0.47 (0.06)	0.46 (0.22)	0.52 (0.13)	
Stress (N mm ⁻²)	8.72 (1.03)	8.04 (0.45)	8.39 (0.35)	
Midshaft cross-sectional area (mm ³)	1.64 (0.10)	2.07 (0.07)	1.93 (0.15)	0.02a, 0.03b
Midshaft wall thickness (mm)	0.179 (0.014)	0.188 (0.008)	0.182 (0.007)	
Maximum load per millimetre of wall (N)	20.12 (2.52)	19.68 (0.79)	20.40 (1.22)	
Stiffness (N mm ⁻¹)	36.79 (5.23)	59.05 (15.54)	56.81 (10.66)	
Energy	3.58 (0.56)	4.35 (2.84)	5.37 (2.00)	

Structural data from femur of offspring. All values shown are mean ± SD (in parentheses)

For p values, a indicates C/HF compared to C/C, b indicates HF/HF compared to C/C, and c indicates HF/HF compared to C/HF.

Male cohort

In contrast to the female samples, male offspring in the HF/HF group showed increased trabecular spacing compared to the other groups ($p = 0.03$ versus C/C group; Table 2). Together with unaltered trabecular thickness, this alteration in bone parameter was also confirmed by a reduction in the bone volume to total volume ratio and reduced number of trabeculae per millimetre, although neither reached a statistically significant level. Interestingly, the HF/HF groups also showed increased trabecular pattern factor values ($p = 0.03$ versus C/C group) and reduced degree of anisotropy value ($p = 0.03$ versus C/C group). The increased trabecular bone pattern factor indicates a less connected structure [35], whilst the reduced degree of anisotropy shows a more isotropic trabecular structure [36].

Bone density

Female cohort

Differences in total femoral bone density in the C/C, C/HF and HF/HF groups are shown in Fig. 1a. In female

offspring in the C/HF group, the femur showed a significant increase in density compared to C/C controls. For the female groups, this range extended from 5,300 to 5,800 Hounsfield units ($p < 0.05$ for each 100 Hounsfield portion within the range). There were no statistically significant differences in density between the C/C and the HF/HF groups. Figure 1c shows representative false colour CT sections of the proximal and distal femur for female offspring (the colours represent the density ranges shown in Fig. 1a below the graph, with density range 3,000–4,499 Hounsfield units shown in white, 4,500–4,999 shown in blue, 5,000–5,499 shown in yellow, 5,500–5,999 shown in red and over 6,000 shown in magenta), with the top images in Fig. 1c showing a horizontal section through the centre of the femoral head and the greater trochanter.

In the C/HF group, the lower density area around the femoral head, seen in C/C controls and the HF/HF groups, is not visible, indicating an increased bone density in this area in this diet group (Fig. 1c, red arrows). In addition, the distance from the edge of the femoral head to the edge of the trochanter is increased in the HF/HF group (Fig. 1c, white arrows). The lower images show a horizontal section through the condyles. The C/HF group displays a higher

