The Role of Developmental Programming in the Pathogenesis of Non-Alcoholic Fatty Liver Disease

Angelina Camilla Mouralidarane

University College London

PhD Research Degree

2012

Declaration

I, Angelina Camilla Mouralidarane, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I can confirm that this has been indicated in the thesis.

Acknowledgements

I would like to thank Dr Jude A Oben, Dr Junpei Soeda, Professor William Rosenberg and Dr Paul D Taylor for their supervision, guidance and support throughout this PhD. I would also like to thank the Fiorina Elliot Charity for funding my studentship and this research.

I would also like to acknowledge the research facilities and resources of the Institute of Liver and Digestive Health, Centre for Fatty Liver and Repair-Regeneration Research (Royal Free Hospital, UCL) and the Division of Women's Health (King's College London) that enabled this study to be carried out.

Finally I would like to thank my family, especially my mother, Christine Mouralidarane, for her support throughout my studies. This thesis is dedicated to my late grandmother, Arlette Aloysius.

Abstract

Obesity induced, non-alcoholic fatty liver disease (NAFLD), describes the spectrum from steatosis-to-cirrhosis. NAFLD is now the commonest cause of chronic liver disease in affluent populations. Its prevalence is increasing in tandem with rising obesity rates. However, given the exponential rise in obesity and NAFLD disease prevalence, availability of cheap energy dense foods may not alone be the sole determinant of these rising rates: maternal obesity – through developmental programming - may also be involved.

A growing body of epidemiological evidence suggests that perinatal factors may contribute to chronic disease in adulthood via programming. Programming is the process whereby an insult/stimulus during a critical period of development induces permanent structural and/or physiological changes. The role of developmental programming in NAFLD is unknown. The aims, therefore were to determine if maternal obesity during gestation and/or lactation transmits a predisposition to NAFLD in offspring, to evaluate the natural progression of such programmed disease, to examine the relative contributions of maternal obesity and the post-natal environment on offspring NAFLD and to investigate the mechanisms therein.

Using a rodent diet induced obesity-programming model, our initial studies confirmed that maternal obesity, through developmental programming, was indeed involved in the pathogenesis of NAFLD with the lactation period most

susceptible to these programming effects. Moreover, there was an interaction between *in utero* exposure to an obesogenic environment and a post-weaning obesogenic environment to cause exacerbated offspring NAFLD. Mechanistically, perturbed hepatic innate immune function and disrupted peripheral circadian rhythms were observed in offspring with NAFLD programmed by maternal obesity. Finally, these results also provide a novel and uniquely pathophysiologically relevant model of NAFLD.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ALT	Alanine Transaminase
ANOVA	Analysis of Variance
α-SMA	Alpha Smooth Muscle Actin
AST	Aspartate Transaminase
ATP	Adenosine Triphosphate
BMAL1	Brain and Muscle Arnt Like 1
BMI	Body Mass Index
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
CLOCK	Circadian Locomotor Output Cycles Kaput
Coll 1α2	Collagen Type 1 Alpha 2
CRY	Cryptochrome
DAB	Di-Aminobenzide Based
DOHaD	Developmental Origins of Health and Disease
ECL	Enhanced Chemiluminescence
ECM	Extra-Cellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
ETC	Electron Transport Chain
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FFA	Free Fatty Acid
H&E	Haematoxylin and Eosin

HBSS	Hank's Balanced Salt Solution
HFD	High Fat Diet
HDL	High Density Lipoprotein
HSC	Hepatic Stellate Cell
IL	Interleukin
IR-β	Insulin Receptor Beta
IRS-1	Insulin Receptor Substrate 1
KC	Kupffer Cell
KCAL	Kilogram Calorie
MCD	Methionine Choline Deficient
MNC	Mononuclear Cell
mRNA	Messenger Ribonucleic Acid
NAFLD	Non-Alcoholic Fatty Liver Disease
NAS	NAFLD Activity Score
NASH	Non-Alcoholic Steatohepatitis
NK	Natural Killer
NKT	Natural Killer T
NPY	Neuropeptide Y
OD	Obesogenic Diet
OffCon	Offspring of Control Dam
OffOb	Offspring of Obese Dam
PAR	Predictive Adaptive Response
PCN	Penicillin
PER	Period

RAC	Rapidly Absorbed Carbohydrates
ROS	Reactive Oxygen Species
RPM	Revolutions per Minute
RPMI	Roswell Park Memorial Institute
RTP	Room Temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SBP	Systolic Blood Pressue
SC	Standard Chow
SCN	Suprachiasmatic Nucleus
SDS-PAGE	Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis
SNS	Sympathetic Nervous System
STREP	Streptomycin
TAG	Triacylglycerol
TBS	Tris Buffered Saline
TGF-β	Transforming Growth Factor Beta
Th1	T Helper 1
Th2	T Helper 2
TNF-α	Tumour Necrosis Factor Alpha
VLDL	Very Low Density Lipoprotein

Publications

Original Research Articles

I. **Mouralidarane A**, Soeda J, Visconti-Pugmire C, Samuelsson AM, Pombo J, Maragkoudaki X, Butt A, Saraswati R, Novelli M, Fusai G, Poston L, Taylor PD, Oben JA (2012). Maternal obesity programs non-alcoholic fatty liver disease via innate immune dysfunction in mice. *Hepatology*. Re-submitted.

II. Oben JA, **Mouralidarane A**, Samuelsson AM, Matthews PJ, Morgan ML, McKee C, Soeda J, Fernandez-Twinn DS, Martin-Gronert MS, Ozanne SE, Sigala B, Novelli M, Poston L, Taylor PD (2010). Maternal obesity during pregnancy and lactation programs the development of offspring non-alcoholic fatty liver disease. *J Hepatol* 52(6):913-20.

III. Oben JA, Patel T, **Mouralidarane A**, Samuelsson AM, Matthews P, Pombo J, Morgan M, McKee C, Soeda J, Novelli M, Poston L, Taylor P (2010). Maternal obesity programs offspring development of non-alcoholic fatty pancreas disease. *Biochem Biophys Res Commun* 394(1):24-8.

Reviews

IV. **Mouralidarane A**, Soeda J, Oben JA (2011). Pathophysiology and clinical management of non-alcoholic fatty liver disease. *Medicine* 39(10):592-96.

V. **Mouralidarane A**, Lin C, Suleyman N, Soeda J, Oben JA. (2010). Practical management of the increasing burden of non-alcoholic fatty liver disease. *Frontline Gastroenterol* 1:149-55.

Abstracts

VI. **Mouralidarane A**, Soeda J, Visconti-Pugmire C, Samuelsson A, Pombo J, Taylor PD, Poston L, Oben JA (2011). Maternal obesity programs offspring innate immune dysregulation in non-alcoholic fatty liver disease. *Hepatology*, 62nd Annual Meeting of the American Association for the Study of Liver Diseases 54(S4): 1149A. (Presidential Poster of Distinction).

VII. **Mouralidarane A**, Soeda J, Samuelsson A, Taylor PD, Poston L, Oben JA (2011). Maternal obesity programs offspring innate immune dysregulation in non-alcoholic fatty liver disease. *Gut*, British Association for the Study of the Liver Annual Meeting. 60(S2):A52-3.

VIII. **Mouralidarane A**, Soeda J, Lin C-I, Samuelsson A, Pombo J, Persaud J, Novelli M, Poston L, **Taylor PD,** Oben JA (2011). Maternal Obesity during Pregnancy and Lactation Programs Development of Non-Alcoholic Fatty Liver Disease in Offspring fed a Postnatal Hyper-Calorific Diet. *Reproductive Sciences*, Society of Gynecologic Investigation Annual Meeting. 18 (S3):216A.

IX. **Mouralidarane A**, Soeda J, Lin C, Samuelsson A, Taylor P, Poston L, Oben JA (2010). Maternal obesity programs offspring non-alcoholic liver disease in mice. Symposium on Cardiovascular and Metabolic Sciences: Processes, disease prevention and therapeutic strategies, UCL.

X. **Mouralidarane A**, Taylor P, Samuelsson A, Morgan ML, McKee C, L Poston, Oben JA (2008). Developmental Programming in the pathogenesis of non-alcoholic fatty liver disease. *Hepatology*, 59th Annual Meeting of the American Association for the Study of Liver Diseases. 58(S1):408A-505A. (Presidential Plenary).

XI. **Mouralidarane A**, Taylor P, Samuelsson A, Morgan ML, McKee C, Poston L, Oben JA (2008) Developmental programming in the pathogenesis of NAFLD. British Association for the Study of the Liver Annual Meeting.

1 General Introduction

1 Introduction

1.1 The Obesity Epidemic

The population prevalence of obesity is rising worldwide, most notably in affluent countries such as the United Kingdom with some 30% of adults, presently obese (1). It is estimated that the incidence of obesity will continue to escalate, reaching 50% by 2050 in the UK alone (1). Childhood obesity rates are similarly rising, with approximately 30% of children, aged between 2 and 15, classed as overweight or obese in the UK (2). This is projected to reach an alarming 60% by 2050 (1). Obesity is associated with significant morbidity and mortality, both independently, and in association with other diseases. Given that many obesity related medical complications begin manifestation in children and young adults, the projected incidence of obesity becomes of even greater concern (3). Besides being a significant contributor to ill-health, it also presents a substantial fiscal burden on healthcare systems with projected costs to the UK National Health Service of £2 billion a year by 2030 (1).

The terms 'overweight' and 'obese' are defined as a 'Body Mass Index' (BMI) greater than 25 kg/m^2 and 30 kg/m^2, respectively. However, these values should be interpreted with caution since the BMI is an indirect measure of adiposity. The National Institute of Clinical Excellence (NICE) recommends that besides BMI, other indices of adiposity, such as waist circumference be used when characterising the extent of obesity and determining associated health risks (4).

The causes of obesity are multiple attributed to complex biological systems and societal frameworks, in which no individual disease determinant predominates. Therefore, it is necessary to invoke both preventative measures and combined interventions to curb the obesity epidemic (3).

Genetic factors, known to predispose individuals to obesity, were likely advantageous in previous resource-deficient environments, where energy conservation was essential to survival. However, the availability of cheap energy-dense foods combined with a sedentary lifestyle generates an adverse energy imbalance; the catalyst behind our modern day obese society (2).

Visceral deposition of adipose tissue in the intra-abdominal cavity is strongly associated with type 2 diabetes, hypertension and hyperlipidaemia. Such complications collectively characterise the metabolic syndrome (3). Unsurprisingly, the incidence of metabolic disorders is rising in tandem with obesity rates. In light of genome wide association studies (GWAS), which have highlighted an individual's genetic susceptibility to obesity, it has also been suggested that the inexorable rise of dysmetabolism is in part attributable to environmental factors (5).

Rates of obesity amongst women of reproductive age are concomitantly rising with a current prevalence, in the USA, of 31.5% in those aged 20-39 (6). The consequences of maternal obesity are not limited to obstetric complications. A growing body of evidence suggests that the nutritional milieu, during critical

periods of development, can permanently alter the offspring's physiology. These alterations have been shown to induce an increased propensity towards dysmetabolism, and its sequelae, in later life. Given that offspring rely upon maternal metabolic signals to guide their early developmental trajectory, in the absence of direct environmental experience, it is plausible that maternal nutrition evokes permanent change in foetal physiology. Such a phenomenon is termed 'Developmental Programming' or the 'Developmental Origins of Health & Disease' (5).

1.2 Developmental Origins of Health and Disease

1.2.1 Maternal Under-nutrition and Developmental Programming: Epidemiological Evidence

Epidemiological research, from the late 1980s, indicates that chronic, degenerative illnesses presenting in adulthood may originate from, or be influenced by, the intra-uterine environment (7). The Hertfordshire study was pivotal to our observations and understanding of the 'foetal origins of disease' hypothesis. It revealed an increased prevalence of cardiovascular disease in offspring with lower birth weights (8). Using a population cohort of 16,000 men and women, born between 1911 and 1930, David Barker and his colleagues also demonstrated an increased associated risk between growth patterns in early life and glucose intolerance and type 2 diabetes (9, 10).

The Dutch Famine of 1944-45, which provided an invaluable opportunity to investigate the effects of a deprived hypo-calorific peri-natal environment, further supported these observations. It was observed that individuals born with low birth weights also had an increased risk of cardiovascular disease in adulthood, as well as its associated comorbidities such as hypertension and stroke. More specifically, Roseboom and colleagues reported that maternal under-nutrition, during mid to late gestation, in humans was associated with lower birth weight (exposed 3175g v unexposed 3373g, $p < 0.05$); length (exposed 49.6cm v unexposed 50.5cm, $p < 0.05$); and a reduced head circumference (exposed 32.2cm v unexposed 32.8cm, $p < 0.05$). Consequently, this reduced birth weight was thought to increase the offspring's susceptibility to development of type 2 diabetes (11).

In the same cohort, it was observed that maternal under-nutrition during early gestation resulted in offspring with higher birth weights. However, this data did not reach statistical significance (exposed 3470g v unexposed 3373g). Additionally, these offspring have been associated with a larger body mass index and greater atherogenic lipid profile in adulthood, as demonstrated by a raised LDL/HDL cholesterol (exposed 3.26 mmol/l v unexposed 2.91 mmol/l); fibrinogen (exposed 3.21g/l v unexposed 3.02g/l) and reduced Factor VII (exposed 117% v unexposed 128% of standard) (7). These associations were replicated in many other cohorts from industrialised and developing countries (12).

1.2.2 Thrifty Phenotype Hypothesis

From these epidemiological studies, the 'thrifty phenotype' hypothesis was borne. Put forward by Barker and his colleagues, it was theorised that the plasticity of biological systems in early development allows for a susceptibility to permanent genetic, and epigenetic alterations as a consequence of nutritional insult (13). Such permanent alterations are thought to occur in order to maximise utilisation and conservation of available nutrients. It was thought that this would confer a survival advantage, should similar nutritionally challenging environments recur. In relation to the development of type 2 diabetes, it was proposed that foetal malnutrition reduced pancreatic beta cell mass and islet function. These changes persist into post-natal life and thus pose significant risks if BMI, or any other factor known to potentiate insulin resistance, were to increase. The notion that the 'thrifty phenotype' is a survival mechanism is further corroborated by evidence demonstrating that both liver and muscle tissue are sacrificed in order to sustain the metabolic requirements imposed by the brain (13).

1.2.3 Predictive Adaptive Response Hypothesis

Mechanistically, these permanent survival changes are also termed, Predictive Adaptive Responses (PARs) (14). If, however, nutritional adequacy is restored after birth, the offspring will have reduced biological capacity to metabolise a normo-calorific or hyper-calorific diet, increasing their propensity to obesity and the metabolic syndrome (13). Essentially, a mismatch occurs between the metabolic load and the offspring's metabolic capacity. A broader application of

this hypothesis implies that disease manifests when the predicted *in utero* environment (plastic phase) does not correlate with the post-natal nutritional status (post-plastic phase).

It has been argued that early post-natal growth patterns are responsible for increased disease risk in adulthood and that, subsequently, such diseases do not originate as a result of an adverse foetal environment (15). However, extensive epidemiological data suggests that both foetal and post-natal environments contribute to increased disease risk. Additionally, given that hyperplasia, the increase in cell number, ceases during early infancy, development of offspring organ structure and physiology is dependent upon foetal development (16).

1.2.4 Maternal Under-nutrition and Developmental Programming: Experimental Evidence

Despite establishing a causal link between foetal nourishment and chronic adult disease, retrospective epidemiological studies pose numerous limitations. In the first instance, the application of these studies to present society may not be clinically relevant. Moreover, much of the evidence concerning the influence of maternal nutrition arises from populations that constitute a minority of the birth cohort and probably incorporate selection bias. Most fundamentally, infant anthropometry is inferred as a proxy for foetal nutrition, although other perinatal factors can affect such measurements (17). Coupled with uncontrollable variables of genetic and environmental origin, experimental models of maternal under-nutrition were developed.

These models, tabulated below (Table 1.1), in essence, suggest an association between maternal under-nutrition and development of metabolic disorders.

Table 1.1 Experimental Rodent Models of Maternal Under-nutrition and Offspring Chronic Disease

Feature of Met Syndrome	Breeding Protocol	Readouts	Reference
Abnormal glucose homeostasis	70% of *ad libitum* from 0-18 days gestation in Wistar rats	Plasma glucose measured 28 weeks (M+F) **↑ plasma glucose**	**Armitage et (33)**
Abnormal glucose & insulin homeostasis	50% of *ad libitum* day 11 until delivery in Wistar rats	Plasma glucose & insulin measured at 80 days (F) **↑ glucose, ↓insulin**	**Holemans et al. (37)**
Hypertension	30% of *ad libitum* during gestation in Wistar rats	Tail cuff measurement at 100 days (F) **↑ blood pressure**	**Vickers et Al. (243)**
Plasma Leptin	30% of *ad libitum* during gestation in Wistar rats	Plasma leptin measured at 175 days (F) **↑ plasma leptin, ↑ leptin receptor pancreatic β cells**	**Vickers et al. (243)**

1.2.5 Maternal Over-nutrition and Developmental Programming: Epidemiological Evidence

As the prevalence of obesity, including maternal obesity reaches epidemic proportions in both the developing and developed world, recent studies have focused on the potential detrimental effects of maternal over-nutrition and offspring health. Numerous epidemiological studies have shown a positive correlation between maternal BMI and gestational diabetes with offspring

adiposity (18, 19). It has been suggested that maternal obesity transmits susceptibility to their offspring, enabling a physiological predisposition for efficacious fat storage compared to offspring of normal weight mothers in animal models (20).

Maternal obesity is associated with elevated concentrations of cholesterol and triglycerides. Such maternal hypercholesterolemia has been significantly correlated with increased lipid accumulation in offspring arterial walls, inducing fatty streak formation, compared with normocholesterolemic mothers. It was therefore suggested that the appearance of advanced atherosclerotic lesions in young adults was a consequence of exposure to maternal obesity during perinatal development (21).

In a retrospective population based study of men and women born between 1948 and 1954 in Aberdeen, Scotland, impaired glucose metabolism and insulin resistance was reported in offspring exposed to high protein, high fat diet in late gestation. It was suggested that maternal obesity, through unknown mechanisms, impaired development of pancreatic ß-cells. Additionally, offspring exhibiting insulin resistance were also found to be hypertensive (22, 23).

Moreover, maternal weight gain and increased BMI between pregnancies have been associated with an increased risk of obesity in offspring, compared to their siblings (24). Interestingly, an interventional study, comparing the risk of

obesity and other adverse consequences amongst offspring born to mothers pre- and post-bariatric surgery, reported ameliorated outcomes in offspring born following maternal weight reduction (25). The importance of these findings is exemplified by the disproportionate risk in offspring born to the same mother, but exposed to differential *in utero* nutritional environments. Such observations therefore support the suggestion that the maternal environment, in addition to, or independent of genetic susceptibilities, is able to physiologically program offspring during development.

Given that obesity amongst women of reproductive age is exponentially rising, the implications of maternal over-nutrition on offspring obesity, and its metabolic complications such as hypertension, type II diabetes and hepatosteatosis, have become pertinent.

1.2.6 Maternal Over-nutrition and Developmental Programming: Experimental Evidence

Unarguably, there is a profound association between maternal over-nutrition and offspring chronic disease, evidenced from epidemiological studies. However, animal models are necessary to investigate the mechanisms by which maternal obesity, and its contributing factors, program offspring disease. A variety of experimental models have been established, of which rodents are most commonly used.

In an early non-human primate model, baboons exposed to maternal over-nutrition in the pre-weaning period, developed elevated serum triglyceride levels and adiposity, through adipocyte hypertrophy, in adulthood (26). In a high fat diet induced obesity model using rhesus macaques, it was reported that foetal offspring developed non-alcoholic fatty liver disease, coupled with lipotoxicity, which persisted into post-natal life (27).

Using sheep models, it has been shown that maternal diet induced obesity evokes alteration in placental morphology and function. More specifically, an increase in placental fatty acid transporter expression was reported, resulting in raised triglyceride levels in foetuses. In the same study, it was additionally reported that placental inflammatory signalling pathways were up-regulated (28, 29). Skeletal muscle is a large glucose store, which if impaired, contributes to insulin resistance and aberrant glucose homeostasis. It has been shown in ewes born to obese dams that myogenesis is down-regulated in foetal skeletal muscle. Altered signalling pathways, involving toll-like receptors, further impede adequate skeletal muscle development, whilst propagating increased fat deposition in offspring exposed to maternal obesity (30, 31).

Rodents are commonly used animals when studying the effects of maternal obesity on offspring health. Rodents have a short gestation period and life expectancy, although gestational development is dissimilar to humans. Rodents are born with an immature neuroendocrine system. Therefore, significant maturation of the hypothalamic-pituitary-adrenal axis does not occur until the

post-natal weaning period (14). These signalling systems, however, are significantly established in humans during late gestation (32).

Dietary restriction of rodents is more achievable than over-feeding, as they stringently regulate food consumption and energy expenditure. Such metabolic homeostasis ensures maintenance of a 'body weight set-point'. Pregnant rodents, fed a high-fat diet with normal carbohydrate levels, reduce their consumption of energy releasing nutrients; thereby regulating calorie intake (33). To overcome this limitation in maternal over-nutrition studies, litter size is reduced to encourage over-feeding in the weaning period (34). Alternatively, making the diet highly palatably, through the addition of sweeteners, is favourable; especially given that the modern day obesogenic diet is high in sugar as well as fat (35).

In a model of maternal diet induced obesity, offspring born to rats fed a high fat diet prior to and throughout pregnancy and lactation had higher body and liver weights with concomitant elevated plasma glucose and triglyceride levels. Additionally, these offspring exhibited insulin resistance compared to offspring of lean or normal weight mothers (36). Such perturbation of metabolic function has been observed in a similar model, using nine-month-old offspring, whereby exposure to maternal obesity during development programmed reduced insulin secretion from pancreatic ß-cells compared to controls. It was proposed that the observed impaired glucose homeostasis was the result of hypertrophied islet cells and mitochondrial abnormalities (37).

Exposure to maternal obesity during *in utero* development has also been shown to cause vascular dysfunction. It was reported by Koukkou et al. that offspring exposed to maternal obesity displayed reduced endothelium dependent relaxation of femoral arteries compared to controls. Moreover, an enhanced vasoconstrictor response was observed in these offspring (38). Vascular function was further examined in the context of gestational diabetes; a common complication of maternal obesity. Unsurprisingly, vascular function was impaired to a greater degree in the presence of both comorbidities (38). An association between exposure to high saturated fatty acids during development and hypertensive phenotypes in adulthood, has also been reported in the rodent (39).

Central adiposity is positively correlated with the development of the metabolic syndrome. Offspring of sprague-Dawley rats fed a diet constituting 40% fat during pregnancy, displayed increased body weights and visceral fat deposits (36). Parallel findings have been reported in rodent models comprising maternal diets of 21% and 24% fat (40). Concurrently, offspring exposed to maternal over-nutrition exhibit abnormal serum lipid profiles, alongside hyperleptinaemia, which are fundamental components of the metabolic syndrome (33). It was postulated that increased fat pad mass in these offspring was a consequence of a reduced basal metabolic rate, as opposed to hyperphagia or reduced locomotor activity. However, studies using more palatable obesogenic diets have reported a strong relationship between

exposure to maternal obesity and offspring hyperphagia and food preference with subsequent weight gain (41).

From experimental evidence, there is an association between offspring of dams fed an obesogenic diet, and hyperphagia, adiposity, insulin resistance, hyperlipidaemia and hypertension in adulthood. These comorbidities collectively encompass the metabolic syndrome, suggesting that an adverse exposure to maternal over-nutrition evokes a dysmetabolic phenotype in offspring.

1.2.7 Maternal Over-nutrition and Developmental Programming: Contributing Factors

1.2.7.1 Maternal Weight Gain

It is difficult to ascertain whether maternal obesity, and its associated comorbidities, or maternal over-nutrition is responsible for the imprinted metabolic phenotype observed in offspring. Over-nutrition inevitably results in obesity and, once established, the hyper-calorific environment is maintained. Consequently the mechanistic relationship between maternal obesity and over-nutrition, with offspring risk of obesity and dysmetabolism, is poorly understood.

However, epidemiological evidence suggests that maternal weight gain imparts risk for large for gestational age babies and offspring obesity at 3 years of age,

independent of maternal BMI and glucose tolerance (42). Moreover, it has been reported that gestational weight gain, at levels greater than recommended by the Institute of Medicine, exponentially increases the risk of producing offspring with greater adiposity and dysmetabolic traits (43). In rodent models, however, it is difficult to isolate the effects of maternal weight gain as the dams are fed an obesogenic diet.

1.2.7.2 Maternal Obesity

A number of rodent models have attempted to delineate the effects of maternal obesity, independent of maternal dietary fat consumption, on offspring propensity to obesity and dysmetabolism. Using a novel programming model, White and colleagues fed dams either: a diet either high or low in fat *ad libitum* or a pre-determined quantity of the high fat diet which wouldn't cause excess weight gain 4 weeks prior to mating, throughout gestation and lactation. Offspring body weight was greater in those born to obese dams, whilst results from offspring pair fed a high fat diet or a low fat diet were similar. Additionally, it was observed that maternal adiposity, and not dietary fat per se, induced hyper-leptinaemia and insulin resistance in offspring (44).

Other groups have corroborated the contention that maternal obesity programs offspring obesity and dysmetabolism, independent of maternal diet. Using an intra-gastric over-feeding model, Shankar et al., were able to demonstrate that exposure to maternal obesity during gestation alone, increased offspring risk of obesity and its associated comorbidities; when later exposed to a hyper-calorific

diet postnatally (45). However, these findings do not suggest a definitive role for maternal obesity over maternal diet per se, but simply highlight their relative contributions to the imprinted phenotype in offspring, and perhaps their differential effects. Experimental evidence suggests that exposure to maternal obesity *in utero* reduces foetal weight, whereas exposure to a hyper-calorific environment in the same developmental period causes abnormal placental growth (46).

1.2.7.3 Maternal Diet

Alterations in maternal nutrition may induce permanent metabolic changes in offspring, namely the propensity to obesity and its adverse sequelae. Using a rodent model of programming, the effects of a maternal high fat diet (45%) either throughout the mother's lifetime, thus rendering the dam obese, or only throughout gestation and lactation; offspring metabolic parameters were investigated. A lifetime exposure to hyper-calorific nutrition in dams produced a similar offspring phenotype, compared with restricted exposure in gestation and lactation only. The resulting phenotype, in offspring, was increased body weights and adiposity (47).

Further experimental evidence from non-human primates corroborate the aforementioned findings, as maternal high fat diet consumption increased foetal free fatty acids with increased risk of insulin resistance postnatally (27). Such findings suggest that the maternal diet has the potential to program offspring phenotype, independent of obesity (Figure 1.1).

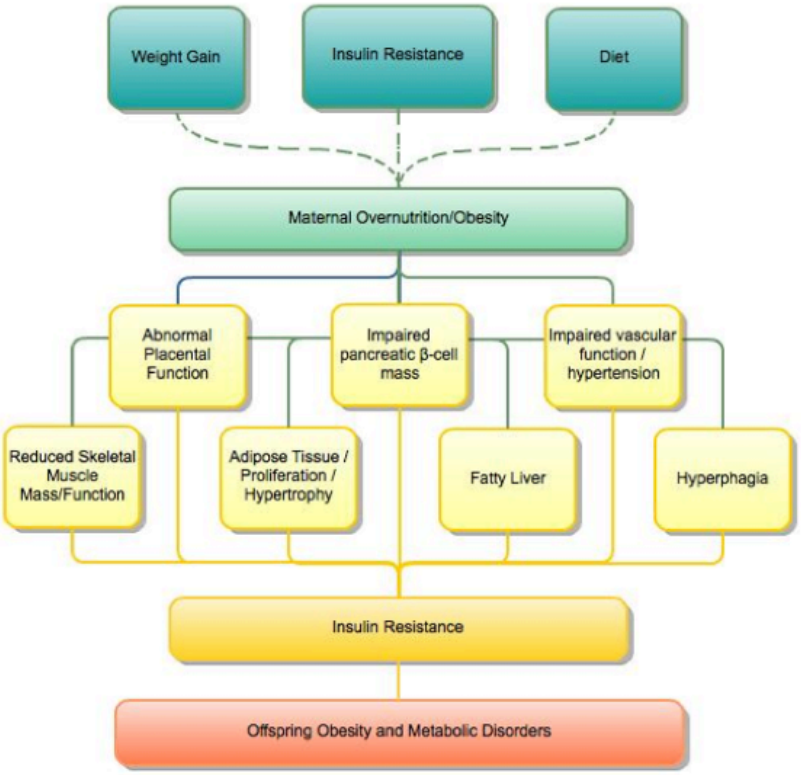


Figure 1.1 Effects of developmental programming by maternal over-nutrition on offspring phenoytpe

1.2.7.4 Maternal Over-nutrition and the Early Postnatal Period

The early postnatal period is another critical developmental window as growth and organ system maturation continues. This period is, therefore, susceptible to maternal influences.

Rapid growth or exposure to an adverse environment in the immediate postnatal period can, therefore, increase risk of obesity and dysmetabolism. Previous studies reported rapid early postnatal growth, following exposure to nutrient deficient *in-utero* environments, leading to adult onset obesity. Similarly, rapid weight gain in this period has been associated with increased

adiposity, hypertension, cardiovascular disease and hepatosteatosis in young adults, independent of birth weight (48).

Essentially, factors that influence early postnatal growth can also potentiate obesity and dysmetabolic risk in adulthood. A common observation is the effect of a nutrient enriched formula feed in increasing the risk of childhood and adult obesity, along with hyper-cholesterolaemia, compared to breastfeeding (49, 50). Epidemiological evidence suggests that the energy dense formula feed causes increased dietary intake at 4 months of age, thereby potentiating obesity risk (49). Therefore, the effects of maternal over-nutrition in programming offspring phenotype are not limited to gestation alone.

1.2.7.5 Maternal Over-nutrition and the Relative Contributions of *In utero* and Postnatal Exposure

Standard rodent models of programming involve dams fed an obesogenic diet throughout the gestation and lactation period. Readouts in offspring, therefore, are a reflection of the interaction between both developmental periods. It is now well established that exposure to maternal over-nutrtion, during both these periods, predisposes offspring to adult onset obesity and dysmetabolic traits (35).

However, by cross-fostering offspring initially born to obese dams, with lean dams in the post-partum suckling period, and vice versa, one is able to investigate the role of the *in utero* environment, independent of the immediate

postnatal period. In such a study, where offspring exposure to maternal obesity was limited to the gestational period, it was observed that there were no changes in offspring body weight or adiposity; as determined by CT analysis, although hyper-leptinaemia, hyper-lipidaemia and insulinaemia were recorded (45). In a similar experimental paradigm, offspring body weight, fat pad mass and hepatosteatosis, were reported in rodents exposed to maternal obesity during gestation alone (51).

Interestingly, a profound dysmetabolic and fatty liver phenotype was reported in the same study in offspring exposed to maternal obesity in the lactation period alone (51). These findings corroborate earlier reports by Gorski et al., in rodent models of diet resistance, and diet induced obesity. Cross-fostering offspring of diet resistant dams to diet induced obese dams during lactation appeared to program for obesity and insulin resistance. Conversely, offspring suckled by diet resistant dams, irrespective of maternal over-nutrition exposure *in utero*, displayed ameliorated insulin sensitivity (52).

Reducing litter size post-partum, to increase food availability during lactation, has also provided evidence of increased obesity risk in offspring exposed to an adverse extra-uterine environment (53). The effects of a hyper-nutritional environment during lactation have been shown to be trans-generational in rodents. Providing offspring with high carbohydrate formula milk post-partum generated adult onset obesity, which was then transmitted to their progeny, despite being weaned on to standard chow (54). Therefore, it could be

suggested that the immediate postnatal environment is able to override genetic and pre-natal factors, ultimately influencing offspring metabolic outcome.

There are additional studies demonstrating accelerated adiposity and weight gain in offspring born to obese dams and maintained on an obesogenic diet, both post-weaning and throughout post-natal life. These findings are met with reports of hyperphagia in rodents exposed to a continued adverse maternal environment post-partum, compared to control offspring or those exposed only during gestation and/or lactation (55). It can, therefore, be suggested that the effects of programming interact with the post-natal diet, and not only the immediate post-partum period to influence offspring risk of obesity and dysmetabolism in adulthood.

1.2.8 Mechanisms of Developmental Programming

1.2.8.1 Programming at the organ or cellular level

Many factors have been, mechanistically, implicated in the development of offspring obesity and dysmetabolism in response to maternal over-nutrition. Experimentally, it has been shown that offspring born to obese dams are hyperphagic irrespective of diet palatability. It was, therefore, suggested that the regulatory appetite centres in the hypothalamus were permanently altered causing adiposity, and its associated comorbidities. More specifically, neuropeptide Y (NPY), an orexigenic hormone, has been reported to be up-

regulated (5, 56). Changes to these neuropeptides are thought to be the consequence of perturbed levels of insulin and leptin.

Leptin is involved in the long-term regulation of food intake. Recent evidence supports the role of leptin in neuronal differentiation, within hypothalamic circuitry, suggesting that programming could be the result of aberrant leptin levels during critical developmental periods (57). Reconstitution of leptin in the ob/ob leptin deficient mouse has been shown to reduce hyperphagia and adiposity through down regulation of NPY, and other orexigenic peptides (58). Moreover, physiological doses of leptin, during lactation, have been reported to protect against high-fat diet-induced leptin and insulin resistance in adult rodents (59).

Insulin, like leptin, possesses neurotrophic actions and is developmentally regulated. It is well documented that insulin administration reduces NPY expression, thereby protecting against diet-induced obesity in adulthood (60). Neonatal hyper-insulinaemia may be a programming cue. This hypothesis is as a result of reports that neonatal insulin treatment induces permanent alterations in hypothalamic structure, associated with adult onset obesity and hyper-insulinaemia (61).

Impaired glucose homeostasis in offspring is observed in many models of maternal over-nutrition and often precedes the development of type II diabetes. Glucose homeostasis is a balance between insulin production by

pancreatic β cells and the responsiveness of peripheral tissues to insulin. A growing body of evidence suggests that programming, by maternal over-nutrition, may be the result of permanent alterations in insulin signalling molecules in insulin responsive tissues, such as skeletal muscle. Permanent alterations in insulin receptor substrate-1 and reduced serine 473 protein kinase B/Akt phosphorylation, the main downstream signalling molecule in insulin signalling, have been reported in offspring of ewes fed an obesogenic diet (31).

Another putative mechanism, by which programming may occur, is via altered adipose physiology. More precisely, altered body composition and excessive fat deposition are observed in offspring exposed to maternal over-nutrition, implicating perturbed adipocyte differentiation and function. It has been reported, at 3 months of age, that offspring born to obese dams have hypertrophic adipocytes with increased PPARγ expression, suggesting increased lipogenesis and reduced lipolysis (35). Additionally, it has been reported that genes governing adipogenesis are up-regulated in peri-conceptional and gestational models of over-nutrition (62). Although the increased fat mass may be compensatory to ensure that lipids are stored in adipocytes, opposed to other metabolic tissues, it adversely propagates insulin resistance and inflammation.

1.2.8.2 Programming at the sub-cellular level

Mitochondria are responsible for generating ATP and oxidising fatty acids. They are also the site of ROS production. If the mitochondrial electron transport

chain (ETC) is over-stimulated, free radicals are produced, stimulating inflammation. In obese mothers, with increased mitochondrial membrane potentials, elevated levels of oxidative phosphorylation and ROS, aberrant mitochondrial function has been reported as early as embryogenesis in mice (63). Disruption of mitochondrial oxidative phosphorylation has been reported in insulin responsive tissues, such as the liver and skeletal muscle, in offspring exposed to maternal over-nutrition (64, 65). Therefore, programming by maternal over-nutrition may be the result of perturbed mitochondrial pathways.

1.2.8.3 Programming and Epigenetic Mechanisms

Epigenetic programming involves changes in gene expression, with the DNA sequence unaltered. Such changes include DNA methylation, histone modification and chromatin packaging. In particular, DNA methylation is most commonly studied. Given that dietary intake is the source of methyl donors and cofactors, it is hypothesised that maternal nutrition could alter epigenetic patterns, thereby increasing offspring susceptibility to chronic disease in adulthood. Additionally, methylation patterns are established during development, making it more plausible that programming, via maternal over-nutrition, could occur through epigenetic modifications (66).

Early evidence from a rodent model describes epigenetic alterations in pancreatic genes of offspring reared in small litters i.e. over-fed. This observation was associated with reduced, glucose stimulated, insulin release

(67). Using a similar experimental paradigm, Plagemann et al., demonstrated hyper-methylation of the insulin receptor and anorexigenic peptide, POMC, promoter regions; correlating with aberrant blood glucose concentrations in offspring (68).

MicroRNAs (short ribonucleic acids found in eukaryotic cells), that target proteins involved in epigenetic regulation, have also been shown to be differentially expressed in offspring exposed to a maternal high fat diet; namely methylCpG binding domain proteins in the liver (69). Despite experimental evidence of an association between maternal over-nutrition and altered epigenetic processes, epidemiological extrapolation of these findings is very limited.

1.2.8.4 Programming and Circadian Rhythms

An important component of metabolic homeostasis is the coordination of daily rhythms in feeding behaviour, energy utilisation, rest and activity. This is achieved through synchronisation of the external environment with internal physiology, made permissible by molecular machinery generating circadian rhythms. A transcriptional/translational feedback loop located within the hypothalamic suprachiasmatic nucleus (SCN), also known as the master clock, is recognised at the pace-setter for all peripheral clocks, located in every cell of the body, although peripheral clocks can under certain conditions, act autonomously (70).

It is well documented that RNA expression of genes encoding lipogenesis, lipolysis and gluconeogenesis is variable over a 24 hour period and it has been suggested that there is a close association between the periodicity of transcription and the programmable effects of the circadian clock (71). Additionally, mice with the *Clock* gene mutation are reported to be hyperphagic, obese and hyper-leptinaemic, re-affirming the link between the molecular circadian clock and metabolic processes (72). Moreover, a high fat diet in rodents has been reported to alter the expression and rhythmicity of canonical circadian clock genes and nuclear receptors, which regulate clock transcription factors (73).

So, therefore, it appears that a hyper-calorific nutritional environment can disrupt molecular circadian rhythms. A similar nutritional environment, during critical periods of development, might permanently alter circadian molecular circuitry in offspring. It is thus plausible that programming via maternal over-nutrition implicates disruption of circadian rhythms mechanistically.

1.3 The Metabolic Syndrome

The metabolic syndrome is a constellation of risk factors, primarily including: abdominal adiposity; hyperglycemia; type II diabetes; hypertension and hyperlipidaemia. A growing body of evidence suggests that excess central adiposity initiates insulin resistance. Consequently, this gives rise to an array of comorbidities comprising the clinical entity - the metabolic syndrome. Obesity itself is considered to be a chronic low grade inflammatory state which

accelerates tissue injury, especially the vasculature to increase cardiovascular risk (74). The unified definition of the metabolic syndrome is summarised in Table 1.2.

Table 1.2 Criteria for Clinical Diagnosis of the Metabolic Syndrome

Measure	Ranges
Increased waist circumference*	Population-specific and country-specific definitions
Increased triglycerides (drug treatment for elevated TG is alternate indicator†)	≥150 mg/dL (1·7mmol/L)
Reduced HDL cholesterol (drug treatment for reduced HDL cholesterol is alternate indicator†)	<40 mg/dL (1·0 mmol/L) in men; <50 mg/dL (1·3 mmol/L) in women
Increased blood pressure (antihypertensive drug treatment in patient with history of hypertension is alternate indicator)	Systolic ≥130 and/or diastolic ≥85 mm Hg
Increased fasting glucose‡ (drug treatment of increased glucose is alternate indicator)	> 100 mg/dL (5·5 mmol/L)

** It is recommended that the IDF cutpoints be used for non-Europeans and either the IDF or AHA/NHLBI cutpoints used for people of European origin until more data are available.*

† Most commonly used drugs for increased triglycerides and reduced HDL cholesterol are fibrates and nicotinic acid. A patient on one of these drugs can be presumed to have high triglycerides and low HDL. Use of high-dose ω-3 fatty acids presumes high triglycerides.

‡ Most patients with type 2 diabetes will have the metabolic syndrome by the proposed criteria.

The prevalence of the metabolic syndrome is increasing proportionally with obesity rates in developed and developing countries. In 2003, it was estimated that the syndrome affected between 24 and 34% of individuals in the US and up to 36% of Europeans (14). A major complication of the metabolic syndrome is non-alcoholic fatty liver disease (NAFLD).

Development of the metabolic syndrome has been attributed to environmental risk factors such as dietary imbalance. However, a growing body of evidence, from population-based and experimental studies, suggest a predisposition to development of the syndrome following exposure to adverse maternal environments during neonatal development (5). In light of this, it becomes necessary to investigate the association between maternal over-nutrition and NAFLD in offspring, for which a paucity of data exists.

1.4 Non-Alcoholic Fatty Liver Disease (NAFLD)

1.4.1 Introduction to NAFLD

NAFLD was first described as a clinical entity by Ludwig and colleagues (75). The term implicitly acknowledges that the characteristics of NAFLD resemble alcoholic fatty liver, but without a history of immoderate alcohol use. An Alcohol consumption of <140g for men and <70g per week for women has been deemed accepted as 'little or no alcohol' by some authorities and in recent times NAFLD has been defined as the hepatic manifestation of the metabolic syndrome (76).

NAFLD is a spectrum of liver disease ranging from hepatosteatosis (fatty liver), to non-alcoholic steatohepatitis (NASH) (fat with inflammation), through to fibrosis, cirrhosis and potentially hepatocellular carcinoma (77). Patients with NAFLD who become cirrhotic usually exhibit a reduction in their degree of

steatosis (78). The terms NASH and NAFLD are often wrongly used as synonymous. More correctly, NASH is a more severe stage of NAFLD.

1.4.2 Prevalence of NAFLD

The population prevalence of NAFLD has been estimated at 7-35%, using alanine transaminase level, ultrasound scanning or magnetic resonance spectroscopy (MRS), as diagnostic techniques; of which MRS is most sensitive for detecting liver fat (79, 80). NAFLD prevalence is about 15% in Asian (81) and 20-30% in Western adults (82). The Dionysos Study, reported 25% of adult Italians with NAFLD, of which 55% had normal transaminase levels. This is suggestive that liver enzymes alone, as a diagnostic marker of NAFLD, underestimate the true population prevalence (83). Estimates of NASH prevalence are at around 2.5% (84, 85), making NASH more widespread than chronic hepatitis B and C, alcoholic liver disease and other metabolic liver diseases. The rising rates of obesity worldwide (86) parallel rising rates of NAFLD. As a result, it is projected that within the next decade a greater number of patients will be transplanted for NAFLD than for end stage chronic hepatitis C (87).

1.4.3 Risk Factors

Risk factors associated with NAFLD are, predominantly, the features of the metabolic syndrome (obesity, type 2 diabetes, insulin resistance, hypertension, and dyslipidaemia). Male sex and increased waist circumference are also well established risk factors (76, 88). NAFLD is primarily associated with increased

intra-abdominal fat mass. However, NAFLD can occur in lean subjects and in patients with forms of lipodystrophia (89). Additionally, NAFLD has been shown to cluster within families (90) and patterns of inter-ethnic variation have also been described (82). There is emerging evidence moreover, that maternal obesity, by means of programming during offspring physiological development, increases susceptibility to NAFLD in adulthood (91).

1.4.4 Natural History of NAFLD

The natural history of NAFLD is imprecisely defined. The present literature, examining NAFLD manifestation, can be divided into two main categories; cohort studies using patients diagnosed ultrasonographically with investigation of their clinical outcomes and sequential biopsy studies investigating histological progression.

An observational study reported that 40 patients with steatosis, over a median follow up period of 11 years, did not progress to NASH or cirrhosis (92). A subsequent study, with 132 patients diagnosed with NAFLD and a mean follow up period of 8 years, found that cirrhosis was more common with ballooning degeneration, Mallory hyaline inclusions and/or fibrosis, than pure steatosis alone (93).

The most recent study from two Scandinavian hospitals, with a mean follow up period of 14 years, demonstrates a low risk of progression to cirrhosis from steatosis alone. However, around 20% of patients with NASH developed

cirrhosis over the 14 years follow-up period. It was found that patients more often died from cardiovascular and liver related co-morbidities. Furthermore, 5.4% of patients, with NASH at inception of the study, developed end-stage liver disease including hepatocellular carcinoma (94).

In a sequential biopsy study, with a mean interval between biopsies of 3.2 ± 3.0 years, fibrosis progression occurred in 37%; whilst 34% remained stable with regression occurring in 29%. Additionally, co-morbid cardiovascular disease and diabetes increased risk of fibrosis progression and mortality after exclusion of cirrhotic patients (95).

It appears that NAFLD disease progression is largely dependent on co-morbid insulin resistance, as evidenced by a large prospective cohort study involving around 1 million patients. This study demonstrated an increased risk of hepatocellular carcinoma in patients with type 2 diabetes and NAFLD (96).

1.4.5 Diagnosis

Presently, there is no single diagnostic test that reliably detects NAFLD. Its diagnosis is dependent on identification of hepatic triglyceride accumulation, at the tissue level, in an obese, insulin resistant and dyslipidaemic patient; with little or no alcohol history. Diagnosis of NAFLD is, largely, by exclusion. Its presence is confirmed in the absence of focal liver lesions, positive serology for hepatitis A, B and C, aberrant autoimmune profile, copper, caeruloplasmin and ferritin (97).

The majority of NAFLD patients have abnormal liver function tests. However, elevated transaminase and gamma glutamyltransferase (GGT) levels lack sensitivity and specificity for NAFLD. It has been reported that alanine transaminase levels may be normal in the presence of hepatosteatosis, as evidenced radiologically (82). Furthermore, aberrant liver enzymes may normalise with disease progression. The aspartate transaminase to alanine transaminase ratio is usually <1 unlike alcoholic liver disease where it is >1. However, reversal of the AST/ALT ratio to >1 implies advanced fibrosis in NAFLD (97).

Serum albumin and bilirubin remain within physiological ranges in NAFLD patients. However, these levels become perturbed with disease progression to cirrhosis. A low level inflammatory state exists in obesity and NAFLD, which is reflected by increased ferritin levels in the presence of normal iron indices. The increased serum ferritin in this pathophysiological setting is believed to be an acute phase reactant (98). Raised anti-smooth muscle and anti-nuclear antibodies, seen in up to 25% of NAFLD patients, are indicative of more severe inflammation and injury (95).

Histological classification of hepatosteatosis is diagnostic of NAFLD. Macrovesicular steatosis is the hallmark histological lesion present in NAFLD. Fat accumulation commences in zone 3 but may spread depending on severity to encompass the entire acinus. The extent of hepatosteatosis is broadly graded into 3 categories; 0-33%, 33-66% and >66%, with >5% considered as

NAFLD. Haemotoxylin and eosin staining illustrate acute or chronic inflammatory cell infiltrates and Mallory's hyaline. Pericellular fibrosis may also be evident on connective tissue staining (97).

Histopathological features of NASH include the presence of lobular inflammation, hepatocellular ballooning, lytic necrosis and/or apoptotic bodies, predominately in zone 3. Moreover, portal inflammation and dispersed lobular lipogranulomas and microgranulomas may be present (98).

The NAFLD Activity Score (NAS) is a quantitative scoring system developed for the grading and staging of histological lesions. It is based on the presence of steatosis, hepatocellular ballooning and lobular inflammation, providing a composite score. A score above 5 is classified as NASH and below 3 is not NASH (98). Fibrosis is scored separately with an attainment of 4 equating to cirrhosis. The NASH Clinical Research Network (CRN) have more recently proposed a scoring system encompassing the entire spectrum of NAFLD, which is also applicable to paediatric NAFLD (99). Typical histological features of NAFLD, and its more severe form, non-alcoholic steatohepatitis (NASH), are tabulated below (Table 1.3).

Table 1.3 Histological Features of Steatohepatitis

Feature	Description
Hepatocellular Injury	• Ballooning • Apoptosis/necrosis • Mallory's hyaline • Giant mitochondria
Inflammation	• Neutrophil polymorphs • Other cells e.g. T-lymphocytes, macrophages
Fibrosis	• Perisinusoidal • Pericellular

1.4.6 Pathogenesis

1.4.6.1 Two Hit Hypothesis

The pathogenesis of NAFLD is poorly understood. Both obesity and insulin resistance are thought crucial to the development of NAFLD. The association between fat accumulation and hepatic injury, until recently, was explained using the 'Two-Hit Hypothesis'. This hypothesis proposed that increased supply of free fatty acids (FFA), due to obesity, and enhanced de novo lipogenesis, from extra-hepatic insulin resistance, led to hepatic fat accumulation – the 'First-Hit'. Subsequently the liver becomes sensitised to 'Second-Hits' such as oxidative, cytokine-mediated and endoplasmic reticulum mediated stress. Hepatic inflammation and injury, as observed in NAFLD, ensue (100). Excess fat deposition within the liver was also thought to arise from a variety of insults. These include impaired FFA oxidation, due to hyperinsulinaemia and mitochondrial damage, reduced export of triacylglycerol (TAG) as very-low-density-lipoproteins (VLDLs), and increased esterification of FFAs to TAGs (101).