Table 2 Bone structure and strength data from male femur

Factor	Diet group			<i>p</i> value
	C/C (mean ± SD) (<i>n</i> =4)	C/HF (mean ± SD) (<i>n</i> =6)	HF/HF (mean ± SD) (<i>n</i> =4)	
Bone structure data				
Body mass (g)	31.90 (1.13)	42.13 (3.90)	43.40 (2.90)	0.004a, 0.02b
Femur length (mm)	15.18 (0.17)	14.80 (0.25)	14.16 (0.49)	0.04a, 0.03b
Bone volume (mm ³)	33.23 (1.26)	32.21 (3.07)	33.38 (3.27)	
Bone surface/bone volume ratio	14.96 (0.64)	14.97 (1.76)	15.32 (3.28)	
Bone volume/total volume ratio	0.60 (0.04)	0.58 (0.05)	0.54 (0.06)	
Trabecular thickness (mm)	0.134 (0.006)	0.135 (0.017)	0.135 (0.026)	
Trabecular spacing (mm)	0.089 (0.010)	0.098 (0.012)	0.111 (0.006)	0.03b
Number of trabeculae per millimetre	4.50 (0.15)	4.31 (0.32)	4.10 (0.39)	
Trabecular bone pattern factor (mm ⁻¹)	-13.2 (1.5)	-11.2 (1.9)	-8.9 (2.3)	0.03b
Degree of Anisotropy	0.40 (0.06)	0.32 (0.08)	0.26 (0.08)	0.03b
Bone strength data				
Maximum load (N)	14.98 (1.07)	17.56 (3.64)	18.58 (0.91)	0.03b
Maximum displacement (mm)	0.44 (0.06)	0.43 (0.06)	0.43 (0.12)	
Stress (N mm ⁻²)	6.41 (0.46)	7.09 (1.09)	7.13 (0.59)	
Midshaft cross-sectional area (mm ³)	2.34 (0.13)	2.47 (0.21)	2.62 (0.19)	
Midshaft wall thickness (mm)	0.21 (0.01)	0.22 (0.02)	0.22 (0.01)	
Maximum Load per mm ² of Wall / N	14.99 (1.50)	16.55 (1.70)	16.67 (0.90)	
Stiffness (N mm ⁻¹)	39.89 (6.64)	47.03 (9.98)	52.13 (13.64)	
Energy	2.78 (0.49)	3.21 (0.86)	3.62 (1.59)	

Structural data from femur of offspring. All values shown are mean ± SD (in parentheses)

For *p* values, a indicates C/HF compared to C/C, b indicates HF/HF compared to C/C, and c indicates HF/HF compared to C/HF

proportion of dense bone evidenced by false colour compared to C/C controls. The HF/HF group displays a further increased level of high-density bone, with higher proportions of dense bone compared to the C/HF group. Unlike the proximal section, there appears to be no difference in size in this distal section.

Male cohort

Figure 1b shows the differences in density of the femur for the different diet groups. In male offspring in both the C/HF and the HF/HF groups, the femur showed a significant increase in density compared to C/C controls. For the male groups, this range extended from 5,200 to 5,800 Hounsfield units ($p < 0.05$ for each 100 Hounsfield portion within the range). Figure 1d shows representative false colour CT sections of the proximal and distal femur for male offspring (the colours represent the density ranges shown in Fig. 1b below the graph, with density range 3,000–4,499 Hounsfield units shown in white, 4,500–4,999 shown in blue, 5,000–5,499 shown in yellow, 5,500–5,999 shown in red and over 6,000 shown in magenta), with the top images in Fig. 1d showing a

horizontal section through the centre of the femoral head and the greater trochanter.

Unlike the female offspring, both the C/HF and HF/HF groups showed similar bone densities, which were increased compared to C/C controls, the control samples showing a lower level of high density bone. Furthermore, in contrast to the female offspring, the distance from the edge of the femoral head to the edge of the trochanter was reduced in the HF/HF group (Fig. 1d, white arrows). The lower images show a horizontal section through the condyles. Both the C/HF and HF/HF groups displayed a higher proportion of higher density bone than C/C controls. Unlike the proximal section, there appeared to be no difference in size in this distal section.

Bone strength analysis—midshaft femur

Female cohort

Table 1 shows the results of mechanical testing of the femoral midshaft. Maximum load at failure tended to be higher in both C/HF and HF/HF dietary groups in female offspring compared to C/C controls, although this did not