A growing body of evidence suggests that steatosis is not a prerequisite for disease development, as inhibition of TAG synthesis has been shown to increase hepatic injury (102).

1.4.6.2 Hepatosteatosis

Hepatosteatosis is a consequence of altered lipid: uptake; synthesis; degradation and secretion (103). More specifically, there are two mechanistic pathways, responsible for hepatosteatosis, initiated by insulin resistance. In the first instance, lipolysis increases the concentration of free fatty acids, which are removed from the circulation by hepatocytes. The mitochondrial ß-oxidation pathway consequently becomes saturated, leading to accumulation of triglycerides in the liver. Fatty acids are inducers and substrates of microsomal lipooxygenases, which may evoke production of reactive oxygen species. These free radicals in turn induce lipid peroxidation of hepatocyte membranes (103).

The alternative pathway is governed by hyperinsulinemia, which augments hepatic fatty acid synthesis by promoting glycolysis. Insulin resistance also diminishes synthesis of apolipoprotein B-100, which is required for the incorporation of triglycerides into very low density lipoproteins (VLDLs) (103). Reduced expression of apolipoprotein B-100 mRNA in the liver has been identified in several NASH patients (104). These putative pathways are supported by epidemiological studies which have demonstrated that insulin resistance is a stronger predictor of NAFLD outcome compared to other risk factors, such as body mass index, central adiposity or glucose intolerance (105).

Although hepatosteatosis may not be a prerequisite for NAFLD development, the resulting lipotoxicity clearly plays a fundamental role in disease progression, as evidenced by the strong correlation between steatosis severity and prognosis (106).

1.4.6.3 Lipid Peroxidation

Studies have demonstrated increased lipid peroxidation, secondary to accumulation of hepatic triglycerides. Consequently, pro-fibrogenic and pro-inflammatory cytokine pathways, with adiponectin and TNF-α being key mediators, are activated (107). Additionally, increased oxidation of FFAs by peroxisomes, the microsomal oxidising system in hepatocytes and mitochondria all contribute to direct liver injury by up-regulation of cytokine gene transcription (108).

1.4.6.4 Oxidative Stress

Experimental evidence suggests a strong association between oxidative stress markers and obesity, insulin resistance and diabetes (109). As expected, therefore, elevated intra-hepatic fatty acids are a source of reactive oxygen species (ROS). Interestingly, the production of ROS and oxidative stress has been observed to precede the onset of insulin resistance. The expression of oxidative stress related genes is increased in the placenta of obese women (110), suggesting that maternal obesity can program offspring susceptibility, to dysmetabolism and NAFLD, through chronic up-regulation of inflammatory and oxidative stress pathways.

Oxidative stress is often observed in tandem with impaired insulin signalling. Up-regulation of lipogenic genes, in correlation with oxidative stress markers, were reported in a rodent programming model of high fat diet induced obesity. These findings are suggestive of proportionate fatty acid accumulation and ROS production (64). The putative role of oxidative stress, in maternal over-nutrition models, is exemplified through observation of reversed, or reduced, dysmetabolic traits in offspring; following anti-oxidant intervention in obese dams (111).

1.4.6.5 Mitochondrial Dysfunction

Free fatty acids undergo mitochondrial β-oxidation producing ROS, thereby illuminating hepatic mitochondria as major sources of oxidative stress. Mitchondrial dysfunction, which is implicated in diabetes and other dysmetabolic disorders, is therefore thought to be pivotal in progressing steatosis to steatohepatitis and fibrosis. Ultrastructural mitochondrial lesions and linear crystalline inclusions in megamitochondria are found exclusively in NASH patients, as opposed to steatotic or healthy individuals. It is thought that this mitochondrial injury is consequent to impaired ATP recovery in the liver (103).

When the oxidative capacity of mitochondria becomes impaired, alternative pathways are recruited to prevent hepatic fatty acid accumulation. Such pathways include peroxisomal ß-oxidation and ω-oxidation in microsomes, which generate additional ROS (112). With regards to maternal obesity,

mitochondrial dysfunction has been reported in embryos which have higher levels of oxidative phosphorylation and ROS production when compared to those of lean mothers (63). Moreover, disruption of the hepatic mitochondrial electron transport chain has been reported in offspring exposed to maternal obesity and is proposed as potential mechanism of programmed NAFLD (64).

1.4.6.6 Cytokine Induction

NAFLD, and ensuing fibrogenesis, may be triggered by cytokines. ROS can stimulate release of pro-inflammatory cytokines such as tumour necrosis alpha (TNF-α), interleukin 6 (IL-6) and the pro-fibrogenic cytokine, transforming growth factor ß (TGF-ß). TNF-α and TGF-ß induce caspase activation and therefore hepatocyte cell death via the apoptotic pathway. As a double edged sword, TNF-α also impairs redox reactions of the electron transport chain, further promoting ROS production(103). Adipocytes are another source of the immunomodulatory cytokine TNF-α, which also been implicated in molecular mechanisms influencing insulin resistance. TNF-α is thought to down-regulate insulin-receptor substrate-1 and reduce expression of GLUT-4, an insulin dependent glucose transport molecule (113). The mechanistic role of TNF-α in NAFLD pathogenesis is highlighted by observation of improved histological features in the ob/ob mouse model where TNF-a antagonism inhibited inflammatory activity; thereby improving NAFLD (114).

1.4.6.7 Activation of Fibrogenesis

1.4.6.7.1 Alpha Smooth Muscle Actin (αSMA)

Alpha smooth muscle actin (αSMA) is an actin isoform, indicative of smooth muscle cell differentiation. It is expressed permanently in activated hepatic stellate cells (HSCs) in fibrocontractive diseases. It is thought that an appreciable threshold of αSMA expression may be a hallmark of liver injury or fibrogenesis. ASMA relative expression in NAFLD is therefore predictive of fibrosis risk (115).

1.4.6.7.2 Transforming Growth Factor Beta (TGF-ß)

Up-regulation of TGF-ß has been demonstrated in human and animal hepatic fibrosis (116). Principal production of TGF-ß occurs in the HSCs, however, platelets, Kupffer cells and inflammatory cells possess TGF-ß synthesising capacity, thereby implicating the hepatic innate immune system in NAFLD pathogenesis and progression to fibrosis. TGF-ß induces transdifferentiation of HSCs to myofibroblasts, evoking increased synthesis of the extracellular matrix (ECM) (117).

1.4.6.7.3 Collagen Type 1α2

mRNA expression of collagen type 1α2 is thought to be up-regulated, at the transcriptional and post-transcriptional level, in activated HSCs (Stefanovic et al., 1997). Alterations in the quantity and composition of the ECM initiate the fibrogenic process. Further supporting this, increased predominance of collage

type 1α2 in chronic fibrosis has been demonstrated in immunoassays of hepatic ECM (118).

1.4.6.8 The Hepatic Innate Immune System

1.4.6.8.1 Introduction

The liver is a major immune organ, predominately synthesising the body's innate immune proteins. The hepatic innate immune system largely comprises of Kupffer (phagocytic cells), natural killer (NK) and natural killer T (NKT) cells. Other components include acute-phase proteins (APP), pattern recognition receptors and complement factors (119). From epidemiological and experimental evidence, it is well documented that innate immunopathogenesis is implicated in hepatic pathophysiology (120). More specifically, it has been suggested that an imbalance of hepatic Th1 cytokines, produced by these innate immune cells, are a common pathogenic mechanism in insulin resistance and NAFLD (121).

1.4.6.8.2 Kupffer Cells

Kupffer cells (KC) constitute the majority of the body's macrophage population. These cells are mature and specialised derivatives of blood monocytes, that originate from bone marrow progenitors. KCs make up approximately 20% of hepatic non-parenchymal cells (i.e cells other than hepatocytes) and reside in liver sinusoids. KCs are responsible for eliminating blood borne pathogens,

clearance of bacteria and generating ROS and pro-inflammatory cytokines, which potentiate immune responses indicative of hepatocyte injury (119).

The characteristics of macrophages include functional polarisation to either an M1 or M2 phenotype. M1 macrophages produce copious amounts of cytokines, including IL-6, IL-12, IL-18, IL-23, TNF-α and ROS, as a result of pro-inflammatory mediator induction. These pro-inflammatory cytokines are, in turn, able to activate polarised Th1 responses.

Th1 cells are a sub-group of T helper lymphocytes capable of producing pro-inflammatory cytokines and inducing cellular immunity. T helper cells are able to regulate immune responses through activation of the Th1 cells and anti-inflammatory cytokine producing cells, Th2 (119). An imbalance of these states or a Th1 predominated cytokine response is thought to be crucial in NAFLD pathogenesis.

M2 macrophages, conversely, produce large quantities of anti-inflammatory cytokines such as IL-10, thereby promoting the Th2 response. Corroborating this, experimental studies show that adipose tissue macrophages from lean animals express the M2 (anti-inflammatory) phenotype and those from obese animals express the M1 (pro-inflammatory) phenotype (119). Additionally, it has been reported that KC ablation improves histological outcome of NASH in a rodent model (122). These findings were preceded by reports of increased KC recruitment and activation in a similar experimental paradigm (123). Therefore,

it can be suggested that KCs play a pivotal role in pathogenesis and are not just a phenomenological observation in the context of chronic liver injury.

1.4.6.8.3 Natural Killer T (NKT) Cells

NKT cells are a unique lymphocytic sub-population, that co-express NK cell surface markers and the T cell receptor (TCR), originating from the thymus and bone marrow (124). NKT cells can be further categorised into 3 sub-classes; class I- classical or invariant, class II- non-classical or non-invariant and class III- NKT-like cells (119). Through their T cell receptor, invariant and non-invariant NKT cells are able to recognise exogenous and endogenous (self-antigen) glycolipids presented by the MHC class I like molecule, CD1d (124).

Upon activation, these cells have the unique ability to produce pro-inflammatory and anti-inflammatory cytokines also known as Th1 and Th2 cytokines, respectively. This results in direct cellular effects and recruitment of other immune cells through indirect downstream signalling pathways. Thus CD1d restricted NKT cells have diverse immunoregulatory properties enabling them to modulate the eventual inflammatory response (124). NKT cells are in turn regulated by KC derived cytokines, dietary factors and sympathetic nervous system outputs such as noradrenaline (121).

Within the murine liver, over 80% of the NKT cell population is of the invariant subtype (iNKT). iNKT cells can be distinguished from the non-invariant sub-population because they express a semi-variant TCR and are activated by the

glycolipid α-galacto-sylceramide (α-GalCer). The TCR of non-invariant NKT cells is far more diverse and the lack of α-GalCer reactivity makes this subtype difficult to study (124). iNKT cells reside within liver sinsuoids and are activated early during an immune response, making these cells key players in liver inflammation and therefore NAFLD pathogenesis. Moreover, greatest expression of CD1d in the liver occurs on hepatocyte cell membranes and given, that hepatocytes are putative regulators of intermediary lipid metabolism, it is plausible that hepatosteatosis and ensuing NAFLD are closely associated with NKT cell defects (125).

Recently, evidence has emerged for abnormalities in the NKT cell population and/or function in NAFLD patients. Results from a variety of diet-induced obesity models of NAFLD have reported an inverse relationship between the degree of injury and NKT cell number. More specifically, it has been shown, in the ob/ob mouse model, that NKT cells are selectively reduced (126) and biopsied NASH livers have declining NKT cell numbers proportionate to the extent of hepatosteatosis (127). Previously, inoculation of a small quantity of NKT cells in leptin deficient mice was shown to induce a 12% reduction in hepatosteatosis within 12 hours, suggesting a putative role for NKT cells in NAFLD pathogenesis (128).

1.4.6.8.4 Natural Killer (NK) Cells

Natural killer (NK) cells are large and granular cytotoxic lymphocytes, of the innate immune system, that play a major role in tumour rejection and viral

infected cells. These cells too originate from bone marrow progenitor cells. NK cells release small cytotoxic granules of proteins, called perforin, that cause the cell to die by either apoptosis or osmotic cell lysis. The cells are activated by cytokines released from viral infected cells, interferon and macrophage-derived cytokines. NK cells can also be activated by the KC ligand, NKG2D (129).

A growing body of evidence suggests that NK cells are involved in various types of liver pathology such as NAFLD and fibrosis. In an experimental model of diet-induced obesity, significantly reduced NK cytotoxic activity, compared to lean animals, was reported with later corroboration in obese human subjects (130, 131). It is thought that NK cells are positively correlated with reduced fibrosis as they induce hepatic stellate cell apoptosis, the liver's principal fibrogenic cell. However, the inverse is true of hepatic injury as NK cells induce hepatocyte apoptosis via interferon γ (IFN-γ) (132).

1.4.6.9 Dietary Constituents

The effect of diet on NAFLD pathogenesis has recently been investigated. Rapidly absorbed carbohydrates (RAC) i.e. those with a high glycaemic index were shown to promote hyperinsulinaemia and hepatic TAG accumulation, compared to slowly absorbed carbohydrates (133). RAC consumption has also been shown to decrease beta oxidation and increase hepatic de novo lipogenesis, inevitably increasing risk of hepatosteatosis (134). Common therapeutics may also induce hepatosteatosis. Examples include non-steroidal anti-inflammatory drugs (NSAIDs), tetracycline, amiodarone, sodium valporate

and anti-virals such as zidovudin and interferon. Fat accumulation in this context arises from inhibition of the mitochondrial beta-oxidation of fatty acids (135).

1.4.6.10 Sympathetic Nervous System

It has also been proposed that the sympathetic nervous system (SNS), both via direct and indirect mechanisms of action, enhance fibrogenesis in NAFLD. Animal models of NASH lacking a functional SNS, either through pharmacological or genetic manipulation or reduced angiotensin-1 signalling, are poorly fibrogenic (136, 137).

1.4.7 Treatment of NAFLD

There is presently no single therapeutic intervention. NAFLD is largely the consequence of obesity, malnutrition and sedentary behaviour and thus primary prevention are lifestyle modifications. However, given the growing body of evidence reporting a foetal origin of adult onset disease, intervention in early life, or even pre-conception, may be effective; if not preventative.

1.4.8 Experimental Models of NAFLD

Experimental models characterising clinical disease presentation can improve our comprehension of aetiology, prognosis and intervention possibilities. Presently, two pathophysiologically imperfect models of NAFLD are in existence, necessitating creation of a more representative animal model. These experimental models, however, have uncovered several basic mechanisms perturbed in dysmetabolism and NAFLD.

1.4.8.1 The ob/ob Mouse Model

This model is generated via mutation of the obesity (ob) gene which disrupts leptin synthesis, a satiety hormone. Leptin is an adipokine that acts on the hypothalamus to decrease appetite and elevate energy expenditure. Consequently, in the absence of leptin, the mouse develops hyperphagia and obesity. The ob/ob mouse goes on to develop a liver phenotype similar to human NAFLD, although poorly fibrogenic. This lack of a robust fibrogenic response, despite evident hepatosteatosis and inflammation, is thought to be due to the absence of leptin and consequent reduced sympathetic nervous system tone (138).

The pitfalls of this model are further highlighted by the hypo-leptinaemic state of the ob/ob mouse as excessive leptin and its resistance is well characterised in human NAFLD. The ob/ob model therefore is of limited utility in the study of NAFLD pathogenesis. However, this insulin resistant model has demonstrated that hepatocytes become steatotic and apoptose at greater rates, propagating development of the more severe form, non-alcoholic steatohepatitis (NASH). It has also been well documented in the ob/ob mouse model that leptin potentiates fibrosis by innate immune cells, through direct activation of hepatic stellate cells and regulation of pro- and anti-fibrogenic cytokine production, resulting in cirrhosis (139).

1.4.8.2 The Methionine Choline Deficient (MCD) Diet Model

A diet deficient in methionine and choline induces depletion of hepatic oxidants, such as glutathione. This increases oxidative stress, which in turn evokes lipid peroxidation and induction of pro-inflammatory cytokines. The MCD diet model is more commonly used to study steatohepatitis (140). Like the ob/ob mouse model, the MCD diet model is imperfect, as these animals do not become obese which is pivotal in human NAFLD; otherwise known as obesity induced liver disease. Additionally, the MCD fed rodents are not insulin resistant, as commonly observed in human NAFLD (141).

1.5 Developmental Programming and NAFLD: Current Knowledge

The role of developmental programming in offspring NAFLD, via maternal over-nutrition, has been long speculated as suggested by reports of Guo et al. Experimentally, they show hepatomegaly, in the context of dysmetabolism, attributable to hepatic fat accumulation in offspring rodents born to obese mothers (142). Such an association between maternal obesity, offspring hyper-triglyceridaemia and propensity to NAFLD development, has been shown in various models (27, 35).

However the role of programming in offspring NAFLD has only been comprehensively investigated using a physiologically relevant model recently, in this PhD, and the novel findings published. Using a rodent model of maternal obesity (crude fat 17.9%, simple sugars 55%, energy 7.8 kcal/g; *ad libitum* 6

weeks prior to and during gestation and lactation), we reported our novel findings of hepatosteatosis, marked liver injury and induction of liver fibrogenesis, in offspring exposed to maternal obesity.

Markers such as interleukin-6 (IL-6), TNF-α, α-smooth muscle actin (ASMA), TGF-β, collagen, hepatic tissue triglyceride content and macrovesicular steatosis were even more pronounced in offspring exposed to maternal obesity only during lactation. Therefore, it was concluded that although exposure to maternal obesity during gestation and lactation programmed a NAFLD phenotype, maternal over-nutrition limited to the lactation period was more critical to disease potentiation. These findings, in part, may explain the inexorable rise of obesity induced liver disease (143).

Using a similar experimental paradigm, the observed phenotype in the aforementioned study corroborates the findings of Bouanane et al. They report a permanent reduction in hepatic β-oxidation and increased lipogenesis, with altered composition of saturated fatty acids in VLDL lipids, as a potential mechanism for hepatosteatosis; as observed in offspring born to high-fat fed dams (144). Additionally, increased lipogenesis, as evidenced by up-regulated SREBP-1 and impaired lipolysis due to PPARa down regulation, has been reported in a rodent model of maternal over-nutrition (145).

However, there is emerging evidence suggesting that maternal obesity is not the only determinant of programmed offspring NAFLD. High calorific intake

during pregnancy, independent of maternal obesity, may also be involved in programming hepatic injury as has recently been shown in non-human primates (146). Similarly, Bayol and colleagues report hepatosteatosis and increased oxidative stress, in offspring born to mothers fed a hyper-calorific 'junk' diet during gestation and lactation, independent of prior maternal obesity (147).

Experimental research indicates that nutritional perturbations in the intra- and extra-uterine environment, prime offspring liver to an increased risk of NAFLD in adulthood. However, the underlying mechanisms responsible for the observed phenotype are unknown. It has been suggested that maternal breast milk factors may act on central neonatal energy homeostasis centres, evoking hyperphagia and subsequent obesity and obesity induced liver disease (143). However, there is a paucity of data on the direct effects of maternal over-nutrition on hepatic function. It has been recently suggested that maternal over-nutrition, during developmental plasticity, perturbs hepatic mitochondrial electron transport chain reactions; increasing oxidative stress and fat accumulation with resultant hepatosteatosis (64).

Additionally, evidence from animal models has demonstrated diet induced alterations in the hepatic epigenomic profile, of offspring exposed to maternal over-nutrition, namely hyper-acetylation and -methylation (148). Godfrey et al., have recently corroborated these findings in a human study, examining the methylation status of umbilical cord tissue DNA from healthy neonates with their adiposity at age 9 years. Consequently, a correlation between perinatal

epigenetic changes and childhood adiposity, owing to altered maternal nutritional status, was reported (149).

In summary, a strong association between exposure to maternal obesity during development and offspring propensity to develop NAFLD exists. Although there is accumulating evidence for this observed correlation, further research is required to dissect the downstream hepatic regulatory pathways perturbed and responsible mechanisms.

1.6 General Hypothesis

NAFLD is an increasingly prevalent disorder and now the most common cause of chronic liver disease in affluent countries. Additionally, obesity, which is regarded as pivotal in the aetiopathogenesis of NAFLD, incorporating a familial component, is rising in tandem. Therefore, a better understanding of the underpinning pathophysiology is required for improved diagnosis and intervention. The growing body of evidence suggesting that perinatal factors affect health outcomes in adult life warrants enquiry into offspring liver phenotypes following exposure to maternal over-nutrition. The prevailing hypothesis is that maternal obesity programs offspring dysmetabolism with a pre-disposition to NAFLD in adulthood, an association presently unknown. Additionally, it is hypothesised that the adverse maternal milieu permanently alters physiological processes governing liver repair

1.7 Aims

The aim of this thesis, therefore, is to determine if maternal obesity during gestation and/or lactation transmits a predisposition to dysmetabolism and NAFLD in offspring and secondarily to investigate the mechanisms therein. In order to delineate the effects of maternal over-nutrition on offspring hepatic outcome, this thesis specifically aims to examine the following:

a. The effect(s) of exposure to maternal obesity on offspring dysmetabolism and NAFLD in adulthood.

b. The independent effect(s) of the *in utero* and immediate post-partum period i.e. lactation, on offspring susceptibility and development of dysmetabolism and NAFLD in adulthood.

c. To develop a uniquely pathophysiologically relevant model of NAFLD in which to further study the interaction between *in utero* exposure and the post-weaning environment in programming development of offspring dysmetabolism and NAFLD in adulthood.

d. To investigate the natural progression of NAFLD in programmed offspring and ascertain whether exposure to maternal obesity primes accelerated disease development, cirrhosis or malignancy.

e. To examine the role of the hepatic innate immune system as a putative mechanistic pathway through which maternal over-nutrition programs offspring propensity to dysmetabolism and NAFLD in adulthood.

f. To examine the role of the peripheral circadian system as an alternative, but putative mechanistic pathway through which maternal over-nutrition programs offspring propensity to dysmetabolism and NAFLD in adulthood.

2 General Methods

2 General Methods

2.1 The Developmental Programming Model

Overview

Using female C57BL/6J mice, dietary manipulation in the pre-conception and post-partum periods offspring exposed to differing *in utero* and post-partum dietary environments were generated to allow investigation of nutritional influences in programming NAFLD in adult offspring.

2.1.1 Pilot Protocols

Experimental diets were rendered obesogenic by varying their fatty acid and sugar contents and further supplemented with soy oil to ensure essential fatty acid requirements. These diets were then further supplemented with sweetened condensed milk fed *ad libitum.* This model has previously been shown to rapidly increase and sustain body weights throughout pregnancy (35).

In a pilot study, two similar obesogenic diets obtained from Special Diet Services, Whitham, UK, varying only in the simple sugar content were fed to female mice post-weaning (n = 5) (Table 2.1). Additionally, the diets were supplemented with a highly palatable condensed milk, rich in sugar. The aim of the pilot study was to ascertain which diet enhanced appetite thereby potentiating accelerated weight gain. A 30% increase in body weight, within 6-8 weeks, was attained in mice fed the SDS obesogenic diet #2.

Table 2.1 Dietary Constituents of Obesogenic Diets

Dietary Constituent	Chow	SDS OB#1	SDS OB#2	OSD Condensed Milk	Condensed milk
Protein	Crude 21.6%	Casein 21%	Casein 21%	16.8%	8.1 %
Polysaccharides	48.80%	18.5%	25%		
Simple sugars	5.40%	17.6%	10%	51.4%	55 %
Fiber	4.50%	5%	5%		
Lipid	Corn oil 4.3%	Soy oil 4.3%, Lard17.9%	Soy oil 4.3%, Lard17.9%	31.8%	9 %
Methionine	0.20%	0.20%	0.20%		
Energy (kcal/g)	3.3	4.6	4.5	4.4	3.3

*All percentage values are given as w/w. *total vitamin/ mineral mix is 3.5%. Note that due to varying moisture and nitrogen free extract (polysaccharide) content, rows will not sum to 100%.* ***Abbreviations****:* ***CHOW*** *is RM 3 from SDS. EARNEST obesogenic#1 is diet 824127 from SDS and EARNEST obesogenic#2 is DIET 824053 from SDS and sweetened condensed milk is from Nestle.*

Similarly, a condensed milk diet from Open Source Diets (D12266B) was additionally piloted in female C57BL/6J mice. This diet is all encompassing, not requiring additional supplementation with mineral mixes or sweetened condensed milk. The contention was that a composite diet would render measurement of food intake and management of animal husbandry more efficient. However, adequate increase in adiposity was not attained (Figure

2.1). Consequently, the chosen experimental diet was *SDS obesogenic diet #2* as above.

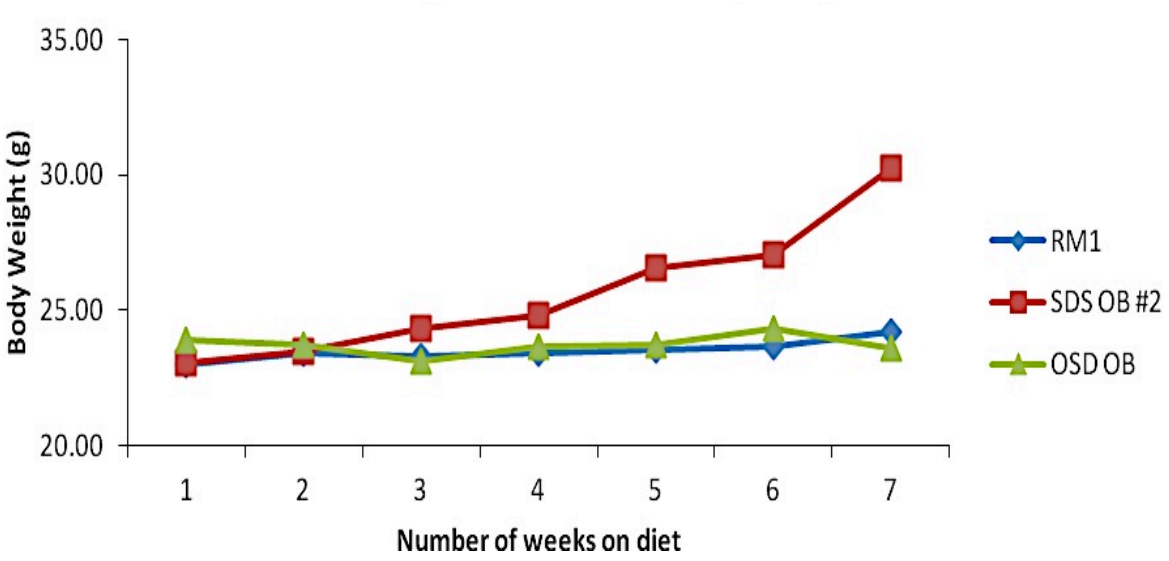


Figure 2.1 Effects of different experimental diets on pre-conception body weights

As discussed, addition of sweetened condensed milk increases palatability of the feed. Moreover, this introduced a high sugar component to the obesogenic diet, typically observed in cheap, energy dense western diets.

2.1.2 Breeding Protocols

Experimental mice C57BL/6J outbred strain (Charles River Laboratories (UK) were used for all animal studies. Female mice of first order parity were allowed seven days to habituate to the animal facilities, during which time, standard chow RM1 (rat and mouse maintenance diet from SDS, Whitham Essex, UK) *ad libitum* was provided. Subsequent to this, female mice, randomly assigned to

the obesogenic arm of the study, were fed an obesogenic diet #2 (SDS Diet Batch 824053) with sweetened condensed milk (Nestle) as above, mixed with mineral mix (SDS Batch 31801, 125 mg/pot) and control female mice, the RM1 diet, *ad libitum*, for 6-8 weeks. Female mice were then entered into the breeding protocol upon attainment of the target weight i.e. 30% increase in weight from baseline. All animals were treated in accordance with The Animals (Scientific Procedures) Act 1986 guidelines.

Breeding males, selected from the same litter, were simultaneously introduced and the animals were allowed to freely mate. Selection of breeding males from one litter minimised genetic variability of offspring. Day 0 of pregnancy was noted by the formation of a vaginal plug. Dams were allowed to re-mate upon if they failed to conceive after first mating. However, if successful conception was not achieved on subsequent matings, the females were fasted overnight and then humanely sacrificed by CO_2 inhalation (schedule-1 method) (Figure 2.2).

Pregnant mice were maintained on their respective diets throughout pregnancy and lactation with maternal weight and dietary intake recorded weekly. Dams were allowed to deliver spontaneously and left undisturbed with their litters for 48 hours. Litter sizes were documented to assess for cannibalisation of pups (Figure 2.2).

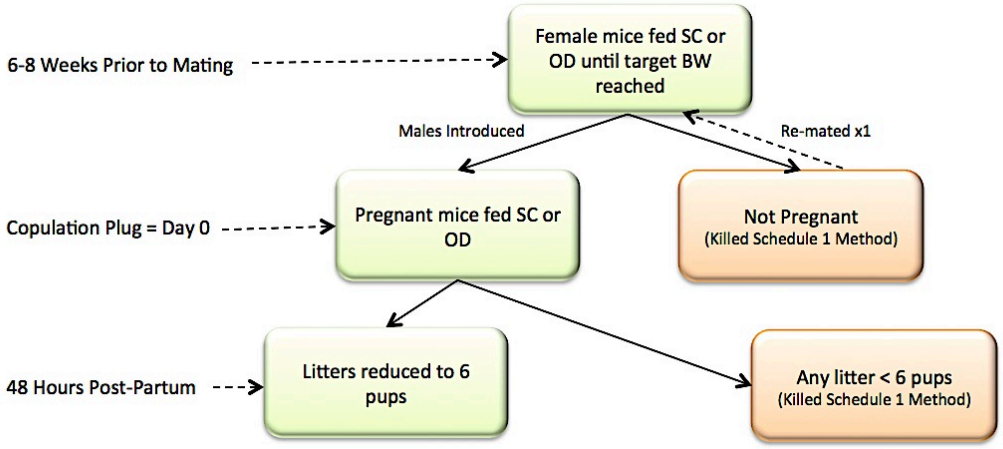


Figure 2.2 Schematic Representation of General Mouse Breeding Protocol

Additionally, this breeding protocol was manipulated to permit investigation of the effects of maternal obesity - during pregnancy and/or lactation and in the post-natal environment on programming offspring dysmetabolism and NAFLD. This was achieved by restricting exposure to the obesogenic diet in separate windows of developmental plasticity.

2.1.2.1 Breeding Protocol - Phase 1

At 48 hours post-partum, the litter size was reduced to 6 pups in an attempt to standardise milk availability during the suckling period. Offspring were suckled by their mothers for 21 days post-partum. Importantly, the mothers were maintanined on the same diet as they had whilst pregnant. Female offspring were then weaned on to a standard chow diet, *ad libitum*, at 3 weeks and continued on this diet until adulthood (3 months of age). Parameters indicative of dysmetabolism and NAFLD were then assayed as detailed (Figure 2.3).

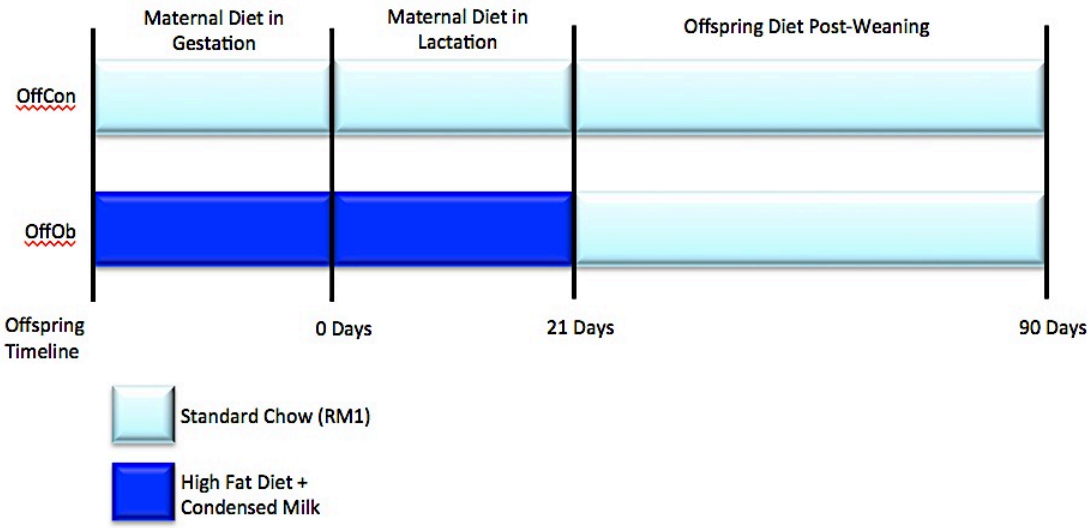


Figure 2.3 Schematic Representation of Breeding Protocol – Phase 1

2.1.2.2 Breeding Protocol – Phase 2

In 'Phase 2' of the breeding protocol, offspring born to dams consuming a control RM1 diet (lean dams) were cross-fostered to obese dams to suckle at 48 hours post-partum. Conversely, offspring born to obese dams were cross-fostered and suckled by lean dams. Therefore, exposure of offspring to maternal over-nutrition was limited to either gestation or lactation, permitting investigation of the effects of the intra-uterine and extra-uterine environments on offspring phenotype. As previous, all offspring were weaned on to a standard chow diet at 3 weeks post-partum and continued on this control diet into adulthood. Readouts were obtained at 3 months of age (Figure 2.4).

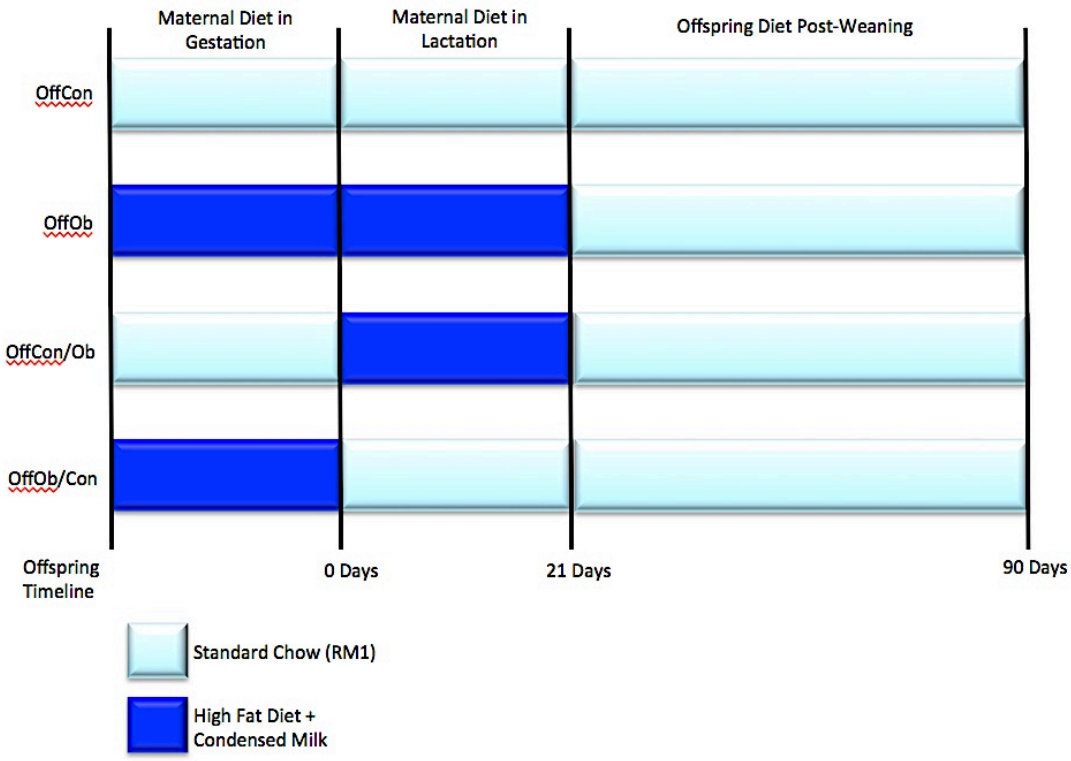


Figure 2.4 Schematic Representation of Breeding Protocol – Phase 2

2.1.2.3 Breeding Protocol – Phase 3

Although, 'Phase 2' of the model allowed investigations of the relative contributions of the intra- and extra-uterine environments on development of offspring hepatic phenotypes, cross-fostering is a stressor and has been shown to increase anxiety states in rodents (150). Additionally, it has been reported that cross-fostering stress may alter complex epigenetic traits in rodent offspring and compromise investigations into the independent effects of maternal over-nutrition (151). Moreover, cross-fostering is non-physiological and its clinical relevance is therefore limited.

In light of the above, a more physiologically applicable model was designed, termed 'Phase 3'. In this model, offspring continued to be suckled by their mothers and were then weaned on to either a control or high fat diet as consumed by the dams during pregnancy and lactation. The rationale for 'Phase 3' is based on the observation that mothers who expose their offspring to over-nutrition *in utero* are likely to nurture them in a similar environment post-partum. Additionally, offspring were continued on their allocated diets in to adulthood with readouts obtained at 3 and 12 months of age, in order to investigate the natural progression of programmed liver disease (Figure 2.5).

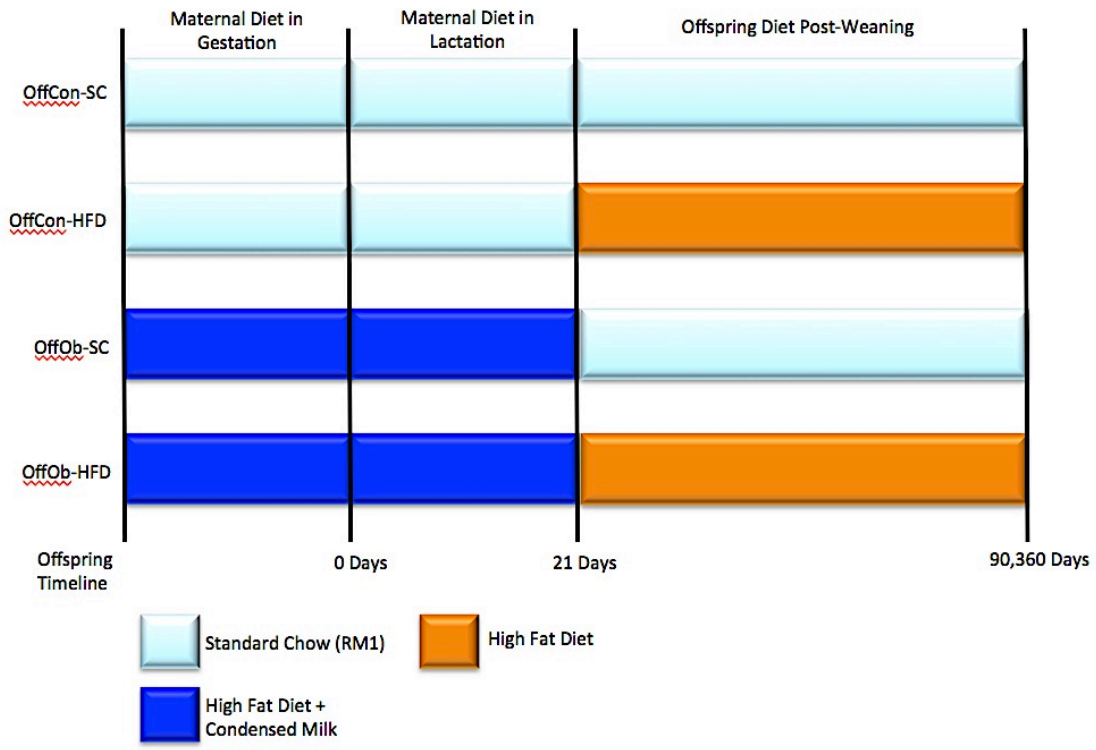


Figure 2.5 Schematic Representation of Breeding Protocol – Phase 3

2.1.2.4 Breeding Protocol – Phase 4

In attempt to generate a uniquely pathophysiological model of NAFLD in which to investigate underlying mechanisms, a further revision of the 'Phase 3' model was implemented. Much of the literature documenting the effects of maternal over-nutrition, focus on the implications of offspring exposure to a high fat diet. However, it is necessary to examine the effects of a high fat and high sugar diet, which is propelling the present obesity epidemic (6). As such, offspring were weaned onto either a control or a high fat diet supplemented with sweetened condensed milk i.e. a truly obesogenic diet (Figure 2.6). Offspring were continued on their respective diets into adulthood and readouts, pertaining to mechanistic pathways, obtained at 6 months.

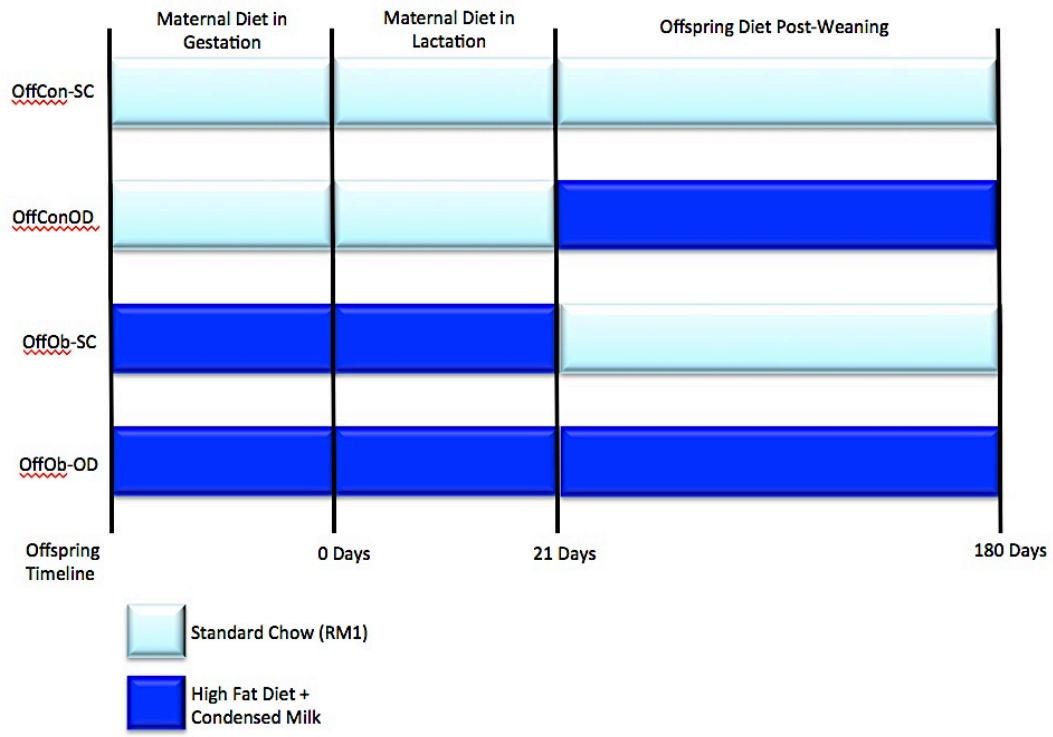


Figure 2.6 Schematic Representation of Breeding Protocol – Phase 4

2.1.3 Reproductive Rates and Cannibalism

Dams entered into the breeding protocol were of first order parity to reduce the risk of cannibalisation, although this encroached upon the desired breeding age of 8-16 weeks. Cannibalism was recorded to be greater in offspring born to control or lean mothers. As expected, fertility rates were reduced in obese dams, alongside compromised successful pregnancies, as the incidence of foetal re-absorption, early spontaneous and complicated deliveries increased (Table 2.2).

Table 2.2 Reproductive Rates and Incidence of Cannibalisation

	Control Dams	Obese Dams
Fertility Rate* (%)	94.4	72.2
Successful Pregnancies (%)	77.8	44.4
Cannibalisation (%)	16.7	5.6

**Calculated as % formation of copulation plugs for all dams entered into the breeding protocol*

Litters were reduced to 6 pups post partum, by random selection, to standardise litter sizes and ensure an adequate milk supply. The remaining offspring were sacrificed by a schedule-1 method on day 2 post-partum. Mothers with litters of less than 4 pups after 48 hours were also not used even if the reduced number was due to cannibalisation.

2.1.4 Maternal Anthropometry

Pre-conception maternal body weights were recorded on a weekly basis to ascertain suitability for entry into the breeding programme. Food intake was measured thrice weekly during condensed milk changes. From this, calorie intake was also calculated. As expected, calorie intake was directly related to maternal obesity (Figure 2.7).

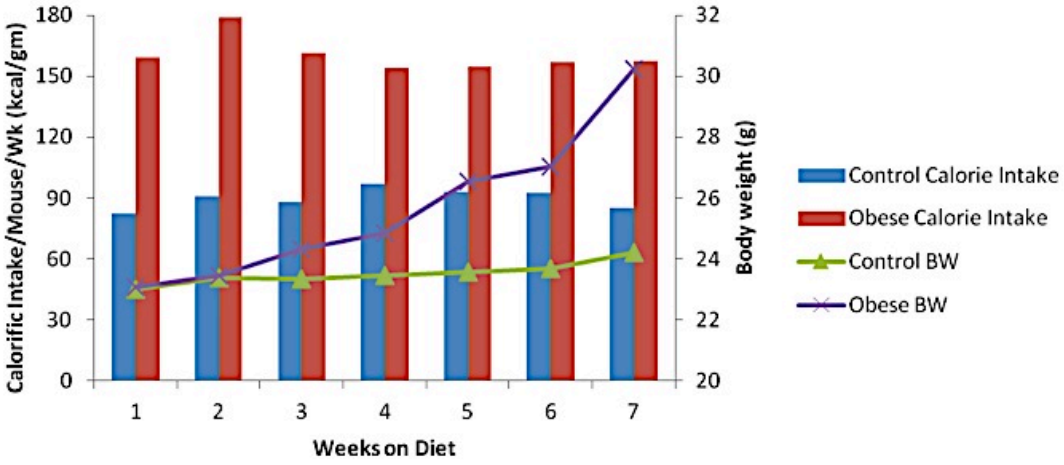


Figure 2.7 Pre-Conception Maternal Body Weights and Calorific Intake

Body weight was similarly recorded throughout gestation. Increments in body weight during this period were used to monitor whether the breeding colonies were undergoing normal pregnancies (Figure 2.8).

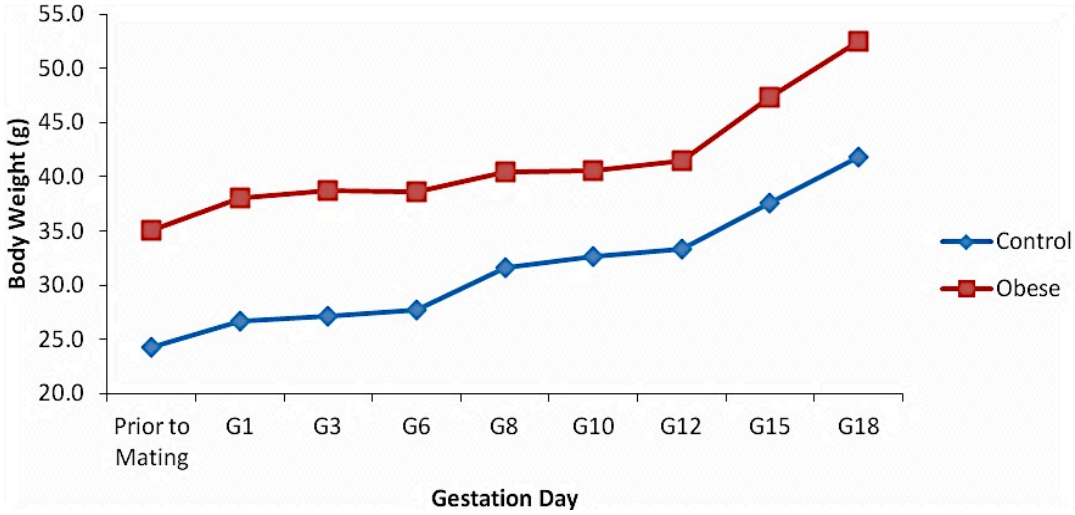


Figure 2.8 Gestational Maternal Body Weights

2.2 Tissue Harvesting

All offspring were housed in a group of 2 or 3 per cage and subjected to a 12 hour light/dark cycle. In 'Phase 1' of the programming model, all offspring were fasted for 24 hours prior to being sacrificed, using CO_2 inhalation and cervical dislocation - schedule 1 method, to allow measurement of fasting insulin and glucose levels. In subsequent phases of the model, offspring were sacrificed in the non-fasted state so that accurate leptin levels could be measured. Organ weights were determined before being preservation.

2.2.1 Liver Tissue Preservation

Liver tissue was differentially preserved for use in gene expression analysis, protein and lipid extraction, flow cytometry, histology and immunohistochemistry.

Formalin Fixing (Histology)

a) Whole liver was dissected using middle orientation on aluminium foil.
b) Liver slices were placed in pre-labelled cassettes and stored in 10% neutral buffered formalin.
c) To ensure adequate saturation of tissue sections with formalin, tissue was stored at 4ºC for at least 24 hours before paraffin embedding.

Cryopreservation (Protein Analysis, Triglyceride Quantification)

a) Approximately 1/5 liver tissue was dissected into 5mm^3 cubes and placed into 2ml cryotubes.
b) The tubes were immediately placed in liquid nitrogen causing instantaneous freezing.
c) All tissue was then stored at -80ºC until further processed.

RNAlater Fixing (Gene Expression Analysis)

a) Approximately 1/5 liver tissue was dissected into 5mm^3 cubes and placed into 1.5ml cryotube containing 1ml of RNAlater solution.
b) Samples were stored at 4°C for at least 24 hours to permit adequate tissue saturation prior to long term storage at -20°C.

2.2.2 Blood Collection

Offspring blood was collected via cardiac puncture or inferior vena cava cannulation using heparinised syringes. The blood was transferred to endotoxin-free eppendorf vials and centrifuged at 3000 x g for 10 minutes at

4˚C. The decanted plasma was then stored in vials at -20˚C until biochemical analysis. Serum alanine transaminase levels were analysed by the clinical biochemistry department, Royal Free Hospital, University College London.

2.3 RNA Extraction

Total RNA was isolated from murine whole liver tissue using TRIzol Reagent (Invitrogen Life Technologies). TRIzol Reagent is a mono-phasic solution of phenol and guanidine isothiocyanate. During tissue homogenisation, the reagent maintains RNA integrity whilst disrupting cells and dissolving cell components. RNA was isolated in accordance with the manufacturer's recommended protocol detailed below.

Homogenisation

50-100mg of murine liver tissue from each sample was homogenised in 1ml of TRIzol Reagent using an autoclaved pestle and mortar. The homogenised sample was transferred to a 1.5ml microfuge tube using a 5ml pipette.

Phase Separation

a) The homogenised samples were incubated for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes.
b) 0.2ml of chloroform was added to the homogenised samples.

c) The samples were vortexed for 15 seconds prior to incubation at room temperature for 2-3 minutes.

d) The samples were then centrifuged at 11,600 x g for 10 minutes at 4˚C. Following centrifugation, the mixture separated into a lower red phenol-chloroform phase, an interphase and an upper aqueous phase. The RNA remained exclusively in the upper aqueous phase.

RNA Precipitation

a) 0.5 ml of isopropyl alcohol was added to precipitate RNA from the aqueous phase.

b) The samples were incubated at room temperature for 10 minutes followed by centrifugation at 11,600 x g for 10 minutes at 4˚C. The RNA precipitate formed a gel-like pellet on the bottom of the tube.

RNA Wash

a) The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol.

b) The sample was vortexed and centrifuged at 7,500 x g for 5 minutes at 4˚C.

Re-dissolution of RNA

a) RNA pellet was briefly air-dried for 5-10 minutes, taking care not to completely dry the pellet and reduce its solubility.

b) RNA pellet was re-dissolved in RNase-free water by passing the solution a few times through a pipette tip prior to incubation at 60˚C for 10 minutes.

The concentration of RNA in ng/µL was determined using a NanoDrop ND-1000 Spectrometer. A "260/280" ratio was also recorded to ensure adequate re-dissolution of RNA and ascertain RNA quality and purity. To ensure accurate loading of RNA during the polymerase chain reactions (PCRs), RNA concentrations were standardised to 100ng/µL by diluting samples with RNAse free H_2O.

2.4 Primer Design

Gene specific primers were designed using nucleotide base sequences of the genes of interest obtained from NCBI Reference Sequence database (www.ncbi.nlm.nih.gov/RefSeq) (Table 2.3). These sequences were analysed for optimum primer sequences using Primer 3 Software. Primer mouse specificity and efficacy was determined using Basic Local Alignment and Search Tool. The primers were designed to effectively anneal to exon-intron-exon complexes or cross exon/exon boundaries of mRNA, in order to prevent co-amplification of genomic DNA. It was also verified, during primer design, that the sequences were not self-complementary or complementary to each other at the 3' ends.

Table 2.3 Primer Sequences, Expected Weights and Annealing Temperatures of Target Genes

Gene	Primer Sequence	Weight	Annealing Temp
IL-6	F: 5'-TTCACAGAGGATACCACTCC-3' R: 5'-GTTTGGTAGCATCCATCATT-3'	203bp	55˚C
TNF-α	F: 5'-TCCAGCTGACTAAACATCCT-3' R: 5'-CCCTTCATCTTCCTCCTTAT-3'	220bp	55˚C
ASMA	F: 5'-ATCTGGCACCACTCTTTCTA-3' R: 5'-GTACGTCCAGAGGCATAGAG-3'	191bp	59˚C
TGF-β	F: 5'-AAAATCAAGTGTGGAGCAAC-3' R: 5'-CCACGTGGAGTTTGTTATCT-3'	224bp	59˚C
Collagen 1-α2	F: 5'-GAACGGTCCACGATTGCATG-3' R: 5'-GGCATGTTGCTAGGCACGAAG-3'	167bp	55˚C
Adrenoceptor α1-D	F: 5' TTGAATTCCTACAGAGACCCACGACCCAG-3' R: 5'-CGGAATTCTTAAATGTCAGTCTCCCGGAG-3'	229bp	55˚C
Adrenoceptor ß-1	F: 5'-ACGCTCACCAACCTCTTCAT-3' R: 5'-AGGGGCACGTAGAAGGGAGAC-3'	440bp	55˚C

2.4.1 Primer Assay

Additionally, ready-to-use Quantitect Primer Assays (Qiagen), which allow accurate real-time quantification of target RNA, using SYBR green based detection, were utilised (Table 2.4). Each assay contains forward and reverse primers that are generated from the NCBI Reference Sequence database,

optimised and bioinformatically validated. The annealing temperatures also remain unchanged across target genes in primer assays. For use in a two-step RT-PCR reaction using a Rotor Gene cycler, the annealing temperature is 55°C.

Table 2.4 Primer Assay References and Annealing Temperatures of Target Genes

Primer	Assay Reference	Annealing Temp
Circadian Locomotor Output Cycles Kaput (CLOCK)	QT00197547	55°C
Brain and Muscle Arnt Like-1 (Bmal-1)	QT00101647	55°C
Period 1 (Per 1)	QT00113337	55°C
Period 2 (Per 2)	QT00198366	55°C
Cryptochrome 1 (Cry 1)	QT00117012	55°C
Cryptochrome 2 (Cry 2)	QT00168868	55°C
REV-ERBα	QT00164556	55°C
IL-12	QT00153643	55°C
IL-18	QT00171129	55°C

2.5 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

2.5.1 Semi-Quantitative RT-PCR

The SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* High Fidelity (Invitrogen Life Technologies) was used to perform semi-quantitative RT-PCR. This one step formulation incorporates DNase treatment, to remove residual DNA from RNA isolation, cDNA synthesis and PCR amplification using gene specific primers. This system comprises two main components: SuperScript™ III RT/ Platinum® *Taq* High Fidelity Enzyme Mix and 2X Reaction Mix. The enzyme mix reduces RNase H activity thereby increasing thermal stability and blocks polymerase activity at ambient temperatures. The 2X Reaction Mix includes Mg^{2+}, deoxyribonucleotide triphosphates (dNTPs), and stabilizers.

Master mixes for the target and reference genes were prepared using the appropriate ratios of the components detailed in Table 2.5 in order to minimise pipetting errors and reagent loss. The master mixes were vortexed briefly prior to centrifugation at 500 x g for 15 seconds. Following aliquot of the appropriate master mix, the RNA template was added to a 0.5 ml amplification tube and placed in a thermal cycler, BIO-RAD iCycler™ 96 Well Reaction Module (Table 2.6). Primer annealing temperatures were adjusted accordingly.

Table 2.5 SuperScript™ III One-Step RT-PCR Components

Component	Volume
2X Reaction Mix	10µl
Template RNA (200ng)	1µl
Target Forward primer (10 µM)	1µl
Target Reverse primer (10 µM)	1µl
Reference F/R primer (10uM of each)	1µl
SuperScript™ III RT/ Platinum® Taq High Fidelity Enzyme Mix	0.5µl
RNase Free Water (GIBCO)	10.5µl
Total	**25µl**

Table 2.6 Thermal Cycling Conditions Specific For SuperScript™ III One-Step RT-PCR and BIO-RAD iCycler

cDNA Synthesis & Pre-denaturation	PCR Amplification	Final Extension
1 cycle:	**35 cycles:**	**1 cycle:**
• 45°C for 30 mins • 95°C for 5 mins	• Denature: 95°C for 30 sec • Anneal: Primer dependent	• 68°C for 5 sec

2.5.1.1 Gel Electrophoresis

Amplification products of the semi-quantitative RT-PCR were prepared for gel electrophoresis. Typically, 1.5% agarose gels were used, whereby 0.4 g of agarose powder was added to 4 ml of 50X TBE (Tris/Borate/EDTA) buffer and 46 ml of water before being heated to generate a molten mixture. Following this, 2.5µL of 10 mg/ml ethidium bromide was added to the agarose and poured into the gel apparatus. The gel was submerged in 1x TAE (Tris/Acetic acid/EDTA) buffer prior to loading and electrophoresis.

A DNA ladder of 50 base pairs (New England Biolabs) was loaded onto the gel with 2µl of 6x loading buffer mixed with 12µl of amplified complementary DNA. The RT-PCR products were then electrophoresed for 1 hour at 90V, 60mA. These settings ensured that the Bromophenol Blue did not run off the gel. The electric field created across the gel causes nucleic acid molecules to separate with smaller molecules migrating faster towards the charged nodes. Gene expression of the target sequence was normalised to the expression of the reference gene i.e. GAPDH, 18s or β-actin to control for variable DNA concentrations in each sample. Subsequent to electrophoresis, the bands were visualised under an ultraviolet transilluminator (UVP Laboratory Products, EPI Chemi II Darkroom) and densitometry measurements obtained using Labworks.

2.5.2 Two-Step Real-Time PCR

2.5.2.1 cDNA Synthesis

Real time PCR provides direct quantification of both DNA and cDNA targets. Real time 2 step RT-PCR of RNA targets was performed following reverse transcription with the Quantitect Reverse Transcription Kit (Qiagen). This kit not only synthesises cDNA but also effectively eliminates genomic DNA. The Quantitect Reverse Transcription procedure comprises of 2 main steps.

Elimination of Genomic DNA

Incubation of purified RNA samples with gDNA wipeout buffer eliminates genomic DNA. This is imperative in instances where primers or probes for target genes designed to eliminate or minimise detection of such DNA are not available.

Reverse Transcription

Using a master mix containing a reverse transcriptase enzyme, RT buffer and RT primer mix, additional RNA denaturation and RNase H digestion are not necessary (Table 2.7). Due to the high affinity of the reverse transcriptase for RNA, cDNA yields from templates with high GC nucleotide content or complex secondary structures are also possible. The RT primer mix ensures that cDNA synthesis occurs along all areas of the RNA transcripts including 5' regions. Collectively, these processes ensure sensitive and higher yields of cDNA.

Table 2.7 Reverse Transcription Master Mix Reaction Components

Component	Volume/Reaction
Quantiscript Reverse Transcriptase	1µl
Quantiscript RT Buffer, 5x	4µl
RT Primer Mix	1µl
Template RNA from gDNA elimination Reaction	14µl
Total	**20µl**

2.5.2.2 Real-time PCR

Real-time PCR was performed using the QuantiTect SYBR Green PCR System with HotStar Taq DNA Polymerase (Qiagen). SYBR Green I is a fluorescent dye, which enables analysis of targets without needing to synthesise target-specific labelled probes. It binds to all double stranded DNA molecules. The HotStar Taq DNA Polymerase enzyme achieves high specificity and sensitivity in the PCR, by preventing the formation of mis-primed products and primer-dimers. The reaction mixture is as detailed in table 2.8.

Table 2.8 Two-Step Real-Time PCR Reaction Setup

Component	Volume/Reaction
2x Quantitect SYBR Green PCR Master Mix	12.5µl
Target/Reference Forward Primer	0.25µl
Target/Reference Reverse Primer	0.25µl
Template cDNA (as per Table 2.5)	1µl
RNase free water	11µl
Total	**25µl**

2.5.2.3 ΔΔCt Method for qRT-PCR Data Analysis

Relative quantification was used to analyse data from real-time PCR experiments. This was achieved through use of the ΔΔCt algorithm. The house-keeping gene, GAPDH, was assumed to be uniformly and constantly expressed in all samples and compared to relative changes in target gene expression. The ΔΔCt algorithm is an approximation method relying upon various assumptions unlike the standard curve and Pfaffl methods. However, the output results are not dissimilar to other non-approximation methods and is easier to apply (152).

2.6 Western Blotting

Western blotting identifies proteins that have been size dependently separated by gel electrophoresis using specific antibodies. Total liver protein was extracted in ice-cold RIPA buffer (150nM sodium chlorise, 1% Triton-X 100, 0.5% sodium deoxycholate, 50mM Tris, pH 8.0 and 0.1% SDS) and lysate protein concentrations, determined using the copper/bicinchoninic assay (Sigma), and

standardised to 2 mg/ml by dilution into Laemmli buffer. RIPA buffer was chosen for its ability to lyse membrane-bound, nuclear and mitochondrial proteins. 20µg total protein was loaded for SDS-PAGE (Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis).

Separated proteins were transferred onto PVDF Immobilon-P membrane (Millipore, Billerica, MA) and incubated in blocking buffer for 1 hr (0.5% non-fat dehydrated milk, 0.5% TBS-Tween). Following thrice washing using wash buffer (0.5% non-fat dehydrated milk, 0.5% TBS-Tween), membranes were incubated for either an hour or overnight depending on the antibody. Antibodies against IRS-1, phospho-IRS1 (Ser307), (Upstate Biotechnology, Lake Placid, USA); IRβ (Santa Cruz Biotechnology, Santa Cruz, USA); ASMA (Sigma, UK) and Collagen Type 1 (Millipore, UK) diluted in wash buffer were applied to the blots. Subsequent incubation with secondary antibodies followed by ECL (enhanced chemiluminescence) detection allowed assessment of protein expression densitometrically using AlphaEase software (Alphaimager).

2.7 Immunohistochemistry

Immunohistochemical detection was performed on paraffin embedded sections treated with proteinase K, a primary rat anti-mouse F4/80 monoclonal antibody (1/100, Serotec) and a secondary rabbit anti-rat immunoglobulin (ImmPRESS anti-rat Ig (Peroxidase) Polymer Detection Kit, Vector Laboratories). Peroxidase

activity was demonstrated using ImmPact DAB (diaminobenzidine-based) peroxidise substrate kit (Vector Laboratories) as detailed in Table 2.9).

Table 2.9 Immunohistochemistry Protocol for Paraffin Embedded Sections

Step	Method
Deparaffinsation	In xylene followed by hydration using descending concentrations of ethanol
Antigen Retrieval	Proteinase K for 5 minutes followed by thrice washing with TBS
Endogenous Peroxidase Quenching	3% endogenous peroxidise quenching solution incubated with slides for 10 minutes at rtp.
Blocking	Blocked using ImmPact normal goat serum for 20 minutes at rtp.
Primary Antibody Incubation	Primary antibody diluted to desired concentration using 1% BSA prior to an hour incubation at rtp.
Secondary Antibody Incubation	Incubate with ImmPress reagent for 30 minutes at rtp.
Colour Development	Add 1µl of ImmPACT DAB chromogen to 1ml ImmPACT Diluent to make the DAB working solution. Add to the slide, incubating up to 20 minutes checking colour intensity under a light microscope.
Counter Staining	When desired colour intensity is reached, immerse in TBS to prevent further development. Counter stain in Vector haematoxylin QS
Dehydration	Dehydrate sections in incremental concentrations of ETOH and coverslip using VectaMount medium

2.8 Histology

Offspring liver sections were formalin fixed and paraffin embedded prior to sectioning. All sections were then stained with haematoxylin and eosin (H&E) using the following protocol:

I. Sections were immersed in filtered Harris Haematoxylin for 60 seconds
II. Sections were rinsed with water until the water remained free of dye.
III. Sections were then immersed in Eosin stain for 2 minutes.
IV. Subsequent to washing, slides were dehydrated in ascending alcohol solutions – 50%, 70%, 80%, 90% (x2) and 100% (x2).
V. To remove impurities, stained slides were soaked in xylene.
VI. Slides were coverslipped with VectaMount gel as an adhesive.

All slides stained with H&E were assessed for hepatosteatosis and inflammation, by an expert liver pathologist blinded to the identity of the groups, using the Brunt-Kleiner NAFLD Activity Score (Table 2.10). NAS is a feature based scoring system of histological lesions observed in NAFLD. A composite score > 5 is considered to be the more severe form, non-alcoholic steatohepatitis (NASH). H&E stains basophilic structures blue and cytoplasm and acidophilic structures light to dark red.

Table 2.10 Brunt-Kleiner NAFLD Activity Score

Histological Feature	Definition	Score
Steatosis	< 5%	0
	5 – 33%	1
	> 33 – 66%	2
	> 66%	3
Lobular Inflammation	No Foci	0
	< 2 foci per 200 x field	1
	2 – 4 foci per 200 x field	2
	> 4 foci per 200 x field	3
Ballooning	None	0
	Few Balloon cells	1
	Many cells/prominent ballooning	2

To assess the presence and degree of fibrosis, paraffin embedded slides were sectioned and stained with Masson's Trichrome (Histopathology Department, Royal Free Hospital, UCL) and scored by a pathologist, blind to the experimental groups (Table 2.11).

Table 2.11 Histological Scoring System for Fibrosis

Definition	Score
None	0
Perisinusoidal or Periportal	1
Persinusoidal & Periportal	2
Bridging Fibrosis	3
Cirrhosis	4

2.9 Hepatic Tissue Triglyceride Extraction

Murine whole liver tissue triglyceride content was determined using an adaptation of the Folch Method (153).

Homogenisation

a) Snap frozen liver tissue weighing between 0.01-0.05g was homogenised in a 2:1 chloroform-methanol mixture to a final dilution of 20 times the volume of the tissue. All samples were homogenised in a 2ml eppendorf with an autoclaved pestle.

b) The mixture was agitated in an orbital shaker at room temperature for 20 minutes then centrifuged at 4.7 Krpm for 30 seconds at 20˚C.

c) The supernatant was transferred to fresh tubes and the pellet was discarded.

Phase Separation

a) The crude extract was washed with 0.2ml 0.9% NaCl solution
b) The solution was weighed, vortexed, and centrifuged at 4.7 Krpm for 60 seconds at 20˚C. Consequently, the solution separated into upper phase and a lower phase without any interfacial fluff.
c) The lower phase contained 92.75% of tissue lipids. The upper phase contained gangliosides (high-molecular-weight, water-soluble glycolipids) and all non-lipid impurities dissolved.

Incubation

The lower phase was incubated at 55°C for 3 hours to allow evaporation of the chloroform and precipitation of the lipid. The lipid was weighed using digital scales accurate to at least 4 decimal places.

Reconstitution and Calculation

a) The solid lipid was reconstituted with 1ml 0.9% NaCl and heated to aid reconstitution.
b) The reconstituted solution was analysed using a Roch Modular P Analyser. Triglyceride concentration (mmol/l) was determined using enzymatic colorimetric assays (UNIMATE 5 TRIG, Roche BC1. Sussex, UK). The readout was then applied to the formula below to ascertain the triglyceride content of hepatic tissue:

$$\frac{\text{TG concentration (mmol/l) x Dilution Factor x Lipid Weight after incubation (g)}}{\text{Weight of homogenized hepatic tissue prior to lipid extraction (g)}}$$

2.10 ELISA Protocols

ELISAs (enzyme linked immunosorbent assay) were used for analysis of serum leptin (CrystalChem, Catalog #90030) and insulin (CrystalChem, Catalog #90080). Using wide range assays, the antibody-coated microplate was affixed to the frame with 95µl of sample diluent dispensed per well. 5µl of serum was

then added to the microplate, prior to incubation at 4°C, for 2 hours. The plates were washed 5 times with wash buffer before the addition of 100µl of anti-insulin enzyme conjugate per well. Following another course of incubation and washing, a further 100µl of enzyme substrate solution, per well, was dispensed.

As wide range assays (0.1-12.8ng/ml) were used, final incubation at room temperature was for 40 minutes. The enzyme reaction was stopped using a stop solution and the A_{450} wavelength measured with A_{630} values subtracted. The resultant insulin and leptin concentrations were calculated used standard curves.

2.11 Radiotelemetry

Radiotelemetric probes (TA11PA-C10, O.D 0.4mm, Data Science International, DSI) were implanted under general anaesthetic (isoflurane in O_2) at 3 months. Due to the small size and light weight of the transmitter, an indwelling catheter was inserted into either the descending aorta, carotid or femoral artery, eliminating the need for flushing and other catheter maintenance issues (Figure 2.9).

Immediately after surgery but prior to recovery from anaesthesia, the mice were given subcutaneous buprenorphine as an analgesic. Following recovery, systolic blood pressure (SBP) was measured for 1 week in freely moving

offspring by reception of data transmitted via digital telemetry (RPC-1, Data Science International, DSI) (Figure 2.9).

Figure 2.9 Radiotelemetry Equipment

PhysioTel® PA-C10 Mouse Transmitter

2.12 Mononuclear Cell (MNC) Isolation

Hepatic mononuclear cells were isolated is accordance with the protocol detailed below. Reagents and their composition are as detailed in Table 2.12.

Homogenisation

a) The supra- and infra-hepatic inferior vena cavae were isolated and the liver perfused with 5ml PBS via the portal vein.

b) The liver was then excised and homogenised in perfusate buffer containing HBSS (Ca^{2+}, Mg^{2+}), collagenase (0.01%) and DNase I (0.001%) using a stomacher (400 circulator, Seward, USA).

Incubation & Filtration

a) The homogenate was incubated at 37°C for 20 minutes in a water bath.

b) The homogenate was then filtered through 70µm cell strainer (BD falcon #352350) and centrifuged at 500rpm for 2 minutes at 4°C.

Optiprep Density Gradient Cell Separation

a) The pelleted cells were re-suspended in RPMI and layered with 24% optiprep and 1ml RPMI, a culture medium.

b) Subsequent to centrifugation, mononuclear cells were isolated at the 40/60% interface. The cells were washed once with a perfusate buffer containing HBSS (free Ca^{2+}, Mg^{2+}), BSA (0.25%) and DNase I (0.001%) and supplemented with complete culture media (RPMI, FBS (foetal bovine serum) (10%), PCN (penicillin) 100U/ml, Strep 100µg/ml, L-glutamine 200mM).

c) Cell types were determined using microscopy.

Cell counting & validation

Following cell isolation, cell viability and number were determined using a NucleoCounter (NC 100, Chemometec, Denmark) as described:

a) 100ul cell fraction was added to reagents A and B as supplied by the manufacturer to cause cell lysis as per the manufacturer's protocol.

b) Using a nucleo-cassette (chemometec, Denmark), a small volume of cell suspension was aspirated and transferred to the automatic counter.

c) Using the readout, total cell number was calculated with the formula:

Cell number readout/ml x 3 x initial cell suspension volume (ml)

d) Cell viability was determined by counting the cell fraction directly and inputting into the following formula:

100 – (Dead cell number/ml / total cell number/ml) x 100

Table 2.12 Reagents used in Mononuclear Cell Isolation

Perfusate buffer 3 (PF3):	50ml	100ml	250ml	500ml
HBSS (+Ca^{+2}, Mg^{+2})	50ml	100ml	250ml	500ml
Collagenase (0.01% final)	0.5ml	1ml	2.5ml	5.0ml
DNase I (0.001%)	50ul	0.1ml	0.25ml	0.50ml

Perfusate buffer 4 (PB4):	50ml	100ml	200ml	500ml
HBSS (free Ca^{+2}, Mg^{+2})	50ml	100ml	200ml	500ml
DNase I (0.001% final)	50ul	0.1ml	0.20ml	0.50ml
BSA (0.25% final)	1.25ml	2.5ml	5.00ml	12.5ml

Complete Cell Media:	500ml
RPMI	435ml
FBS (10%)	50ml
(PCN 100U/ml/	
Strep 100mg/ml)	10ml

L-Glutamine 200mM 5 ml

2.13 Flow Cytometric Analysis

Cell preparations (as per 2.12) were stained with CD3-FITC/NK1.1-PerCp and F4/80 clone BM8-PerCP-Cy5.5 antibodies (Biolegend), for identification of Natural Killer T (NKT) and Kupffer Cells (KC) respectively. Cells were incubated with Fc block (anti mouse CD16/32) at 4°C for 10 minutes to prevent non-specific Fc-mediated adherence of antibodies, thereby assuring that any staining is due to the interaction between the antigen portion of the desired antibody and the cell (Figure 2.10). This was followed by addition of the antibodies of interest for 20 minutes.

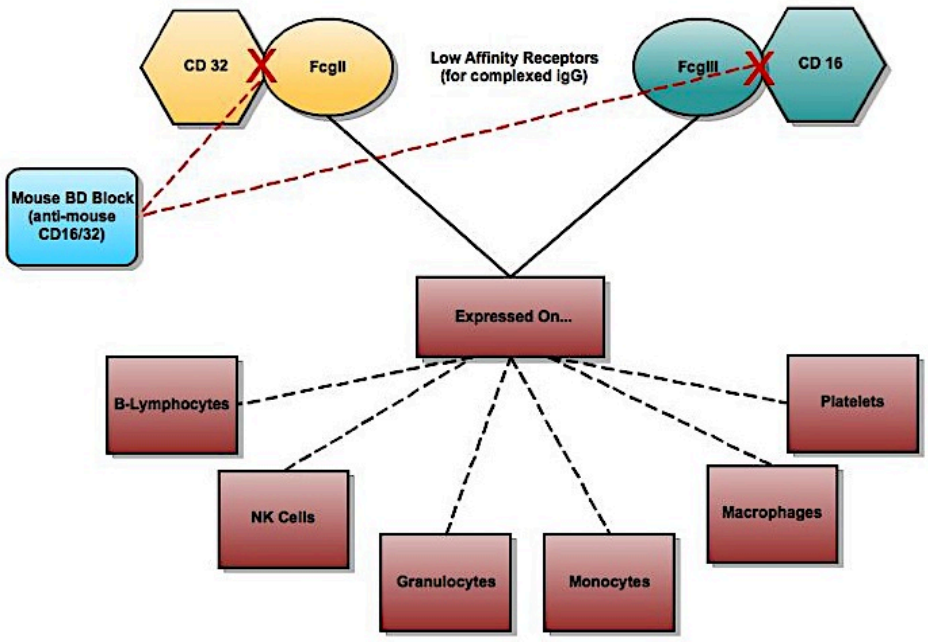


Figure 2.10 Mechanism of Action of BD Fc Block in Immunophenotyping

After incubation, 1ml FACS buffer (Biolegend) was added and the vials centrifuged at 2000rpm for 5 minutes. Cells were then re-suspended in a final volume of 100µl FACS buffer and analysed by flow cytometry (BD LSR II). Quantification of data was performed using FlowJo 5.6.1. The relative fluorescence chart for the conjugates used is tabulated below (Table 2.13).

Table 2.13 BD Fluorescence Chart

Relative Brightness	Image	Regent	Filter
Bright		Per-CP-Cy5.5	695/40
Moderate		FITC	530/30
Dim		Per-CP	695/40

2.14 Kupffer Cell Phagocytosis

Fluorescent microspheres are commonly used in phagocytosis studies. For the purposes of the KC phagocytic assay, Fluoresbrite@YG Microspheres (1.00µm) were used. These yellow green microspheres have excitation and emission

spectra similar to FITC and one of the brightest commercially available microspheres available (Figure 2.11).

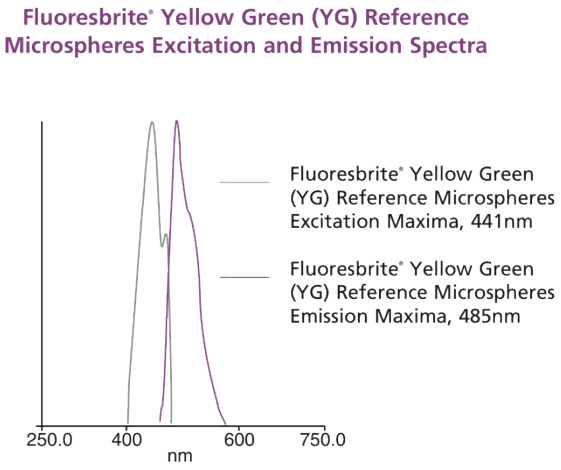


Figure 2.11 Fluoresbrite YG Microshperes Excitation and Emission Spectra

Microspheres were incubated with total MNC suspension for 20 minutes at 37°C. The reaction was then stopped by addition of 2ml ice cold PBS. Following centrifugation at 2000rpm for 5 minutes at 5°C, the cell preparations were washed and incubated with TruStain FcX (anti-mouse CD16/32) and stained with F4/80 antibody to identify the Kupffer cell population within the MNC fraction. The cell suspension was then reconstituted in 100µl of FACS buffer and subsequently analysed using flow cytometry as detailed in 2.13.

2.15 Kupffer Cell Reactive Oxygen Species (ROS) Detection

KC derived ROS was assayed using the Total ROS Detection Kit (ENZO-51011) (Table 2.14 and 2.15). in summary, cell preparations were stimulated using 20µg/ml LPS (E.coli 0111:B4, Sigma Aldrich) and incubated for 30 minutes at 37°C. Samples were then washed and centrifuged at 2000rpm for 5 minutes at 4°C. The pelleted cells were re-suspended in ROS detection solution, incubated with TruStain FcX (anti-mouse CD16/32) and stained with F4/80 antibody to identify the KC population in the MNC fraction (AbD Serotec). The cell suspension was then reconstituted in 100µl of FACS buffer and subsequently analysed using flow cytometry as detailed in 2.13.

Table 2.14 Total ROS Detection Kit Reagents

Reagent	Quantity
Oxidative Stress Detection Reagent (Green)	300 nmol
ROS Inducer (Pyocyanin)	1 µmol
ROS Inhibitor (N-actetyl-L-cysteine)	2 x 10 mg
Wash Buffer Salts	1 pack

Table 2.15 Total ROS Detection Kit Reagent Preparation

Reagent Preparations
Positive Control • Reconstitute ROS Inducer in 100µL anhydrous DMF to yield a 10mM stock solution. • Final concentration of 200-500M as per manufacturer's recommendations • ROS induction occurs within 20-30 minutes upon pyocyanin treatment
Negative Control • Reconstitute ROS inhibitor in 123L of deionised water to yield a 0.5M stock • N-acetyl-L-cysteine is not readily soluble and so requires vortexing • Final concentration of 5mM as per manufacturer's recommendation • Endogenous fluorescence of untreated cells determined in advance of assay
Wash Buffer • Prepare 1x Wash Buffer by dissolving 1 pack in 1L of deionised water • Warm to room temperature before use
ROS Detection Solution • For every 10mL of 1xWash Buffer or culture medium, add 2µL of Oxidative Stress Detection Reagent and mix gently
Cell Preparations • Ensure cells are in the log phase of growth before use
Preparation for Flow Cytometry (Suspension Cells) • Cell density not to exceed 1x106 cells/mL • Induce the cells with an experimental test agent • A separate positive control sample should be treated with the ROS inducer • A negative control sample should be established by treatment with the ROS Inhibitor

2.16 Statistical Analysis

2.16.1 Unpaired Student's t-test

The unpaired student's t-test was used to compare the mean differences between 2 independent but identically distributed data sets. Although randomisation is not a prerequisite for this test, rodents were still randomly assigned to either the control or treatment arm. The paired student's t-test was considered unsuitable as repeated measures, from the same sample, were not being analysed. All data are expressed as mean ± SEM with $p < 0.05$ assigned statistical significance.

2.16.2 One-Way Analysis of Variance (ANOVA)

The one-way ANOVA was used to compare means of two or more data sets that are independent but identically distributed. One-way ANOVA tests the null hypothesis that the samples in 2 or more groups are from the same population. All data are expressed as mean ± SEM with a $p < 0.05$ assigned statistical significance.

2.16.3 Factorial or Two-Way Analysis of Variance (ANOVA)

A two-way ANOVA was used to study the interaction effects between two or more variable factors across the data sets. $p < 0.05$ was considered statistically significant.

2.16.4 Cosinor Analysis

In living organisms, there are several phenomena that are always present and measurable although variable as a function of time. As such, the magnitude of any given phenomena reaches that same level following a given period of time, an example being 24 hours (circadian). Effectively a rhythm is produced which can be quantitatively assessed using cosinor analysis.

Cosinor analysis quantifies the best fitting sinusoidal wave in parameters that represent rhythms within a circadian period, thereby evaluating the period for its mean level, extent of oscillation and timing of the oscillatory crest. Again, $p < 0.05$ was considered statistically significant.

3 Investigation of Dysmetabolism and NAFLD in Offspring Exposed to Maternal Obesity in Pregnancy and Lactation

3. Investigation of dysmetabolism and NAFLD in offspring exposed to maternal obesity in pregnancy and lactation

3.1 Introduction

Non-alcoholic fatty liver disease, NAFLD, is presently the commonest cause of chronic liver dysfunction in affluent countries. The spectrum of NAFLD ranges from hepatosteatosis (fatty liver), through non-alcoholic steatohepatitis (NASH, i.e. fat with inflammation) to fibrosis, cirrhosis and potentially hepatocellular carcinoma. The predominant cause of NAFLD is obesity. Its pathogenesis implicates increased oxidant stress with consequent activation of matrix producing cells and propagation of fibrogenesis (154). Some 23-34% of the United States population is estimated to have NAFLD, and about 2.5% the more severe form of the disease, NASH (155).

The rising rates of obesity and NAFLD may be partially explained by increasing availability of cheap energy dense foods, perhaps compounded by a developmental programming effect of maternal obesity (156). In support of this, obesity amongst women of reproductive age is similarly increasing with some 31.5% of women, aged 20-39 years, in the USA reported to be obese (6).

Earlier studies have elicited relationships between *in utero* under-nutrition, low birthweight and adult hypertension, type 2 diabetes and metabolic dysfunction (157-159), leading to the hypothesis that nutritional insults during early

development may induce permanent alterations in plastic regulatory systems to induce adult disease (157). Such physiological perturbations are the consequences of developmental programming. Developmental programming is the process whereby a stimulus or insult during a critical period of development initiates permanent structural and/or physiological changes as protective adaptive responses (160). The developmental over-nutrition hypothesis, conversely, suggests that maternal obesity predisposes offspring to obesity and metabolic dysfunction in adulthood (161-163).

In support of this developmental over-nutrition hypothesis, reports of elevated cholesterol and triglyceride concentrations in the plasma of obese pregnant women have been positively correlated with lipid accumulation in offspring arterial vasculature, early fatty streak development and advanced atherosclerosis in young adults (164). Additionally, epidemiological studies have reported impairment of glucose-insulin metabolism with accompanying hypertension in offspring exposed to high protein and fat intake in late gestation (165, 166).

Such epidemiological findings have led to the development of experimental models of maternal over-nutrition to understand pathogenesis, multiple adverse sequelae and determine involved mechanisms. A growing body of evidence from these models of maternal diet induced obesity suggest a programmed dysmetabolic phenotype in offspring. More specifically, reports of insulin resistance, hyper-leptinaemia and abnormal glucose homeostasis have

been documented in offspring born to obese mothers (167, 168). Furthermore, cardiovascular perturbations, including hypertension and vascular dysfunction have been reported in offspring of obese mothers (169, 170).

The aims therefore, were to test the hypothesis that maternal diet induced obesity in pregnancy and lactation transmits a predisposition to offspring dysmetabolism and NAFLD in adulthood and determine involved mechanisms.

3.2 Methods

3.2.1 Animal Experimentation

Female C57BL/6J mice (n = 20 per group) (Charles River Laboratories, UK), of first order parity, approximately 100 days old were allowed 7 days to habituate following transfer to the facility and fed standard laboratory chow (RM1, Special Dietary Services, energy 3.5 kcal/g) *ad libitum*.

Following acclimatisation, female mice were randomised to either a control or experimental group and fed standard chow or a semi-synthetic energy-rich, highly palatable obesogenic diet (Special Dietary Services, energy 4.5 kcal/g) supplemented with sweetened condensed milk (Nestle) admixed with mineral mix (AIN93G, Special Dietary Services, 125 mg/pot) *ad libitum*.

Dams were maintained on their respective diets for 6-8 weeks. Upon attainment of an average 30% increase in body weight of the experimental

group, mice were entered into the breeding protocol. All animals were treated in accordance with The Animals (Scientific Procedures) Act, UK, 1986 guidelines.

Pregnant dams were continued on their pre-conception diets throughout pregnancy and lactation. During pregnancy, maternal weight and dietary intake were recorded weekly. Dams were allowed to deliver spontaneously and litter size standardised. All female offspring (n = 10) born to lean (OffCon) and obese (OffOb) dams were weaned onto standard chow at 3 weeks postpartum. Markers of dysmetabolism and NAFLD were assayed at 3 months of age (Figure 2.3).

3.2.2 Offspring Calorific Intake and Body Weight

Pelleted food intake was measured weekly to assess calorific intake. Bodyweight of offspring aged 3 months was measured prior to sacrificing using a schedule-1 method. Weights were recorded using a balance accurate to 4 decimal places.

3.2.3 Tissue Collection

Offspring liver tissue was differentially preserved for use in gene expression analysis, protein and lipid extraction and histology. Briefly, whole liver tissue was snap frozen in liquid nitrogen or dissected using middle orientation and stored in 10% neutral buffered formalin prior to paraffin embedding. Offspring blood was collected via cardiac puncture or inferior vena cava cannulation using

heparinised syringes. The blood was centrifuged at 3000 x g for 10 minutes at 4˚C and decanted plasma stored at -20˚C until biochemical analysis.

3.2.4 Serum Analysis

ELISAs were used for analysis of serum leptin (Biovender R&D 1301) and insulin (CrystalChem, Catalog #90080). Wide range assays were used for adequate detection. Aspartate transaminase (AST) was measured by the local clinical biochemistry department.

3.2.5 Radiotelemetry

Remote radiotelemetric probes (TA11PA-C10, O.D 0.4 mm, Data Science International) were implanted under general anaesthetic (isofluorane in O_2) and an indwelling catheter was inserted into the left carotid artery. Following recovery, offspring were subjected to 30 minutes restraint and 120 minutes recovery during which time, the systolic blood pressure (SBP) was measured and expressed as a change from baseline.

3.2.6 Liver Tissue Triglyceride

Whole liver tissue triglyceride was determined by an adaptation of the Folch Method using an enzymatic colorimetric assay (UNIMATE 5 TRIG, Roche BC1. Sussex, UK). Approximately 0.05g of liver tissue per sample was used and all results normalised to sample quantity.

3.2.7 Gene Expression of Liver Injury Markers

Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was performed using SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* High Fidelity (Invitrogen Life Technologies). Gene specific primers were designed for interleukin-6 (Il-6) and tumour necrosis factor-α (TNF-α). Expression of target genes was normalised to GAPDH expression.

3.2.8 Protein Expression of Insulin Signalling Molecules

Total liver protein was extracted in ice-cold RIPA buffer (150nM sodium chlorise, 1% Triton-X 100, 0.5% sodium deoxycholate, 50mM Tris, pH 8.0 and 0.1% SDS), lysate protein concentrations, determined using the copper/bicinchoninic assay (Sigma), and standardised to 2 mg/ml by dilution into Laemmli buffer. 20µg total protein was loaded for SDS-PAGE followed by overnight incubation with antibody against IRS-1, phospho-IRS1 (Ser307), IRβ, (Santa Cruz Biotechnology, Santa Cruz, USA). Protein expression was assessed densitometrically using AlphaEase software (AlphaImager).

3.2.9 Liver Histology

Offspring liver sections at 3 months were stained with haematoxylin and eosin (H&E) and assessed for steatosis, by an expert liver pathologist blinded to the identity of the groups. Steatosis was characterised into 4 groups, extrapolated from the steatosis scoring methods as per the Brunt-Kleiner NAFLD Activity Score (Table 3.1).

Table 3.1 Histological Assessment of Steatosis

Parameter	Score
< 5%	0
5 – 33%	1
> 33 – 66%	2
> 66%	3

3.2.10 Breast Milk Leptin

Breast milk samples were obtained from suckling dams after anaesthesia via direct aspiration of the mammary glands. Leptin content was determined using ELISA (Biovender R&D 1301).

3.2.11 Statistical Analysis

Multiple comparisons on a single data set were performed using ANOVA and expressed as mean ± SEM unless otherwise stated. $p < 0.05$ was regarded as significant. Sample size per group; n = 5, 1 from each litter.

3.3 Results

3.3.1 Exposure to maternal obesity during gestation and lactation does not influence offspring adiposity and calorific intake at 3 months

At 3 months post-partum, offspring exposed to maternal diet induced obesity throughout gestation and lactation had similar bodyweights as offspring of lean dams (OffCon; 21.9 ± 0.45 v. OffOb; 22.4 ± 0.21 gm). Corroboratively, calorific intake between these two groups was comparable (OffCon; 70.95 ± 3.51 v. OffOb; 78.87 ± 4.10 kcal/gm), as was serum leptin (OffCon; 1036 ± 46.02 v.

OffOb; 1266 ± 129.60 pg/ml) (Figure 3.1). It is important to note that post-weaning, all offspring were maintained on a standard chow to permit investigation of the effects of maternal obesity during periods of developmental plasticity only. In summary therefore, at 3 months post-partum, no statistically significant difference was observed between offspring of OffCon and OffOb in body weights, calorific intake and serum leptin.

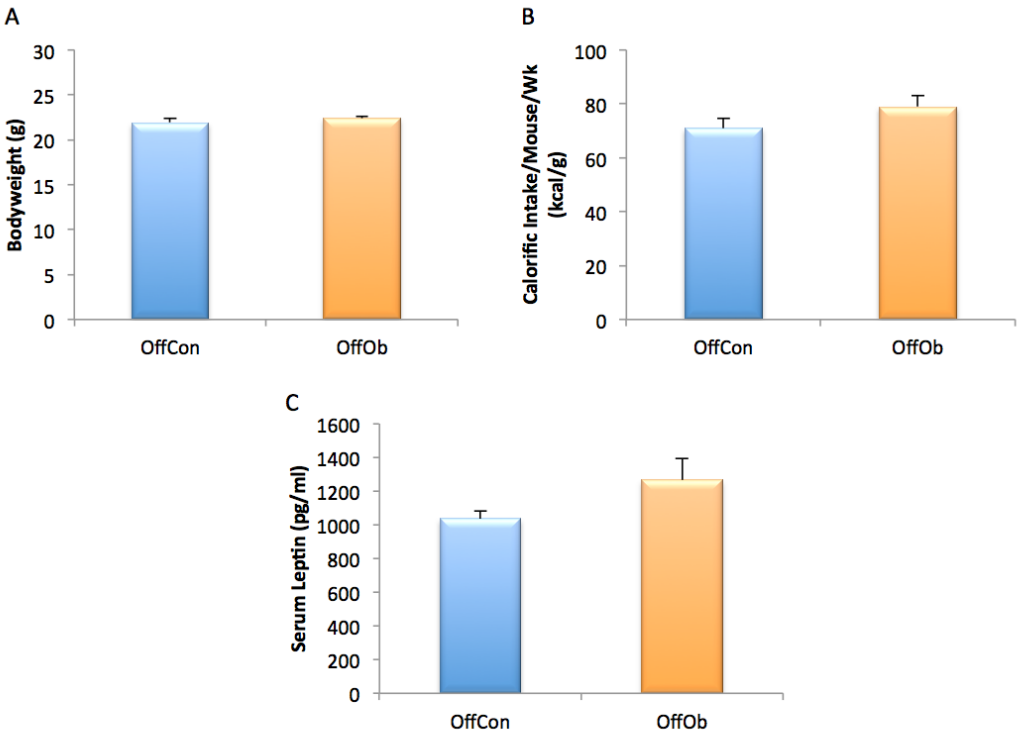


Figure 3.1 Offspring Adiposity and Calorific Intake at 3 months post-partum: (A) Bodyweight, (B) Calorific Intake per week and (C) Serum Leptin Concentrations. A similar pattern was observed in all measured indices of adiposity, with no statistically significant differences observed (unpaired student's t-test, $p < 0.05$) between offspring of lean suckled by lean (OffCon)

and offspring of obese suckled by obese (OffOb). n = 4 -6/group, values shown are mean ± SEM.

3.3.2 Offspring exposure to maternal obesity during gestation and lactation induces hyper-insulinaemia and increased SNS tone at 3 months

Serum insulin concentration was measured as a biochemical indicator of metabolic perturbation. Insulin levels were significantly increased in offspring exposed to maternal obesity compared to offspring of lean dams (OffCon; 0.1533 ± 0.001 v. OffOb; 0.1267 ± 0.083 µg/l). Additionally, the mean nocturnal systolic blood pressure was raised alongside the response to restraint stress in OffOb, indicating increased sympathetic nervous system (SNS) tone (Figure 3.2).

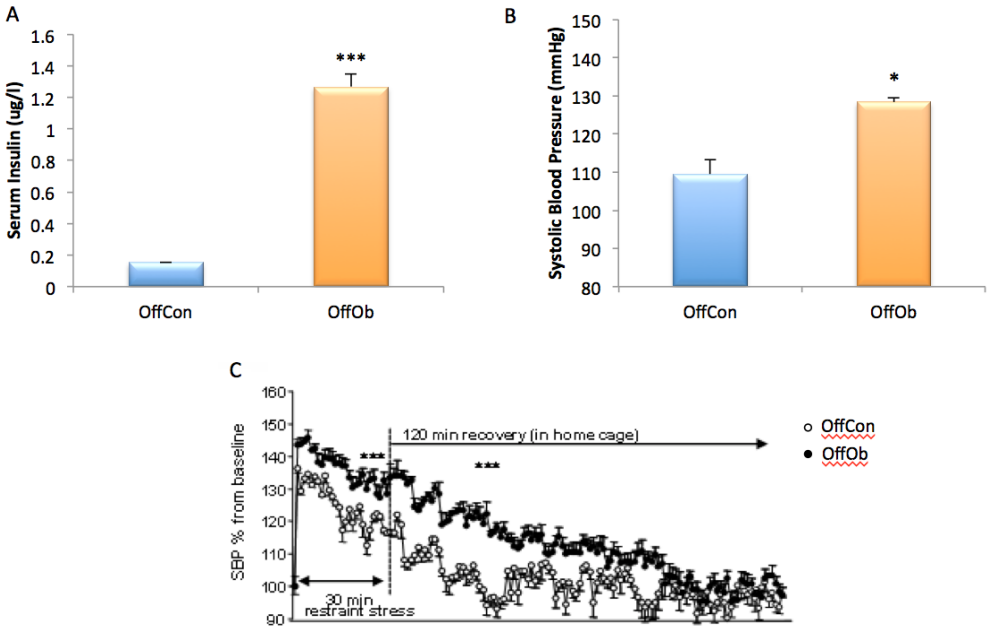


Figure 3.2 Serum Insulin and Systolic Blood Pressure responses at 3 months post-partum: (A) Serum Insulin Concentrations, (B) Nocturnal Systolic Blood

Pressure and (C) Cardiovascular Reactivity to Stress. Offspring of obese dams displayed hyper-insulinaemia and raised SNS tone compared to controls. Offspring of lean suckled by lean (OffCon) and offspring of obese suckled by obese (OffOb); n = 4 – 6/group, values shown as mean ± SEM; * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, unpaired student's t-test.

3.3.3 Offspring exposure to maternal obesity during gestation and lactation reduces hepatic insulin signalling at 3 months

Expression of IRβ in the livers of offspring exposed to maternal obesity throughout gestation and lactation was not altered compared to offspring of lean dams. However, expression of IRS-1 protein was significantly decreased in the offspring of obese dams, while phosphorylation of IRS-1 at Ser 307, an inhibitory signal of insulin action was significantly increased. Reduced liver IRS expression and hyper-phosphorylation of IRS serine residues are key factors in the development of insulin resistance and type 2 diabetes (Figure 3.3).

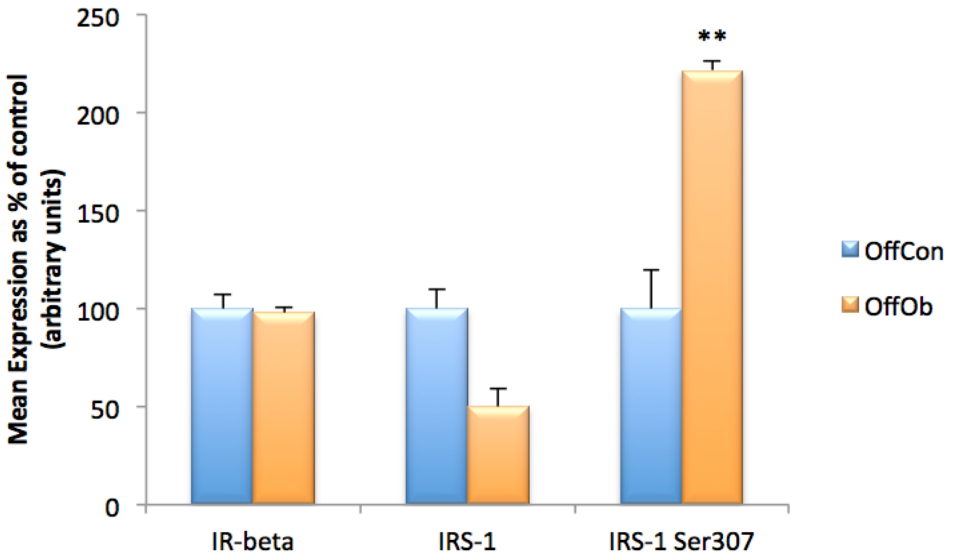


Figure 3.3 Impaired hepatic insulin signalling at 3 months post-partum: Maternal obesity led to a post receptor defect in insulin signalling in offspring. IR-beta was unaffected, IRS-1 down-regulated and IRS-1 Ser307 up-regulated. Offspring of lean suckled by lean (OffCon) and offspring of obese suckled by obese (OffOb); n = 4 – 6/group, values are expressed as a % of control ± SEM; * $p < 0.05$, ** $p < 0.001$; unpaired student's t-test.