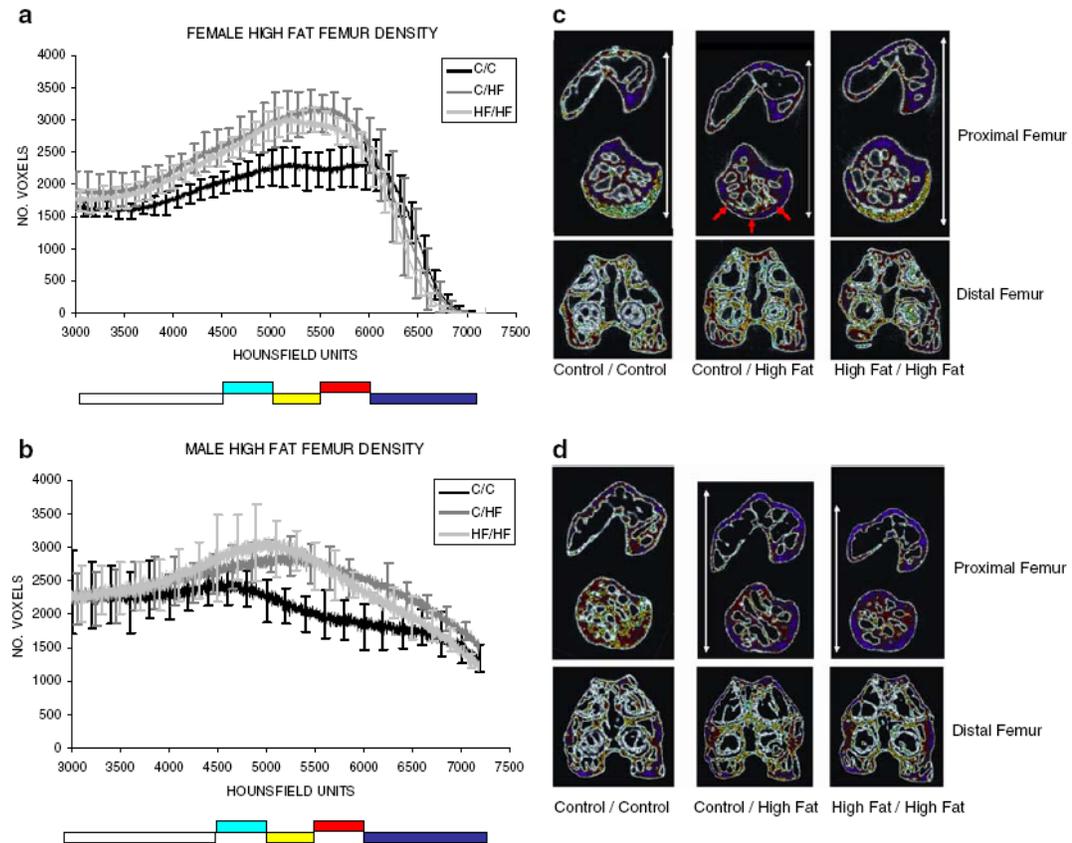


Fig. 1 CT analysis of femur. Each voxel within the reconstructed volume has a specific bone density. The graph shows the result of plotting the voxel bone density (in Hounsfield units) against the number of voxels with that density, thus giving a bone density plot for the bone within the volume. Colours below the graph represent the colours assigned for a bone density range. These colours are used in the images in c and d. **a** Plot of mean femoral trabecular density for female offspring. **b** Plot of mean femoral trabecular density for male offspring. For a–d, $n=4-6$. Error bars represent 95% confidence

limits. **c** False colour representative CT sections for female offspring. **d** False colour representative CT sections for male offspring. For c and d, images show variation in voxel density through horizontal section through centre of femoral head and greater trochanter (*top panel*) and horizontal section through the condyles (*bottom panel*). Hounsfield units 3,000–4,499 are shown in *white*, Hounsfield units 4,500–4,999 are shown in *blue*, 5,000–5,499 are shown in *yellow*, 5,500–5,999 are shown in *red*, and Hounsfield units over 6,000 are shown in *purple*

quite reach significance (p values both 0.06). Although there was a significant increase in cross-sectional area in both the C/HF and HF/HF groups compared to C/C controls ($p = 0.02$ and 0.03 , respectively). However, there were no significant differences in maximum displacement or stress for female offspring. The increase in cross-sectional area was due to an increase in lumen area rather than an increase in wall thickness. The software calculated the wall thickness by subtracting the mean radius of the midshaft from the mean radius of the lumen. Mean stiffness remained higher in the HF/HF female offspring, but not to a significant level ($p = 0.06$).