3.3.4 Offspring exposure to maternal obesity during gestation and lactation induces hepatosteatosis at 3 months

Histological evidence of hepatic macrovesicular steatosis was observed in OffOb compared to OffCon at 3 months post-partum. Morphometrical quantification of the stained surface was performed on microphotographs taken from ten random field sections per liver, corroborating these findings. Additionally, liver tissue triglyceride content was raised, although not statistically significant (OffCon; 4.76 ± 0.89 v. OffOb; 7.22 ± 0.53 mmol/l) (Figure 3.4).

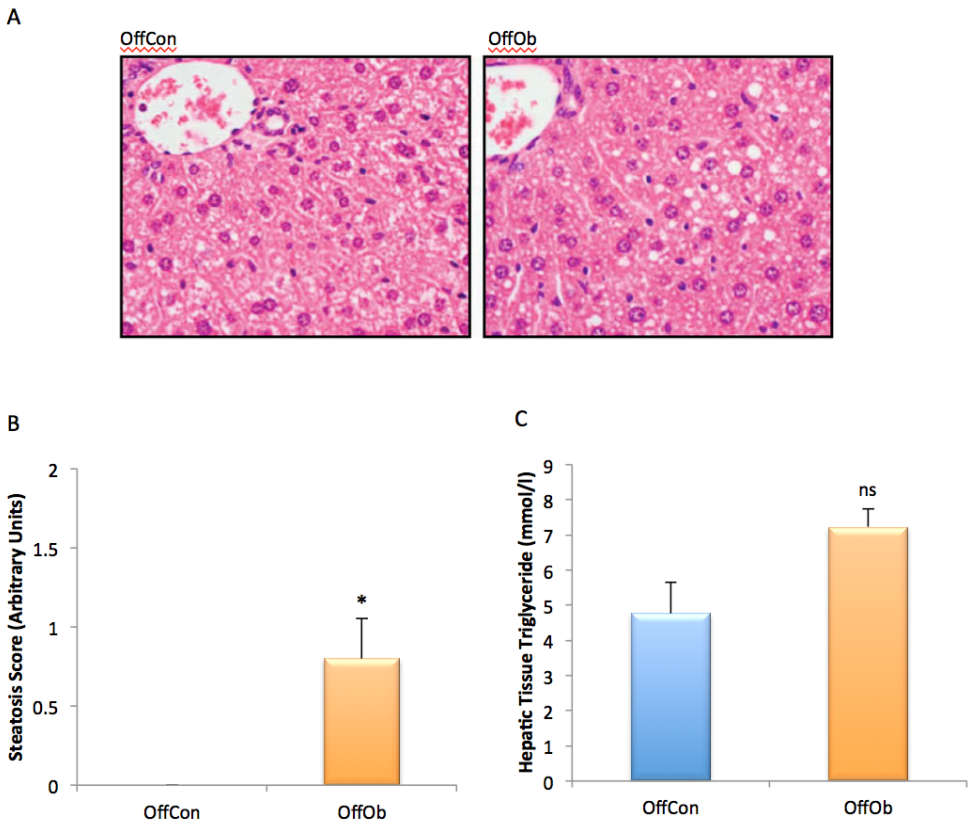


Figure 3.4 Histological and Biochemical Evidence of Hepatosteatosis at 3 months post-partum: (A) Representative H&E sections, (B) Steatosis Score and (C) Hepatic Tissue Triglyceride. Maternal obesity led to offspring hepatosteatosis compared to control offspring, although hepatic triglyceride content was not statistically significant between the groups. Offspring of lean suckled by lean (OffCon) and offspring of obese suckled by obese (OffOb); n = 4 – 6/group, values shown as mean ± SEM; * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$; unpaired student's t-test.

3.3.5 Offspring exposure to maternal obesity during gestation and lactation induces moderate liver injury at 3 months

Serum aspartate aminotransaminase (AST), a putative biochemical indicator of liver injury, was significantly raised in offspring exposed to maternal obesity compared to offspring of lean dams (OffCon; 214.8 ± 21.0 v. OffOb; 416.7 ± 11.5 IU/L). Additionally, relative mRNA expression of IL-6 and TNF-α, cytokines implicated in NAFLD pathogenesis, was raised in OffOb compared with OffCon (Figure 3.5).

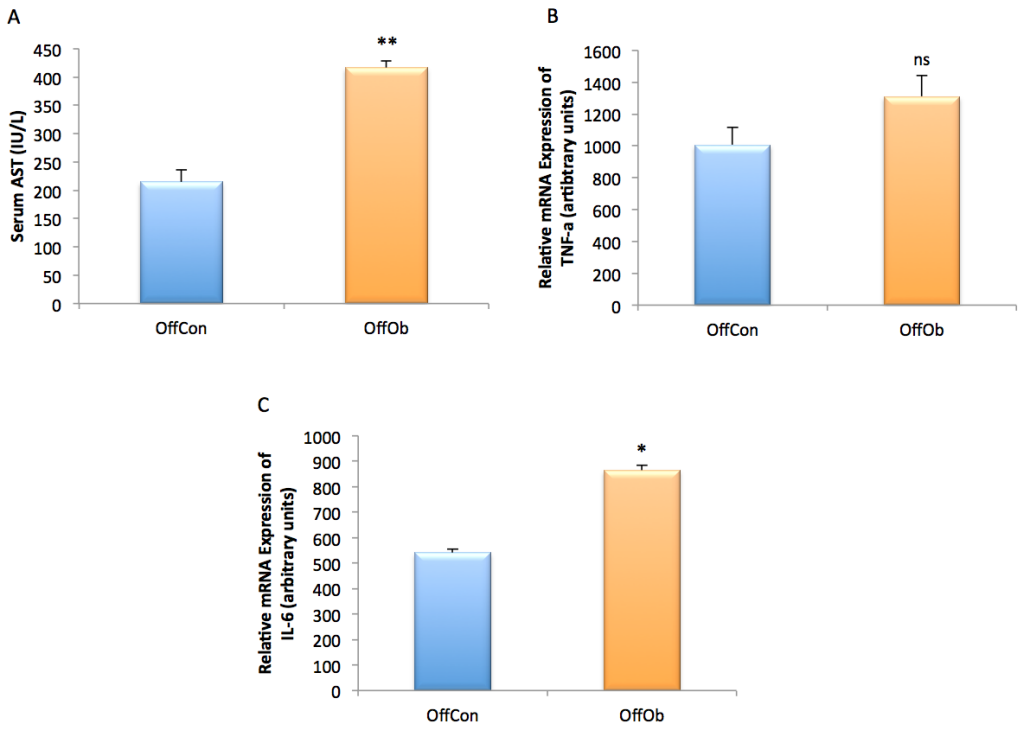


Figure 3.5 Biochemical Evidence of Liver Injury at 3 months post-partum: (A) Serum AST, (B) mRNA TNFα expression and (C) mRNA Il-6 expression. Maternal obesity led to up-regulation of hepatic pro-inflammatory markers and, AST, an indicator of liver injury compared to control offspring. Offspring of lean

suckled by lean (OffCon) and offspring of obese suckled by obese (OffOb); n = 4 – 6/group, values shown as mean ± SEM; * $p < 0.05$, ** $p < 0.001$; unpaired student's t-test.

3.3.6 Breast milk leptin as a mediator of programmed offspring dysmetabolism and NAFLD

Mechanistically, it was thought that the hyperleptinaemic status of the obese mother during gestation and lactation could alter physiological functioning of the developing offspring. In order to investigate this possibility, breast milk content of leptin in the obese and lean dams were measured and found to be markedly raised in OffOb (OffCon; 3527 ± 142.3 v. OffOb; 36468 ± 5082.4 pg/ml) (Figure 3.6).

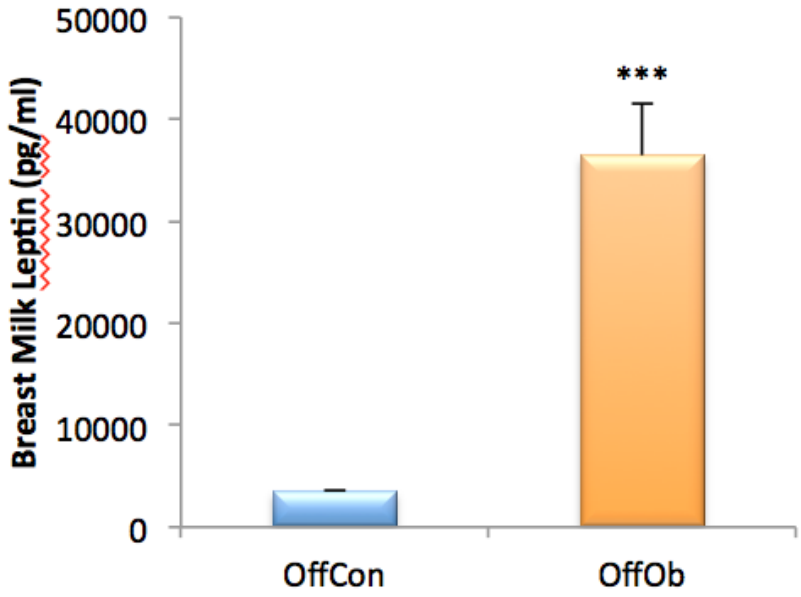


Figure 3.6 Maternal Breast Milk Leptin: Breast milk leptin was raised in obese dams compared to lean dams. Offspring of lean suckled by lean (OffCon) and

offspring of obese suckled by obese (OffOb); n = 4 – 6/group, values shown as mean ± SEM; *** $p < 0.0001$; unpaired student's t-test.

3.4 Discussion

This study has demonstrated that offspring exposure to maternal obesity in infancy induces dysmetabolism with priming of NAFLD in adulthood. This was evidenced by hyper-insulinaemia and impaired insulin signalling, hypertension, raised SNS tone, increased serum AST and up-regulation of pro-inflammatory cytokines implicated in NAFLD aetiopathogenesis.

3.4.1 Offspring Adiposity and Calorific Intake

Adiposity remained unaffected in female offspring exposed to maternal obesity at 3 months as calorific intake, bodyweight and the adipokine, leptin, were all similar to control values. Conversely, it has been reported that offspring anthropometry in those exposed to maternal obesity is significantly adverse compared to offspring of lean dams (35). These readouts however, are not directly comparable as both male and female offspring were collectively represented and as such, incorporate gender influences of programming by maternal obesity. Here, to exclude any potential gender induced variability, the effects of programming by maternal obesity on hepatic outcome in either female and/or male offspring have been investigated separately. However, in support of the aforementioned findings, it has been previously reported that offspring adiposity in those exposed to maternal obesity only become apparent

at 1 year in rodents (14). It may therefore be argued that investigation of offspring adiposity at 3 months is experimentally premature.

Irrespective, obesity is considered a fundamental criterion in the characterisation of the dysmetabolic syndrome although adiposity is a stronger correlate in developmental programming (14). Epidemiologically, a longitudinal cohort study of children aged up to 11 years has reported an increased odds ratio for development of the dysmetabolic syndrome in those born large for gestational age (18). Despite the association between overt adiposity and dysmetabolism, it is shown here, for the first time, that maternal obesity programs offspring dysmetabolism and hepatic injury independent of obesity. So therefore, the observed phenotype is not a secondary consequence of programmed offspring obesity and hyperphagia, but rather a direct effect of programming on the liver by maternal obesity.

3.4.2 Offspring Dysmetabolism

Hyper-insulinaemia was observed in OffOb compared to OffCon. It is thought that the increased fetal nutrient supply from exposure to maternal obesity during development plasticity impairs glucose induced insulin release from pancreatic ß-cells through enlargement of secretory granules (37). Consequently, an imbalance in glucose and insulin homeostasis evokes abnormally higher levels of insulin in adversely exposed offspring. Similar findings have been reproduced in rodent models of maternal over-nutrition,

whereby offspring born to and suckled by an obese dam have significantly elevated levels of insulin compared to controls (35, 36).

3.4.3 Offspring Hypertension

Offspring exposure to maternal obesity induced raised systolic blood pressure compared to control offspring. These findings are corroborated in similar rodent models of developmental programming by maternal obesity (35, 40). It is thought that perturbed cardiovascular function is due to sympathetic activation, altered vascular responses, hormonal changes and elevation of inflammatory mediators (171). It has been suggested that exposure to maternal obesity accelerates maturation and permanently up-regulates the hypothalamo-pituitary-adrenal (HPA) axis in rodents. Consequently, disruption of the offspring endocrine status induces metabolic dysfunction and raised SNS tone (32).

3.4.4 Offspring Liver Injury

The current paradigm for the pathogenesis of NAFLD, invokes insulin resistance as the primary driver for steatosis in the livers of obese individuals (172), with IL-6 and TNF-α involved in the inflammatory responses (173). Increased hepatic expression of these inflammatory cytokines could also be related to the observed altered insulin signalling observed in OffOb, as they induce an increase in Ser 307 phosphorylation on IRS-1. It is shown here that OffOb insulin levels are raised with evidence of insulin resistance as demonstrated by a reduction of

IRS-1 in the presence of normal levels of IR-β (173, 174).

Serum AST was significantly raised in OffOb compared to OffCon, indicating the presence of liver injury. Although limited studies examining the effects of maternal over-nutrition on development of liver injury exist, it is accepted that dysmetabolism precedes liver injury which is consistent with the present findings (36).

3.4.5 Offspring Hepatosteatosis

Hepatic tissue triglyceride content was raised in offspring exposed to maternal obesity compared to controls, although the difference was not statistically significant. Mild hepatosteatosis was evidenced by histological assessment and quantification. Given that hepatic triglycerides originate as non-esterified fatty acids, a product of adipose tissue, it is in keeping with the recorded bodyweights (175). These findings are the first reports of quantified hepatosteatosis in NAFLD programmed by maternal obesity, although associations between offspring exposure to maternal obesity and serum cholesterol and triglycerides have been reported (35, 64). The observed insulin resistance may be mechanistically responsible for the ensuing hepatosteatosis through increased lipolysis and saturation of the mitochondrial ß-oxidation pathway, generating reactive oxygen species; or through enhancing de novo hepatic lipogenesis through promotion of glycolysis (64, 103).

3.4.6 Breast Milk Derived Leptin

This experimental protocol involved weaning all offspring onto standard chow ensuring, that any phenotypic changes were the result of maternal nutritional differences during pregnancy and lactation only. The findings here confirm that the observed development of the dysmetabolic and NAFLD phenotype in offspring of obese dams is driven by exposure to maternal obesity, with maternal breast milk factors, being possible mechanistic candidates via their inducing this phenotype, through effects on plastic central neonatal energy homeostasis centres (176).

Breast milk leptin in obese dams has been shown here to be elevated compared to that in lean dams. Moreover, oral leptin is absorbed by the neonatal stomach (177), is biologically active and involved in energy homeostasis (59). It is plausible, therefore, that exposure of the developing neonatal hypothalamic appetite regulating centres, to raised levels of absorbed breast milk leptin mediates the observed phenotype by engendering a leptin resistant state to further promote hyper-insulinaemia (178). Given the technical difficulties involved in extracting mouse breast milk leptin, its role in developmental programming has not been further delineated. However, the role of leptin has been shown to alter energy homeostatic mechanisms through attenuated leptin induced appetite suppression and signalling in a similar experimental paradigm involving rats with hyperphagic and dysmetabolic traits (179).

3.5 Conclusion

In conclusion, offspring exposure to maternal obesity during gestation and lactation programs a dysmetabolic and NAFLD phenotype, perhaps through the effects of breast milk leptin on plastic neonatal hypothalamic centres or perturbed hepatic insulin signalling. Therefore, developmental programming may be involved in the rising rates of NAFLD. The critical periods of neonatal development, whether *in utero* or lactation, responsible for the observed phenotype, remains unknown. Chapter 4 investigates the relative contributions of the *in utero* and lactation periods on the development of offspring dysmetabolism and NAFLD, in an attempt to better understand the pathophysiological pathways involved in liver disease programmed by maternal obesity.

4 Investigation of Dysmetabolism and NAFLD in Offspring Exposed to Maternal Obesity During Gestation or Lactation:

Identification of the Critical Developmental Period Responsible for Observed Phenotypes

4. Investigation of dysmetabolism and NAFLD in offspring exposed to maternal obesity during gestation or lactation – identification of the critical developmental period responsible for observed phenotypes

4.1 Introduction

Rising rates of obesity and non-alcoholic fatty liver disease, NAFLD may be partially explained by the increasing availability of cheap energy dense foods, perhaps compounded by a developmental programming effect of maternal obesity (51, 64, 180). NAFLD is now the commonest cause of chronic liver disease in affluent countries, with 23-34% of the US population estimated to have NAFLD, and about 2.5% the more severe form of the disease, non-alcoholic steatohepatitis (NASH) (155). Obesity amongst women of reproductive age is similarly rising with some 31.5% of women, aged 20-39 years, in the USA reported to be obese (6).

Earlier studies elicited relationships between *in utero* mal-nutrition and adult onset metabolic and cardiovascular dysfunction (5, 20, 181), leading to the hypothesis that nutritional insults during early development may induce permanent alterations in plastic regulatory systems to induce adult disease (157). It has now been shown for the first time that maternal obesity predisposes offspring to metabolic dysfunction with induction of NAFLD in adulthood, as described in Chapter 3 (51, 182). These findings were subsequently corroborated in a similar experimental paradigm (64). The

relative contributions of the gestation and lactation periods to the programmed phenotype are, however, unknown. Standard rodent models of programming, as in Chapter 3, involve dams fed an obesogenic diet throughout gestation and lactation such that readouts in offspring are a reflection of the interaction between both developmental periods. By cross-fostering offspring born to lean dams to be suckled by obese dams, in the immediate post-partum period, and vice versa, allows an investigation of the independent roles of the *in utero* and *ex utero* environment, on the observed adult phenotype.

The importance of the post-natal environment in influencing offspring obesity and insulin resistance has been demonstrated in a cross-fostering experimental paradigm involving rats. It has previously been reported that offspring born to lean dams but cross-fostered to genetically obese dams to suckle, programs for obesity and insulin resistance in adulthood. Conversely, offspring born to diet induced obese dams, but suckled by a lean dam maintained on a low fat diet, improved insulin resistance in the context of persisting obesity (52).

Reducing litter size post-partum, to increase food availability during lactation, has also provided evidence of increased obesity and dysmetabolism risk in offspring. In an attempt to investigate the long term consequences of early post-natal over-feeding, normal litter size of Wistar rats (n = 10) was reduced to 3 pups per dam during the lactation period. These offspring displayed hyperphagia, hypertension and impaired glucose tolerance in adulthood (53) as a consequence of alterations to the hypothalamic energy circuitry (54).

Additionally, it has been suggested that offspring exposure to over-nutrition in lactation is trans-generational as rodents fed carbohydrate rich formula milk during this period develop adult onset obesity, with transmission to the F2 progeny despite consumption of a standard chow diet post-weaning (183). Therefore, it could be suggested that the lactation period is critical and that changes during this period may compound or even over-ride genetic and pre-natal factors to determine the eventual offspring metabolic and hepatic phenotype.

Insulin resistance, as the initiating defect in the pathogenesis of NAFLD (172), was assayed alongside other implicated mediators such as tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (103). Consequent activation of matrix producing cells and propagation of fibrogenesis can ensue. Therefore, markers of hepatic fibrogenesis and indices of sympathetic nervous system (SNS) activation, known to regulate hepatic fibrosis in NAFLD (138), were also investigated.

The aim in this current series of experiments was, therefore, to test the hypothesis that maternal obesity in either gestation or lactation transmits varying predispositions to offspring dysmetabolism and NAFLD in adulthood and to then identify the most critical developmental period.

4.2 Methods

4.2.1 Animal Experimentation

Female C57BL/6J mice (n = 20 per group) (Charles River Laboratories, UK), of first order parity, approximately 100 days old were allowed 7 days to habituate following transfer to the facility and fed standard chow RM1 (7% simple sugars, 3% fat, 50% polysaccharide, 15% protein [w/w] RM1, Special Dietary Services, energy 3.5 kcal/g) *ad libitum*.

Following acclimatisation, the dams were randomised to either a control or experimental group and fed standard chow or a semi-synthetic energy-rich, highly palatable obesogenic diet (10% simple sugars, 20% animal lard 28% polysaccharide, 23% protein [w/w], Special Dietary Services, energy 4.5 kcal/g) supplemented with sweetened condensed milk (approx 55% simple sugar, 8% fat, 8% protein, w/w, Nestle) admixed with mineral mix (AIN93G, Special Dietary Services, 125 mg/pot) *ad libitum*.

Dams were maintained on their respective diets for 6-8 weeks and macro-nutrients and calorific intake were calculated from measured daily intake of pellets and milk (approx 16% fat, 33% simple sugars, 15% protein, energy 4.0kcal/g). Upon attainment of an average 30% increase in body weight of the experimental group, mice were entered into the breeding protocol. Males from the same litter were selected to minimise genetic variability. All animals were

treated in accordance with The Animals (Scientific Procedures) Act, UK, 1986 guidelines.

Day 0 of pregnancy was determined by formation of a vaginal plug. Dams failing to become pregnant after development of a copulation plug were allowed to re-mate. Pregnant dams were continued on their pre-conception diets throughout pregnancy and lactation. During pregnancy, maternal weight and dietary intake were recorded weekly. Dams were allowed to deliver spontaneously and left undisturbed with their litters for 48 hours.

All offspring (n = 20) were weaned onto standard chow at 3 weeks postpartum. A subgroup of offspring (n = 5) born to lean dams were cross-fostered and suckled by obese dams (OffCon/Ob). Conversely, a subgroup of offspring (n = 5) born to obese dams were cross-fostered and suckled by lean dams (OffOb/Con) (Figure 2.4). All readouts were compared to the control group, offspring of lean suckled by lean dams (OffCon/Con). Offspring food intake and body weight were measured weekly, biochemical analysis, markers of liver injury and fibrogenesis and liver histology were determined at 3 months.

4.2.2 Offspring Calorific Intake and Body Weight

Pelleted food intake was measured weekly to assess calorific intake. Bodyweight of offspring aged 3 months was measured prior to sacrificing using a schedule-1 method. Weights were recorded using a balance accurate to 4 decimal places.

4.2.3 Tissue Collection

Offspring liver tissue was differentially preserved for use in gene expression analysis, protein and lipid extraction and histology. Briefly, whole liver tissue was snap frozen in liquid nitrogen or dissected using middle orientation and stored in 10% neutral buffered formalin prior to paraffin embedding. Offspring blood was collected via cardiac puncture or inferior vena cava cannulation using heparinised syringes. The blood was centrifuged at 3000 x g for 10 minutes at 4˚C and decanted plasma stored at -20˚C until biochemical analysis.

4.2.4 Serum Analysis

ELISAs were used for analysis of serum leptin (Biovender R&D 1301) and insulin (CrystalChem, Catalog #90080). Wide range assays were used for adequate detection. Aspartate transaminase (AST) was measured by the local clinical biochemistry department.

4.2.5 Radiotelemetry

Remote radiotelemetric probes (TA11PA-C10, O.D 0.4 mm, Data Science International) were implanted under general anaesthetic (isofluorane in O_2) and an indwelling catheter was inserted into the left carotid artery. Following recovery, offspring were subjected to 30 minutes restraint and 120 minutes recovery during which time, the systolic blood pressure (SBP) was measured and expressed as a change from baseline.

4.2.6 Liver Tissue Triglyceride

Whole liver tissue triglyceride was determined by an adaptation of the Folch Method using an enzymatic colorimetric assay (UNIMATE 5 TRIG, Roche BC1. Sussex, UK). Approximately 0.05g of liver tissue per sample was used and all results normalised to sample quantity.

4.2.7 Renal Catecholamines

Harvested kidneys were snap-frozen and stored at -80ºC. The left kidney from each animal was homogenised on ice with 0.1M hydrochloric acid (Sigma-Aldrich, UK) containing 1mM EDTA (BDH Chemicals Ltd., Poole, England) and 4mM sodium metabisulfite. Renal NE content was assessed by ELISA (ALPCO Immunoassays, Salem, NH, USA) as per the manufacturer's instructions.

4.2.8 Gene Expression of Liver Injury and Fibrogenic Markers

Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was performed using SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* High Fidelity (Invitrogen Life Technologies). Gene specific primers were designed for IL-6, TNF-α, adrenoceptor α1-D and ß1, α–Smooth Muscle Actin (α-SMA) and collagen Type 1-α2 (Col 1-α2) (Table 2.3). Expression of target genes was normalised to GAPDH expression.

4.2.9 Protein Expression of Fibrogenic Markers

Total liver protein was extracted in ice-cold RIPA buffer (150nM sodium chlorise, 1% Triton-X 100, 0.5% sodium deoxycholate, 50mM Tris, pH 8.0 and

0.1% SDS), lysate protein concentrations, determined using the copper/bicinchoninic assay (Sigma), and standardised to 2 mg/ml by dilution into Laemmli buffer. 20μg total protein was loaded for SDS-PAGE followed by overnight incubation with antibody against ASMA (Sigma) and Collagen Type 1 (Millipore). Protein expression was assessed densitometrically using AlphaEase software (AlphaImager).

4.2.10 Liver Histology

Offspring liver sections at 3 months were formalin fixed and paraffin embedded prior to sectioning. All sections were then stained with haematoxylin and eosin (H&E) and assessed for steatosis, lobular inflammation and hepatocellular ballooning according to the NAFLD Activity Score (NAS), by an expert liver pathologist blinded to the identity of the groups (Table 2.10).

4.2.11 Statistical Analysis

Multiple comparisons on a single data set were performed using ANOVA and expressed as mean ± SEM unless otherwise stated. $p < 0.05$ was regarded as statistically significant. Sample size per group; n = 5, 1 from each litter.

4.3 Results

4.3.1 Offspring exposure to maternal obesity during lactation only induces hyperphagia and adiposity at 3 months

Offspring exposed to maternal obesity during lactation only were significantly heavier than offspring exposed to maternal obesity *in utero* or control conditions (OffCon/Con; 20.80 ± 0.46 v. OffCon/Ob; 23.85 ± 0.85 v. OffOb/Con; 21.95 ± 0.47 gm) at 3 months post-partum. In corroboration of this, calorie intake was significantly increased ($p < 0.001$) in OffCon/Ob compared to all other groups (OffCon/Con; 73.01 ± 0.93 v. OffCon/Ob; 91.67 ± 0.98 v. OffOb/Con; 69.20 ± 1.00 kcal/g), as was serum leptin (OffCon/Con; 1037 ± 43.12 v. OffCon/Ob; 19334 ± 2231 v. OffOb/Con; 2448 ± 192.7 pg/ml) (Figure 4.1).

Statistically significant hyperphagia and accompanying adiposity was observed in offspring exposed to maternal obesity in lactation alone. Given that all offspring were weaned on to a standard chow diet, the observed effects were the result of maternal obesity during gestation or lactation only.

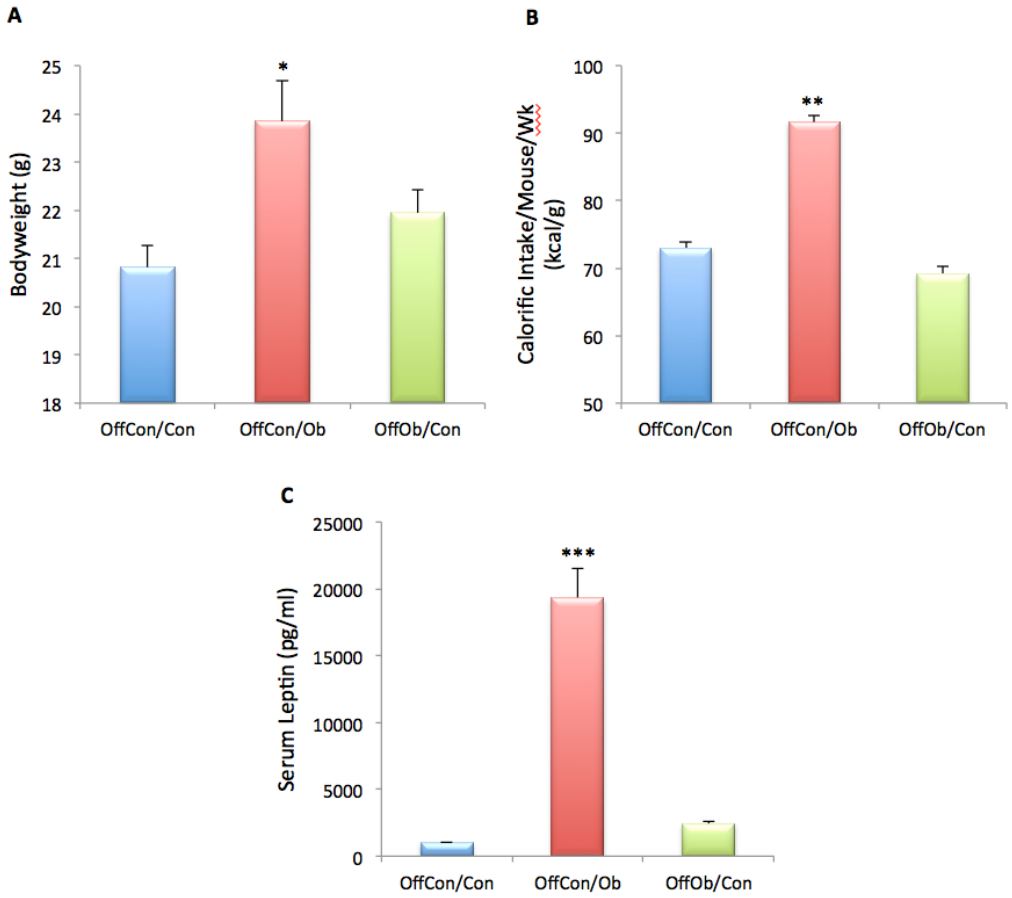


Figure 4.1 Offspring Adiposity and Calorific Intake at 3 months: (A) Bodyweight, (B) Calorific Intake and (C) Serum Leptin Concentrations. Hyperphagia and adiposity was observed in offspring exposed to maternal obesity in lactation only (OffCon/Ob). Offspring of lean suckled by lean - OffCon/Con; offspring of lean suckled by obese – OffCon/Ob and offspring of obese suckled by lean – OffOb/Con. n = 4-6 per group, values shown are mean ± SEM; * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, one-way ANOVA.

4.3.2 Offspring exposure to maternal obesity in lactation induces hyper-insulinaemia and hypertension at 3 months

Insulin levels were significantly raised in offspring exposed to maternal obesity in lactation compared to those exposed to maternal obesity only in gestation or to control conditions (OffCon/Con; 0.15 ± 0.01 v. OffCon/Ob; 2.15 ± 0.09 v. OffOb/Con; 0.42 ± 0.05 µg/L). Similarly, the mean nocturnal systolic blood pressure was raised in OffCon/Ob compared to all other groups, indicating that the perturbed metabolic function was due to exposure of over-nutrition in lactation alone (Figure 4.2).

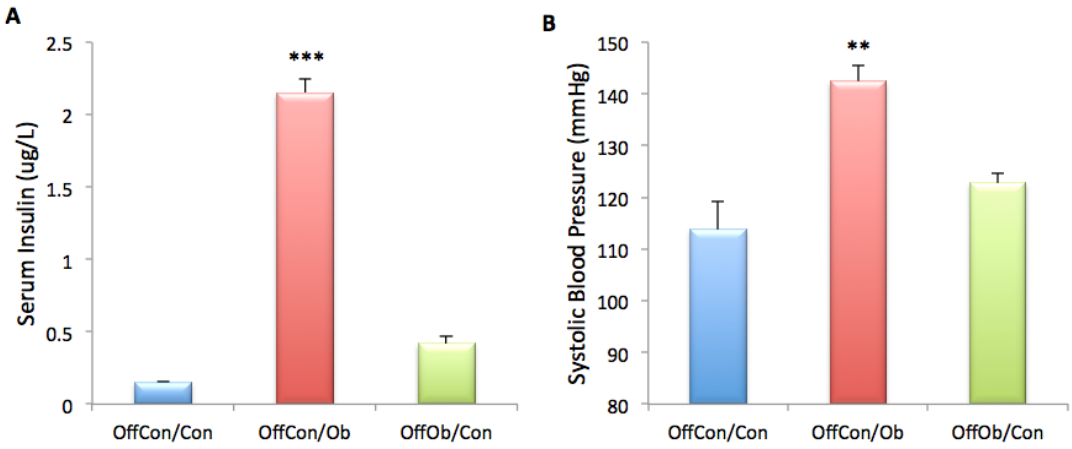


Figure 4.2 Serum Insulin and SBP responses at 3 months: (A) Serum Insulin and (B) Nocturnal Systolic Blood Pressure (SBP). Offspring exposed to maternal obesity in lactation exhibited hyper-insulinaemia and raised SNS tone compared to OffOb/Con and OffCon/Con. Offspring of lean suckled by lean - OffCon/Con; offspring of lean suckled by obese – OffCon/Ob and offspring of obese suckled by lean – OffOb/Con. n = 4-6 per group, values shown are mean ± SEM; * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, one-way ANOVA.

4.3.3 Offspring exposure to maternal obesity during lactation induces profound hepatosteatosis at 3 months

Histological evidence of profound hepatic macrovesicular steatosis was observed in offspring exposed to maternal obesity in lactation alone compared to OffCon/Con and OffOb/Con at 3 months. Morphometrical quantification of steatosis was performed on microphotographs from 10 random field sections per liver, and corroborated the histological findings (OffCon/Con; 0.0 ± 0.0 v. OffCon/Ob; 1.8. ± 0.26 v. OffOb/Con; 1.0 ± 0.45 arbitrary units) (Figure 4.3).

In tandem, hepatic tissue triglyceride content was significantly increased in OffCon/Ob compared to all other groups (OffCon/Con; 4.76 ± 0.89 v. OffCon/Ob; 28.76 ± 2.09 v. OffOb/Con; 3.41 ± 0.38 mmol/l). Therefore exposure to maternal obesity in lactation is more important in inducing offspring hepatosteatosis than exposure to maternal obesity during gestation (Figure 4.3).

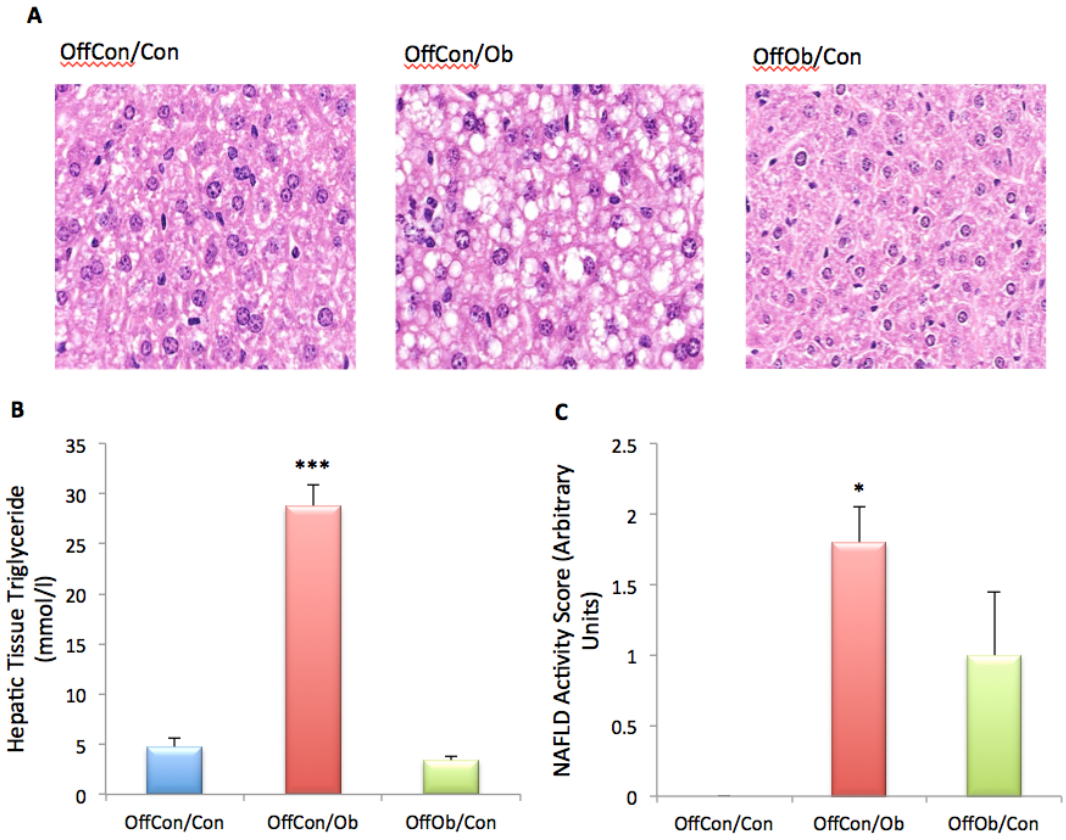


Figure 4.3 Histological and Biochemical Evidence of Hepatosteatosis at 3 months: (A) Representative H&E sections, (B) Hepatic Tissue Triglyceride and (C) NAFLD Activity Score. Exposure to maternal obesity in lactation only, led to profound hepatosteatosis compared to exposure in gestation alone or a normo-calorific environment throughout gestation and lactation. Offspring of lean suckled by lean - OffCon/Con; offspring of lean suckled by obese – OffCon/Ob and offspring of obese suckled by lean – OffOb/Con. n = 4-6 per group, values shown are mean ± SEM; * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, one-way ANOVA.

4.3.4 Offspring exposure to maternal obesity in lactation induces profound liver injury at 3 months

Serum aspartate transaminase (AST) (OffCon/Con; 214.8 ± 21.0 v. OffCon/Ob; 359.1 ± 9.6 v. OffOb/Con; 166.5 ± 23.7 IU/L), IL-6 mRNA (OffCon/Con; 2.8 ± 0.5 v. OffCon/Ob; 18.1 ± 2.0 v. OffOb/Con; 4.4 ± 0.2 arbitrary units) and TNF-α mRNA (OffCon/Con; 25.2 ± 3.4 v. OffCon/Ob; 63.6 ± 8.2 v. OffOb/Con; 15.4 v. 6.9 arbitrary units), as putative markers of liver injury, were all raised in offspring exposed to maternal obesity in lactation compared to offspring exposed only in gestation or to control conditions. Therefore, exposure to maternal obesity in lactation is more important in inducing offspring liver injury than exposure to maternal obesity during gestation (Figure 4.4).

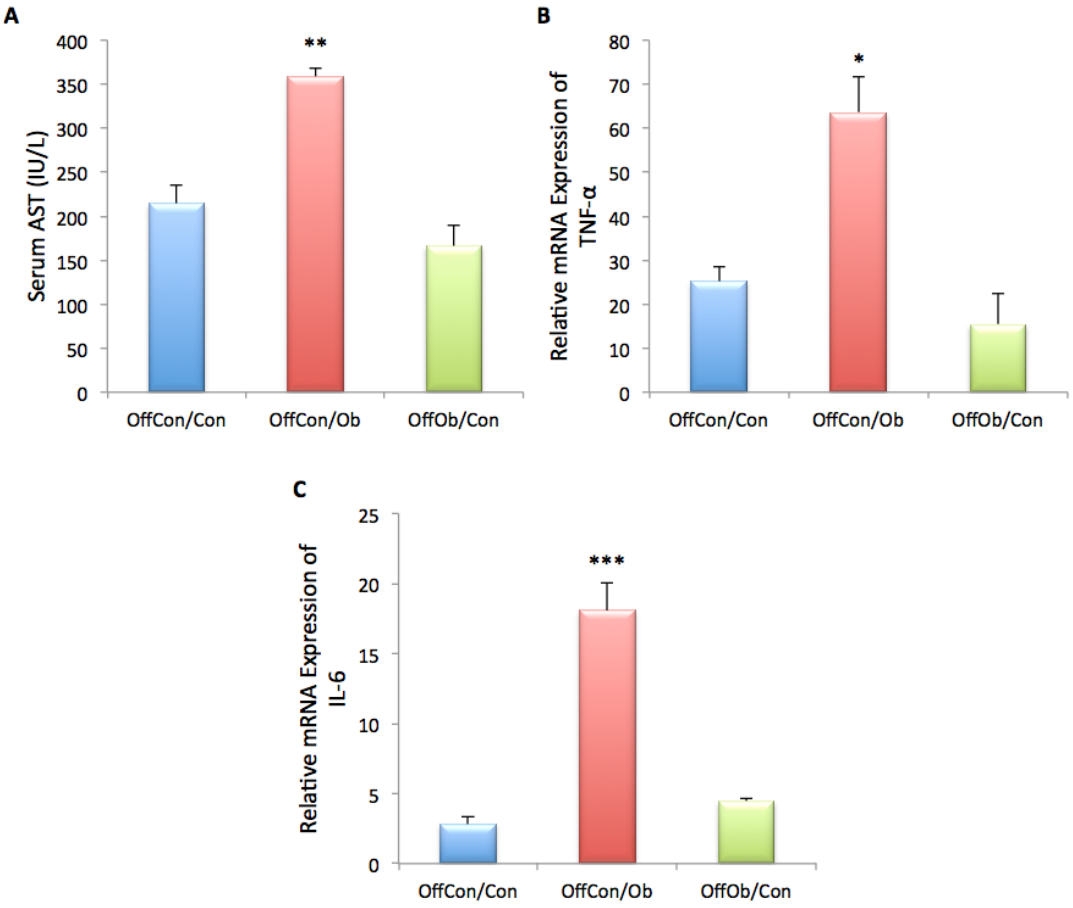


Figure 4.4 Biochemical Evidence of Liver Injury at 3 months: (A) Serum AST, (B) mRNA TNF-α and (C) mRNA IL-6. Maternal obesity in lactation (OffCon/Ob) led to an up-regulation of hepatic pro-inflammatory markers and AST. Offspring of lean suckled by lean - OffCon/Con; offspring of lean suckled by obese – OffCon/Ob and offspring of obese suckled by lean – OffOb/Con. n = 4-6 per group, values shown are mean ± SEM; * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, one-way ANOVA.

4.3.5 Offspring exposure to maternal obesity in lactation activates hepatic fibrogenesis at 3 months

Offspring exposed to maternal obesity in lactation had significant up-regulation of α-SMA gene expression, a marker of hepatic stellate cell activation – the liver's principal fibrogenic cells (OffCon/Con; 72.4 ± 1.6 v. OffCon/Ob; 91.7 ± 2.8 v. OffOb/Con; 52.6 ± 0.6 arbitrary units). These observations were corroborated at the protein level by Western Blot analysis, densitised to permit quantitative analysis (OffCon/Con; 37.2 ± 5.7 v. OffCon/Ob; 194.6 ± 15.8 v. OffOb/Con; 92.1 ± 7.4 arbitrary units).

In tandem, the relative expression of collagen 1α2 mRNA was also raised in OffCon/Ob compared to all other groups (OffCon/Con; 17.6 ± 1.9 v. OffCon/Ob; 25.7 ± 3.9 v. OffOb/Con; 14.4 ± 0.9 arbitrary units). This was also corroborated at the protein level (OffCon/Con; 13.0 ± 10.9 v. OffCon/Ob; 1029 ± 59.6 v. OffOb/Con; 16.8 ± 3.4 arbitrary units). Therefore, exposure to maternal obesity in lactation has a significant effect on programming offspring susceptibility to hepatic fibrogenesis in the context of NAFLD (Figure 4.5).

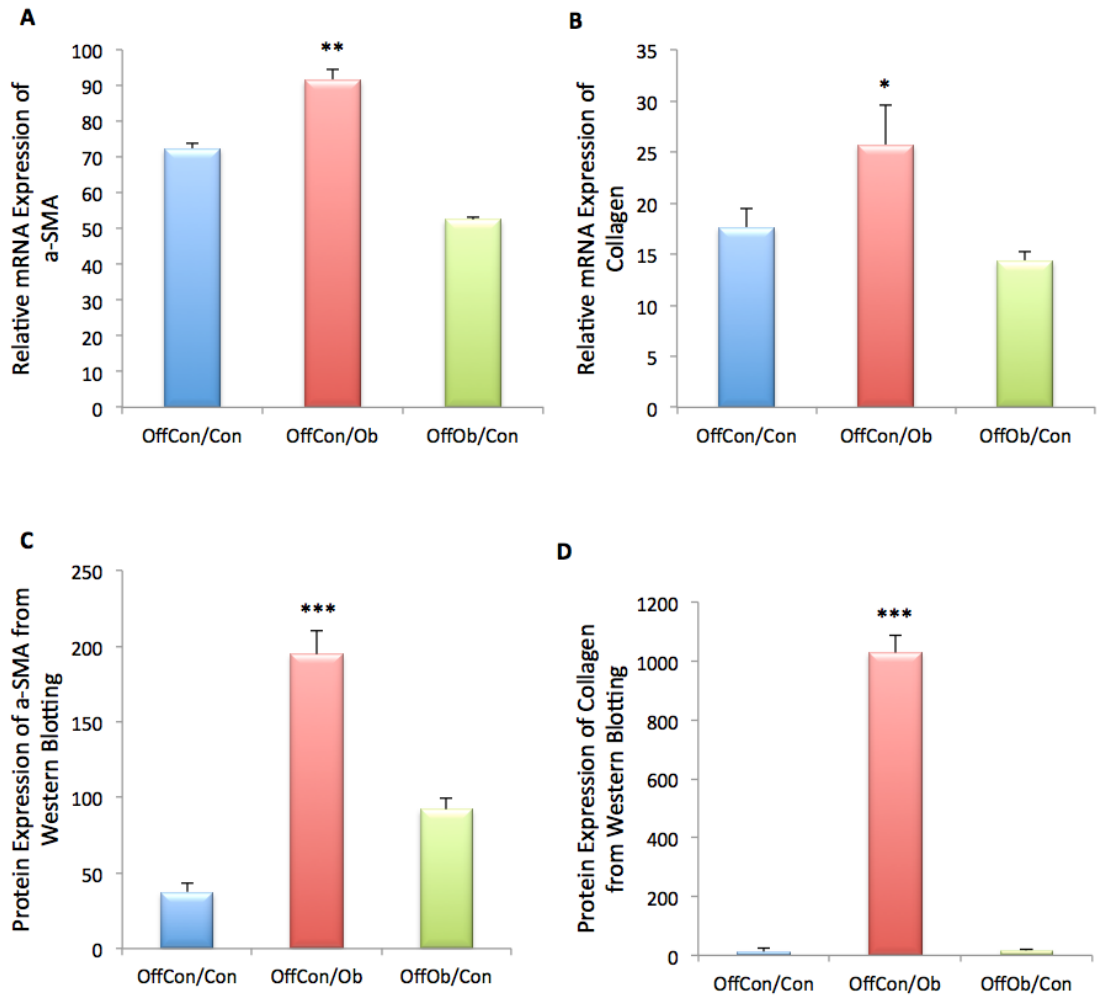


Figure 4.5 Biochemical Evidence of Liver Fibrogenesis at 3 months: (A) α-SMA mRNA, (B) Collagen type 1 mRNA, (C) α-SMA protein and (D) Collagen type 1 protein. Maternal obesity in lactation (OffCon/Ob) led to up-regulation of pro-fibrogenic markers at the mRNA and protein levels compared to all other groups. Offspring of lean suckled by lean - OffCon/Con; offspring of lean suckled by obese – OffCon/Ob and offspring of obese suckled by lean – OffOb/Con. n = 4-6 per group, values shown are mean ± SEM; * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, one-way ANOVA.

4.3.6 Offspring exposure to maternal obesity in lactation induces SNS activation at 3 months

Offspring exposure to maternal obesity only in lactation led to up-regulation of hepatic α-1D (OffCon/Con; 0.063 ± 0.002 v. OffCon/Ob; 0.138 ± 0.016 v. OffOb/Con; 0.065 ± 0.010 arbitrary units) and β-1 (OffCon/Con; 0.0022 ± 0.0002 v. OffCon/Ob; 0.0032 ± 0.0004 v. OffOb/Con; 0.0010 ± 0.0001 arbitrary units) SNS adrenoreceptors in the liver compared to those offspring exposed to maternal obesity only in gestation or control conditions. In tandem, renal catecholamine content was raised (Figure 4.6). As SNS activation is a putative driver of hepatic fibrogenesis, these findings are in keeping with gene expression analysis of pro-fibrotic mediators shown in Figure 4.5.

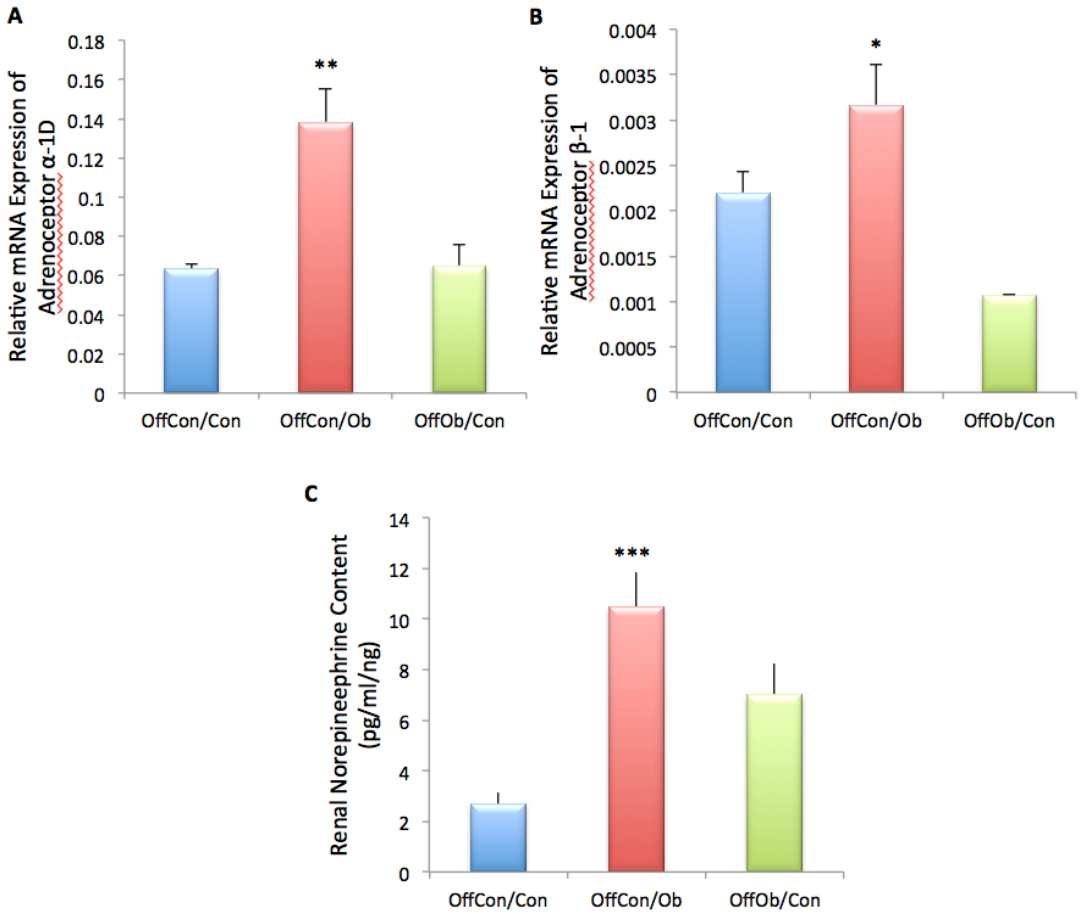


Figure 4.6 Liver Adrenoreceptor Expression and Renal Catecholamine Content at 3 months: (A) Liver adrenoreceptor α-1D mRNA, (B) Liver adrenoreceptor β-1 mRNA and (C) Renal NE. All markers of SNS activation were significantly raised in OffCon/Ob. Offspring of lean suckled by lean - OffCon/Con; offspring of lean suckled by obese – OffCon/Ob and offspring of obese suckled by lean – OffOb/Con. n = 4-6 per group, values shown are mean ± SEM; * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, one-way ANOVA.

4.4 Discussion

It was described in Chapter 3 that offspring exposure to maternal obesity, throughout gestation and lactation, (compared to exposure to a normal intra-uterine and peri-natal environment), induced dysmetabolism and hepatic injury in the form of NAFLD. It has since been shown that offspring exposure to maternal over-nutrition during gestation and lactation programs NAFLD (64, 180). To determine the period of development most responsible for the observed phenotype, we engendered a cross-fostering protocol, which involved the switching of offspring born to lean dams to obese dams to suckle and vice versa.

These current studies show that offspring exposure to maternal obesity only in lactation more aggressively programs insulin and leptin resistance, marked liver injury, raised SNS tone and induction of liver fibrogenesis compared to exposure during gestation. So, therefore it may be suggested that the most critical period of development responsible for the observed programmed phenotype in offspring of obese dams is the immediate post-partum period.

4.4.1 Offspring Adiposity and Calorific Intake

Offspring exposure to maternal obesity in lactation programmed hyperphagia resulting in increased bodyweight. That our experimental protocol involved weaning all offspring onto standard chow ensured that any phenotypic changes were the result of maternal nutritional differences during gestation or lactation

only and any increases in food intake were the result of differential appetite and not food palatability. The importance of assessing the energy content of the offered diet is highlighted by the fact that maternal obesity per se, may not be the only determinant of programmed NAFLD, as a high calorie intake during pregnancy independent of maternal obesity may also be involved in programming hepatic injury as recently shown in non-human primates (27).

4.4.2 Offspring Liver Injury

The current paradigm for the pathogenesis of NAFLD, invokes insulin resistance as the primary driver for steatosis in the livers of obese individuals (172), with IL-6 and TNF-α involved in the inflammatory responses (174). More specifically, TNF-α propagates hepatic inflammation and injury by impairing redox reactions within the electron transport chain, allowing the accumulation of more reactive oxygen species.

Increased expression of TNF-α and IL-6 were observed in the context of hyper-insulinaemia in offspring exposed to maternal obesity in lactation alone compared to those exposed in gestation or not at all. Additionally, the liver enzyme, aspartate aminotransferase, was significantly raised in offspring exposed to maternal obesity in lactation compared to gestation. Therefore, profound liver injury is observed in offspring adversely exposed to maternal over-nutrition in the immediate post-partum period.

4.4.3 Offspring Hepatosteatosis

Here, hepatic tissue triglyceride content was increased in offspring exposed to maternal obesity during lactation compared to gestation or control. There are two mechanistic pathways responsible for hepatocellular steatosis, initiated by insulin resistance. Firstly, lipolysis increases the concentration of free fatty acids which are removed from the circulation by hepatocytes. The mitochondrial ß-oxidation pathway consequently becomes saturated leading to accumulation of triglycerides in the liver. Fatty acids are inducers and substrates of microsomal lipooxygenases which may evoke production of reactive oxygen species. These free radicals can induce lipid peroxidation of hepatocyte membranes. The second pathway is governed by hyper-insulinemia, which has been observed in offspring exposed to maternal obesity in lactation. Subsequent promotion of glycolysis increases hepatic fatty acid synthesis and diminishes hepatic production of apolipoprotein B-100 which is required for the incorporation of triglycerides into very low density lipoproteins (VLDLs) (103).

Present findings of increased hepatic tissue triglyceride content in offspring exposed to maternal obesity in lactation were corroborated by histological assessment of steatosis using H&E staining and morphometric analysis. The gold standard technique employed in the diagnosis of NAFLD is histological evaluation secondary to liver biopsy. A fundamental assessment criterion for the inference of NAFLD is steatosis (184). Its manifestation is typically macro-vesicular and it frequently leads to hepatic injury with accumulation of

inflammatory cells (185). It is therefore, not surprising that the markers of liver injury, IL-6 and TNF-α, are too up-regulated in offspring livers with evident fatty infiltration.

4.4.4 Offspring Liver Fibrogenesis

Hepatic fibrogenesis is a consequence of hepatic injury which if unhindered, as occurs with persistent injury in NAFLD, may lead to cirrhosis. Fibrogenesis is the result of over-exuberant production and deposition of matrix proteins secreted by injury activated hepatic stellate cells (HSCs), a phenotype characterised by abundant expression of α-SMA and collagen type-1, the predominant matrix constituent in fibrosis (186). α-SMA is expressed by activated HSCs and as such is considered a hallmark of hepatic injury or fibrogenesis (115). It has been shown here that the relative expression of α-SMA both at the mRNA and protein level, are significantly raised in offspring exposed to maternal obesity in lactation. Additionally, gene and protein expression of collagen type 1 was subsequently up-regulated in offspring exposed to maternal obesity in lactation.

SNS signalling components have been implicated in hepatic fibrogenesis mediated by adrenoreceptor expressing HSCs (138, 140, 187) expressing adrenoreceptors. Given that hypertension, a component of the metabolic syndrome, is reflective of SNS over-activity (35) and shown to be present in these offspring, changes in SNS signalling components were investigated. The results showed that exposure to maternal obesity in lactation induces SNS

activation in offspring as evidenced by raised systolic blood pressure, increased hepatic expression of adrenoreceptors and renal catecholamine content. This may in part, therefore, be responsible for the observed pro-fibrogenic phenotype as evidenced by up-regulation of α-SMA and collagen.

4.4.5 Offspring Hyper-leptinaemia

Leptin is similarly implicated in fibrogenesis through its activation of HSCs, since animals lacking leptin are poorly fibrogenic (138, 187, 188). Fibrogenic HSCs in these leptin deficient animals do however, respond to catecholamine neurotransmitters which are known to be released by leptin from adipocytes and HSCs themselves (138, 187). The observed hyper-leptinaemia in offspring exposed to a hyper-calorific diet in the post-partum period, may be secondary to the described hyper-insulinaemic state, since insulin can induce adipocytes to secrete leptin via an adipoinsular axis (189). Alternatively or additionally, raised adult leptin levels may have been induced by the heightened norepinephrine, which has been shown to induce the release of leptin in adipocytes (190).

4.4.6 Relative Contributions of the *in utero* and lactation periods

The more robust dysmetabolic and NAFLD phenotype observed in offspring exposed to an adverse *ex utero* environment only, confirms the relative predominance of this period in programming offspring NAFLD. However, offspring exposure to an adverse *in utero* environment only had negligible effects on hepatic outcome, suggesting that exposure during this period initiates protective adaptive mechanisms in preparation for a similar post-natal

environment as a survival advantage (157, 191). Evidence of such adaptation has been reported for endothelial function which is preserved in rodent offspring exposed to maternal over-nutrition, although hypertension ensued (191).

Consequently, those offspring exposed to an adverse *ex utero* environment only are more vulnerable to the post-natal hyper-calorific environment and respond with an enhanced dysmetabolic and liver phenotype. These findings moreover, confirm that development of the dysmetabolic and NAFLD phenotype is largely driven by exposure to maternal obesity in the post-partum period with maternal breast milk factors being possible candidates inducing this phenotype.

4.4.7 A Novel Experimental Model of NAFLD

A derivative of this study is the creation of a near complete pathophysiologically relevant model of NAFLD as it demonstrates features of the human disease which all current available experimental models lack (192). The model created here has biochemical evidence of liver injury, biochemical and histological evidence of steatosis, hyper-leptinaemia, hyper-insulinaemia, hypertension, raised inflammatory cytokines and induction of fibrogenesis - with activation of the SNS and up-regulation of its signalling components. The creation of this model may aid further studies in NAFLD including drug discovery for treatment of NAFLD induced fibrosis. The findings here are especially relevant given the rising rates of maternal obesity and increasing prevalence of adult and paediatric NAFLD (6, 155).

4.4.8 Effects of cross-fostering on offspring phenotype

There is however, a fundamental criticism of the series of experiments involving cross-fostering of offspring born to lean dams for obese dams to suckle and vice versa, which is that this practice is non-physiological. Moreover, there is emerging experimental evidence indicating that cross-fostering can induce a permanent state of anxiety in mice as well as alter genetic and epigenetic effects on complex traits (193). Additionally, the stress of cross-fostering cannot be controlled for by fostering litters as studies in both mice and piglets demonstrate variable levels of distress in resident and fostered offspring (194).

However, for the purposes of investigating the aetiopathogenesis of NAFLD programmed by maternal obesity, the present experimental paradigm is preferred as it allows a dissection of the gestation and lactation periods. Through these studies, it is shown that the critical period most susceptible to programming effects in rodents is lactation. Caution has to be exercised however, in extrapolating these findings to humans given that this immediate post-partum period is of developmental equivalence to late gestation in humans as rodents are born with an immature neuroendocrine system (32, 33).

4.5 Conclusion

In conclusion, exposure to maternal obesity during gestation and lactation programs dysmetabolism and hepatic injury, with the most critical period of offspring susceptibility to programming effects by maternal obesity being

lactation. This may be because maturation of the neuroendocrine system, HPA axis, hepatocyte differentiation and hepatic maturation predominantly occur at this time (32, 195).

Given the inherent non-physiological nature of the cross-fostering studies above, the next series of experiments will investigate the role of maternal obesity on offspring development of dysmetabolism and NAFLD using a wholly physiologically relevant model. In these subsequent experiments, offspring will not be cross-fostered post-partum but be maintained on their pre-weaning diet in to adulthood to permit validation of previous findings and allow investigation of the interactions between the environments in gestation, lactation and post-weaning.

5 Investigation of Dysmetabolism and NAFLD in Offspring Exposed to Maternal Obesity in the Context of a Post-Weaning Obesogenic Environment:

A Pathophysiologically Relevant Disease Model

5 Investigation of dysmetabolism and NAFLD in offspring exposed to maternal obesity in the context of a post-weaning hyper-calorific environment – A Pathophysiologically Relevant Disease Model

5.1 Introduction

A growing body of evidence suggests that the rising rates of non-alcoholic fatty liver disease, NAFLD, may be partially attributable to the developmental programming effect of maternal obesity (51, 64, 180). This is unsurprising given that NAFLD is the hepatic manifestation of the metabolic syndrome, which in turn has been shown to be programmable following exposure to maternal over-nutrition (5, 20, 22, 23, 37, 39). Programming, therefore, is a fundamental risk factor as obesity amongst women of reproductive age (20-39 years) is presently 31.5%, rising in tandem with NAFLD prevalence of 23-34% in the US (6, 155).

Standard rodent models of programming involve dams fed an obesogenic diet throughout the gestation and lactation period. Readouts in offspring, therefore, are a reflection of the interaction between both developmental periods. However, by cross-fostering offspring initially born to obese dams, with lean dams in the post-partum suckling period, and vice versa, one is able to investigate the role of the *in utero* environment, independent of the immediate post-natal period as in Chapter 4. In order to delineate the influences of maternal over-nutrition in these developmental periods, all offspring were weaned on to a normal chow diet. Arguably, this is of limited physiological

relevance as offspring exposed to maternal diet induced obesity during development are likely to be nurtured in a similar hyper-calorific environment post-partum. Therefore, it is necessary to investigate the interaction of the programmed phenotype with a post-weaning hyper-calorific diet.

There are studies demonstrating accelerated adiposity and weight gain in offspring born to obese dams and maintained on an obesogenic diet, both post-weaning and throughout post-natal life. These findings are met with reports of hyperphagia in rodents exposed to a continued adverse environment post-partum, compared to control offspring or those exposed only during gestation and/or lactation (55). It can, therefore, be suggested that the effects of programming interact with the post-natal diet, and not only the immediate post-partum period to influence offspring risk of obesity, dysmetabolism and potentially NAFLD in adulthood.

As the initiating defect in the pathogenesis of NAFLD is insulin resistance (172) and implicated factors include tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (103), these mediators were assayed alongside markers of hepatic fibrogenesis.

Here, the aims were to investigate the interactions, if any, between programmed dysmetabolism and NAFLD by maternal diet induced obesity, and a post-weaning hyper-calorific diet in early and late adulthood.

5.2 Methods

5.2.1 Animal Experimentation

Female C57BL/6J mice (n = 20 per group) (Charles River Laboratories, UK), of first order parity, approximately 100 days old were allowed 7 days to habituate following transfer to the facility and fed standard chow RM1 (7% simple sugars, 3% fat, 50% polysaccharide, 15% protein [w/w] RM1, Special Dietary Services, energy 3.5 kcal/g) *ad libitum*.

Following acclimatisation, female mice were randomised to either a control or experimental group and fed standard chow or a semi-synthetic energy-rich, highly palatable obesogenic diet (10% simple sugars, 20% animal lard 28% polysaccharide, 23% protein [w/w], Special Dietary Services, energy 4.5 kcal/g) supplemented with sweetened condensed milk (approx 55% simple sugar, 8% fat, 8% protein, w/w, Nestle) admixed with mineral mix (AIN93G, Special Dietary Services, 125 mg/pot) *ad libitum*.

Dams were maintained on their respective diets for 6-8 weeks and macronutrient and calorific intake were calculated from measured daily intake of pellets and milk (approx 16% fat, 33% simple sugars, 15% protein, energy 4.0kcal/g). Upon attainment of an average 30% increase in body weight of the experimental group, mice were entered into the breeding protocol. Males from the same litter were selected to minimise genetic variability. All animals were treated in accordance with The Animals (Scientific Procedures) Act, UK, 1986 guidelines.

Day 0 of pregnancy was confirmed by formation of a vaginal plug. Dams failing to become pregnant after development of a copulation plug were allowed to re-mate. Pregnant dams were continued on their pre-conception diets throughout pregnancy and lactation. During pregnancy, maternal weight and dietary intake were recorded weekly. Dams were allowed to deliver spontaneously and left undisturbed with their litters for 48 hours.

Subgroups of female offspring (n = 20) born to and suckled by lean dams or born to and suckled by obese dams were weaned on to either normo-calorific or semi synthetic obesogenic diet without condensed milk supplementation (Figure 2.5). This is different to previously used models as all offspring were weaned on to only a standard chow diet. All readouts were compared to the control group, offspring of lean suckled by lean dams and weaned onto standard chow (OffCon-SC). Offspring body weight, fat pad mass, biochemical analysis, markers of liver injury and fibrogenesis and liver histology were determined at 3 and 12 months.

5.2.2 Body Weight and Inguinal Fat Pad Mass

Offspring bodyweight was measured prior to sacrificing using a schedule-1 method. Inguinal fat pads were excised and weights recorded using a balance accurate to 4 decimal places.

5.2.3 Tissue Collection

Offspring liver tissue was differentially preserved for use in gene expression analysis, protein and lipid extraction and histology. Briefly, whole liver tissue was snap frozen in liquid nitrogen, preserved in RNAlater or dissected using middle orientation and stored in 10% neutral buffered formalin prior to paraffin embedding. Offspring blood was collected via cardiac puncture or inferior vena cava cannulation using heparinised syringes. The blood was centrifuged at 3000 x g for 10 minutes at 4˚C and decanted plasma stored at -20˚C until biochemical analysis.

5.2.4 Serum Analysis

ELISA was used for analysis of serum leptin (Biovender R&D 1301). Wide range assays were used for adequate detection. Alanine transaminase was measured by the local clinical biochemistry department.

5.2.5 Liver Tissue Triglyceride

Whole liver tissue triglyceride was determined by an adaptation of the Folch Method using an enzymatic colorimetric assay (UNIMATE 5 TRIG, Roche BC1. Sussex, UK). Approximately 0.05g of liver tissue per sample was used and all results normalised to sample quantity.

5.2.6 Gene Expression of Liver Injury and Fibrogenic Markers

Real Time Polymerase Chain Reaction (RT-PCR) was performed using QuantiTect SYBR Green PCR System with HotStar Taq DNA Polymerase (Qiagen). Gene

specific primers were designed for IL-6, TNF-α, α–Smooth Muscle Actin (α-SMA), transforming growth factor-β (TGF-β) and collagen Type 1-α2 (Col 1-α2). Expression of target genes was normalised to GAPDH expression.

5.2.7 Liver Histology

Offspring liver sections at 3 and 12 months were formalin fixed and paraffin embedded prior to sectioning. All sections were then stained with haematoxylin and eosin (H&E) and Masson's Trichrome and assessed for steatosis and fibrosis, respectively, by an expert liver pathologist blinded to the identity of the groups (Table 2.10 and 2.11).

5.2.8 Statistical Analysis

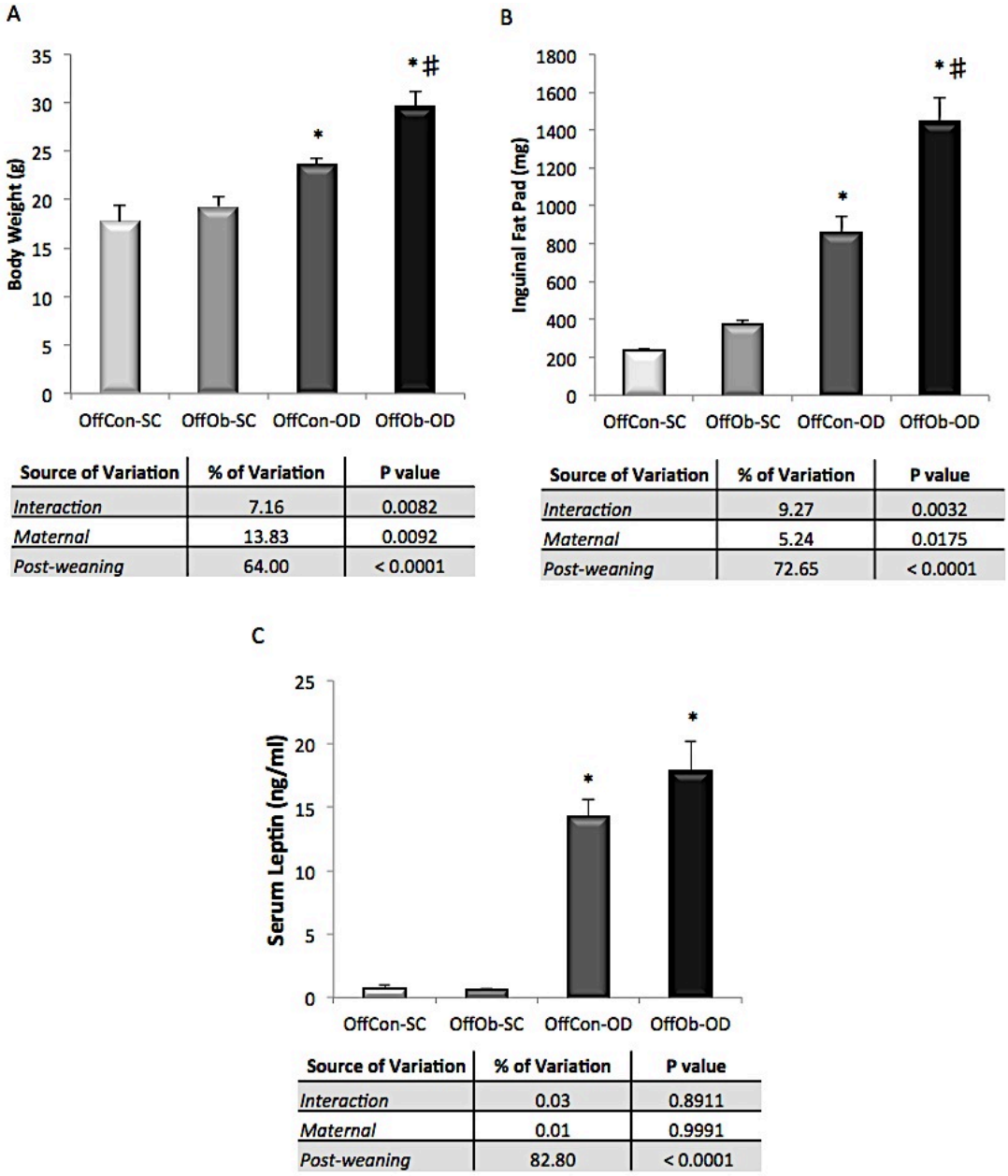
Multiple comparisons on a single data set were performed using ANOVA followed by Tukey's *post hoc* test and expressed as mean ± SEM unless otherwise stated. Interactions between maternal obesity and the post-natal diet were investigated using two-way (2x2 factorial) ANOVA through data analysis software (GraphPad Prism 5.0). $p < 0.05$ was regarded as significant. Sample size per group; n = 5, 1 from each litter.

5.3 Results

5.3.1 Offspring exposure to maternal obesity and a post-natal obesogenic diet accelerates adiposity with concomitant hyper-leptinaemia at 3 months.

Bodyweight was significantly increased in offspring of control dams fed a post-natal obesogenic diet, which was accelerated by prior exposure to maternal obesity, compared to those fed a control diet (OffCon-SC; 17.78 ± 1.61 v. OffCon-OD; 23.60 ± 0.69 v. OffOb-OD; 29.69 ± 1.44 gm). As previously observed, offspring exposed to maternal obesity during gestation and lactation had similar bodyweights as those fed a control diet at 3 months post-partum. There was an independent effect of maternal obesity on offspring bodyweight with a significant interaction between maternal obesity and the post-natal diet.

Corroboratively, offspring inguinal fat pad mass (OffCon-OD; 855.53 ± 87.90 v. OffOb-OD; 1440.80 ± 133.13 mg) and serum leptin (OffCon-OD; 14.29 ± 1.35 v. OffOb-OD; 17.90 ± 2.35 ng/ml) were significantly raised in tandem, in OffCon-OD with exacerbation in offspring additionally exposed to maternal obesity (Figure 5.1). Moreover, a two-way ANOVA revealed an independent effect of maternal obesity on offspring inguinal fat pad mass with significant interaction between maternal influences and the post-natal diet for offspring bodyweight and inguinal fat pad mass. Therefore and as expected, a post-natal obesogenic diet induced offspring adiposity which was exacerbated by prior exposure to maternal obesity in gestation and lactation.



Source of Variation	% of Variation	P value
Interaction	7.16	0.0082
Maternal	13.83	0.0092
Post-weaning	64.00	< 0.0001

Source of Variation	% of Variation	P value
Interaction	9.27	0.0032
Maternal	5.24	0.0175
Post-weaning	72.65	< 0.0001

Source of Variation	% of Variation	P value
Interaction	0.03	0.8911
Maternal	0.01	0.9991
Post-weaning	82.80	< 0.0001

Figure 5.1 Offspring Adiposity and Serum Leptin at 3 months: (A) Bodyweight, (B) Inguinal Fat Pad Mass and (C) Serum Leptin Concentrations. The measured indices of adiposity were exacerbated in offspring with exposure to maternal obesity and a post-natal obesogenic diet. Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese

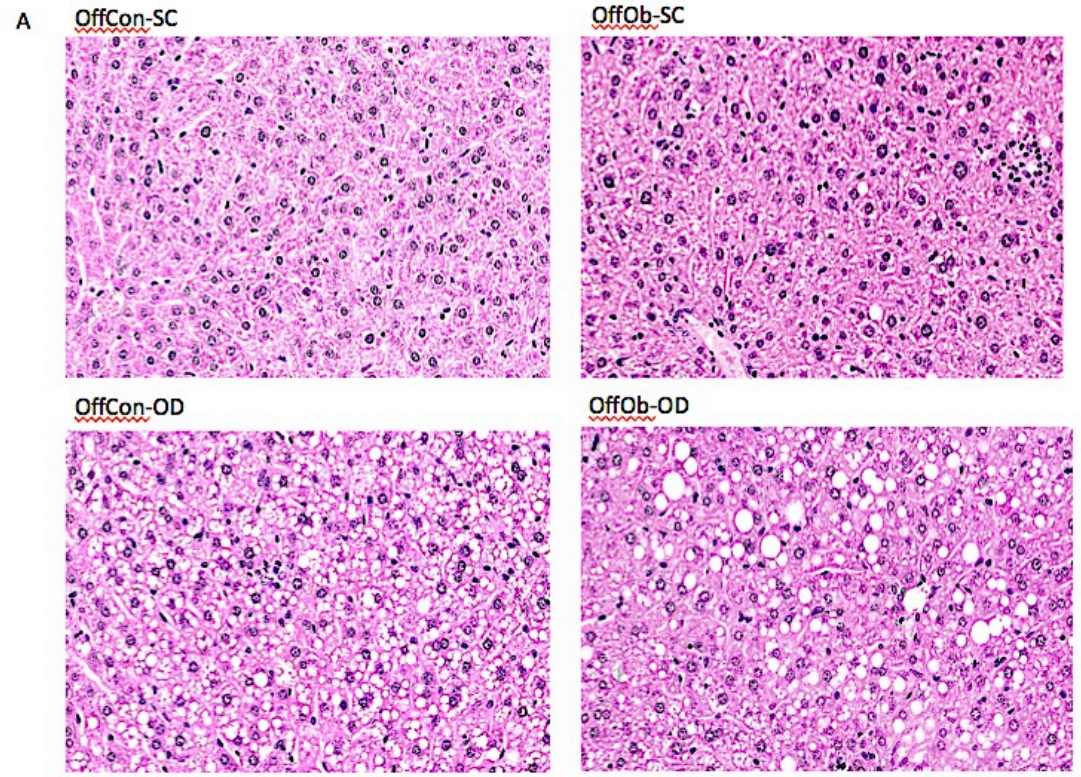
weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. n = 4-6 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.01$). # Mean values significantly greater than OffCon-OD ($p < 0.01$).

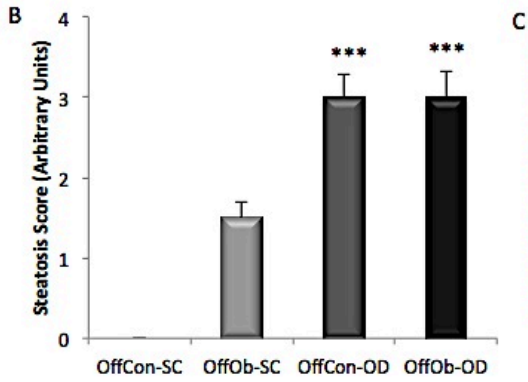
5.3.2 Offspring exposure to maternal obesity and a post-natal obesogenic diet exacerbates hepatosteatosis at 3 months

Histological evidence of hepatosteatosis was observed in OffCon-OD compared to OffCon-SC at 3 months post-partum. More profound hepatosteatosis was observed in offspring exposed to maternal obesity in addition to being fed an obesogenic diet in post-natal life. Morphometric quantification of the stained surface was performed on representative liver sections, corroborating these findings. Additionally, there was an independent effect of maternal obesity on the NAFLD Activity Score with evidence of an interaction between the maternal and post-weaning environments.

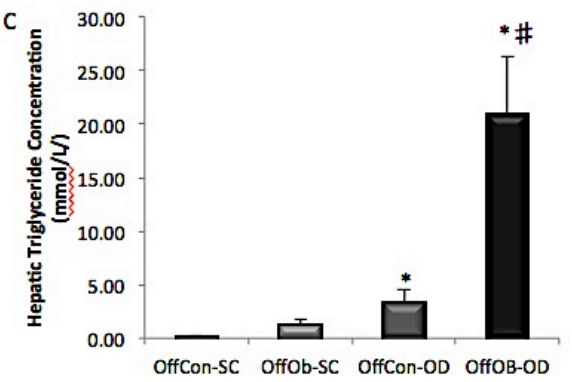
An interesting observation is that fat accumulation in the livers of OffCon-OD was predominantly micro-vesicular, as reported by the histopathologist, whereas OffOb-OD presented with macro-vesicular steatosis, as commonly observed in NAFLD. These observations are in tandem with the hepatic tissue triglyceride content which was most marked in OffOb-OD (OffCon-OD; 3.27 ± 1.22 v. OffOb-OD; 20.95 ± 5.30 mmol/l/) (Figure 5.2). Similarly, an independent effect of maternal obesity on offspring hepatic TG content was observed with

evidence of a significant interaction between maternal obesity and the post-natal diet. So therefore, maternal obesity induces offspring hepatosteatosis which is exacerbated in the context of a post-weaning hyper-calorific diet.





Source of Variation	% of Variation	P value
Interaction	9.14	0.0009
Maternal	82.23	< 0.0001
Post-weaning	12.76	0.0002



Source of Variation	% of Variation	P value
Interaction	21.61	0.0053
Maternal	27.48	0.0025
Post-weaning	41.08	0.0006

Figure 5.2 Histological and Biochemical Evidence of Hepatosteatosis at 3

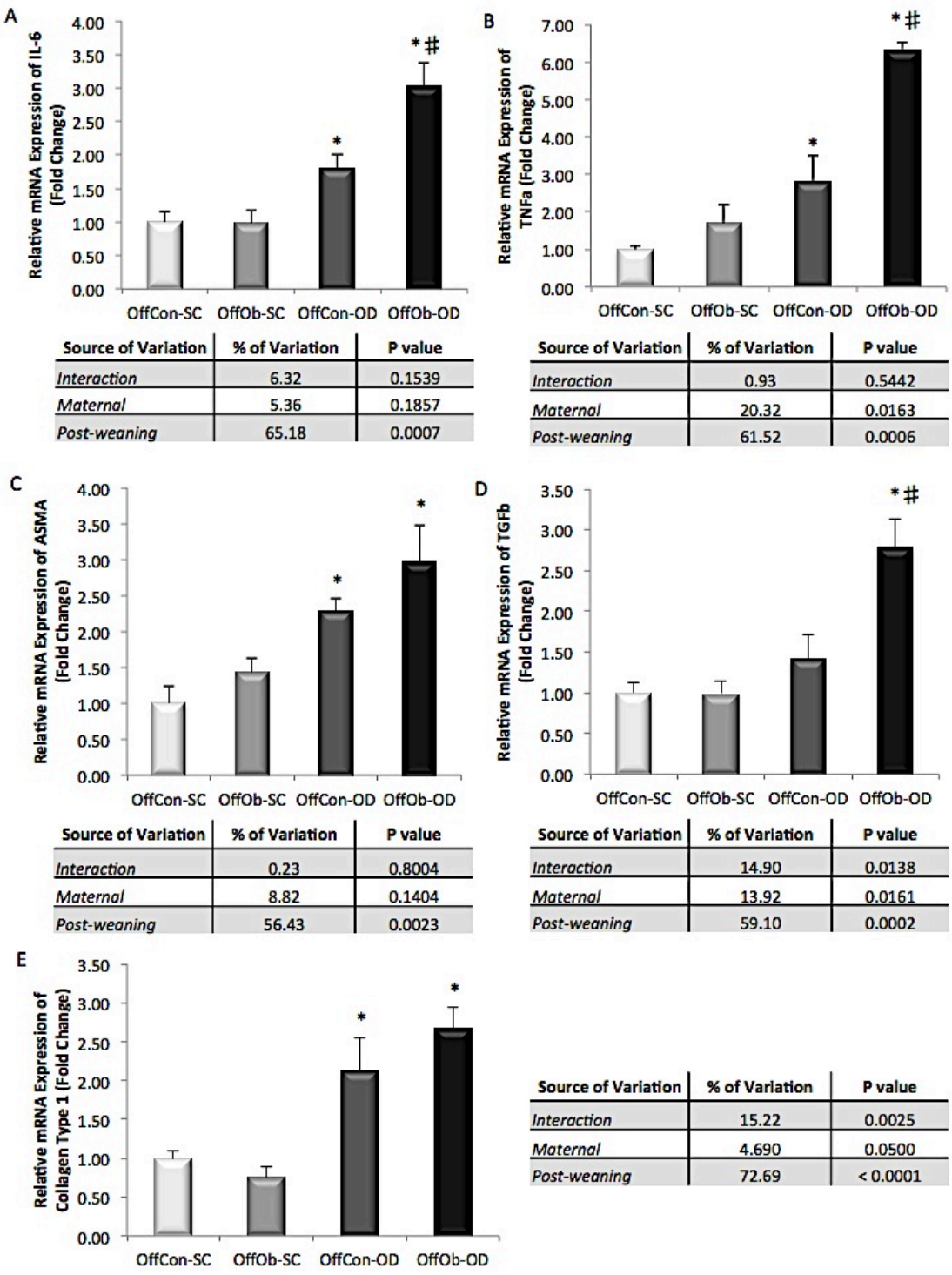
months: (A) Representative H&E sections, (B) Steatosis Score and (C) Hepatic Tissue Triglyceride. Offspring exposure to maternal obesity in the context of a post-weaning hyper-calorific diet significantly exacerbated hepatosteatosis. Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. n = 4-6 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.01$). # Mean values significantly greater than OffCon-OD ($p < 0.01$).

5.3.3 Offspring exposure to maternal obesity and a post-natal obesogenic diet worsens liver injury and exacerbates up-regulation of pro-fibrogenic markers at 3 months

The relative mRNA expression of IL-6 and TNF-α, chemokines implicated in NAFLD pathogenesis, was significantly increased in offspring of control fed a post-natal obesogenic diet compared to controls. Prior offspring exposure to maternal obesity exacerbated this injury as evidenced by greater increases in relative expression (OffCon-SC; 1.00 ± 0.15 v. OffCon-OD; 1.81 ± 0.20 v. OffOb-OD; 3.03 ± 0.34 arbitrary units) (Figure 5.3). There was an independent effect of maternal obesity on TNF-α expression.

Gene expression of α-SMA, TGF-β and collagen type 1, as markers of hepatic fibrogenesis were too up-regulated in offspring exposed to a post-natal

obesogenic diet. Importantly, the expression of these markers was further increased in offspring exposed to maternal obesity in addition to a post-natal obesogenic diet (Figure 5.3). The role of maternal obesity in programming offspring NAFLD is confirmed by the independent maternal effects on TGF-β and collagen expression with evidence of interaction between maternal and post-natal diets for these markers. So therefore, offspring exposure to maternal obesity worsens hepatic injury in the context of a post-weaning hyper-calorific diet.



Source of Variation	% of Variation	P value
Interaction	6.32	0.1539
Maternal	5.36	0.1857
Post-weaning	65.18	0.0007

Source of Variation	% of Variation	P value
Interaction	0.93	0.5442
Maternal	20.32	0.0163
Post-weaning	61.52	0.0006

Source of Variation	% of Variation	P value
Interaction	0.23	0.8004
Maternal	8.82	0.1404
Post-weaning	56.43	0.0023

Source of Variation	% of Variation	P value
Interaction	14.90	0.0138
Maternal	13.92	0.0161
Post-weaning	59.10	0.0002

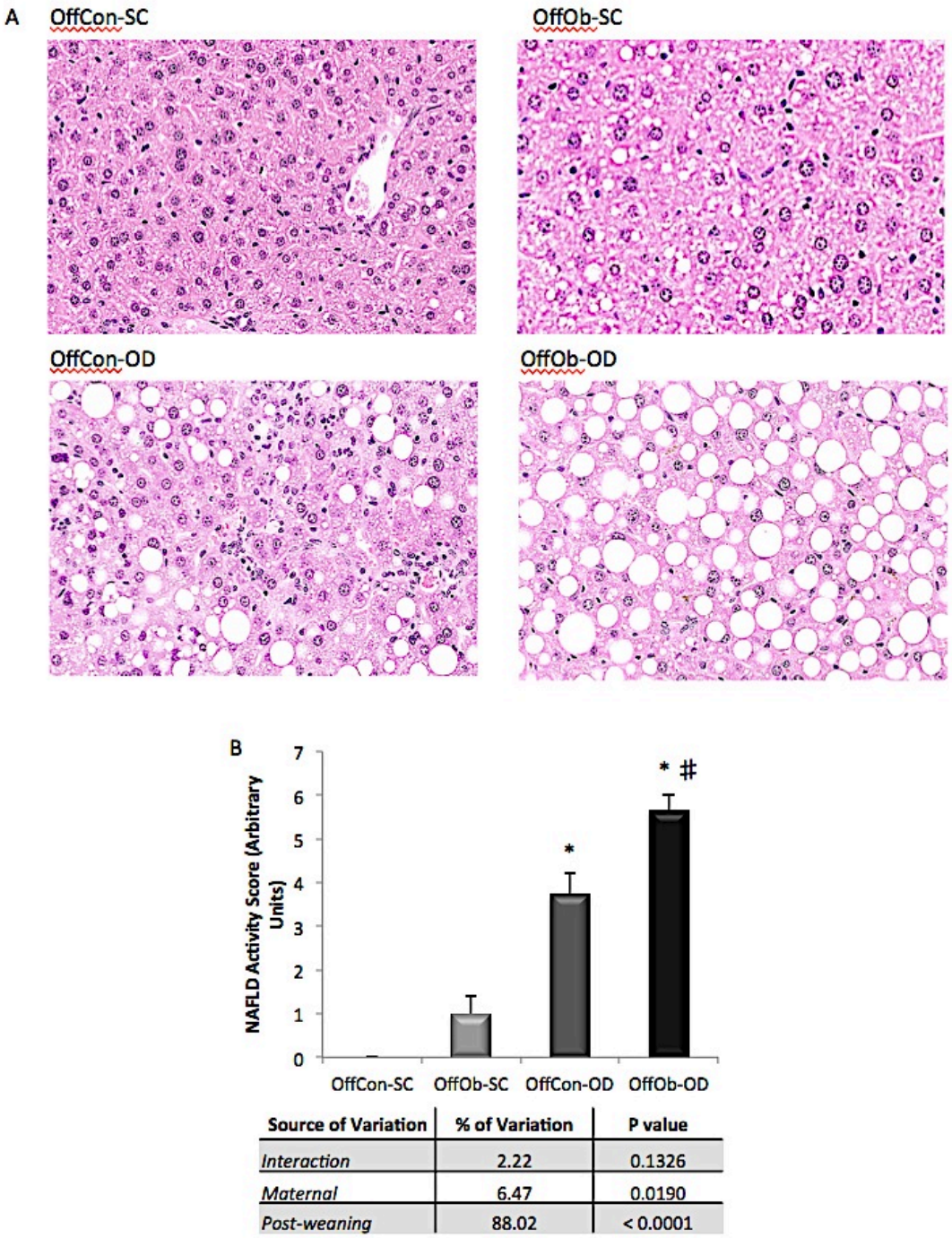
Source of Variation	% of Variation	P value
Interaction	15.22	0.0025
Maternal	4.690	0.0500
Post-weaning	72.69	< 0.0001

Figure 5.3 Biochemical Evidence of Liver Injury and Fibrogenesis at 3 months: (A) mRNA IL-6 expression (B) mRNA TNF-α expression, (C) mRNA α-SMA, (D) mRNA TGF-β and (E) mRNA Collagen type 1. Maternal obesity in the context of

a post-weaning hyper-calorific diet worsened liver injury and enhanced induction of hepatic fibrogenesis. Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. n = 4-6 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.01$). # Mean values significantly greater than OffCon-OD ($p < 0.01$).

5.3.4 Offspring exposure to maternal obesity and a post-natal obesogenic diet profoundly accelerates hepatosteatosis at 12 months

Histological assessment of hepatosteatosis revealed greater fat infiltration in offspring exposed to a post-natal obesogenic diet (OffCon-OD) compared to controls (OffCon-SC) at 12 months than at 3 months. Prior exposure to maternal obesity followed by a post-natal obesogenic diet resulted in disproportionately exacerbated hepatosteatosis at 12 months compared to 3 months. In parallel with hepatosteatosis, there was at 12 months, clear evidence of histological liver injury as confirmed by the increased Brunt-Kleiner NAFLD Activity Score, confirming the presence of non-alcoholic steatohepatitis (NASH) (Figure 5.4). Additionally, an independent effect of maternal obesity on liver injury as denoted by the NAFLD Activity Score was observed.



Source of Variation	% of Variation	P value
Interaction	2.22	0.1326
Maternal	6.47	0.0190
Post-weaning	88.02	< 0.0001

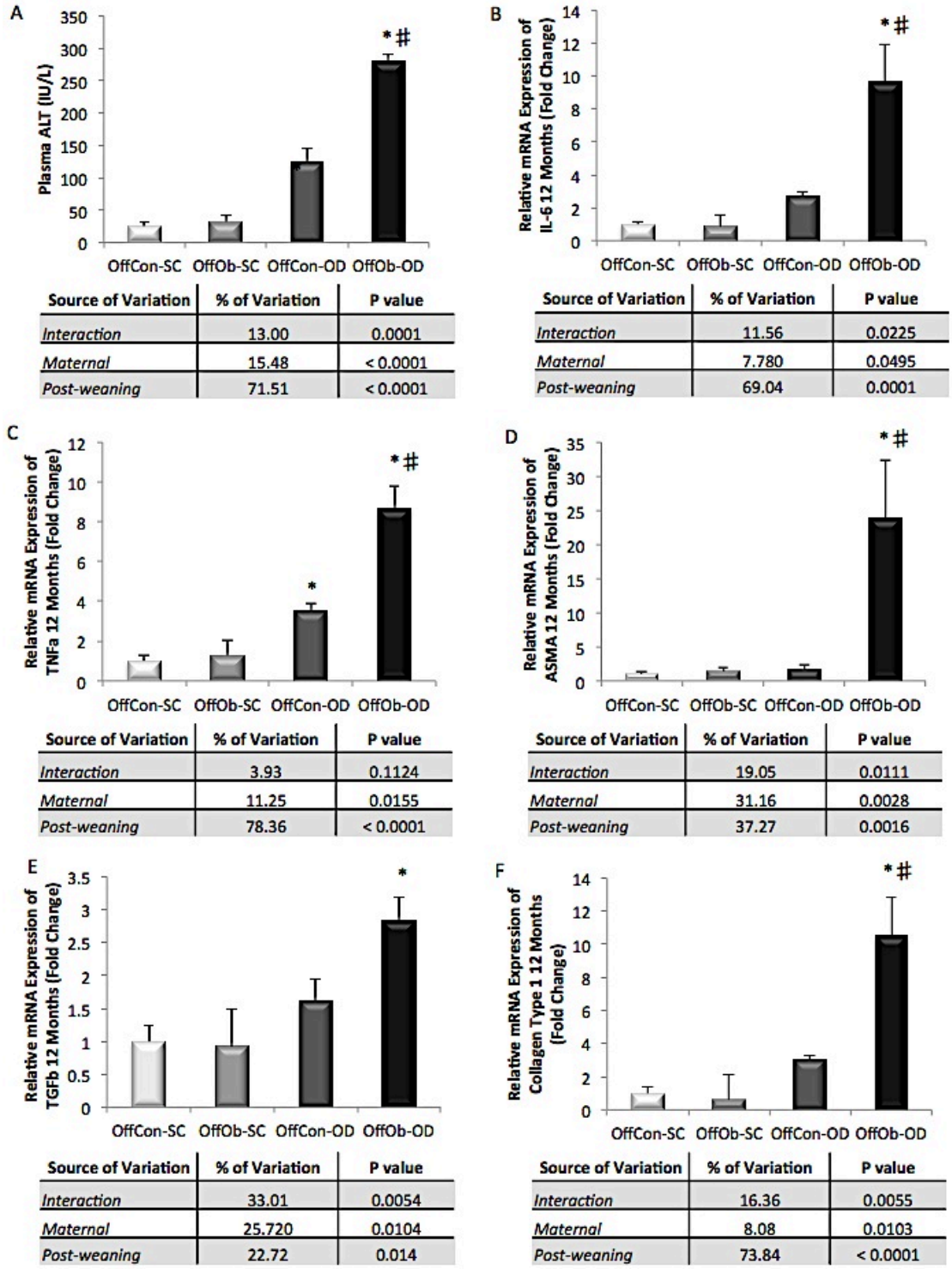
Figure 5.4 Histological Evidence of Hepatosteatosis and NASH at 12 months: (A) Representative H&E sections and (B) NAFLD Activity Score. Offspring exposure to maternal obesity in the context of a post-weaning hyper-calorific diet significantly exacerbated hepatosteatosis and development of NASH.

Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. n = 4-6 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.01$). # Mean values significantly greater than OffCon-OD ($p < 0.01$).

5.3.5 Offspring exposure to maternal obesity and a post-natal obesogenic diet induces profound liver injury and fibrogenesis at 12 months

At 12 months, serum ALT as a marker of liver injury was markedly and significantly raised in offspring exposed to a post-natal obesogenic diet only (OffCon-OD). ALT was further increased in offspring exposed additionally to maternal obesity (OffOb-OD). These observations were paralled by findings of increased expression of IL-6 and TNF-α mRNA in the same groups. Importantly, there were independent effects of maternal obesity on ALT levels and IL-6 and TNF-α expression with evidence of interaction between the maternal and post-weaning environments for ALT and IL-6. As with observations at 3 months, ASMA, TGF-β and collagen were also all profoundly up-regulated in offspring exposed to maternal obesity in addition to a post-natal obesogenic diet (Figure 5.5). Similarly, main effects were observed for both maternal and post-natal diets with a significant interaction between these two variables for all fibrogenic markers. So therefore, maternal obesity clearly induces offspring hepatic

fibrogenesis which is markedly enhanced in the context of a post-weaning obesogenic diet.