Male cohort

Table 2 shows the results of mechanical testing of the femoral midshaft. As with the female offspring, the maximum load at failure tended to be higher in both C/HF and HF/HF dietary groups in male offspring compared to C/C controls, which was significant in the HF/HF group ($p = 0.03$). However, there were no significant differences in maximum displacement or stress for male offspring. As with the female offspring, the C/HF and HF/HF groups tended to have an increased cross-sectional area, but these differences were not significant in the male offspring

compared to C/C controls. Interestingly, male offspring from all dietary groups had a mean wall thickness of around 0.22 mm, whereas female offspring from all dietary groups had a mean wall thickness of around 0.18 mm. The maximum load per square millimetre of wall area was lower in male offspring from all dietary groups (15 N/mm²) compared to female offspring from all dietary groups (around 20 N/mm²).

Histological analysis of adiposity in the distal femur

Female cohort

Representative images of gross morphology of the distal femur were acquired after staining with Sirius red (for collagenous bone matrix) and Alcian blue (for cartilage proteoglycans; Fig. 2). Adipocytes are visualised as white circles within the bone marrow. Figure 2a shows a section

from control offspring. A few clusters of small white circles indicative of adipocyte lipid accumulation within the marrow space. Figure 2b shows a similar section from C/HF female offspring. The section shows a similar number of adipocyte clusters compared with C/C controls (Table 3). However, the size of the lipid vesicles are much larger than in the C/C controls (13.4 vs 6.7 μ m). Most of the lipid accumulation appears below the growth plate shown in blue in both C/HF and C/C groups. Figure 2c shows a section from HF/HF female offspring with a marked increase in the number (473 vs 107, $p = 0.001$) and size (11.5 vs 6.7 μ m, $p = 0.01$) of lipid accumulation above the growth plate and comparable lipid accumulation to C/HF below the growth plate.

Male cohort

Representative images of gross morphology of the distal femur were acquired after staining with Sirius red and

Fig. 2 Increasing marrow adiposity with dietary fat in female offspring. All slides are stained with Alcian blue and Sirius red. Pictures on left show low magnification image ($\times 50$) of section through distal femur. For all samples, bars represent 0.5 mm. Pictures on the right show high magnification image ($\times 100$) of a section from the image on the left. Bar represents 0.2 mm. $n = 4-6$. **a** Images from representative C/C female. **b** Images from representative C/HF female. Note the increased level of adiposity compared to controls. **c** Images from representative HF/HF female. Note the increased level of adiposity compared to the C/HF group



Table 3 Adipocyte number and size data

Factor	Diet group			<i>p</i> value
	C/C (mean ± SD)	C/HF (mean ± SD)	HF/HF (mean ± SD)	
Females	<i>n</i> =5	<i>n</i> =5	<i>n</i> =5	
No. of adipocytes per low-power field	107.2 (15.5)	279.8 (91.7)	472.6 (156.8)	0.001b
Diameter of adipocytes	6.7 (1.2)	13.4 (2.9)	11.5 (4.8)	0.04a, 0.01b
Males	<i>n</i> =4	<i>n</i> =6	<i>n</i> =4	
No. of adipocytes per low-power field	18.8 (10.9)	73.2 (48.2)	279.3 (110.6)	>0.001b, 0.001c
Diameter of adipocytes	5.0 (1.2)	7.1 (1.9)	7.8 (1.9)	

Mean number and mean diameter of adipocytes from low-power magnification view of horizontal section through offspring distal femur as shown in Figs. 2 and 3. Standard deviation is shown in parentheses. Diet groups are mothers fed control diet and offspring on control diet (C/C), mother fed control diet and offspring fed high-fat diet (C/HF) or both mother and offspring fed high-fat diet (HF/HF).

For *p* values, a indicates C/HF compared to C/C, b indicates HF/HF compared to C/C, and c indicates HF/HF compared to C/HF.

Alcian blue and are shown in Fig. 3. The C/HF male offspring showed increased levels of lipid within the marrow, which was further increased in the HF/HF males. Figure 3a shows a section from control offspring. Adipocyte lipid accumulation within the marrow space is almost completely absent in the C/C males. Figure 3b shows a similar section from C/HF male offspring. Table 3 shows no increases in the number or diameter of adipocytes compared to C/C males. Figure 3c shows a section from HF/HF male offspring. Below the growth plate, there is a similar amount of lipid as in the C/HF male offspring. In contrast to what was seen in the female offspring, in the HF/HF males (Table 3), there was a significant increase in the number, but not diameter, of lipid compared to both C/C and C/HF groups.

Discussion

This study has investigated the effects of maternal HF feeding coupled with post-weaning HF diet on the morphology, quantity and quality of bone in the offspring.

Whilst HF/HF female offspring displayed increased body mass, this was not to the extent of the C/HF females. It is possible that this was due to altered energy intake. However, other studies using similar high-fat diet manipulations in mice have found no differences in energy intake [37–39]. Thus, it appears that a maternal HF diet alters the basal metabolic activity of the offspring, similar to that observed in rats [40]. In addition, in a previous study of older offspring [41], we found that the C/HF and HF/HF offspring (regardless of gender) had higher proportions of total body fat than controls, although female HF offspring had higher levels than males (all controls 9% fat, male C/HF and HF/HF approximately 15% fat, female C/HF and HF/HF approximately 29% fat), confirming the increased

number and size of adipocytes seen in the distal femur in the C/HF and HF/HF offspring in the present study.

The C/HF female offspring displayed increased bone volume, trabecular spacing, load to fracture, cross-sectional area of the femoral midshaft and bone density compared to those on the C diet. In contrast, HF/HF females only showed reduced femur length and increased load to fracture and cross-sectional area of the midshaft, but not bone density. These data suggest that the C/HF group increased the cross-sectional area of the femur to withstand the greater mass of the animal. As femur length compared to C/C group was maintained in the C/HF offspring, this resulted in an increase in the total amount of bone present in the femur and, interestingly, increased trabecular spacing. In contrast, the HF/HF females appeared to maintain the same bone parameters seen in the C/C group. Hence, to increase the cross-sectional area of the midshaft needed to sustain the extra mass of the animal resulted in a reduction in femur length. There was also an increase in the SMI and fractal dimension values, indicating a more rod-like structure and a more complex surface structure. Hence, although both the C/HF and HF/HF groups showed increased mass compared to controls, they displayed different solutions to alter bone structure to compensate; the C/HF group maintained femur length and increased bone strength by accumulating more bone at the midshaft, but altered trabecular spacing. The HF/HF group also increased bone strength by accumulating more bone at the midshaft, but in contrast to the C/HF group, total bone volume and trabecular thickness and spacing were maintained, and hence, the projected bone length was reduced to provide sufficient bone mineral for the additional midshaft wall thickness. In addition, the HF/HF group appears to have altered the trabecular structure. Exposure to the HF environment in utero thus seems to induce a conservation of bone mass in the offspring, perhaps in prediction of exposure