Source of Variation	% of Variation	P value
Interaction	13.00	0.0001
Maternal	15.48	< 0.0001
Post-weaning	71.51	< 0.0001

Source of Variation	% of Variation	P value
Interaction	11.56	0.0225
Maternal	7.780	0.0495
Post-weaning	69.04	0.0001

Source of Variation	% of Variation	P value
Interaction	3.93	0.1124
Maternal	11.25	0.0155
Post-weaning	78.36	< 0.0001

Source of Variation	% of Variation	P value
Interaction	19.05	0.0111
Maternal	31.16	0.0028
Post-weaning	37.27	0.0016

Source of Variation	% of Variation	P value
Interaction	33.01	0.0054
Maternal	25.720	0.0104
Post-weaning	22.72	0.014

Source of Variation	% of Variation	P value
Interaction	16.36	0.0055
Maternal	8.08	0.0103
Post-weaning	73.84	< 0.0001

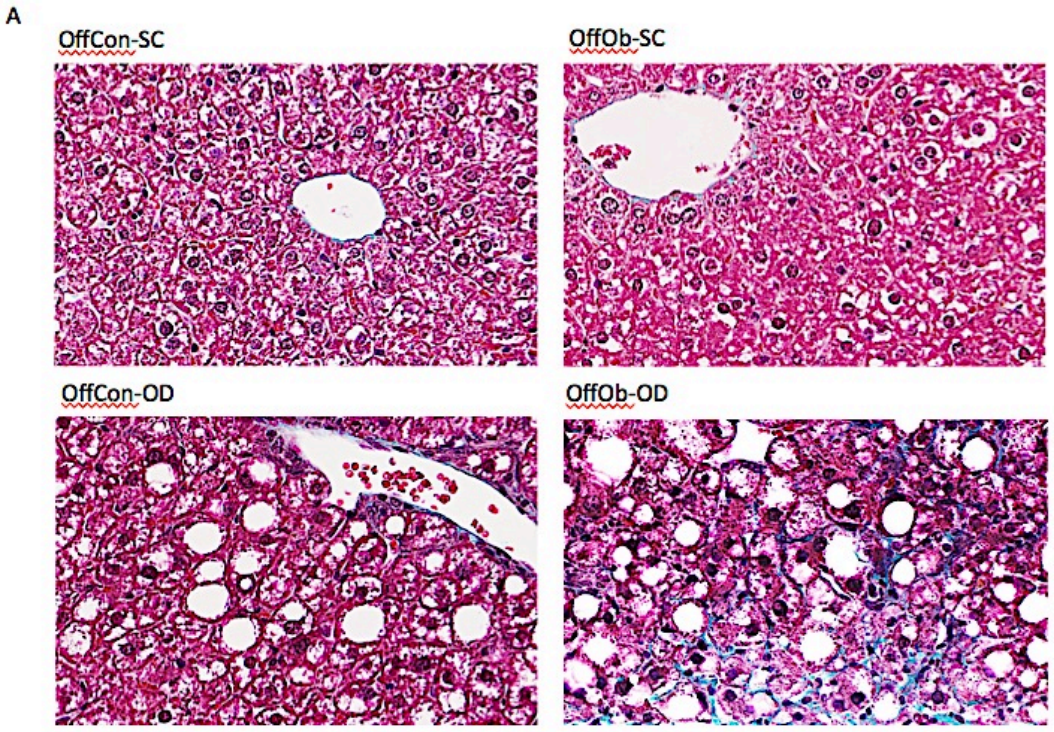
Figure 5.5 Biochemical Evidence of Liver Injury and Fibrogenesis at 12 months:

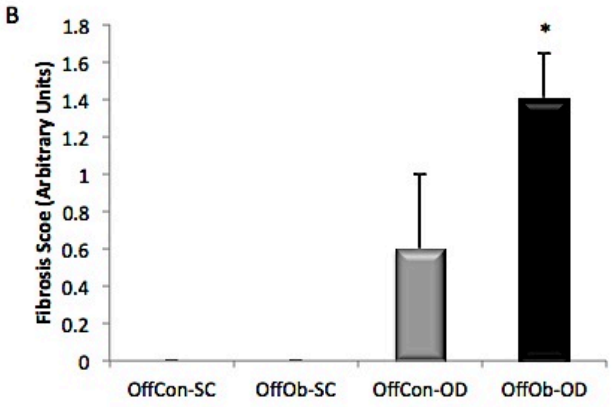
(A) Serum ALT, (B) IL-6, (C) TNF-α, (D) α-SMA, (E) TGF-β and (F) Collagen type 1.

Maternal obesity in the context of a post-weaning hyper-calorific diet accelerated liver injury and fibrogenesis. Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. n = 4-6 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.01$). # Mean values significantly greater than OffCon-OD ($p < 0.01$).

5.3.6 Offspring exposure to maternal obesity and a post-natal obesogenic diet induces evident fibrosis at 12 months

Biochemical evidence of fibrogenic pathway activation was corroborated by clear findings of pericellular fibrosis, on Masson Trichrome staining, in offspring exposed to a maternal obesity and a post-natal obesogenic diet. Minimal fibrosis was observed in offspring fed only a post-natal obesogenic diet as confirmed by the fibrosis score (OffConOD; 0.6 ± 0.4 v. OffOb-OD; 1.4 ± 0.2, arbitrary units) (Figure 5.6). So therefore, maternal obesity programs offspring NAFLD, which in the context of a post-weaning hyper-calorific diet, accelerates disease development resulting in irreversible hepatic fibrosis.





Source of Variation	% of Variation	P value
Interaction	7.27	0.1074
Maternal	7.27	0.1074
Post-weaning	45.45	0.0006

Figure 5.6 Histological Evidence of Hepatic Fibrosis at 12 months: (A) Representative Masson's Trichrome sections and (B) Fibrosis Score. Evident fibrosis was observed in OffOb-OD. Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese weaned on to

standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. n = 4-6 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.01$). # Mean values significantly greater than OffCon-OD ($p < 0.01$).

5.4 Discussion

A growing body of evidence implicates a role for maternal obesity in programming offspring NAFLD (Chapter 1) (51, 64, 180). Additionally, it has been previously observed that offspring exposed to maternal obesity in the immediate post-natal environment have a more profound dysmetabolic and NAFLD phenotype at 3 months (Chapter 2) (51). Using a more physiologically relevant and translational model, it is now shown that the maternal and post-natal environments interact to exacerbate the progression of NAFLD throughout adulthood.

A post-natal obesogenic diet causes offspring obesity with consequent hepatosteatosis and liver injury. Importantly, prior exposure of offspring to maternal obesity followed by an obesogenic diet results in an enhanced phenotype. At 3 and 12 months there was evidence of offspring obesity, hepatosteatosis and up-regulation of IL-6 and TNF-α and ASMA, TGF-β and collagen as biochemical markers of liver injury and fibrosis, respectively. Therefore, it may be suggested that in the liver, developmental programming sensitises the organ to deleterious effects in the context of continued insult

postnatally. Such phenotypic propagation has been reported in similar rodent models of maternal diet induced obesity investigating hepatic injury (64, 180, 196). It has been suggested that the interaction of the maternal and post-natal environments induce hepatic mitochondrial dysfunction and lipogenesis gene priming, evoking offspring NAFLD (64).

5.4.1 Offspring Adiposity

Exposure to a post-weaning obesogenic diet significantly increased bodyweight in offspring compared to controls. Importantly, prior exposure to maternal obesity, exacerbated weight gain. A similar pattern was observed for offspring inguinal fat pad mass, corroborating bodyweight data. A two-way ANOVA revealed statistically significant interaction between maternal obesity and the post-weaning obesogenic diet, confirming the additive effect of programming by maternal obesity in generating the enhanced phenotype at 3 months. These findings are supported by a previous report of accelerated adiposity in offspring with additional exposure to maternal obesity (55).

5.4.2 Offspring Liver Injury

The current paradigm for the pathogenesis of NAFLD, invokes insulin resistance as the primary driver for steatosis in the livers of obese individuals (172), with IL-6 and TNF-α involved in the inflammatory responses (174). Gene expression of IL-6 and TNF-α were both significantly increased in offspring fed an obesogenic diet post-weaning. However, relative expression of these inflammatory mediators was significantly greater in offspring with prior

exposure to maternal obesity at 3 and 12 months post-partum. Expectedly, hepatic injury was more marked at 12 months compared to 3 months. As observed with indices of adiposity, a significant difference between control offspring and those exposed to only maternal obesity was not observed although an upward trend was noted in OffOb-SC. So therefore, these novel findings demonstrate that the exacerbated phenotype of OffOb-OD was the result of programmed NAFLD adversely interacting with the hyper-calorific post-weaning environment in early and late adulthood, as confirmed by two-way ANOVA analysis.

5.4.3 Offspring Hepatosteatosis

It is well documented that saturation of hepatic lipid metabolism initiates and/or propagates liver injury (103). As previous, the most profound phenotype was observed in offspring exposed to maternal obesity and additionally fed an obesogenic diet post-weaning at 3 and 12 months. Hepatic tissue triglyceride content was significantly greater in this group (OffOb-OD) compared to offspring fed only an obesogenic diet post-weaning.

Interestingly, micro-vesicular presentation of hepatocellular steatosis was observed in OffCon-OD as evidenced histologically at 3 months. Steatosis in NAFLD is predominantly macro-vesicular causing cellular damage and potentiating pathogenetic mechanisms (197). It is therefore unsurprising that the extent of liver injury is greater in offspring presenting with larger lipid vesicles. The steatosis score, however, does not differentiate between vesicle

size and so an identical score based on morphometrical analysis was observed for OffCon-OD and OffOb-OD at 3 months. At 12 months however, hepatic injury was more profound and steatosis in addition to hepatocellular ballooning and lobular inflammation were observed. Therefore a composite NAFLD activity score was produced. A score greater than 5 was reported for OffOb-OD which is indicative of NASH, the more severe form of NAFLD associated with greater mobidity and mortality. Therefore, exposure to maternal obesity in the context of a post-weaning hyper-calorific diet is responsible for NAFLD progression to NASH.

5.4.4 Offspring Liver Fibrogenesis

Fibrogenesis is the result of over-exuberant production and deposition of matrix proteins secreted by injury activated hepatic stellate cells (HSCs), a phenotype characterised by abundant expression of α-SMA, TGF-β and collagen type-1, the predominant matrix constituent in fibrosis (186). Fibrogenic induction can occur following unhindered and continuous liver injury as observed in NAFLD. Both α-SMA, which is expressed permanently in activated HSCs, and collagen mRNA expression were increased in offspring fed a post-weaning obesogenic diet at 3 months. This increase was enhanced in offspring with prior exposure to maternal obesity although a significant difference between OffCon-OD and OffOb-OD was not reported using one-way ANOVA at 3 months. Moreover, two-way ANOVA analysis confirmed that increased gene expression of collagen was the result of interaction between maternal obesity and the post-natal diet.

At 12 months, more profound induction of hepatic fibrogenesis was observed in offspring exposed to maternal obesity and fed a post-weaning hyper-calorific diet. The exponential increase in gene expression of these fibrogenic markers was due to the interaction of maternal obesity with the post-weaning diet.

TGF-β, a pro-fibrogenic cytokine, was only found to be significantly up-regulated in offspring exposed to maternal obesity and fed an obesogenic diet postnatally at 3 and 12 months. Moreover, it was confirmed through two-way ANOVA, that the observed phenotype was due to maternal as well as post-weaning effects. Therefore, it could be suggested that in these current studies, priming of offspring livers through exposure to maternal obesity is predominantly responsible for the observed fibrogenesis.

5.4.5 Offspring Hyper-leptinaemia

Leptin, an adipokine, increased in tandem with fat pad mass although there was no statistically significant difference between OffCon-OD and OffOb-OD or significant interaction identified through two-way ANOVA analysis. Leptin is also implicated in fibrogenesis through its activation of HSCs, since animals lacking leptin are poorly fibrogenic (138, 187, 188). Therefore, the observed hyper-leptinaemia at 3 months, in OffCon-OD and OffOb-OD, may be in part, responsible for induction of hepatic fibrogenesis.

5.4.6 Relative Contributions of the peri-natal and post-weaning periods

As anticipated, all indices of adiposity, hepatic injury and fibrogenesis were raised in offspring fed an obesogenic diet post-weaning. A more aggressive dysmetabolic and NAFLD phenotype was observed in offspring with prior exposure to maternal obesity. Importantly, readouts for OffCon-SC and OffOb-SC were similar as previously reported in Chapter 3. Therefore, it cannot be argued that the disproportionately enhanced phenotype observed in offspring exposed to maternal obesity and continued on an obesogenic diet throughout post-natal life, compared to OffCon-OD, is simply due to longer exposure of a hyper-calorific environment.

By randomising offspring post-weaning to either one of two post-natal diets, one was able to investigate any possible interactions of maternal obesity in the context of a post-weaning obesogenic diet using two-way ANOVA analysis. Interactions of maternal obesity with the post-weaning diet were subsequently confirmed. Additionally, through investigation of early and late adulthood, it has now been shown that the natural progression of NAFLD is accelerated and worsened in offspring exposed to maternal obesity in gestation and lactation.

5.4.7 A More Pathophysiological Experimental Model of NAFLD

Continued exposure of offspring to maternal obesity and a hyper-calorific diet has provided a complete pathophysiologically relevant model of NAFLD. This novel model of NAFLD encompasses prominent features of the human disease along with biochemical and histological evidence of hepatosteatosis, liver injury

and fibrosis, raised inflammatory cytokines and hyper-leptinaemia, unlike the ob/ob and MCD mouse models of NAFLD which are leptin deficient and underweight with normal insulin homeostasis, respectively.

5.5 Conclusion

In conclusion, a post-natal obesogenic diet, in the context of prior exposure to maternal obesity, leads to heightened dysmetabolic and obesity related liver sequelae, of greater severity than that induced by the obesogenic diet alone. Therefore, maternal obesity through developmental programming, via changes induced during *in utero* and early peri-natal development, may be compounding the effects of excess calories consumed as an adult, leading to a worsened obesogenic and liver phenotype with irreversible fibrosis.

Having developed a novel and pathophysiologically complete model of NAFLD, Chapters 6 and 7 will investigate the mechanisms by which developmental programming via maternal obesity occurs.

6 Investigation of Mechanisms through which Maternal Obesity Programs Offspring NAFLD:

A Role for Hepatic Innate Immunity

6. Investigation of Mechanisms through which Maternal Obesity Programs Offspring NAFLD – A Role for Hepatic Innate Immunity

6.1 Introduction

The rising population prevalence of NAFLD (155) may be partially explained by the increasing availability of cheap energy dense foods. Additionally, this may be compounded by maternal obesity influencing eventual offspring liver phenotype, through dysmetabolic changes induced during *in utero* and perinatal development, as previously reported in Chapters 3 and 4 (51) and subsequently reported by other groups (64, 180).

Programmed metabolic and hepatic abnormalities by peri-natal over-nutrition may then be amplified in the context of a post-natal hyper-calorific environment. This can cause development of the more severe form, NASH, as reported in Chapter 3 or promote insulin resistance and perturb hepatic mitochondrial function as has been reported by other groups (20, 64, 198).

Hepatosteatosis and insulin resistance are considered to be the initiating factors in the pathophysiological cascade underpinning NAFLD. Its pathogenesis is propagated by the adipokines, tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (199) through generation of reactive oxygen species, ROS (200). Importantly, the hepatic innate immune system has also been implicated in liver pathophysiology, through contribution to the pro-inflammatory

response mediated by the cytokines, IL-12 and IL-18 (120). More specifically, it has been suggested that an imbalance of hepatic Th1 cytokines, produced by innate immune cells, is a common pathogenic mechanism in insulin resistance and NAFLD (121).

The hepatic innate immune system largely comprises of Kupffer (phagocytic), natural killer (NK) and natural killer T (NK-T) cells. Other components include acute-phase proteins (APP), pattern recognition receptors and complement factors (119). Kupffer cells (KC) constitute the majority of the body's macrophage population. They make up approximately 20% of hepatic non-parenchymal cells (i.e cells other than hepatocytes) and reside in liver sinusoids. KCs are responsible for eliminating blood borne pathogens, clearance of bacteria and generating ROS and pro-inflammatory cytokines, which potentiate immune responses indicative of hepatocyte injury (119).

NK-T cells are a unique lymphocytic sub-population that possess the ability to produce both pro- and anti-inflammatory cytokines, thereby modulating the eventual inflammatory response. Invariant NK-T cells or iNK-T cells are predominately found in the livers of the C57BL/6J mouse strain and are able to recognise lipids and glycolipids presented by CD1d molecules. As such, this cell type is implicated in metabolic liver disease (201). NKT cell autoreactivity is also regulated by KC derived cytokines, dietary factors through CD1d mediated antigen presentation and sympathetic nervous system outputs such as noradrenaline (121, 201).

Attenuation of hepatosteatosis post Kupffer cell (KC) ablation in a NASH model has been reported (202). It is thought that KC-mediated cytokine production impairs lipid peroxidation and propagates insulin resistance, culminating in hepatosteatosis (203). Moreover, KC activation and defective phagocytosis has been reported in the induction and potentiation of NAFLD (204, 205). Additionally, Natural Killer T (NK-T) cells which can mediate an anti-inflammatory response, are selectively reduced in the ob/ob mouse model of NAFLD (126). Therefore, investigation of mechanisms that associate lipid metabolism with inflammation, such as the hepatic innate immune system may divulge the mechanistic pathway by which maternal obesity programs offspring NAFLD.

Here, the aims were to investigate the mechanistic role of the hepatic innate immune system in a pathophysiologically relevant model of programmed offspring NAFLD via maternal diet induced obesity.

6.2 Methods

6.2.1 Animal Experimentation

Female C57BL/6J mice (n = 20 per group) (Charles River Laboratories, UK), of first order parity, approximately 100 days old were allowed 7 days to habituate following transfer to the facility and fed standard laboratory chow (RM1, Special Dietary Services, energy 3.5 kcal/g) *ad libitum*.

Following acclimatisation, female mice were randomised to either a control or experimental group and fed standard chow or a semi-synthetic energy-rich, highly palatable obesogenic diet (Special Dietary Services, energy 4.5 kcal/g) supplemented with sweetened condensed milk (Nestle) admixed with mineral mix (AIN93G, Special Dietary Services, 125 mg/pot) *ad libitum*.

Dams were maintained on their respective diets for 6-8 weeks. Upon attainment of an average 30% increase in body weight of the experimental group, mice were entered into the breeding protocol. All animals were treated in accordance with The Animals (Scientific Procedures) Act, UK, 1986 guidelines.

Pregnant dams were continued on their pre-conception diets throughout pregnancy and lactation. During pregnancy, maternal weight and dietary intake were recorded weekly. Dams were allowed to deliver spontaneously and litter size standardised. Subgroups of female offspring (n = 20) born to and suckled by lean dams or born to and suckled by obese dams were weaned on to either normo-calorific or semi synthetic obesogenic diet with condensed milk supplementation, constituting a diet high in sugar and fat (Figure 2.6). All readouts were compared to the control group, offspring of lean suckled by lean dams and weaned onto standard chow (OffCon-SC). Readouts were obtained at 6 months.

6.2.2 Tissue Collection

Offspring liver tissue was differentially preserved for use in gene expression and FACS analysis, immunohistochemistry and histology. Briefly, whole liver tissue was snap frozen in liquid nitrogen, preserved in RNAlater solution or dissected using middle orientation and stored in 10% neutral buffered formalin prior to paraffin embedding.

6.2.3 Liver Mononuclear Cell (MNC) Fraction Isolation

After sacrifice, the liver was perfused with 5ml PBS via the portal vein and homogenised. Total liver cells were then re-suspended in perfusate buffer containing HBSS (Ca^{2+}, Mg^{2+}), collagenase (0.01%) and DNase I (0.001%). Following filtration through 70µm cell strainer, the pelleted cells were re-suspended in RPMI and layered with 24% optiprep. Subsequent to centrifugation, mononuclear cells were isolated at the 40/60% interface. The cells were washed once with a perfusate buffer containing HBSS (free Ca^{2+}, Mg^{2+}), BSA (0.25%) and DNase I (0.001%) and supplemented with complete culture media (RPMI, FBS (10%), PCN 100U/ml, Strep 100µg/ml, L-glutamine 200mM). Cell types were determined using microscopy.

6.2.4 Reactive Oxygen Species Detection

KC derived ROS was assayed using the Total ROS Detection Kit (ENZO-51011). In brief, cell preparations were stimulated using LPS (E.coli 0111:B4, Sigma Aldrich) and incubated for 30 minutes at 37°C. Samples were then washed and cells re-suspended in ROS detection solution, incubated with TruStain FcX (anti-mouse

CD16/32) and stained with F4/80 antibody (AbD Serotec). Subsequent flow cytometric analysis is detailed below.

6.2.5 Kupffer Cell Phagocytosis

Microspheres (Fluoresbrite@YG Microspheres 1.00µm) were incubated with total MNC suspension for 20 minutes at 37°C. Reaction was stopped by addition of 2ml ice cold PBS. The cell preparations were then washed and incubated with TruStain FcX (anti-mouse CD16/32) and stained with F4/80 antibody. Subsequent flow cytometric analysis is detailed below.

6.2.6 FACS (Fluorescent Activated Cell Sorting) Analysis

Cell preparations were stained with CD3-FITC/NK1.1-PerCp and F4/80 clone BM8-PerCP-Cy5.5 antibodies (AbD Serotec) for identification of NKT and KC respectively. Cells were incubated at 4°C for 20 minutes followed by addition of 1ml FACS buffer (Biolegend) and centrifugation. Cells were re-suspended in a final volume of 100µl FACS buffer and analysed by flow cytometry (BD LSR II). Quantification of data was performed using FlowJo 5.6.1.

6.2.7 Gene Expression of Pro-inflammatory Cytokines

Real Time Polymerase Chain Reaction (RT-PCR) was performed using QuantiTect SYBR Green PCR System with HotStar Taq DNA Polymerase (Qiagen). Quantitect Primer Assays (Qiagen) using SYBR green based detection were used for IL-12 and IL-18. Expression of target genes was normalised to GAPDH expression.

6.2.8 Histology

Offspring liver sections at 6 months of age were formalin (10%) fixed and paraffin embedded prior to sectioning. All sections were then stained with haematoxylin and eosin (H&E) and Masson's Trichrome to assess steatosis and inflammation and fibrosis, respectively. The Brunt-Kleiner NAFLD Activity Score was used to quantitatively assess the degree of injury by an expert liver pathologist blinded to the identity of the groups.

6.2.9 Immunohistochemistry

Immunohistochemical detection of KC was performed on paraffin embedded sections treated with proteinase K, a primary rat anti-mouse F4/80 monoclonal antibody (1/100, Serotec) and a secondary rabbit anti-rat immunoglobulin (ImmPRESS anti-rat Ig (Peroxidase) Polymer Detection Kit, Vector Laboratories). Peroxidase activity was demonstrated using ImmPact DAB (diaminobenzidine-based) peroxidise substrate kit (Vector Laboratories). The slides were counter-stained with hematoxylin.

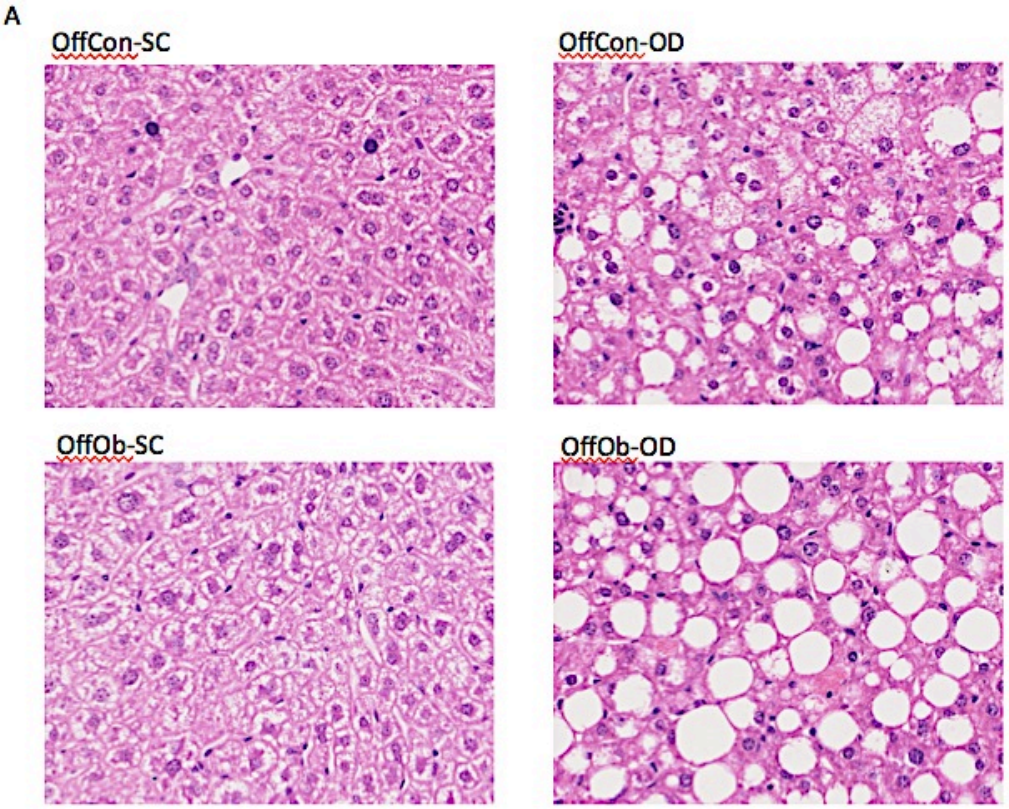
6.2.10 Statistical Analysis

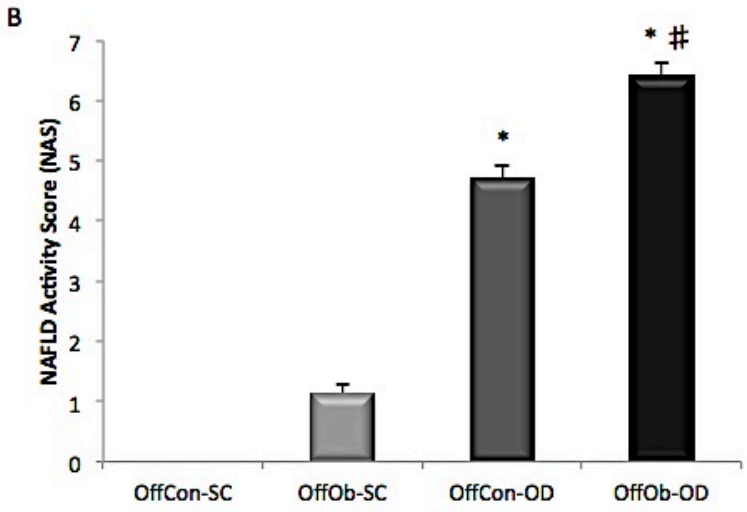
Multiple comparisons on a single data set were performed using ANOVA followed by Tukey's *post hoc* test and expressed as mean ± SEM unless otherwise stated. Interactions between maternal obesity and the post-natal diet were investigated using two-way (2x2 factorial) ANOVA through data analysis software (GraphPad Prism 5.0). $p < 0.05$ was regarded as significant. Sample size per group; n = 5, 1 from each litter.

6.3 Results

6.3.1 Offspring exposure to maternal obesity and a post-natal obesogenic diet induces histological evidence of non-alcoholic steatohepatits at 6 months.

To validate the experimental model prior to investigation of potential mechanisms, histological assessment of hepatosteatosis and liver injury was performed. Greater fat infiltration in offspring exposed to a post-weaning obeogenic diet (OffCon-OD) compared to controls (OffCon-SC) was observed. Prior exposure to maternal obesity followed by a post-weaning obesogenic diet resulted in exacerbated and profound hepatosteatosis at 6 months. In parallel with hepatosteatosis, there was clear evidence of histological liver injury as confirmed by the increased Brunt-Kleiner NAFLD Activity Score in OffOb-OD (Figure 6.1). Moreover, an independent effect of maternal obesity on the NAFLD Activity Score was observed with two-way ANOVA analysis.





Source of Variation	% of Variation	P value
Interaction	2.22	0.1326
Maternal	6.47	0.0190
Post-weaning	88.02	< 0.0001

Figure 6.1 Histological Evidence of Hepatic Injury and Steatosis at 6 months: (A) Representative H&E sections and (B) NAFLD Activity Score. Offspring

exposure to maternal obesity in the context of a post-weaning hyper-calorific diet significantly exacerbated hepatosteatosis and liver injury. Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. n = 4-6 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.01$). # Mean values significantly greater than OffCon-OD ($p < 0.01$).

6.3.2 Offspring exposure to maternal obesity and a post-natal obesogenic diet significantly impairs Kupffer cell function at 6 months

The hepatic non-parenchymal cell fraction was investigated in order to study the mechanisms underlying the observed offspring steatohepatitis phenotype. Focussing on KCs, the population, as determined by FACS analysis and corroborated by immunohistochemical staining, was significantly increased in offspring exposed to maternal obesity and fed an obesogenic diet post-weaning (OffOb-OD). Although an upward trend in KC number was observed for offspring only fed a post-weaning obesogenic diet, the increase was not of statistical significance. Importantly, KC numbers observed in OffCon-SC and OffOb-SC were similar, confirming that the exponential increase in OffOb-OD was not just due to longer hyper-calorific exposure (Figure 6.2).

Despite their increased numbers, KC phagocytic function was impaired in offspring exposed to maternal obesity and fed an obesogenic diet post-weaning compared to all other groups. In contrast, KC ROS production was significantly increased upon lipopolysaccharide stimulation in the same group (Figure 6.2). Two-way ANOVA analysis confirmed significant interactions between the maternal and post-weaning obesogenic environments for KC derived ROS and phagocytosis, thereby suggesting that maternal obesity is a prerequisite for impaired KC functionality that manifests in the context of a post-weaning obesogenic diet.

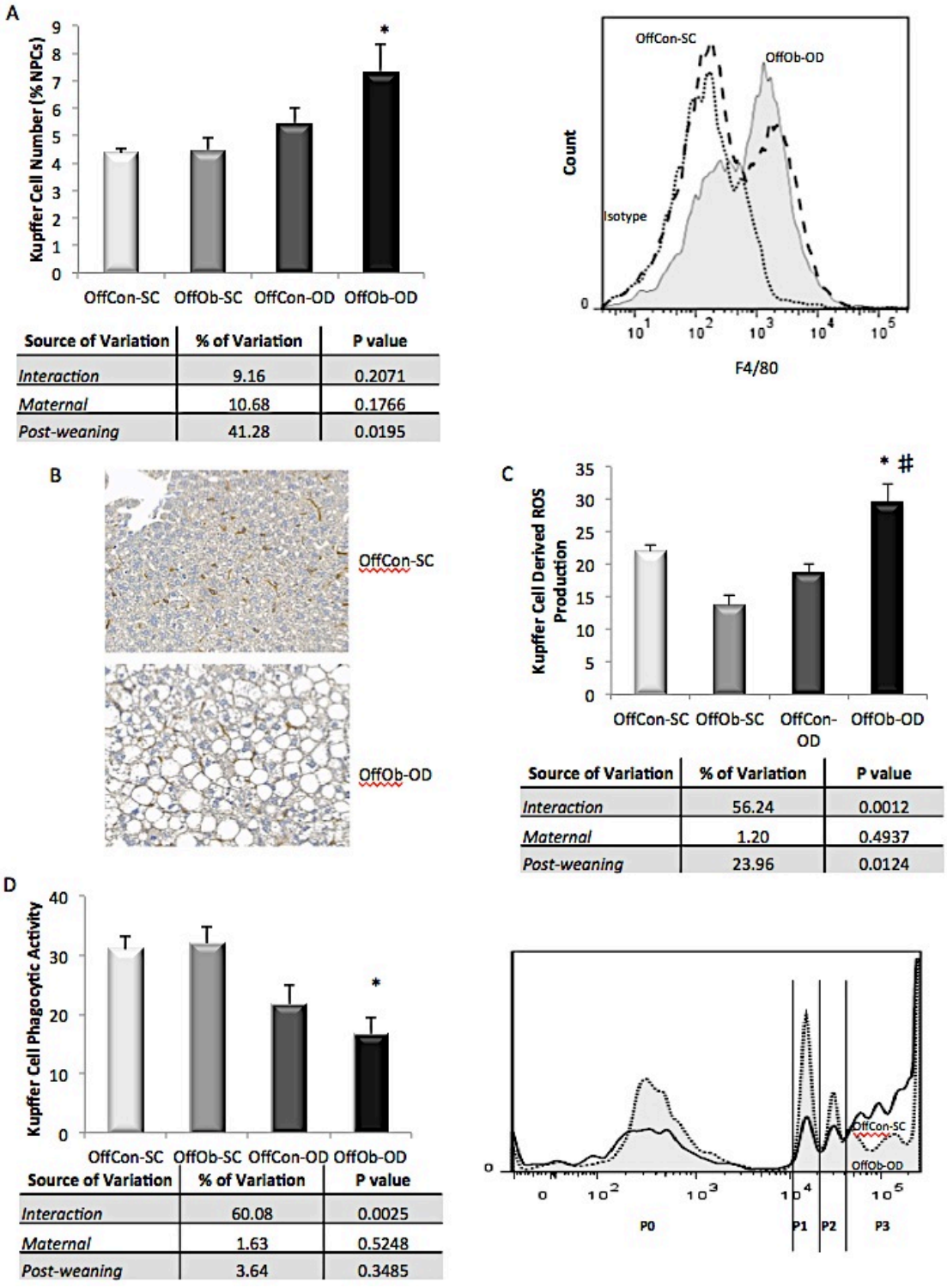
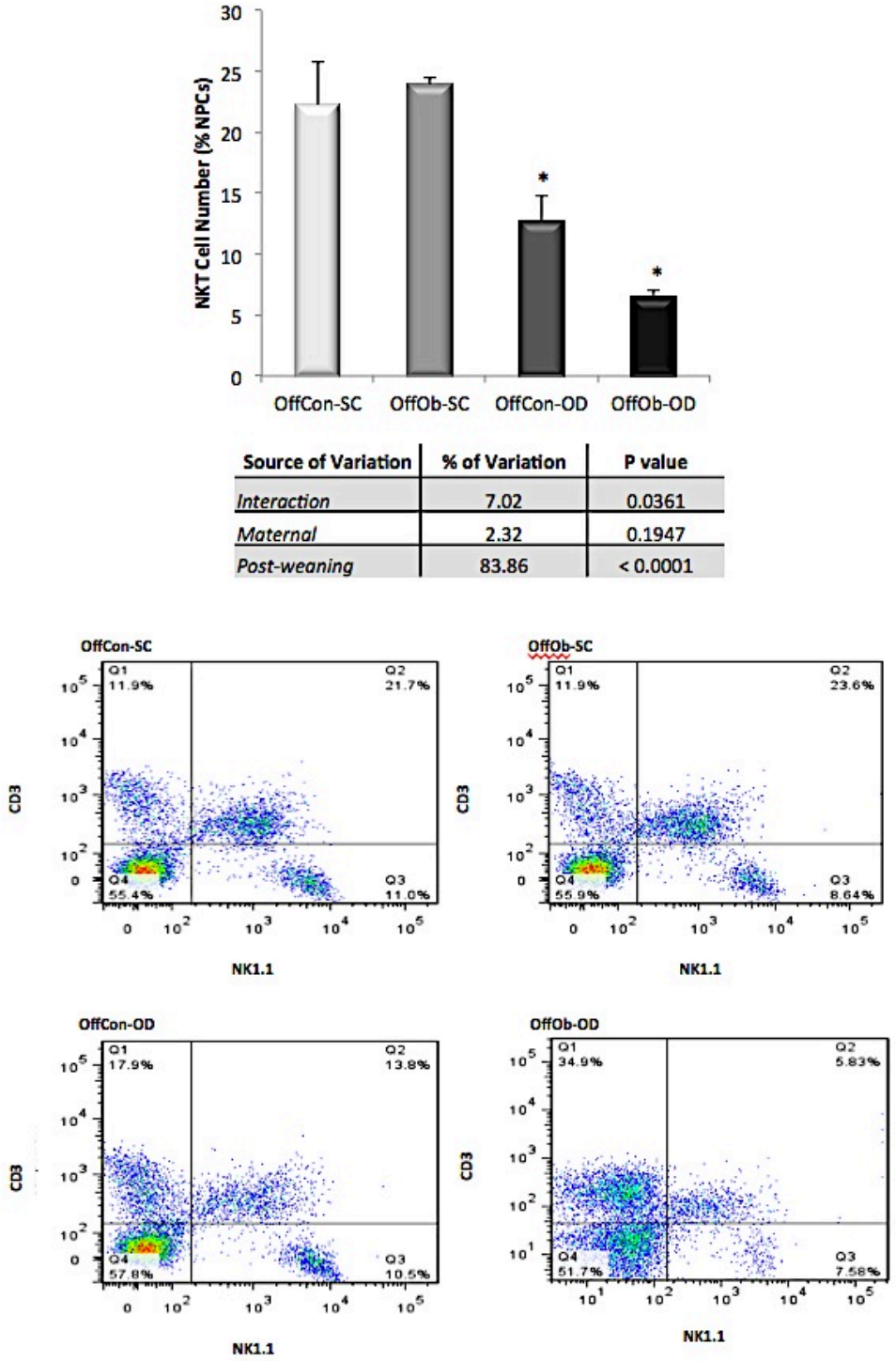


Figure 6.2 Kupffer cell function at 6 months: (A) KC population assay, (B) Immunohistochemical detection of KCs, (C) KC derived ROS and (D) KC phagocytic activity. KC functionality is profoundly impaired in OffOb-OD.

Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. n = 4-6 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.01$). # Mean values significantly greater than OffCon-OD ($p < 0.01$).

6.3.3 Offspring exposure to maternal obesity and a post-natal obesogenic diet significantly attenuates the NK-T cell population at 6 months

NK-T cell number as determined by FACS analysis was significantly reduced in offspring fed a post-natal obesogenic diet (OffCon-OD). Prior exposure to maternal obesity in addition, enhanced this attenuation (OffOb-OD) (Figure 6.3). Moreover, main effects were observed for both maternal and post-natal diets with a significant interaction between these 2 variables for offspring NKT cell number (two-way ANOVA analysis).



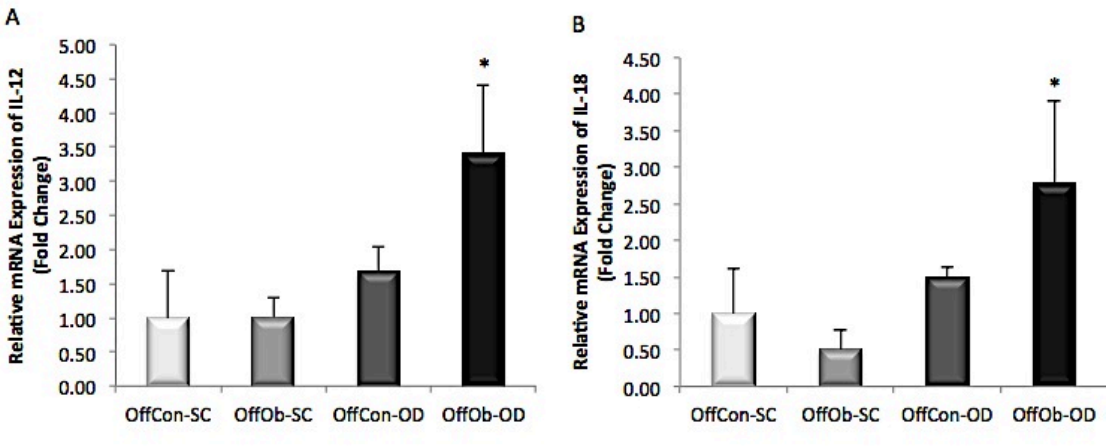
Source of Variation	% of Variation	P value
Interaction	7.02	0.0361
Maternal	2.32	0.1947
Post-weaning	83.86	< 0.0001

Figure 6.3 NK-T cell population assay at 6 months: Reduced NK-T cell numbers observed in OffOb-OD. Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic

diet – OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. n = 4-6 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.01$). # Mean values significantly greater than OffCon-OD ($p < 0.01$).

6.3.4 Offspring exposure to maternal obesity and a post-natal obesogenic diet induces up-regulation of Th1 polarised cytokines

The pro-inflammatory cytokines, IL-12 and IL-18 were significantly up-regulated in offspring exposed to maternal obesity and fed an obesogenic diet post-weaning (OffOb-OD). These pro-inflammatory cytokines, produced by KCs of the M1 (pro-inflammatory) phenotype, corroborate the observation of increased KC number in the same group. Additionally, there was an independent effect of maternal obesity on IL-12 expression with evidence of an interaction between maternal obesity and the post-natal diet forIL-18 expression (Figure 6.4).



Source of Variation	% of Variation	P value
Interaction	4.19	0.1334
Maternal	14.57	0.0132
Post-weaning	65.62	0.0001

Source of Variation	% of Variation	P value
Interaction	22.71	0.0044
Maternal	0.02	0.9262
Post-weaning	59.35	0.0001

Figure 6.4 Th1 Polarised Cytokines at 6 months: (A) IL-12 and (B) IL-18. Cytokines were up-regulated in OffOb-OD. Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. n = 4-6 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.01$). # Mean values significantly greater than OffCon-OD ($p < 0.01$).

6.4 Discussion

Previous chapters have shown that offspring exposed to maternal obesity during gestation and lactation develop a dysmetabolic phenotype with evidence

of liver injury at 3 months and that exposure limited to the immediate post-natal environment profoundly exacerbates NAFLD (51). In a more physiologically relevant and translational model, it was reported that the maternal and post-natal environments interacted to further propagate progression of NAFLD. It is here shown that the hepatic innate immune system may be involved in offspring NAFLD development programmed by maternal obesity.

Prior to mechanistic investigation, histological evidence of NAFLD was confirmed and quantitative morphometrical analysis using the NAFLD Activity Score was used to assess the extent of injury. Offspring exposed to maternal obesity and fed an obesogenic diet post-weaning developed NASH, the more severe form of NAFLD compared to all other groups. Such findings have been corroborated by other groups using a high fat diet in a similar experimental paradigm (64, 180).

Here it is now shown that innate immune dysregulation is profound in the group fed an obesogenic diet with prior exposure to maternal obesity. These novel findings suggest that maternal obesity program offspring susceptibility to NAFLD via perturbation of the innate immune system.

6.4.1 Kupffer Cells

The current paradigm for the pathogenesis of NAFLD holds fat accumulation and insulin resistance as prerequisites for disease development. These insults

then sensitise the liver to secondary injurious effects driven by pro-inflammatory and pro-fibrotic cytokines (206). Therefore, investigation of mechanisms that associate lipid metabolism with inflammation, such as the hepatic innate immune system may divulge the underlying pathophysiology.

Moreover, KC activation and defective phagocytosis have been reported in the induction and potentiation of NAFLD (204, 205). It has been suggested that steatosis may disrupt sinusoid microcirculation and hepatocellular clearance of microbial antigens, all of which activate KCs (207). KCs, the liver's resident macrophages, account for 80-90% of the total fixed tissue macrophage population in the body (208) and were up-regulated here in offspring fed a post-natal obesogenic diet with prior exposure to maternal obesity. These findings are corroborated in a rat model of NASH where KCs are recruited and activated following exposure to a high fat diet (209).

6.4.2 Reactive Oxygen Species (ROS)

KCs are also able to directly generate ROS via the NADPH oxidase-dependent and the xanthine oxidase-dependent pathways (210). KC derived ROS is well documented in alcoholic liver disease (ALD) (205), which bears histological resemblance to NAFLD. As such, KC mediated ROS has been implicated in NAFLD pathogenesis. Direct evidence of elevated ROS production by KCs, rising proportionately with increased KC numbers in programmed offspring exposed to a post-natal obesogenic diet, is reported here. Moreover, two-way ANOVA analysis confirmed the presence of significant interactions between the

maternal and post-natal environments in generating the observed phenotype. These findings are in keeping with previous reports of gross hepatic ROS production mediating NAFLD in a rodent model (211).

6.4.3 Th1 Polarised Cytokines

KCs possess the ability of functional polarisation to release pro-inflammatory (M1 phenotype) or anti-inflammatory (M2 phenotype) cytokines (203). Such M1 phenotypic cytokines include IL-12 and IL-18, both of which we report here to be up-regulated in offspring exposed to maternal obesity plus an obesogenic diet post-weaning. Additionally, it has been shown here that there is an independent effect of maternal diet on IL-12 expression with evidence of an interaction between maternal obesity and the post-natal diet for IL-18 expression. It is also well documented that these M1 phenotypic cytokines are up-regulated in response to increased oxidation of free fatty acids (154).

More recently, M1 macrophage activation has been directly correlated with the severity of NASH in an MCD mouse model through comparison of disease progression in M1 (C57BL/6J) and M2 (Balb/c) macrophage biased rodents (212). Additionally, IL-18 has been shown to regulate NAFLD progression through modulation of the gut microbiota (213). These findings confirm a contributory role of macrophage responses in NAFLD.

IL-12 is the most potent soluble activator of Th1 cells (214), which are a sub-group of T helper lymphocytes capable of producing further pro-inflammatory

cytokines and inducing cellular immunity. T helper cells are thus able to regulate immune responses through activation of these cells and the anti-inflammatory cytokine producing cells, Th2 (119). The evident Th1 polarised state may therefore be largely contributable to the observed NASH phenotype in OffOb-OD.

6.4.4 Kupffer Cell Phagocytic Function

Rodents possess at least two KC subtypes, determined by the extent of phagocytic capacity. Reduced phagocytic function in the context of increased KC number is reported here. These findings are corroborated by previous studies in rodent models of obesity and NAFLD and in patients with biopsy proven NASH (215, 216). Additionally, main effects were observed for both maternal and post-natal diets with a significant interaction between these two variables for impaired KC phagocytosis. Impaired KC phagocytosis evokes over-production and sensitivity to the aforementioned key inflammatory mediators, enhancing hepatic inflammation and further propagating injury in NAFLD. Moreover, reduced clearance of dead cells and lipopolysaccharide generate a hyper-endotoxaemic state to potentiate pro-inflammatory pathways (205).

6.4.5 NK-T Cells

Regarding NK-T cells, these are presently thought to modulate inflammatory responses by achieving a balance between Th-1 and Th-2 polarisation states within the liver. NK-T cell depletion has been associated with worsened NAFLD phenotypes, not only in this present study but also in the ob/ob leptin deficient

mice (126) and in models of diet-induced NAFLD (217). Here, it is shown that maternal obesity interacts with the post-weaning diet to influence NK-T cell numbers. The mechanism under-pinning the inverse relationship between hepatosteatosis and NK-T cells or their subtypes is unclear. We report here an increase in the expression of IL-12, in offspring exposed to maternal obesity and/or a post-natal obesogenic diet. This increase in IL-12 expression is of pathogenic relevance because IL-12 has been shown to promote NK-T cell death (127).

However, it is also known that fatty acids from impaired lipid metabolism or high dietary intake of fat induce invariant and non-variant NK-T cell depletion via altered CD1d antigen presentation by hepatocytes (121, 201). It is not known how these fatty acids alter endogenous lipid ligands although it has been suggested by Ichi and colleagues, that alterations in liver ceramides, which are likely antigens for NK-T cells, may be responsible (218). Additionally, it has been suggested that hepatosteatosis reduces hepatic CD1d expression as lipid loading reduces trafficking of CD1d to the cell surface, further reducing antigen presenting capabilities to NK-T cells in the ob/ob mouse model (125). So therefore, reduction of NK-T cell mediated anti-inflammatory cytokine responses may be the result of impaired CD1d dependent antigen presentation to NK-T cells in addition to KC mediated cytokine depletion of NKT cells, potentiating NAFLD progression.

Given that CD1d is also necessary for maintaining NKT cell homeostasis (125), measurement of hepatocyte CD1d expression would provide further information about the mechanistic association between programmed NAFLD and selective NKT cell depletion. This study relies upon NK1.1 immunoselection to innumerate NKT cell number. However, given that NK1.1 cell surface expression varies with cell maturation, activation and positively selects all NKT cell subtypes, these preliminary findings and their putative association in programmed NAFLD must be confirmed in NKT cells co-expressing the invariant TCR i.e. identification of the CD1d restricted population.

6.5 Conclusion

In conclusion, it has been shown that a post-natal obesogenic diet, in the context of prior exposure to maternal obesity, leads to heightened hepatic injury indicative of non-alcoholic steatohepatitis, of greater severity than that induced by the obesogenic diet alone. Therefore, maternal obesity through developmental programming, via changes induced during gestation and lactation, may be compounding the effects of a hyper-calorific post-weaning environment, causing a worsened hepatic phenotype.

Offspring exposed to maternal obesity and a post-natal obesogenic diet, uniquely have significant disturbance of the hepatic innate immune system, with reduced NK-T cell populations, probably secondary to demonstrated increased IL-12 expression, along with increases in Kupffer cell numbers, which

have perturbed phagocytic function but raised ROS production, further propagating liver injury.

The mechanisms of programming are unknown, although it is accepted that its aetiopathogeneis is multifactorial. Given the complex pathogenic pathways implicated in programmed offspring NAFLD by maternal over-nutrition, it is likely that changes at the cellular, sub-cellular and epigenetic level are responsible. Having preliminarily investigated cellular responses of the hepatic innate immune system to maternal obesity, Chapter 7 will investigate the mechanistic role of circadian rhythms at the transcriptional level.

7 Investigation of Mechanisms through which Maternal Obesity Programs Offspring NAFLD:

A Role for Molecular Circadian Rhythms

7 Investigation of Mechanisms through which Maternal Obesity Programs Offspring NAFLD – A Role for Molecular Circadian Rhythms

7.1 Introduction

The biological circadian clock is a transcriptional mechanism that synchronises physiological and behavioural processes to co-ordinate daily rhythms in feeding behaviour, rest/activity and energy utilisation within a 24 hour light/dark cycle (72, 219). The molecular machinery encoding the biological clock, contained in almost every cell of the body, involves a transcriptional/translational negative feedback loop in which the transactivation of E-box containing genes by CLOCK (circadian locomotor output kaput cycles) and BMAL1 (brain and muscle anrt like 1) is inhibited by the repressors, Periods and Cryptochromes. Regulatory accessory pathways involving REV-ERB-α and other members of the nuclear receptor family stabilise the circadian clock through repressive and inductive activities on BMAL1 (219).

This cyclical process occurs in the suprachiasmatic nucleus (SCN) of the hypothalamus, as well as in most mammalian cells, to achieve metabolic homeostasis. The molecular machinery in the SCN is referred to as the master clock and is the pace-setter for all peripheral clocks. The master clock is predominantly entrained by light, given the direct nervous connection between

the retina and the hypothalamus via the retino-hypothalamic-tract (RHT). In addition to light entrainment via the SCN, peripheral clocks have been shown to be entrainable by nutrients and temperature (70, 220). The synchronisation of the central and peripheral clocks is achieved through autonomic activity, body temperature and hormones (221, 222). Glucocorticoid signalling, as an example of such a systemic cue, has been shown to regulate transcription of a few canonical clock genes in peripheral cells (223, 224) as well as synchronise the liver clock *in vivo* (225).

The association between the molecular circadian clock and metabolism has been highlighted in numerous experimental models including homozygous CLOCK mutant mice which have been reported to be hyperphagic, obese and hyper-leptinaemic and develop hepatosteatosis (72). Moreover, a high fat diet in rodents has been reported to alter the expression and rhythmicity of canonical circadian clock genes and nuclear receptors, which regulate clock transcription factors in peripheral tissues (73). Additionally, expression of genes encoding lipogenesis, lipolysis and gluconeogenesis have been shown to posses rhythmic variation over a circadian period, suggesting that there is a close association between metabolism and the periodicity of transcription programmed by the circadian clock (71, 226-228).

Interestingly, an increased susceptibility to obesity, dysmetabolism, cardiovascular disease and cancer has been observed in shift workers exposed to prolonged periods of darkness or unnatural exposure to light and dark (229-

231). It was suggested that the observed increased risk was due to the inhibited synchronisation of the biological master clock with environmental cues, causing central and systemic disruption of normal circadian rhythms (230).

A growing body of evidence demonstrates the effects of disrupted circadian rhythms on metabolic homeostasis, but much less evidence documenting how metabolic processes alter the circadian clock exists. In a diet induced obesity rodent model, Bass and colleagues demonstrated behavioural and molecular disruption of circadian rhythms (72). These findings are supported by earlier observations of metabolic transcripts binding to promoter regions of CLOCK and BMAL1 and demonstration of transcription factors involved in adipogenesis such as REV-ERB-α, regulating the molecular clock machinery (232).

So, therefore, an established association between nutrient status and pathways governing circadian transcription exists. It is thus plausible that the hyper-calorific maternal milieu could alter circadian molecular circuitry in the liver, increasing offspring susceptibility to metabolic liver disease such as NAFLD. Here, the aims were to investigate the mechanistic role of hepatic circadian rhythms in a pathophysiologically relevant model of programmed offspring NAFLD induced via maternal obesity and a post-weaning hyper-calorific, obesogenic diet.

7.2 Methods

7.2.1 Animal Experimentation

Female C57BL/6J mice (n = 20 per group) (Charles River Laboratories, UK), of first order parity, approximately 100 days old were allowed 7 days to habituate following transfer to the facility and fed standard chow RM1 (Special Dietary Services, energy 3.5 kcal/g) *ad libitum*.

Following acclimatisation, female mice were randomised to either a control or experimental group and fed standard chow or a semi-synthetic energy-rich, highly palatable obesogenic diet (Special Dietary Services, energy 4.5 kcal/g) supplemented with sweetened condensed milk (Nestle) admixed with mineral mix (Special Dietary Services, 125 mg/pot) *ad libitum*. All mice were maintained on a 12:12 LD cycle.

Dams were maintained on their respective diets for 6-8 weeks and macronutrient and calorific intake were calculated from measured daily intake of pellets and milk Upon attainment of mean 30% increase in body weight of the experimental group, mice were entered into the breeding protocol. All animals were treated in accordance with The Animals (Scientific Procedures) Act, UK, 1986 guidelines.

Pregnant dams were continued on their pre-conception diets throughout pregnancy and lactation. During pregnancy, maternal weight and dietary intake

were recorded weekly. Subgroups of male and female offspring (n = 20) born to and suckled by lean dams or born to and suckled by obese dams were weaned on to either normo-calorific or semi synthetic obesogenic diet with condensed milk supplementation, constituting a diet high in sugar and fat as per Phase 4 of the model (Figure 2.6). All readouts were compared to the control group, offspring of lean suckled by lean dams and weaned onto standard chow (OffCon-SC). At 6 months, offspring were sacrificed at 4 hourly intervals across the 24 hour LD cycle to obtain liver tissue.

7.2.2 Body and Liver Weights

Offspring bodyweight was measured prior to sacrificing using a schedule-1 method. Livers were excised and weights recorded using a balance accurate to 4 decimal places.

7.2.3 Tissue Collection

Offspring liver tissue was differentially preserved for use in gene expression analysis, histology and methylation analysis. Briefly, whole liver tissue was snap frozen in liquid nitrogen, preserved in RNAlater solution or dissected using middle orientation and stored in 10% neutral buffered formalin prior to paraffin embedding. Offspring blood was collected via cardiac puncture or inferior vena cava cannulation using heparinised syringes. The blood was centrifuged at 3000 x g for 10 minutes at 4˚C and decanted plasma stored at -20˚C until biochemical analysis.

7.2.4 Serum Analysis

Alanine transaminase was measured by the local clinical biochemistry department.

7.2.5 Liver Tissue Triglyceride

Whole liver tissue triglyceride was determined by an adaptation of the Folch Method using an enzymatic colorimetric assay (UNIMATE 5 TRIG, Roche BC1. Sussex, UK). Approximately 0.05g of liver tissue per sample was used and all results normalised to sample quantity.

7.2.6 Gene Expression of Pro-inflammatory and Fibrogenic Cytokines and Canonical Circadian Transcripts

Real Time Polymerase Chain Reaction (RT-PCR) was performed using QuantiTect SYBR Green PCR System with HotStar Taq DNA Polymerase (Qiagen). Gene specific primers were designed for IL-6, TNF-α, α–Smooth Muscle Actin (α-SMA), transforming growth factor-β (TGF-β) and collagen Type 1-α2 (Col 1-α2) (Table 2.3). Quantitect Primer Assays (Qiagen) using SYBR green based detection were used for CLOCK, BMAL1, Period 1, Period 2, Cryptochrome 1, Cryptochrome 2 and REV-ERB-α (Table 2.4). Expression of target genes was normalised to GAPDH expression.

7.2.7 Histology

Offspring liver sections at 6 months of age were formalin (10%) fixed and paraffin embedded prior to sectioning. All sections were then stained with haematoxylin and eosin (H&E) and Masson's Trichrome to assess steatosis and inflammation and fibrosis, respectively. The Brunt-Kleiner NAFLD Activity Score was used to quantitatively assess the degree of injury by an expert liver pathologist blinded to the identity of the groups.

7.2.8 Statistical Analysis

Multiple comparisons on a single data set were performed using one-way and two-way (2x2 factorial) ANOVA followed by Tukey's *post hoc* test and expressed as mean ± SEM unless otherwise stated. Cosinor analysis was used to determine rhythmicity within a 24 hour period, evaluating the mesor (circadian rhythm adjusted mean based on the parameters of a cosine function), timing of the oscillatory crest and amplitude. $p < 0.05$ was regarded as significant. Sample size per group; n = 5, 1 from each litter.

7.3 Results

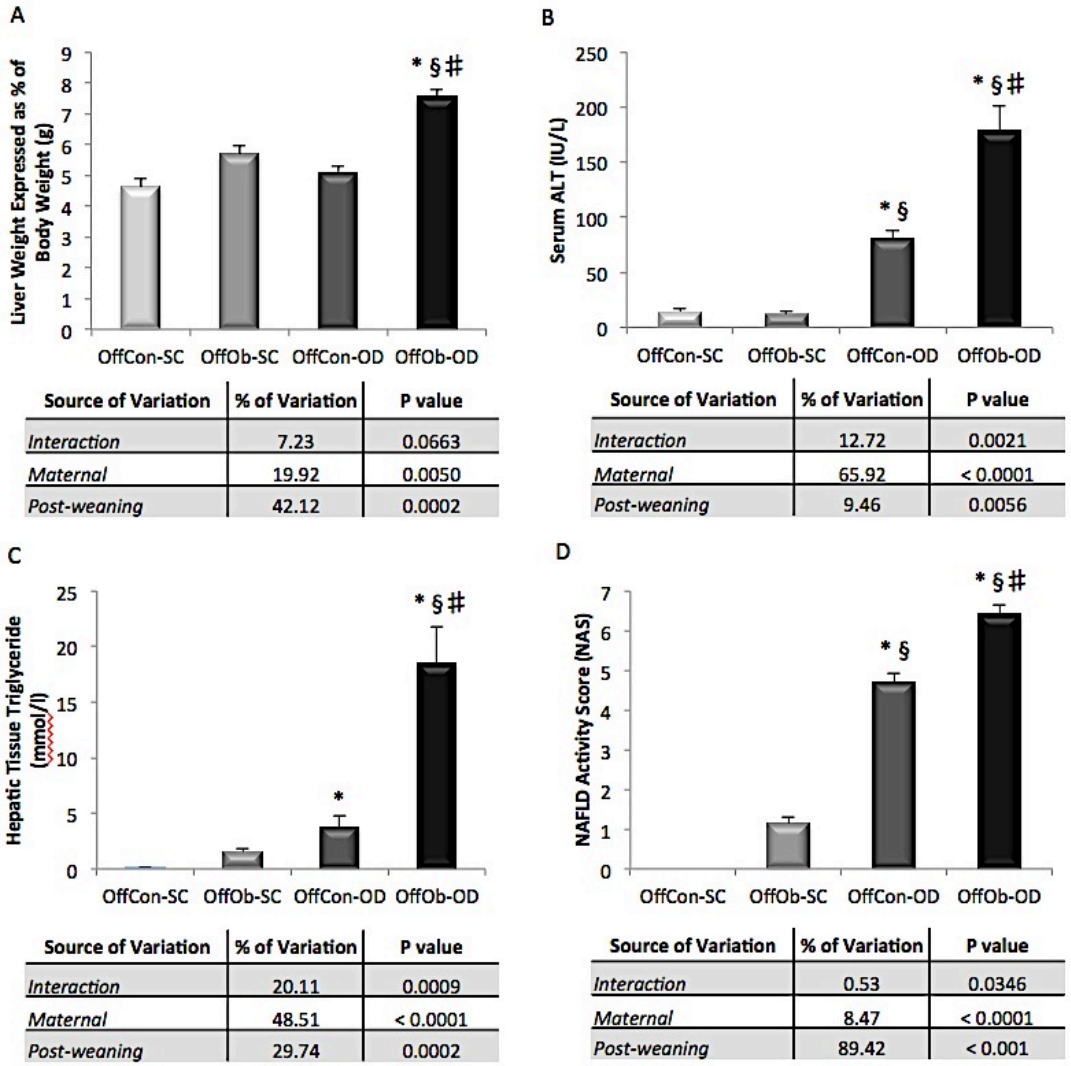
7.3.1 Offspring exposure to maternal obesity and a post-natal obesogenic diet induces non-alcoholic steatohepatitis (NASH) and hepatic fibrosis at 6 months

To validate the experimental model prior to investigation of potential mechanisms, biochemical and histological assessment of hepatosteatosis and liver injury was performed. Main effects were observed for both maternal and

post-natal diets with a significant interaction between these two variables for offspring liver weight, serum ALT, hepatic TG and NAS.

Greater fat infiltration in offspring exposed to a post-weaning obesogenic diet (OffCon-OD) compared to controls (OffCon-SC) was observed. Prior exposure to maternal obesity followed by a post-weaning obesogenic diet resulted in exacerbated and profound hepatosteatosis with development of NASH as evidenced by histological liver injury, confirmed by the increased Brunt-Kleiner NAFLD Activity Score > 5. Additionally, serum ALT, hepatomegaly and hepatic tissue triglyceride concentrations were significantly raised in offspring exposed to both periods of hyper-nutritional insult (Figure 7.1).

Moreover, these offspring developed pericellular fibrosis at 6 months as confirmed by Masson's Trichrome staining for collagen (Figure 7.2). The disproportionately enhanced fatty liver phenotype in OffOb-OD is due to interactions between maternal obesity and the post-weaning obesogenic diet as confirmed by two-way ANOVA for serum ALT, hepatomegaly and hepatic triglyceride content ($p < 0.05$).



Source of Variation	% of Variation	P value
Interaction	7.23	0.0663
Maternal	19.92	0.0050
Post-weaning	42.12	0.0002

Source of Variation	% of Variation	P value
Interaction	12.72	0.0021
Maternal	65.92	< 0.0001
Post-weaning	9.46	0.0056

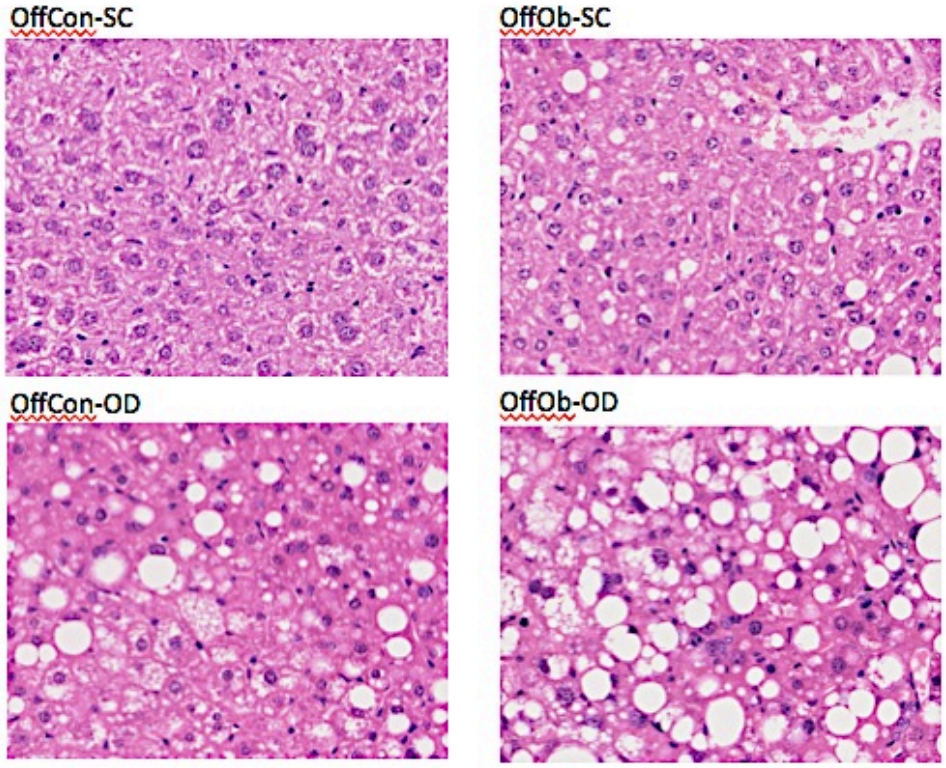
Source of Variation	% of Variation	P value
Interaction	20.11	0.0009
Maternal	48.51	< 0.0001
Post-weaning	29.74	0.0002

Source of Variation	% of Variation	P value
Interaction	0.53	0.0346
Maternal	8.47	< 0.0001
Post-weaning	89.42	< 0.001

Figure 7.1 Biochemical Evidence of Hepatic Injury and Steatosis at 6 months: (A) Liver Weight, (B) Serum ALT, (C) Hepatic Triglyceride Content and (D) NAFLD Activity Score. Offspring exposure to maternal obesity in the context of a post-weaning hyper-calorific diet significantly exacerbated hepatosteatosis and liver injury. Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet –

OffOb-OD. n = 5 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.05$). #Mean values significantly greater than OffCon-OD ($p < 0.05$). §Mean values significantly greater than OffOb-SC ($p < 0.05$).

A


OffCon-SC
OffOb-SC
OffCon-OD
OffOb-OD

B

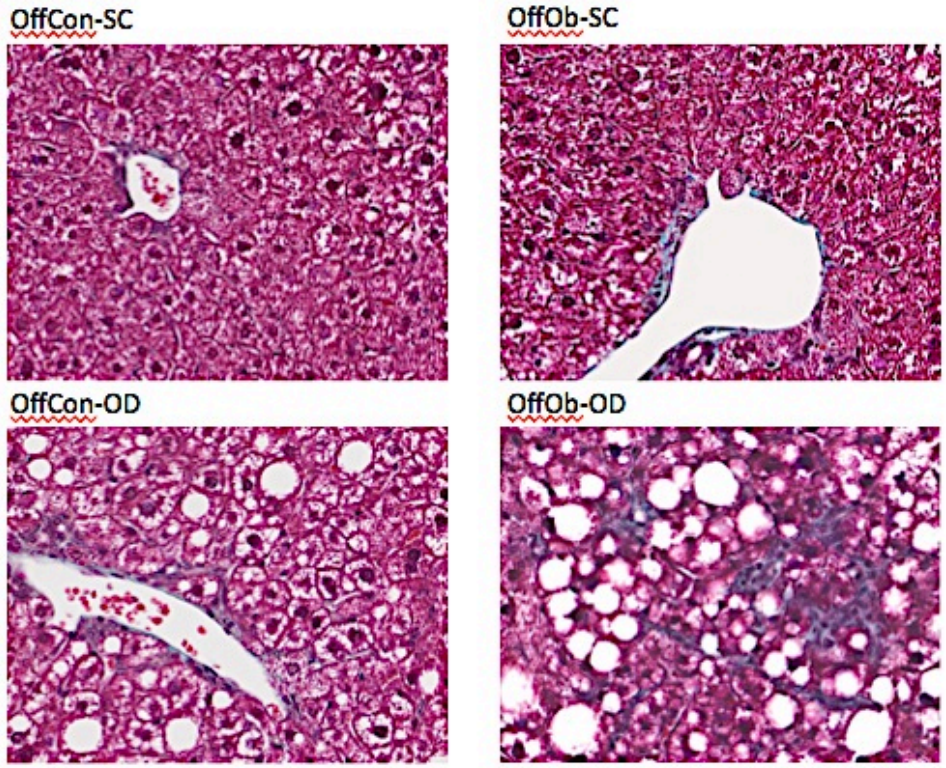

OffCon-SC
OffOb-SC
OffCon-OD
OffOb-OD

Figure 7.2 Histological Evidence of Hepatic Steatosis and Fibrosis at 6 months: (A) Representative H&E sections and (B) Representative Masson's Trichrome sections. Offspring exposure to maternal obesity in the context of a post-weaning hyper-calorific diet significantly exacerbated hepatosteatosis and fibrosis. Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. n = 4-6 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.05$). #Mean values significantly greater than OffCon-OD ($p < 0.05$). §Mean values significantly greater than OffOb-SC ($p < 0.05$).

7.3.2 Offspring exposure to maternal obesity and a post-weaning obesogenic diet disrupts rhythmic expression of canonical circadian activators in the liver

mRNA transcripts of BMAL1, an activator of the transcriptional-translation feedback loop governing biological circadian rhythms, was significantly perturbed following exposure to a post-weaning obesogenic diet. Gene expression was further attenuated following additional exposure to maternal obesity. Moreover peak expression of BMAL1 transcripts in control offspring and those exposed to only maternal obesity were observed at CT20 or at the beginning of the light phase of the 24 hour period. However, amplitudes for OffCon-OD and OffOb-OD were recorded at CT12 and CT16, respectively, or in the dark phase (Figure 7.3).

Exposure to a post-weaning obesogenic diet had negligible affects on rhythmic transcription of CLOCK over the 24 hour period as compared to controls. Interestingly, exposure to maternal obesity alone induced a significant increase in mRNA expression at CT16 or at the end of the dark phase. Additional exposure to maternal obesity induced a biphasic circadian rhythm of CLOCK gene expression with maxima observed at CT12 (dark phase) and CT20 (light phase) (Figure 7.3). Moreover, two-way ANOVA analysis revealed a significant interaction between the maternal and post-natal environments for CLOCK gene expression at CT16 ($p = 0.0127$). So therefore, exposure to maternal obesity and a post-weaning obesogenic diet significantly disrupts normal circadian rhythms of activating canonical clock genes in offspring with induced NAFLD.

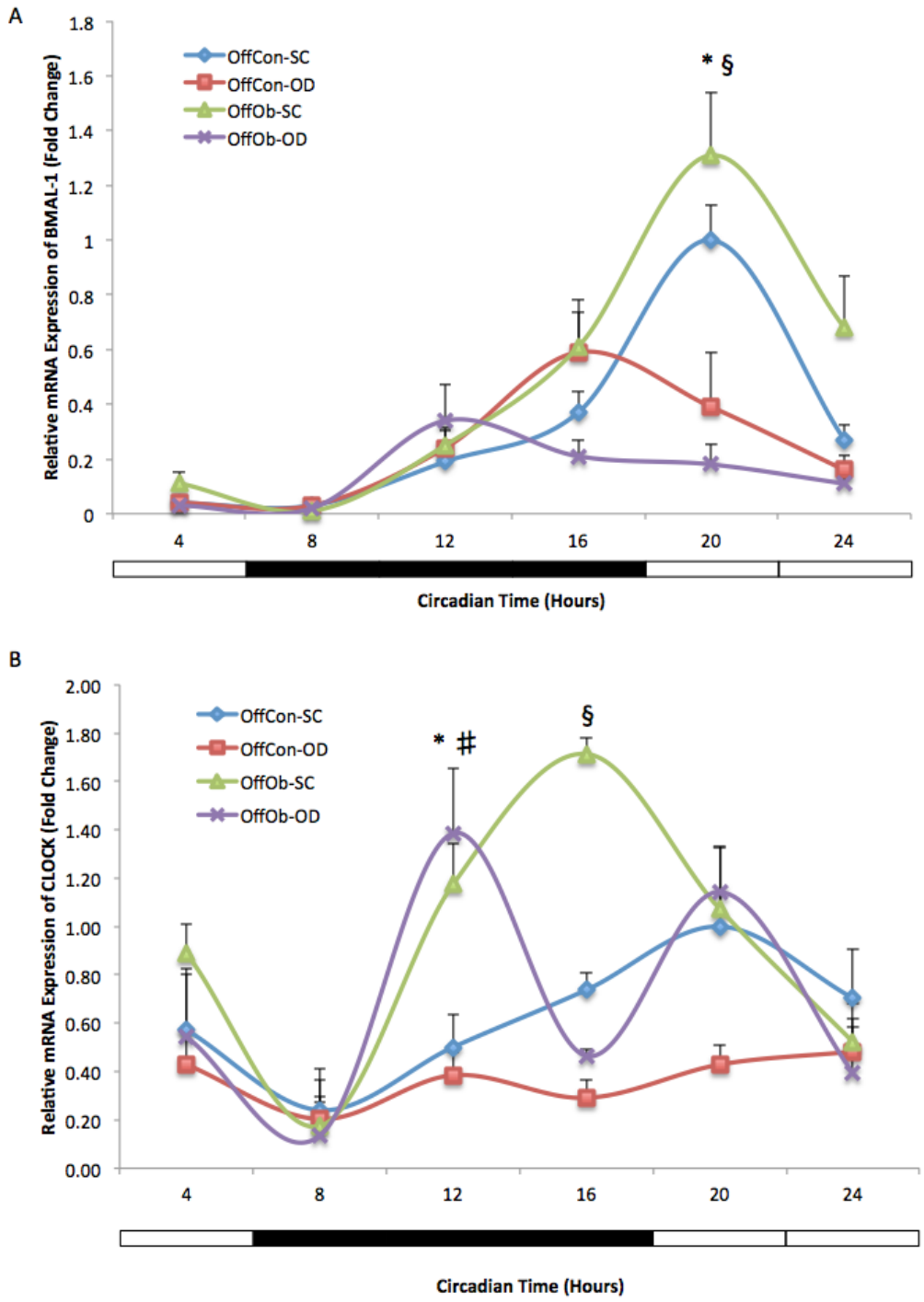


Figure 7.3 Hepatic circadian gene expression of BMAL1 and CLOCK are disrupted in offspring with programmed NAFLD: (A) BMAL1 mRNA and (B) CLOCK mRNA. Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet –

OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. Solid bars - dark, clear bars - light. n = 5 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.05$). #Mean values significantly greater than OffCon-OD ($p < 0.05$). §Mean values significantly greater than OffOb-SC ($p < 0.05$).

7.3.3 Offspring exposure to maternal obesity and a post-weaning obesogenic diet disrupts rhythmic expression of canonical circadian repressors in the liver

The Cryptochrome (Cry) and Period (Per) genes are negative regulators of the core clock molecular network, repressing BMAL and CLOCK co-transcription and therefore cellular circadian rhythmicity. Cry 1 was significantly attenuated in offspring exposed to a post-weaning obesogenic diet and/or maternal obesity at CT20 compared to OffOb-SC and OffCon-SC. Additionally, peak expression of this circadian transcript in OffCon-OD and OffOb-OD was observed at CT12 or the dark phase unlike control offspring in which the amplitude was observed in the light phase (CT20) (Figure 7.4).

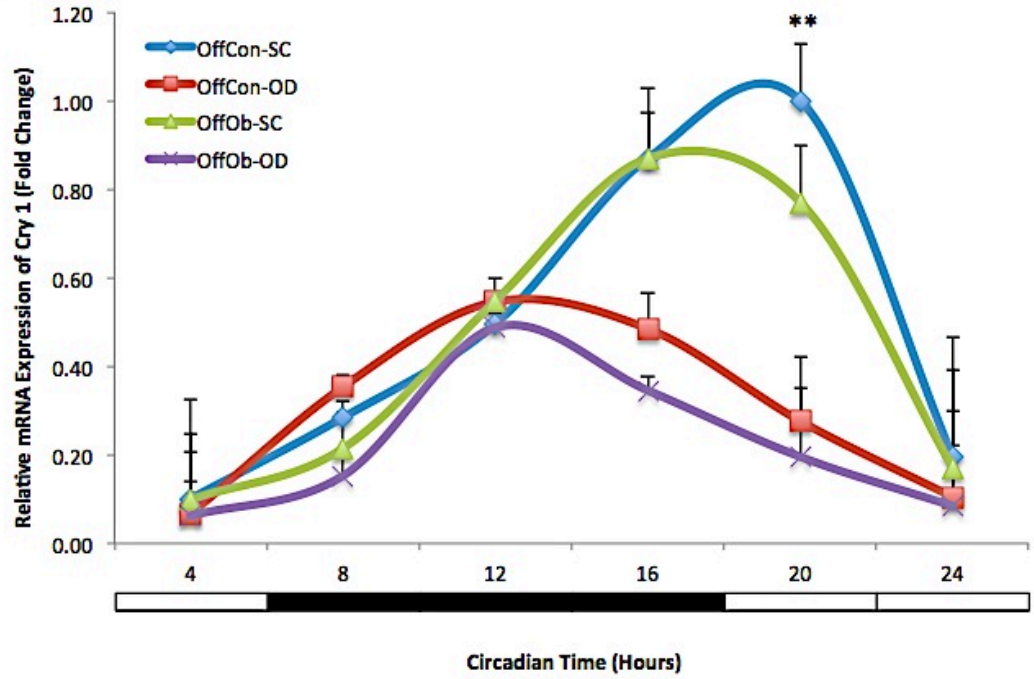
Cry 2 however, showed minimal diurnal or circadian variation in transcription over the 24 hour period in OffCon-SC and OffCon-OD. Exposure to maternal obesity however, induced a significant increase in expression at CT12, which was profoundly exacerbated in offspring additionally exposed to a post-weaning obesogenic diet at CT16. There was an independent effect of maternal obesity

on Cry 2 mRNA expression at CT16 ($p = 0.0143$) with evidence of interaction between the maternal and post-natal diets ($p = 0.0428$) (Figure 7.4).

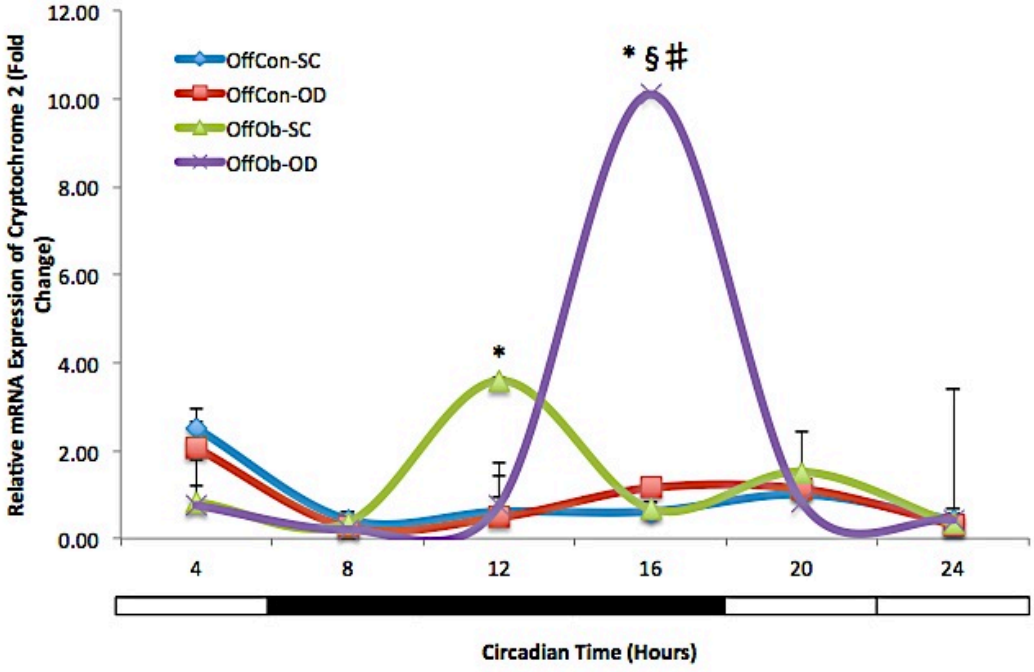
Similar rhythmic disturbance following exposure to maternal obesity was observed for Per1 transcription. OffOb-SC displayed increased expression compared to all other groups at CT12 (dark phase). The control group similarly peaked at this time point, although relative transcript expression was significantly less. Alternatively, exposure to a post-weaning obesogenic diet induced peak transcription in the light phase at CT20 with enhanced expression in offspring exposed to maternal obesity in addition (Figure 7.4). There was an independent effect of maternal obesity on Per1 mRNA expression at CT20 ($p = 0.0028$) with evidence of a significant interaction between maternal and post-natal diets ($p = 0.0008$).

Offspring exposure to maternal obesity and a post-weaning obesogenic diet significantly increased gene expression of Per2 at CT12 compared to those fed a hyper-calorific diet post-weaning only or the control group. Again, an independent effect of maternal obesity on rhythmic transcription of Per2 at this time point was observed ($p < 0.0001$) (Figure 7.4). So therefore, exposure to maternal obesity and/or an obesogenic diet post-weaning, significantly perturbs normal rhythmic expression of the period and cryptochrome genes over a 24 hour period. More specifically, gene expression of Per1, Per2 and Cry 2 is enhanced whilst Cry1 is attenuated at given CT points within the sampled 24 hour period. This is attributable to independent maternal effects and significant interactions between the maternal and post-natal diets.

A


1.20
1.00
0.80
0.60
0.40
0.20
0.00
Relative mRNA Expression of Cry 1 (Fold Change)
OffCon-SC
OffCon-OD
OffOb-SC
OffOb-OD
**
4
8
12
16
20
24
Circadian Time (Hours)

B


12.00
10.00
8.00
6.00
4.00
2.00
0.00
Relative mRNA Expression of Cryptochrome 2 (Fold Change)
OffCon-SC
OffCon-OD
OffOb-SC
OffOb-OD
* § #
*
4
8
12
16
20
24
Circadian Time (Hours)

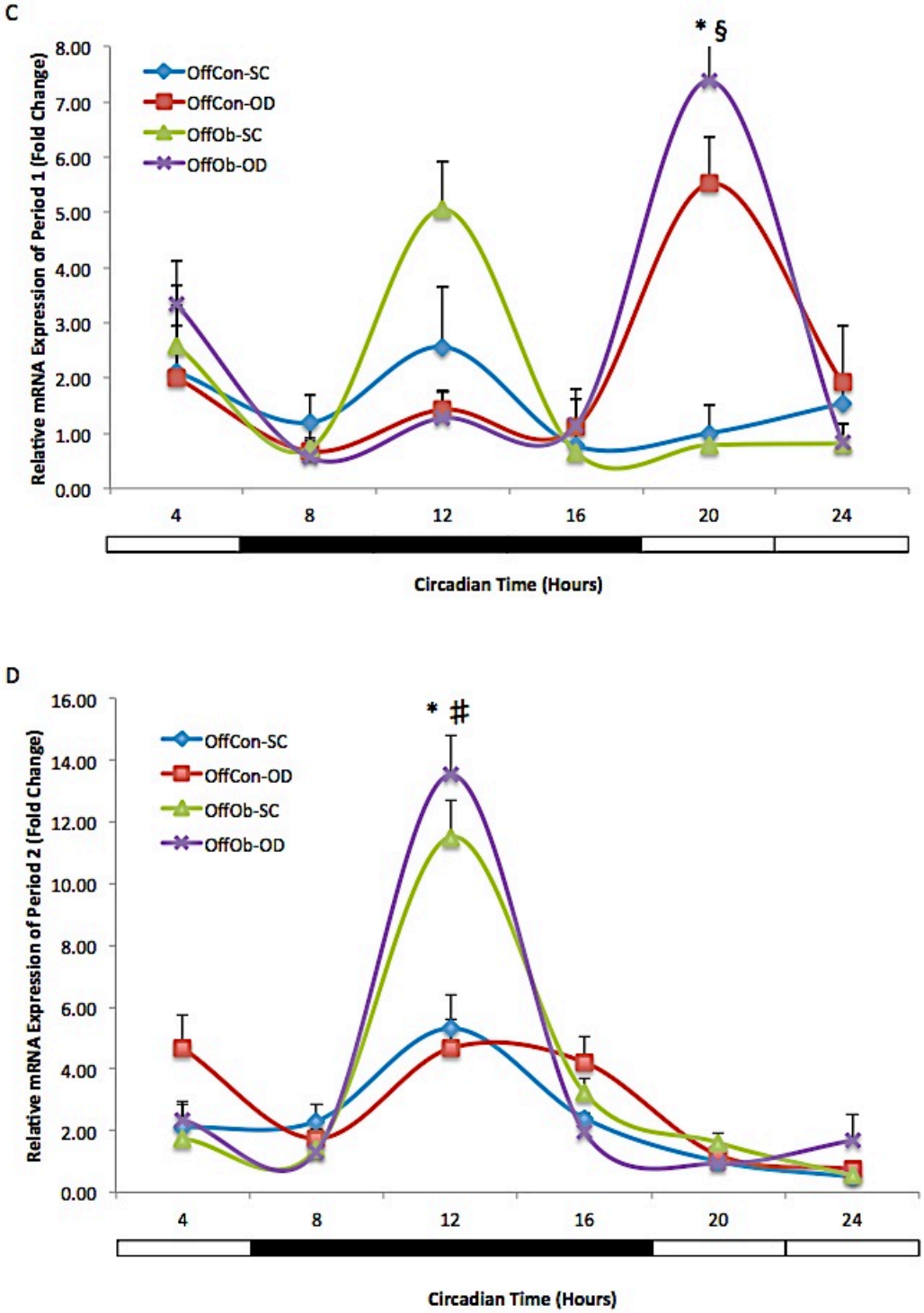


Figure 7.4 Hepatic circadian gene expression of Period and Cryptochrome is disrupted in offspring with programmed NAFLD: (A) Cry1 mRNA, (B) Cry2 mRNA, (C) Per1 mRNA and (D) Per2 mRNA. Offspring of lean suckled by lean

weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. Solid bars - dark, clear bars – light. n = 5 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.05$). #Mean values significantly greater than OffCon-OD ($p < 0.05$). §Mean values significantly greater than OffOb-SC ($p < 0.05$).

7.3.4 Offspring exposure to maternal obesity and/or a post-weaning obesogenic diet attenuates rhythmic expression of REV-ERB-α in the liver

REV-ERB-α is an accessory regulator of the molecular clock through repressive induction of BMAL1. Exposure to maternal obesity and/or an obesogenic diet significantly attenuated REV-ERB-α expression at CT4 compared to OffCon-SC and OffOb-SC. Rhythmic expression of this gene was otherwise similar across all groups within the 24 hour period. These findings corroborate the decreased BMAL1 expression observed in OffCon-OD and OffOb-OD (Figure 7.5).

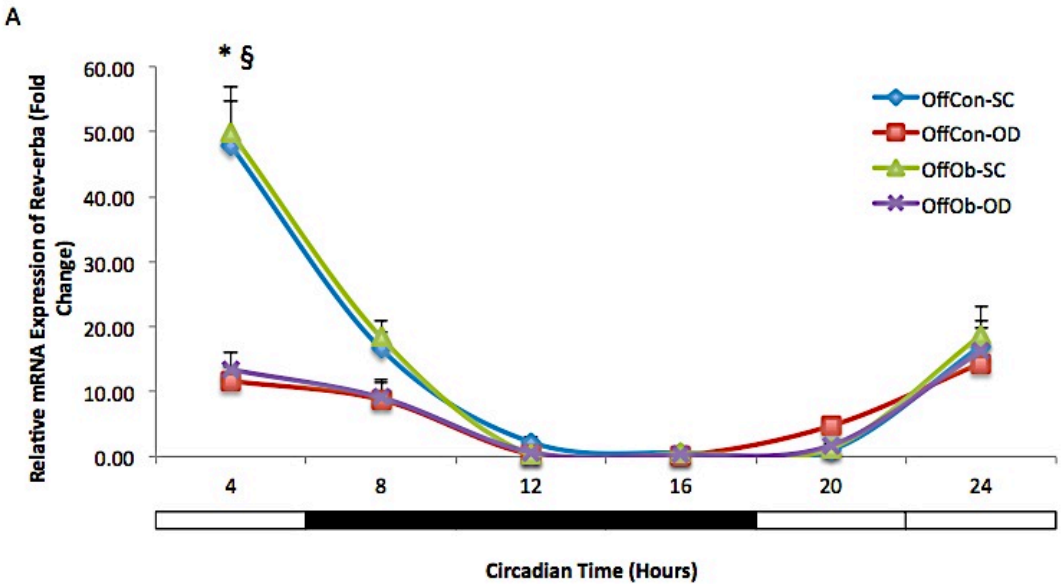


Figure 7.5 Hepatic circadian gene expression of REV-ERB-α is disrupted in offspring exposed to a post-weaning obesogenic diet: (A) REV-ERB-α mRNA. Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. Solid bars - dark, clear bars – light. n = 5 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.05$). #Mean values significantly greater than OffCon-OD ($p < 0.05$). §Mean values significantly greater than OffOb-SC ($p < 0.05$).

7.3.5 Offspring exposure to maternal obesity and a post-weaning obesogenic diet induces phase advances in the circadian expression of CLOCK, BMAL1 and Per2 in the liver

Offspring exposure to maternal obesity and a post-weaning obesogenic diet induces phase advances in the rhythmic expression of CLOCK and BMAL1 compared to controls. More specifically, transcription of CLOCK occurs approximately 3.3 hours earlier in OffOb-OD compared to OffCon-SC ($p < 0.05$). Interestingly, exposure to a hyper-calorific diet post-weaning alone has no effect on phase shifts as evidenced by a similar phase advance of 3 hours in OffOb-OD compared to OffCon-OD ($p < 0.05$) with no significant difference in phase shift between OffCon-OD and OffCon-SC (Figure 7.6). Therefore, the insult of maternal obesity is necessary to induce a phase shift in hepatic CLOCK transcription.

Similarly, a phase advance of approximately 1.9 hours was observed for the transcription of BMAL1 in OffOb-OD compared to OffCon-SC ($p < 0.05$). A significant shift in phase was not observed in offspring exposed to only a post-weaning obesogenic diet or maternal obesity, further affirming the interactions between the *in utero* and post-natal environments in generating the observed NAFLD phenotype (Figure 7.6).

Additionally, a significant phase advance of 2 hours for the transcription of Per2 was observed in offspring exposed to maternal obesity and a post-weaning obesogenic diet compared to those exposed only to maternal obesity ($p < 0.05$).

This phase shift was not due to the post-weaning diet alone as a significant difference between OffOb-OD and OffCon-OD was not observed (Figure 7.6). This supports the preceding findings of an interaction between the maternal and post-natal environments responsible for the observed phenotype.

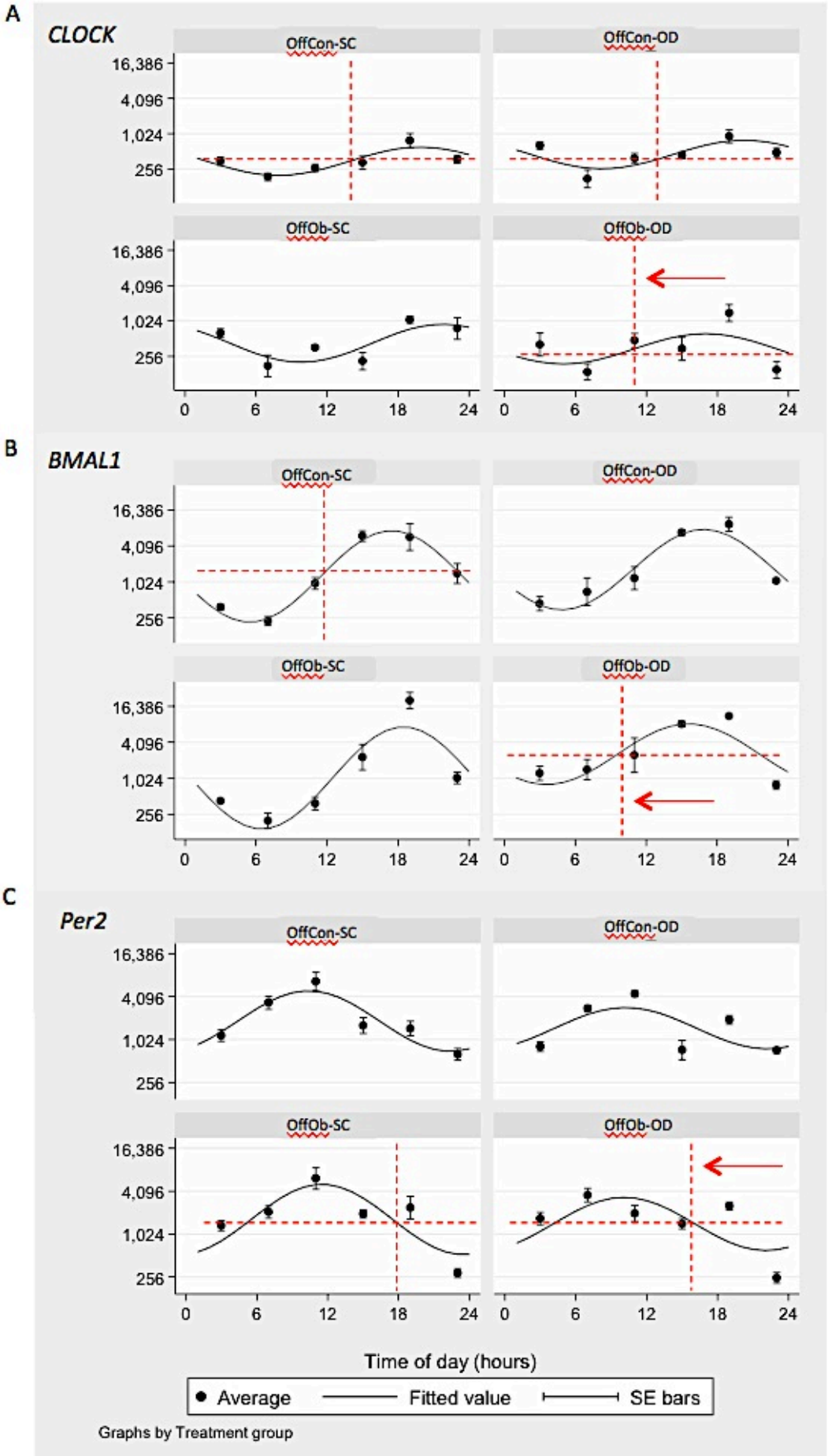

A
CLOCK
OffCon-SC
OffCon-OD
OffOb-SC
OffOb-OD
16,386
4,096
1,024
256
0
6
12
18
24
B
BMAL1
C
Per2
Time of day (hours)
Average
Fitted value
SE bars
Graphs by Treatment group

Figure 7.6 Maternal obesity and a post-weaning obesogenic diet induces phase advances of core canonical clock genes in offspring with programmed NAFLD: (A) CLOCK mRNA, (B) BMAL1 mRNA and (C) Per2 mRNA. Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. n = 5 per group, cosinor analysis.

7.3.6 Offspring exposure to maternal obesity and a post-weaning obesogenic diet disrupts rhythmic expression of hepatic inflammatory markers implicated in NAFLD pathogenesis

Interleukin-6 (IL-6), a pleiotropic and pro-inflammatory cytokine implicated in NAFLD pathogenesis is shown here for the first time, to have a circadian secretion pattern in murine liver. OffCon-SC and OffOb-SC display similar biphasic circadian secretion patterns whereas offspring exposed to a post-weaning obesogenic diet display only one peak or nadir at CT12. Given the discrete differences in maxima and minima of circadian IL-6 expression in OffCon-OD and OffOb-OD, it could be suggested that there is constitutive expression of this pro-inflammatory cytokine (Figure 7.7).

Tumour necrosis factor alpha (TNF-α) however, did not exhibit circadian secretion patterns in the liver, although peak expression of this transcript was observed at CT20 in offspring exposed to maternal obesity and a post-weaning

obesogenic diet compared to all other groups ($p < 0.05$). Moreover, there was an independent effect of maternal obesity on peak transcription ($p = 0.0063$) as revealed by two-way ANOVA analysis (Figure 7.7).

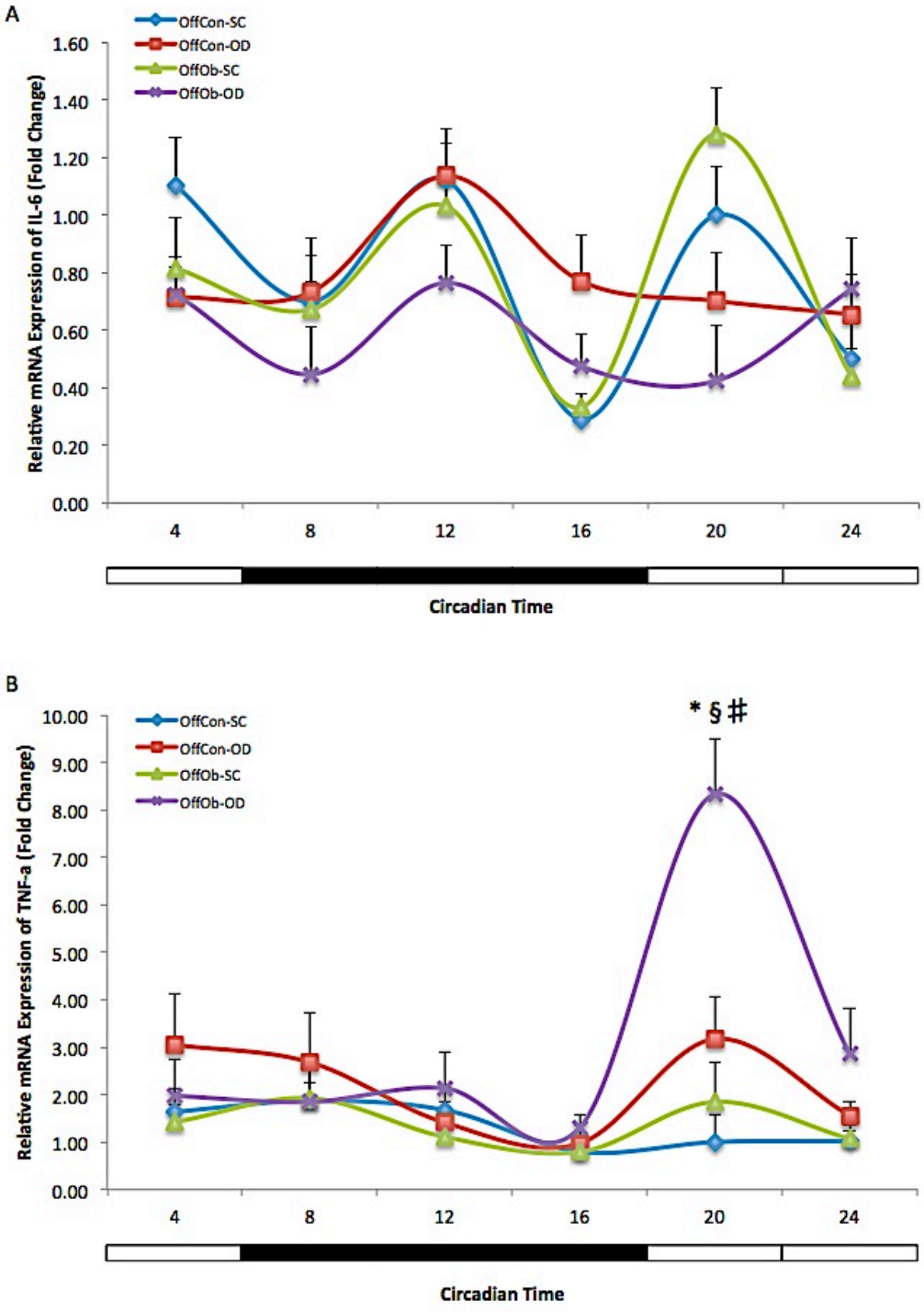


Figure 7.7 Maternal obesity and a post-weaning obesogenic diet disrupts

rhythmic expression of hepatic inflammatory markers in offspring: (A) IL-6 mRNA and (B) TNF-α mRNA. Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. Solid bars - dark, clear bars – light. n = 5 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.05$). #Mean values significantly greater than OffCon-OD ($p < 0.05$). §Mean values significantly greater than OffOb-SC ($p < 0.05$).