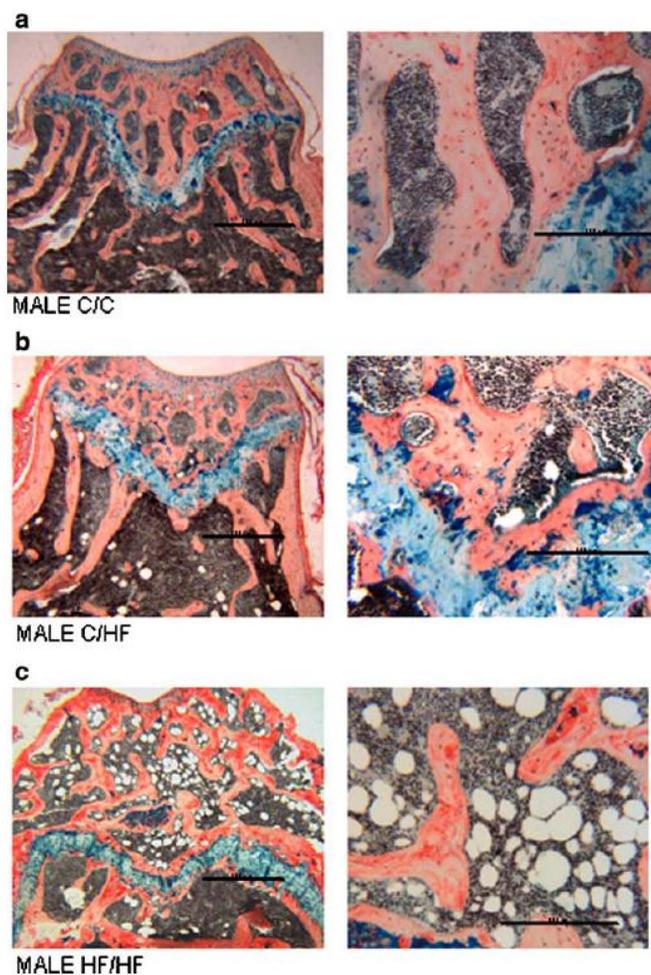


Fig. 3 Increasing marrow adiposity with dietary fat in male offspring. All slides are stained with Alcian blue and Sirius red. *Pictures on left* show low magnification image ($\times 50$) of section through distal femur. For all samples, *bars* represent 0.5 mm. *Pictures on the right* show high magnification image ($\times 100$) of a section from the image on the

left. *Bar* represents 0.2 mm, $n=4-6$. **a** Images from representative C/C male. **b** Images from representative C/HF male. Note the increased level of adiposity compared to controls. **c** Images from representative HF/HF male. Note the increased level of adiposity compared to the C/HF group

to an unbalanced diet postnatally in which micronutrients such as calcium might be limited, especially as a high-fat diet reduces intestinal absorption of calcium [42, 43]. This was followed by a (re)modelling of bone structure to meet the weight-bearing demands on the skeleton.

The HF/HF male offspring showed increased mass compared to controls, but in contrast to the female group, this increase was similar to the C/HF group. However, as seen with female offspring, there was also a reduced femur length and an increased load at fracture of the midshaft,

with the increased cross-sectional area at this point. Although the male HF/HF group showed increased trabecular spacing (not seen in the female group), they also displayed a detrimental alteration in trabecular structure (reduced connectivity) as was seen in the female HF/HF offspring (more rod-like structure).

Our study allowed us to examine whether any PAR occur for the skeleton. For male offspring, feeding a high-fat diet (C/HF compared to C/C diet) increased mass and bone density and reduced femur length. However, with a

prior maternal high-fat diet (HF/HF compared to C/HF diet), there were no additional alterations in bone structure or density. Hence, any PAR cue from the mother does not appear to be utilised in male offspring. In contrast, whilst feeding a high-fat diet to female offspring (C/HF group) increased mass, bone volume, bone density, trabecular spacing, maximum midshaft load and midshaft cross-sectional area, with the addition of a maternal high-fat diet (HF/HF compared to C/HF diet), the response was to reduce mass (although not to the level of controls), reduce femur length and reduce bone volume, bone density, trabecular thickness and spacing to control levels. These data are consistent with the operation of a PAR in terms of bone structure in the female offspring.

Whilst this study has provided a useful framework for investigating the effects of HF dietary exposure in utero and during postnatal life on the bone quality and quantity of the offspring, a number of caveats must be noted. In the first instance, this is an animal model and therefore the effects of the diets cannot be directly transposed to humans. Rather, these studies provide an indication as to the possible effects of such dietary manipulations on skeletal physiology which may suggest future avenues for research of underlying mechanisms. These results provide pilot data for a future, larger study which will include evaluation of the developing bone using in vivo micro-CT or calcein labelling as well as measurement of bone serum parameters such as IGF-1, alkaline phosphatase, calcium, etc. In order to further analyse the lipid accumulation within tissues, it will be necessary to alter the embedding process used. The methodology used here appears to have removed lipid-containing cells. Thus, although we were able to determine increased lipid accumulation by the increase in total area of the lipid containing cells, we were not able to determine the number of individual cells within that accumulation.

In conclusion, these studies demonstrate effects of high-fat maternal diet during pregnancy, with or without a high-fat diet in offspring post-weaning, on the bone quality and quantity of those offspring. The skeleton is subject to the processes of developmental plasticity, responding to environmental cues within a non-pathological range at critical points in the life course, as do other body components. Whilst such adaptive responses may theoretically confer adaptive advantage, for example in terms of reproductive fitness, they may confer greater risk of chronic disease such as osteoporosis in human populations exposed to increasingly rich diets and with greater longevity. These studies indicate the importance of early life interventions that will be needed, ultimately, to promote the health of subsequent generations.

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Conflicts of interest None.

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Appendix -xi: Published Work and Awards from this work

Peer reviewed published papers

- 1) Lanham SA, Roberts C, Hollingworth T, Sreekumar R, Elahi MM, Cagampang FR, Hanson MA, Oreffo RO. Maternal high-fat diet: effects on offspring bone structure. *Osteoporos Int.* 2010; 21: 1703-1704
- 2) Elahi MM, Cagampang FR, Mukhtar D, Anthony FW, Ohri SK, Hanson MA. Long-term maternal high-fat feeding from weaning through pregnancy and lactation predisposes offspring to hypertension, raised plasma lipids and fatty liver in mice. *Br J Nutr.* 2009;1-6. 102: 514-9.
- 3) Elahi MM, Cagampang FR, Anthony FW, Curzen N, Ohri SK, Hanson MA. Statin treatment in hypercholesterolemic pregnant mice reduces cardiovascular risk factors in their offspring. *Hypertension* 2008; 51: 939-44

National and International Meetings Abstracts

- 1) Elahi MM, Cagampang F, Ohri SK, Hanson MA. Long term statin treatment in hypercholesterolemic pregnant mice reduces cardiovascular and metabolic risk in their offspring also fed a high fat diet post-weaning.

Poster presentation (P-9A-387) at the 6th World Congress on Developmental Origins of Health and Disease, 19-22 November 2009, Santiago, Chile. *J Develop Origins Health & Disease*, Vol 1, No.1 (Supplement), 289; November 2009.

- 2) Lanham SA, Roberts C, Hollingworth T, Sreekumar R, Elahi MM, Cagampang FR, Hanson MA, Oreffo ROC. Maternal high fat diet- Deleterious effects on offspring bone structure.

Poster presentation (P-8C-379) at the 6th World Congress on Developmental Origins of Health and Disease, 19-22 November 2009, Santiago, Chile. *J Develop Origins Health & Disease*, Vol 1, No.1 (Supplement), 284; November 2009.

3) Elahi MM, Cagampang FR, Anthony FW, Curzen N., Ohri SK, Hanson MA. Statin treatment in hypercholesterolemic pregnant mice reduces cardiovascular risk factors in their offspring, published in Hypertension Journal, 2009 chaired by Professor Tim Elliott, School of Medicine

Prize Oral presentation for Wessex Medical Research Prize for Postgraduate Research in Biomedical Sciences 2009 at the MHLS Faculty Postgraduate Conference University of Southampton, 2-3 June, 2009. Abstract Book MHLS Faculty, 23: June, 2009.

4) Elahi MM, Cagampang FR, Mukhtar D, Ohri SK, Hanson MA. Statin treatment in female mice has sex specific effects on cardiovascular risk factors and circulating endothelial progenitor cells in their offspring.

Poster Presentation at the Society for Gynaecologic Investigation 56th Annual Scientific Meeting, Reproductive Biology Session, 17-21 March, 2009. Reproductive Sciences, vol. 16, No.3 (Supplement), 412; March 2009

5) Elahi MM, Mukhtar D, Kahraman N, Cagampang FR, Ohri, SK, Hanson MA. Statin Therapy Improves Blood Pressure and Lipid Profiles in Hypercholesterolemic Mothers but not C-reactive proteins levels or Endothelial Progenitor Cell Expression.

Oral presentation at the 5th International Congress on Developmental Origins of Health & Disease Perth, Western Australia. 6-9 November 2007. Early Human Development, 2007; 83 (Suppl-1): 3B-6, S60

6) Elahi MM, Mukhtar D, Cagampang FR, Ohri, SK, Hanson MA. High Fat High Cholesterol diet consumption in Pregnancy Attenuates Bone Marrow- Derived Circulating Endothelial Progenitor Cells and Increases the Risk of Cardiovascular Disorders in the Offspring.

Oral presentation at the 5th International Congress on Developmental Origins of Health & Disease Perth, Western Australia. 6-9 November 2007. Early Human Development, 2007; 83 (Suppl-1): 5D-6, S74

7) Hollingworth T, Lanham SA, Roberts C, Hanson M, Elahi M, Cagampang F, Cooper C, Oreffo ROC. Effects of maternal dietary cholesterol on offspring bone development.

Poster presentation at the 5th International Congress on Developmental Origins of Health & Disease Perth, Western Australia. 6-9 November 2007. *Early Human Development*, 2007; 83 (Suppl-1): P2-95, S155

8) Elahi MM, Mukhtar D, Cagampang FR, Ohri, SK, Hanson MA. High Fat High Cholesterol diet consumption in Pregnancy Attenuates Bone Marrow- Derived Circulating Endothelial Progenitor Cells and Increases the Risk of Cardiovascular Disorders in the Offspring.

Poster presentation at the British Heart Foundation Site Visit to Institute of Developmental Sciences, Southampton, UK, 23-24 July 2007.

9) Elahi MM, Cagampang FR, Mukhtar D, Ohri, SK, Hanson MA. The influence of the pre and postnatal hypercholesterolemia on the development of cardiovascular dysfunction in Adult Mouse Offspring.

Poster presentation at the Perinatal Physiology: From Uterus to Brain Meeting, University of Edinburgh, UK, 12-13 February, 2007.

10) Elahi MM. Effect of Post Weaning Hypercholesterolemic Diet on Adiposity, Blood Pressure and C-Reactive Protein in Adult Mice Offspring.

Oral Presentation at the Lab Meeting, Division of Developmental Origin of Health and Disease, University of Southampton, UK, 9 November 2006

11) Elahi MM. Developmental Plasticity of Stem Cells.

Oral Presentation at the Lab Meeting, Division of Developmental Origin of Health and Disease, University of Southampton, UK, 5 October 2006

12) Elahi MM, Cagampang FR, Anthony FR, Curzen N, Ohri SK, Hanson MA. Statins during pregnancy and lactation in mice on hypercholesterolaemic diet prevents obesity, hypertension and sedentary behaviour in adult offspring.

Oral presentation at the 4th world Congress on Developmental Origins of Health & Disease (DOHaD 2006) in Utrecht, Netherlands, 13-16 September 2006. *Early Human Development*, 2006; 82 (8): J-08, 559

13) Elahi MM. Developmental Origins of Atherogenesis: Can Interventions Aimed at Improving Later Health be Targeted in Utero?

Oral Presentation at the Lab Meeting, Division of Developmental Origin of Health and Disease, University of Southampton, UK, 23 March 2006

Honours and Awards

Recipient of First Prize for Wessex Medical Research for Postgraduate Research in Biomedical Sciences 2009 for outstanding published paper competition, School of Medicine, University of Southampton, 2009, United Kingdom.

Honor Fell Travel Award by the British Society of Cell Biology for selected presentation DOHaD, 2009, Santiago, Chile.

Recipient of Conference attendance fund from School of Medicine, University of Southampton, February 2009 towards attending the SGI Glasgow, United Kingdom.

Recipient of the World DOHaD Society Award for the oral presentation at the DOHaD, 2007, Perth, Western Australia

Honor Fell Travel Award by the British Society of Cell Biology for selected presentation DOHaD, 2007, Perth, Western Australia

SoM, University of Southampton Postgraduate Conference Attendance travel award for DOHaD, 2007, Perth, Western Australia

Recipient of Biochemical Society Travel Prize for presentation at DOHaD, 2007, Perth, Western Australia.

SoM, University of Southampton Postgraduate Conference Attendance travel award for 4th International Congress on Developmental Origins of Health and Disease, 2006