7.3.7 Offspring exposure to maternal obesity and a post-weaning obesogenic diet disrupts rhythmic expression of hepatic fibrogenic markers implicated in NAFLD pathogenesis

The rhythmic expression of alpha smooth muscle actin (α-SMA), a marker of hepatic stellate cell activation – the liver's principal fibrogenic cell, was perturbed in offspring exposed to maternal obesity and fed a post-weaning obesogenic diet as evidenced by significantly increased transcription at CT8 and CT20 compared to all other groups ($p < 0.05$). Moreover, there was an independent effect of maternal obesity on α-SMA transcription at CT8 ($p = 0.0237$).

Similarly, these offspring exposed to maternal obesity and a post-weaning obesogenic diet displayed marked increases in transcription of transforming

growth factor beta (TGF-β) and collagen in the dark phase of the circadian cycle compared to all other groups ($p < 0.05$). Additionally, there was an independent effect of maternal obesity for collagen expression at CT16 ($p = 0.0772$). So therefore, exposure to maternal obesity in the context of a post-weaning obesogenic diet disrupts effects rhythmic transcription of these fibrogenic markers (Figure 7.8).

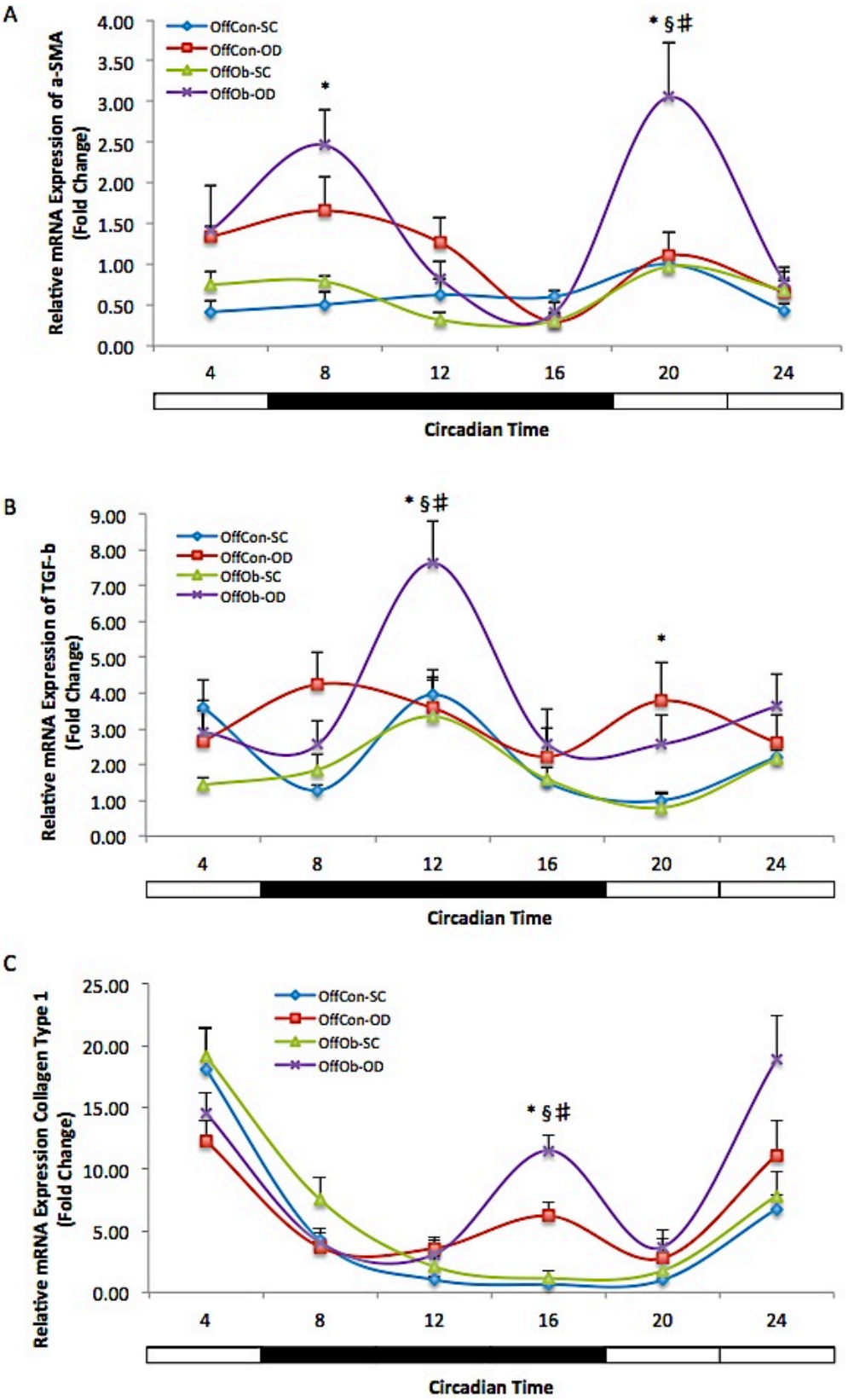


Figure 7.8 Maternal obesity and a post-weaning obesogenic diet disrupts

rhythmic expression of hepatic fibrogenic markers in offspring: (A) α-SMA mRNA, (B) TGF-β mRNA and (C) collagen type 1 mRNA. Offspring of lean suckled by lean weaned on to standard chow – OffCon-SC; offspring of lean suckled by lean weaned on to obesogenic diet – OffCon-OD; offspring of obese suckled by obese weaned on to standard chow – OffOb-SC and offspring of obese suckled by obese weaned on to obesogenic diet – OffOb-OD. Solid bars - dark, clear bars – light. n = 5 per group, values shown are mean ± SEM, one-way ANOVA. *Mean values significantly greater than OffCon-SC ($p < 0.05$). #Mean values significantly greater than OffCon-OD ($p < 0.05$). §Mean values significantly greater than OffOb-SC ($p < 0.05$).

7.4 Discussion

Disruption of central and peripheral circadian rhythms in humans and rodents has been shown to cause metabolic disorders including impaired glucose homeostasis and hyperlipidaemia (73, 219, 233). This suggests a causal association between rhythmic circadian transcription and translation of canonical clock genes and metabolism. To investigate whether programmed offspring NAFLD was caused by rhythmic disruption of hepatic circadian transcripts, 24 hour relative mRNA expression of all canonical clock genes in whole murine liver tissue was assayed and compared to appropriate controls and experimental groups.

7.4.1 CLOCK and BMAL1

The biological circadian clock is a transcription-translation negative feedback loop which is activated by the hetero-dimerisation of CLOCK and BMAL1. In this pathophysiologically relevant model of programmed NAFLD in offspring, it is shown here for the first time that exposure to maternal obesity increased temporal expression of CLOCK mRNA transcripts, peaking at CT16 or in the dark phase. Moreover, additional insult with continued exposure of a hyper-calorific diet in post-natal life induced a biphasic circadian pattern of expression with peaks observed in both the light and dark phases. This time dependent cycling of CLOCK has been observed in other diet induced obesity rodent models (71, 73).

Rhythmic disruption of CLOCK transcription in the liver may therefore be partially responsible for the observed dysmetabolic and fatty liver phenotype. Such a causal association is supported by earlier reports of adiposity, impaired lipid metabolism and insulin resistance in CLOCK mutant mice (71, 72). Although a high fat diet has been shown to alter rhythmic expression of CLOCK in adipose and hepatic tissue, it is clear that these present observations are not phenomenological as offspring exposed to only an obesogenic diet displayed no alteration in time-dependent transcription of CLOCK compared to controls.

The rhythmic circadian expression of BMAL1, a co-activator of the circadian molecular network, was similarly disrupted following exposure to maternal obesity and a post-weaning obesogenic diet. Peak transcription of BMAL1 in

OffOb-OD was observed at CT12 or in the dark phase, although overall fold change was significantly attenuated compared to controls. Interestingly, exposure to an obesogenic diet post-weaning also caused peak transcription to occur in the dark opposed to the light phase alongside controls. Observations of arrhythmic BMAL1 transcription are in keeping with previous reports of impaired adipogenesis and hepatic carbohydrate metabolism in rodents with BMAL1 ablation (234, 235).

Moreover, profound perturbation of rhythmic expression of CLOCK and BMAL1 in offspring exposed to maternal obesity and an obesogenic diet post-weaning was evidenced by phase advances in transcription of approximately 3.3 and 1.9 hours, respectively. Additionally, a higher baseline constitutive expression of CLOCK and BMAL1 was observed from greater than control values for minima as determined by cosinor analysis. Again these observations were recorded only in offspring exposed to both an adverse maternal and post-natal obesogenic environment, affirming that hypercaloriaemia is not predominantly responsible for altered circadian cycles and that programming via maternal obesity plays a pivotal role.

7.4.2 Period and Cryptochrome

Period (Per1 and Per2) and cryptochrome (Cry 1 and Cry 2) genes are repressors of the circadian molecular machinery through inactivation of CLOCK:BMAL1. In this present study, rhythmic expression of cryptochrome within a circadian period in the murine liver was observed as with previous experimental studies

(236). Offspring exposure to a post-weaning obesogenic diet independent of maternal obesity attenuated transcription of Cry1 with peak expression occurring in the dark phase opposed to the light phase alongside controls. Interestingly, a post-weaning obesogenic diet had negligible effects on Cry2 transcription which was comparable to control values. Exposure to maternal obesity induced a significant increase in Cry2 transcription in offspring which was further enhanced in the context of a post-weaning obesogenic diet in the dark phase at CT12 and CT16, respectively. Therefore and most importantly, offspring exposure to maternal obesity significantly induces arrhythmic expression of Cry2 and as such may be in part responsible for the observed NAFLD phenotype. This is further supported by reports of cryptochrome mediated regulation of hepatic gluconeogenesis and cAMP signalling, re-affirming the association between core clock genes and their control of metabolic pathways (236).

Both period genes similarly expressed circadian rhythmic patterns in the liver. Offspring exposure to maternal obesity enhanced Per1 transcription at CT12 compared to OffCon-OD and OffCon-SC. Exposure to a post-weaning obesogenic diet and/or maternal obesity induced arrhythmic Per1 transcription with peak values attained at CT20 or the light phase. Most profound alteration of circadian rhythm was observed in OffOb-OD compared to all other groups. This is the first report of Per1 time-dependent transcription in the murine liver although its role in metabolic regulation has been demonstrated through its effects on phospholipid biosynthesis in fibroblasts *ex vivo* (237).

As observed with Cry2, a post-weaning obesogenic diet had insignificant effects on Per2 circadian secretion patterns in the liver. Most importantly, offspring exposure to maternal obesity induced profound increases in Per2 transcription at CT12 with an enhanced expression in those additionally fed an obesogenic diet post-weaning. Moreover, offspring exposure to maternal obesity induced a phase advance of Per2 transcription. It is here demonstrated that temporal changes in Per2 expression are not the result of nutrient excess at the time of analysis in the offspring but rather a permanently altered component of the molecular circadian clock induced by maternal obesity, affecting hepatic function and metabolic control. Moreover, independent effects of maternal obesity on Per1 and Per2 transcription, as revealed by two-way ANOVA analysis, is confirmatory of a mechanistic pathway by which offspring NAFLD is programmed by maternal obesity.

Per2 is an important intermediary between metabolic and circadian pathways as it has been shown to control lipid metabolism through regulation of PPARγ. Importantly, it has been reported that Per2 deficient mice display profound reduction of triacylglycerol and non-esterified fatty acids (238) and so Per2 acts to inhibit lipid metabolism, potentiating hepatosteatosis as observed here in offspring with programmed NAFLD. Moreover, Per2 has been reported as a risk factor for the metabolic syndrome (239). These previous reports corroborate our findings of increased Per2 transcription in offspring with the most severe phenotype.

7.4.3 REV-ERB-α

The nuclear receptor, REV-ERB-α, acts within an accessory feedback loop to negatively regulate clock function. Offspring fed an obesogenic diet post-weaning displayed a phase delay in REV-ERB-α transcription, of 1.1-1.8 hours, independent of maternal obesity. Additionally, exposure to maternal obesity in the context of a post-weaning obesogenic diet significantly reduced REV-ERB-α transcription at CT4. These findings of reduced relative mRNA expression in profoundly hepatosteatotic offspring are corroborated by observations of hyperlipidaemia in REV-ERB-α null mice (240). It may therefore be suggested that the more profound phenotype observed in offspring programmed via maternal obesity and fed an obesogenic diet post-weaning, is in part caused by reduced, rhythmic REV-ERB-α transcription in the liver.

7.4.4 Impact of circadian desynchronisation on hepatic inflammatory and fibrogenic pathways

A corollary of the aforementioned findings is circadian desynchronisation in the liver induced by offspring exposure to maternal obesity in the context of a post-weaning obesogenic diet. It is evident from previous reports and present findings that these canonical clock genes affect metabolic physiology. It is, additionally now shown here that these circadian genes regulate hepatic inflammatory and fibrogenic pathways implicated in NAFLD pathogenesis.

Rhythmic expression of IL-6 in offspring liver and programmed NAFLD are novel findings. However, offspring exposure to maternal obesity and/or an

obesogenic diet post-weaning ablates normal biphasic rhythmic circadian transcription of IL-6 (241). Additionally, significantly enhanced TNF-α transcription is observed at CT20 in OffOb-OD compared to all other groups. Unsurprisingly, this rhythmic disruption occurs in offspring with the most profound circadian desynchronisation.

Similarly, circadian secretion patterns for the fibrogenic markers, α-SMA, TGF-β and collagen type 1, are shown for the first time in murine liver, NAFLD and programmed NAFLD. Offspring exposure to maternal obesity in the context of a post-weaning obesogenic diet most significantly perturbs rhythmic expression of these fibrogenic markers. Moreover, relative mRNA transcription is enhanced in this group (OffOb-OD) compared to controls or those fed only an obesogenic diet post-weaning. Peak transcription of α-SMA precede that of TGF-β and collagen, as observed in the light and dark phase, respectively. So therefore, canonical clock genes regulate hepatic pro-inflammatory and pro–fibrogenic pathways, increasing susceptibility to NAFLD with development of fibrosis.

7.5 Conclusion

Nutrient excess or a hyper-calorific milieu during *in utero* development interacts with the post-natal obesogenic environment to induce permanent changes in the hepatic molecular circadian clock. The resulting circadian desynchrony not only affects metabolic homeostasis as demonstrated here and previously by

other groups, but also identifies hepatic pro-inflammatory and pro–fibrogenic pathways involved in NAFLD pathogenesis as 'clock output pathways'. Mechanistically, it may be suggested that programming via maternal obesity alters physiological functioning of the hepatic molecular circadian network during development plasticity in offspring, causing rhythmic disruption and increased expression of inflammatory and fibrogenic markers implicated in NAFLD pathogenesis.

8 General Discussion

8 General Discussion

8.1 Initial Aims

The aims of this PhD were to determine if maternal obesity during gestation and/or lactation predisposed offspring to development of dysmetabolism and NAFLD and secondarily to investigate the responsible mechanisms. The following aims were specifically examined:

a. The effect(s) of exposure to maternal obesity on offspring dysmetabolism and NAFLD in adulthood.

b. The independent effect(s) of the *in utero* and immediate post-partum period i.e. lactation, on offspring susceptibility and development of dysmetabolism and NAFLD in adulthood.

c. To develop a uniquely pathophysiologically relevant model of NAFLD in which to further study the interaction between *in utero* exposure and the post-weaning environment in programming development of offspring dysmetabolism and NAFLD in adulthood.

d. To investigate the natural progression of NAFLD in programmed offspring and ascertain whether exposure to maternal obesity primes accelerated disease development, cirrhosis or malignancy.

e. To examine the role of the hepatic innate immune system as a putative mechanistic pathway through which maternal over-nutrition programs offspring propensity to dysmetabolism and NAFLD in adulthood.

f. To examine the role of the peripheral circadian system as an alternative, but putative mechanistic pathway through which maternal over-nutrition programs offspring propensity to dysmetabolism and NAFLD in adulthood.

The rationale for these investigations originate from observations of global paediatric and adult obesity pandemics alongside increases in the prevalence of obesity in women of childbearing age (1, 6). In tandem, the prevalence of NAFLD is rising and is now the most common chronic liver disease worldwide (176, 242, 243). Obesity is pivotal in the aetiopathogenesis of NAFLD and through systematic investigation, this study here, shows that exposure to maternal obesity is also a significant contributory factor.

8.2 The Developmental Programming Model

Four phases of the maternal over-nutrition programming model were developed in this study. In attempts to investigate the long-term effects of programming via maternal obesity, offspring were initially weaned on to a normo-calorific diet. In a non-physiological experimental paradigm, we were

able to delineate the contributory roles of the *in utero* versus the *ex utero* environment. So that observed findings could be extrapolated to the clinical scenario and potential therapeutic and preventative applications realised, the element of cross-fostering was removed and alternative post-weaning diets introduced.

Further refinement of this model included feeding of a composite obesogenic diet, unlike other maternal over-nutrition models which use either high fat or sugar and thus are atypical of a western diet (5). A derivative of these experimental phases is the introduction of a novel and pathophysiologically relevant model of NAFLD.

Experimental obesity models are either nutritionally induced or genetically determined. In this study we used only a nutritionally induced obesity model as obese leptin receptor deficient rodents are sterile and therefore unsuitable for inclusion. Moreover, leptin has been implicated in programming via maternal over- and under-nutrition through actions on peripheral and central systems (244). Therefore, leptin deficiency would render the model pathophysiologically incomplete with biased outputs.

An epidemiological correlate of this study is now needed given that a strong association between maternal obesity and offspring NAFLD has been now established here. It was not sought to investigate the role of human maternal obesity on offspring NAFLD in this present study, as the effects of nutrient

excess during pre-natal development were unknown and the interactions between pre- and post-natal environments on various homeostatic mechanisms would not be easily discernible from such studies due to the lack of appropriate controls e.g. severity of obesity and dietary content during gestation and lactation.

8.3 Developmental Programming of Offspring NAFLD by Maternal Obesity

The association between maternal obesity, offspring hyper-triglyceridaemia and propensity to NAFLD development, has been shown in various experimental models (27, 35). Other groups have speculated on the development of NAFLD following exposure to maternal obesity and their observations in offspring of hepatomegaly and serum hyper-cholesterolaemia (36).

Bouanane and colleagues previously reported a permanent reduction in hepatic β-oxidation and increased lipogenesis, with altered composition of saturated fatty acids in VLDL lipids, as a potential mechanism for hepatosteatosis in offspring born to high-fat fed dams (144). In parallel, increased lipogenesis, as evidenced by up-regulated SREBP-1 and impaired lipolysis due to PPARa down regulation, has been reported in a rodent model of maternal over-nutrition (145).

However, the role of maternal obesity in programmed offspring NAFLD has only now been comprehensively investigated. Not only has a novel association between programming via maternal obesity and offspring NAFLD been reported, but the lactation period has been additionally identified as the most crucial developmental window in which offspring physiology and/or structure is adversely changed. Continued feeding of a hyper-calorific diet post-weaning disproportionately exacerbates NAFLD due to interactions between the pre- and post-natal environments.

Maternal obesity however, may not be the only determinant of programmed offspring NAFLD as a high calorific intake during pregnancy, independent of maternal obesity, has been shown to program hepatic injury in non-human primates (146). Corroboratively, Bayol and colleagues report hepatosteatosis and increased oxidative stress, in offspring born to mothers fed a hyper-calorific 'junk' diet during gestation and lactation, independent of prior maternal obesity (147).

8.4 Examination of the mechanisms involved in developmental programming of offspring NAFLD by maternal obesity

Experimental research indicates that nutritional perturbations in the intra- and extra-uterine environment increase oxidative stress and enhance lipogenesis, priming offspring liver to an increased risk of NAFLD in adulthood (145, 181). However, the underlying mechanisms responsible for programmed offspring

NAFLD were unknown. It had been suggested that maternal breast milk factors may act on central neonatal energy homeostasis centres, evoking hyperphagia and subsequent obesity and NAFLD (143). However, there is a paucity of data on the direct effects of maternal obesity on hepatic function. It has been recently suggested in a diet induced obesity model of programming that hepatic mitochondrial electron transport chain reactions are altered; increasing oxidative stress and fat accumulation with resultant hepatosteatosis in rodents (64).

Additionally, alterations in the hepatic epigenomic profile of offspring exposed to maternal over-nutrition in the form of hyper-acetylation and –methylation have been reported (148). Godfrey et al., have recently corroborated these findings in a human study, reporting a positive correlation between peri-natal epigenetic changes, induced my maternal nutritional imbalance and childhood adiposity (149).

The results presented here, in these experiments, now invoke a role for hepatic insulin signalling, the hepatic innate immune system and altered biological circadian rhythms in programmed NAFLD via maternal obesity. Insulin resistance is considered crucial to the development of NAFLD. Consistent with previous reports of insulin resistance in NAFLD (245, 246), it is here shown that hepatic insulin signalling is impaired in offspring exposed to maternal obesity (51) and may therefore be responsible for the ensuing hepatosteatosis and NAFLD development. In parallel, hepatic innate immune dysfunction is

observed in offspring with programmed NAFLD. It is thought that such perturbation is responsible for propagating liver injury in the context of hepatosteatosis in those exposed to maternal obesity and fed an obesogenic diet post-weaning. However, in order to understand the mechanistic role of the innate immune cells in programmed NAFLD pathogenesis, it is necessary to further investigate the NK-T cell population given their ability to exclusively recognise lipids and glycolipids. This could involve examination of CD1d expression by hepatocytes and ligand presentation to NK-T cells.

It is well understood that many pathogenic processes are multi-factorial especially those involved in metabolic homeostasis. Therefore, assumption that a single mechanistic pathway is responsible for the programmed phenotypes would be incorrect. Peripheral biological circadian rhythms were therefore also investigated. It is shown, here for the first time, that maternal obesity in the context of a post-weaning obesogenic diet alters biological circadian rhythms and the normal rhythmicity of output pathways implicated in NAFLD pathogenesis. Given that these implicated pathways are perturbed in parallel with disease severity and that the post-weaning obesogenic diet predominantly contributes to offspring phenotype, as determined by two-way ANOVA analysis, it is necessary to investigate a time point prior to disease on-set. Such suitable time-points could include gestational day 18 or 21 days post-partum, immediately after weaning, to confirm mechanistic involvement of these putative pathways.

These identified potential mechanisms are not however, an exhaustive list. Emerging evidence suggests that the lipotoxicity observed in hepatosteatosis induces endoplasmic reticulum (ER) stress, propagating NAFLD disease progression. ER stress has now been documented in several experimental models of steatosis (247) and epidemiological studies of NAFLD patients (248). Most recently, ER stress has been implicated in programmed NAFLD pathogenesis across three generations in a high fat diet induced obesity model. It was reported by Li and colleagues that NAFLD manifestation was progressive in consecutive generations characterised by altered histone methylation (249).

8.5 The Relative Contributions of Programming by Maternal Obesity and the Post-Weaning Obesogenic Environment in Offspring NAFLD

There are several lines of evidence suggesting a role for maternal obesity during gestation and lactation in programming offspring susceptibility to dysmetabolism in adulthood (20, 37, 244). It is here shown, that offspring exposure to maternal obesity during development increases susceptibility to NAFLD in adulthood, with the lactation period most crucial to these programming effects. Given that all offspring were weaned on to a standard chow diet in earlier experimental paradigms (Chapters 3 and 4), confirms that the observed phenotype is due to adverse exposure during critical developmental periods. It cannot be argued that disease development is offspring exposed to maternal obesity is simply due to experience of a hyper-calorific environment for a specified period of time, as offspring exposed to

maternal obesity in gestation only displayed phenotypes comparable to controls (Chapter 4).

In later experimental paradigms which involve offspring weaned on to an obesogenic diet, it is apparent from comparison of appropriate controls and two-way ANOVA analysis, that the eventual phenotype is largely and disproportionately induced by the post-weaning hyper-calorific environment. This however, does not negate a role for developmental programming by maternal obesity as there are clear main effects of maternal obesity on offspring development of adiposity, hepatosteatosis, inflammatory and fibrogenic markers, innate immune dysfunction and altered biological circadian rhythms. It is also important to note that there are interactions between the maternal and post-weaning environments which may be propagating the NAFLD disease state in offspring. Such a contention is supported by earlier reports of accelerated adiposity and hyperphagia in rodents exposed to maternal obesity and maintained on an obesogenic diet throughout post-natal life (55).

The findings in this thesis clearly support a role for maternal obesity in increasing offspring susceptibility to NAFLD. However, the relative contribution of developmental programming by maternal obesity in NAFLD pathogenesis is modest. Given that the rates of obesity amongst women of reproductive age are rising alongside offspring obesity with experimental evidence of transgenerational programming effects, it is plausible that the role of

programming in offspring NAFLD may have more putative transgenerational consequences.

8.6 Examination of Gender Differences in Developmental Programming

Sex specific differences in the incidence of adverse events during gestation and early neonatal life are well documented. Such examples include male gender being an independent risk factor for stillbirth (250) and gestation length determined by fetal sex (251). It is therefore plausible that sexual dimorphic patterns of developmental programming exist. The literature is however, conflicting dependent upon the pathogeic pathway being investigated.

Offspring of dams exposed to stress during late gestation display aberrant hypothalamic-pituitary-adrenal (HPA) responses to stress, which is limited to only females (252). Conversely, pre-natal exposure to IL-1β induced more significant alterations in psychomotor development and behaviour in males compared to females (253). Additionally, offspring exposure to maternal over-nutrition has been shown to induce hypertension and decrease renal rennin activity in males and reduce vascular compliance and endothelial dysfunction in females (33, 39, 254-256).

A growing body of evidence suggesting that male and female fetuses adapt differentially to developmental stressors such as maternal obesity. It is also suggested that sex steroids influence development of programmed disease

states in an organ dependent manner. The majority of current knowledge stems from investigation of programmed cardiovascular events, but the impact of sexual dichotomy on hepatic programmed disease development is unknown.

Apart from mechanistic investigation of the biological circadian clock in hepatic programming, only female offspring have been included in this study. Due to the vast number of rodents required in the circadian experimental paradigm, it was decided to use both male and female offspring in accordance with Home Office Guidelines for the humane use of animals. However, prior to execution of this study, sex specific differences in programmed offspring NAFLD were investigated. There were no significant differences in phenotypic presentation of NAFLD in male versus female offspring as evidenced by hepatic markers of injury and fibrogenesis implicated in disease pathogenesis (Appendix 9.1).

8.7 Future Research Directions

8.7.1 Translation to the human situation

There are presently no parallel reports of programmed offspring NAFLD via maternal obesity in humans. The need to investigate the role of maternal obesity on offspring hepatic outcome is pivotal given the exponential rise in paediatric NAFLD, now the most frequent chronic liver disease in children and adolescents (257). Moreover, the prevalence of adult NAFLD is rising in tandem (242). From investigation in a human model, potential preventative and

therapeutic interventions can be identified in an attempt to curb the present and projected population prevalence of NAFLD and its adverse sequelae.

Developmental programming is the response mounted by offspring to various environmental stimuli during critical developmental periods resulting in permanent alterations in structure, physiology and/or the epigenome. As such, an instructive and confirmatory human study design could involve the use of monozygotic twins in a co-twin case controlled paradigm, enabling an examination of the environmental and epigenetic contributions in programmed offspring NAFLD pathogenesis. Preliminary investigation into feasibility of such a study has revealed that the largest global twin database exists at King's College London within the Department of Twin Research and Genetic Epidemiology, housing records from over 10,000 mono- and di-zygotic twins including detailed metabolic read-outs. With detailed reports of maternal anthropometry and peri-natal experiences, such a study would be invaluable to our current understanding of experimental associations between programming and offspring NAFLD.

8.7.2 Programming, NAFLD and the Gut Microbiome

The gut microbiota protects the host organism against digestive and extra-digestive diseases by regulating metabolism, immunity and forming an intestinal barrier between the host and the external environment (258). Interestingly, gut microflora has been found to be altered in chronic liver disease. More recently, it has been shown to influence fat storage and energy utilisation, playing a

direct role in the development of insulin resistance and metabolic disease (259, 260). In addition, it has been reported that obese individuals have fewer Bacteroidetes and more Firmicutes than their lean counter-parts, resulting in altered energy harvesting from ingested food (261). It is therefore unsurprising that the gut microbiome has been implicated in NAFLD pathogenesis.

In this study, a role for innate immune dysfunction in programmed offspring NAFLD has been reported. This may be in response to an altered gut microbiome. It is thus plausible that maternal obesity via developmental programming could permanently alter the offspring gut microbiome, increasing susceptibility to NAFLD development and disease progression. Mechanistically, this could improve our understanding of programmed NAFLD aetiopathogenesis.

8.8 Conclusion

A significant body of knowledge has established that exposure to maternal obesity during neonatal development has long-term health consequences on the adult organism. From this, the 'Developmental Origins of Health and Disease' hypothesis was borne. From experimental and clinical investigation of this hypothesis, associations between maternal obesity, cardiovascular and metabolic diseases have been identified. However, implications for development of hepatic disease were scarcely investigated until now.

In this present study, a confirmatory role for developmental programming in offspring NAFLD via maternal obesity and the responsible critical window of development is reported. The downstream regulatory pathways perturbed by such adverse exposure have been interrogated with contributory roles identified for the hepatic insulin signalling, innate immune system and biological circadian clock network. The role for maternal obesity in programming offspring NAFLD may therefore explain the inexorable rise in disease prevalence (242, 257).

Reference List

1. Government Office for Science. Foresight Tackling Obesities: future choices. 2007.

2. Hurt RT, Frazier TH, McClave SA, Kaplan LM. Obesity epidemic: overview, pathophysiology, and the intensive care unit conundrum. JPEN J Parenter Enteral Nutr 2011 Sep;35(5 Suppl):4S-13S.

3. Kopelman P. Symposium 1: Overnutrition: consequences and solutions. Foresight Report: the obesity challenge ahead. Proc Nutr Soc 2010 Feb;69(1):80-85.

4. NHS: The Information Centre. Statistics on Obesity, Physical Activity and Diet in England 2011. 2012.

5. Alfaradhi MZ, Ozanne SE. Developmental programming in response to maternal overnutrition. Front Genet 2011;2:27.

6. Flegal KM, Carroll MD, Kit BK, Ogden CL. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. JAMA 2012 Feb 1;307(5):491-497.

7. Roseboom TJ. The fetal origins hypothesis. Twin Res 2001 Oct;4(5):iii.

8. Barker DJ, Osmond C. Diet and coronary heart disease in England and Wales during and after the second world war. J Epidemiol Community Health 1986 Mar;40(1):37-44.

9. Barker DJ. In utero programming of chronic disease. Clin Sci (Lond) 1998 Aug;95(2):115-128.

10. Hales CN, Barker DJ, Clark PM, Cox LJ, Fall C, Osmond C, et al. Fetal and infant growth and impaired glucose tolerance at age 64. BMJ 1991 Oct 26;303(6809):1019-1022.

11. Barker DJ. Adult consequences of fetal growth restriction. Clin Obstet Gynecol 2006 Jun;49(2):270-283.

12. Stein CE, Fall CH, Kumaran K, Osmond C, Cox V, Barker DJ. Fetal growth and coronary heart disease in south India. Lancet 1996 Nov 9;348(9037):1269-1273.

13. Hales CN, Barker DJ. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. Diabetologia 1992 Jul;35(7):595-601.

14. Armitage JA, Khan IY, Taylor PD, Nathanielsz PW, Poston L. Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? J Physiol 2004 Dec 1;561(Pt 2):355-377.

15. Singhal A, Lucas A. Early origins of cardiovascular disease: is there a unifying hypothesis? Lancet 2004 May 15;363(9421):1642-1645.

16. Wells JC. The thrifty phenotype: An adaptation in growth or metabolism? Am J Hum Biol 2011 Jan;23(1):65-75.

17. Paneth N, Susser M. Early origin of coronary heart disease (the "Barker hypothesis"). BMJ 1995 Feb 18;310(6977):411-412.

18. Boney CM, Verma A, Tucker R, Vohr BR. Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. Pediatrics 2005 Mar;115(3):e290-e296.

19. Laitinen J, Power C, Jarvelin MR. Family social class, maternal body mass index, childhood body mass index, and age at menarche as predictors of adult obesity. Am J Clin Nutr 2001 Sep;74(3):287-294.

20. Li M, Sloboda DM, Vickers MH. Maternal obesity and developmental programming of metabolic disorders in offspring: evidence from animal models. Exp Diabetes Res 2011;2011:592408.

21. Napoli C, D'Armiento FP, Mancini FP, Postiglione A, Witztum JL, Palumbo G, et al. Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. Intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions. J Clin Invest 1997 Dec 1;100(11):2680-2690.

22. Campbell DM, Hall MH, Barker DJ, Cross J, Shiell AW, Godfrey KM. Diet in pregnancy and the offspring's blood pressure 40 years later. Br J Obstet Gynaecol 1996 Mar;103(3):273-280.

23. Shiell AW, Campbell DM, Hall MH, Barker DJ. Diet in late pregnancy and glucose-insulin metabolism of the offspring 40 years later. BJOG 2000 Jul;107(7):890-895.

24. Villamor E, Cnattingius S. Interpregnancy weight change and risk of adverse pregnancy outcomes: a population-based study. Lancet 2006 Sep 30;368(9542):1164-1170.

25. Kral JG, Biron S, Simard S, Hould FS, Lebel S, Marceau S, et al. Large maternal weight loss from obesity surgery prevents transmission of obesity to children who were followed for 2 to 18 years. Pediatrics 2006 Dec;118(6):e1644-e1649.

26. Lewis DS, Bertrand HA, McMahan CA, McGill HC, Jr., Carey KD, Masoro EJ. Preweaning food intake influences the adiposity of young adult baboons. J Clin Invest 1986 Oct;78(4):899-905.

27. McCurdy CE, Bishop JM, Williams SM, Grayson BE, Smith MS, Friedman JE, et al. Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates. J Clin Invest 2009 Feb;119(2):323-335.

28. Zhu MJ, Du M, Nathanielsz PW, Ford SP. Maternal obesity up-regulates inflammatory signaling pathways and enhances cytokine expression in the mid-gestation sheep placenta. Placenta 2010 May;31(5):387-391.

29. Zhu MJ, Ma Y, Long NM, Du M, Ford SP. Maternal obesity markedly increases placental fatty acid transporter expression and fetal blood

triglycerides at midgestation in the ewe. Am J Physiol Regul Integr Comp Physiol 2010 Nov;299(5):R1224-R1231.

30. Tong JF, Yan X, Zhu MJ, Ford SP, Nathanielsz PW, Du M. Maternal obesity downregulates myogenesis and beta-catenin signaling in fetal skeletal muscle. Am J Physiol Endocrinol Metab 2009 Apr;296(4):E917-E924.

31. Yan X, Zhu MJ, Xu W, Tong JF, Ford SP, Nathanielsz PW, et al. Up-regulation of Toll-like receptor 4/nuclear factor-kappaB signaling is associated with enhanced adipogenesis and insulin resistance in fetal skeletal muscle of obese sheep at late gestation. Endocrinology 2010 Jan;151(1):380-387.

32. Boullu-Ciocca S, Dutour A, Guillaume V, Achard V, Oliver C, Grino M. Postnatal diet-induced obesity in rats upregulates systemic and adipose tissue glucocorticoid metabolism during development and in adulthood: its relationship with the metabolic syndrome. Diabetes 2005 Jan;54(1):197-203.

33. Armitage JA, Taylor PD, Poston L. Experimental models of developmental programming: consequences of exposure to an energy rich diet during development. J Physiol 2005 May 15;565(Pt 1):3-8.

34. Plagemann A, Heidrich I, Gotz F, Rohde W, Dorner G. Obesity and enhanced diabetes and cardiovascular risk in adult rats due to early postnatal overfeeding. Exp Clin Endocrinol 1992;99(3):154-158.

35. Samuelsson AM, Matthews PA, Argenton M, Christie MR, McConnell JM, Jansen EH, et al. Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. Hypertension 2008 Feb;51(2):383-392.

36. Guo F, Jen KL. High-fat feeding during pregnancy and lactation affects offspring metabolism in rats. Physiol Behav 1995 Apr;57(4):681-686.

37. Taylor PD, McConnell J, Khan IY, Holemans K, Lawrence KM, Asare-Anane H, et al. Impaired glucose homeostasis and mitochondrial abnormalities in offspring of rats fed a fat-rich diet in pregnancy. Am J Physiol Regul Integr Comp Physiol 2005 Jan;288(1):R134-R139.

38. Koukkou E, Ghosh P, Lowy C, Poston L. Offspring of normal and diabetic rats fed saturated fat in pregnancy demonstrate vascular dysfunction. Circulation 1998 Dec 22;98(25):2899-2904.

39. Langley-Evans SC. Intrauterine programming of hypertension in the rat: nutrient interactions. Comp Biochem Physiol A Physiol 1996 Aug;114(4):327-333.

40. Khan IY, Taylor PD, Dekou V, Seed PT, Lakasing L, Graham D, et al. Gender-linked hypertension in offspring of lard-fed pregnant rats. Hypertension 2003 Jan;41(1):168-175.

41. Bayol SA, Farrington SJ, Stickland NC. A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a greater propensity for obesity in rat offspring. Br J Nutr 2007 Oct;98(4):843-851.

42. Oken E, Taveras EM, Kleinman KP, Rich-Edwards JW, Gillman MW. Gestational weight gain and child adiposity at age 3 years. Am J Obstet Gynecol 2007 Apr;196(4):322-328.

43. Fraser A, Tilling K, Macdonald-Wallis C, Sattar N, Brion MJ, Benfield L, et al. Association of maternal weight gain in pregnancy with offspring obesity and metabolic and vascular traits in childhood. Circulation 2010 Jun 15;121(23):2557-2564.

44. White CL, Purpera MN, Morrison CD. Maternal obesity is necessary for programming effect of high-fat diet on offspring. Am J Physiol Regul Integr Comp Physiol 2009 May;296(5):R1464-R1472.

45. Shankar K, Harrell A, Liu X, Gilchrist JM, Ronis MJ, Badger TM. Maternal obesity at conception programs obesity in the offspring. Am J Physiol Regul Integr Comp Physiol 2008 Feb;294(2):R528-R538.

46. Akyol A, Langley-Evans SC, McMullen S. Obesity induced by cafeteria feeding and pregnancy outcome in the rat. Br J Nutr 2009 Dec;102(11):1601-1610.

47. Howie GJ, Sloboda DM, Kamal T, Vickers MH. Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet. J Physiol 2009 Feb 15;587(Pt 4):905-915.

48. Ekelund U, Ong KK, Linne Y, Neovius M, Brage S, Dunger DB, et al. Association of weight gain in infancy and early childhood with metabolic risk in young adults. J Clin Endocrinol Metab 2007 Jan;92(1):98-103.

49. Ong KK, Emmett PM, Noble S, Ness A, Dunger DB. Dietary energy intake at the age of 4 months predicts postnatal weight gain and childhood body mass index. Pediatrics 2006 Mar;117(3):e503-e508.

50. Singhal A, Cole TJ, Fewtrell M, Lucas A. Breastmilk feeding and lipoprotein profile in adolescents born preterm: follow-up of a prospective randomised study. Lancet 2004 May 15;363(9421):1571-1578.

51. Oben JA, Mouralidarane A, Samuelsson AM, Matthews PJ, Morgan ML, McKee C, et al. Maternal obesity during pregnancy and lactation programs the development of offspring non-alcoholic fatty liver disease in mice. J Hepatol 2010 Jun;52(6):913-920.

52. Gorski JN, Dunn-Meynell AA, Hartman TG, Levin BE. Postnatal environment overrides genetic and prenatal factors influencing offspring obesity and insulin resistance. Am J Physiol Regul Integr Comp Physiol 2006 Sep;291(3):R768-R778.

53. Plagemann A, Harder T, Rake A, Voits M, Fink H, Rohde W, et al. Perinatal elevation of hypothalamic insulin, acquired malformation of hypothalamic galaninergic neurons, and syndrome x-like alterations in adulthood of neonatally overfed rats. Brain Res 1999 Jul 31;836(1-2):146-155.

54. Srinivasan M, Mitrani P, Sadhanandan G, Dodds C, Shbeir-ElDika S, Thamotharan S, et al. A high-carbohydrate diet in the immediate postnatal life of rats induces adaptations predisposing to adult-onset obesity. J Endocrinol 2008 Jun;197(3):565-574.

55. Bayol SA, Simbi BH, Bertrand JA, Stickland NC. Offspring from mothers fed a 'junk food' diet in pregnancy and lactation exhibit exacerbated adiposity that is more pronounced in females. J Physiol 2008 Jul 1;586(13):3219-3230.

56. Beck B, Burlet A, Nicolas JP, Burlet C. Hypothalamic neuropeptide Y (NPY) in obese Zucker rats: implications in feeding and sexual behaviors. Physiol Behav 1990 Mar;47(3):449-453.

57. Bouret SG, Draper SJ, Simerly RB. Trophic action of leptin on hypothalamic neurons that regulate feeding. Science 2004 Apr 2;304(5667):108-110.

58. Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science 1995 Jul 28;269(5223):546-549.

59. Pico C, Sanchez J, Oliver P, Miralles O, Ceresi E, Palou A. Role of leptin present in maternal milk in the control of energy balance during the post-natal period. Genes Nutr 2007 Oct;2(1):139-141.

60. Air EL, Strowski MZ, Benoit SC, Conarello SL, Salituro GM, Guan XM, et al. Small molecule insulin mimetics reduce food intake and body weight and prevent development of obesity. Nat Med 2002 Feb;8(2):179-183.

61. Harder T, Plagemann A, Rohde W, Dorner G. Syndrome X-like alterations in adult female rats due to neonatal insulin treatment. Metabolism 1998 Jul;47(7):855-862.

62. Muhlhausler BS, Duffield JA, McMillen IC. Increased maternal nutrition stimulates peroxisome proliferator activated receptor-gamma, adiponectin, and leptin messenger ribonucleic acid expression in adipose tissue before birth. Endocrinology 2007 Feb;148(2):878-885.

63. Igosheva N, Abramov AY, Poston L, Eckert JJ, Fleming TP, Duchen MR, et al. Maternal diet-induced obesity alters mitochondrial activity and redox status in mouse oocytes and zygotes. PLoS One 2010;5(4):e10074.

64. Bruce KD, Cagampang FR, Argenton M, Zhang J, Ethirajan PL, Burdge GC, et al. Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression. Hepatology 2009 Dec;50(6):1796-1808.

65. Shelley P, Martin-Gronert MS, Rowlerson A, Poston L, Heales SJ, Hargreaves IP, et al. Altered skeletal muscle insulin signaling and mitochondrial complex II-III linked activity in adult offspring of obese mice. Am J Physiol Regul Integr Comp Physiol 2009 Sep;297(3):R675-R681.

66. Waterland RA, Jirtle RL. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. Nutrition 2004 Jan;20(1):63-68.

67. Waterland RA, Garza C. Early postnatal nutrition determines adult pancreatic glucose-responsive insulin secretion and islet gene expression in rats. J Nutr 2002 Mar;132(3):357-364.

68. Plagemann A, Roepke K, Harder T, Brunn M, Harder A, Wittrock-Staar M, et al. Epigenetic malprogramming of the insulin receptor promoter due to developmental overfeeding. J Perinat Med 2010 Jul;38(4):393-400.

69. Zhang J, Zhang F, Didelot X, Bruce KD, Cagampang FR, Vatish M, et al. Maternal high fat diet during pregnancy and lactation alters hepatic expression of insulin like growth factor-2 and key microRNAs in the adult offspring. BMC Genomics 2009;10:478.

70. Kohsaka A, Bass J. A sense of time: how molecular clocks organize metabolism. Trends Endocrinol Metab 2007 Jan;18(1):4-11.

71. Oishi K, Miyazaki K, Kadota K, Kikuno R, Nagase T, Atsumi G, et al. Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes. J Biol Chem 2003 Oct 17;278(42):41519-41527.

72. Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E, et al. Obesity and metabolic syndrome in circadian Clock mutant mice. Science 2005 May 13;308(5724):1043-1045.

73. Kohsaka A, Laposky AD, Ramsey KM, Estrada C, Joshu C, Kobayashi Y, et al. High-fat diet disrupts behavioral and molecular circadian rhythms in mice. Cell Metab 2007 Nov;6(5):414-421.

74. Ma K, Jin X, Liang X, Zhao Q, Zhang X. Inflammatory mediators involved in the progression of metabolic syndrome. Diabetes Metab Res Rev 2012 Mar 2.

75. Ludwig J, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. Mayo Clin Proc 1980 Jul;55(7):434-438.

76. Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, et al. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. Diabetes 2001 Aug;50(8):1844-1850.

77. Bugianesi E, Leone N, Vanni E, Marchesini G, Brunello F, Carucci P, et al. Expanding the natural history of nonalcoholic steatohepatitis: from

cryptogenic cirrhosis to hepatocellular carcinoma. Gastroenterology 2002 Jul;123(1):134-140.

78. Powell EE, Cooksley WG, Hanson R, Searle J, Halliday JW, Powell LW. The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. Hepatology 1990 Jan;11(1):74-80.

79. Ruhl CE, Everhart JE. Relation of elevated serum alanine aminotransferase activity with iron and antioxidant levels in the United States. Gastroenterology 2003 Jun;124(7):1821-1829.

80. Szczepaniak LS, Nurenberg P, Leonard D, Browning JD, Reingold JS, Grundy S, et al. Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. Am J Physiol Endocrinol Metab 2005 Feb;288(2):E462-E468.

81. Fan JG, Farrell GC. Epidemiology of non-alcoholic fatty liver disease in China. J Hepatol 2009 Jan;50(1):204-210.

82. Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, et al. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. Hepatology 2004 Dec;40(6):1387-1395.

83. Bedogni G, Miglioli L, Masutti F, Tiribelli C, Marchesini G, Bellentani S. Prevalence of and risk factors for nonalcoholic fatty liver disease: the Dionysos nutrition and liver study. Hepatology 2005 Jul;42(1):44-52.

84. Bellentani S, Marino M. Epidemiology and natural history of non-alcoholic fatty liver disease (NAFLD). Ann Hepatol 2009;8 Suppl 1:S4-S8.

85. Yu AS, Keeffe EB. Nonalcoholic fatty liver disease. Rev Gastroenterol Disord 2002;2(1):11-19.

86. Mokdad AH, Ford ES, Bowman BA, Dietz WH, Vinicor F, Bales VS, et al. Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. JAMA 2003 Jan 1;289(1):76-79.

87. Charlton M. Nonalcoholic fatty liver disease: a review of current understanding and future impact. Clin Gastroenterol Hepatol 2004 Dec;2(12):1048-1058.

88. Bugianesi E, Gastaldelli A, Vanni E, Gambino R, Cassader M, Baldi S, et al. Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms. Diabetologia 2005 Apr;48(4):634-642.

89. Wanless IR, Lentz JS. Fatty liver hepatitis (steatohepatitis) and obesity: an autopsy study with analysis of risk factors. Hepatology 1990 Nov;12(5):1106-1110.

90. Willner IR, Waters B, Patil SR, Reuben A, Morelli J, Riely CA. Ninety patients with nonalcoholic steatohepatitis: insulin resistance, familial tendency, and severity of disease. Am J Gastroenterol 2001 Oct;96(10):2957-2961.

91. Oben JA, Mouralidarane A, Samuelsson AM, Matthews PJ, Morgan ML, McKee C, et al. Maternal obesity during pregnancy and lactation programs the development of offspring non-alcoholic fatty liver disease in mice. J Hepatol 2010 Jun;52(6):913-920.

92. Teli MR, James OF, Burt AD, Bennett MK, Day CP. The natural history of nonalcoholic fatty liver: a follow-up study. Hepatology 1995 Dec;22(6):1714-1719.

93. Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. Gastroenterology 1999 Jun;116(6):1413-1419.

94. Ekstedt M, Franzen LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, et al. Long-term follow-up of patients with NAFLD and elevated liver enzymes. Hepatology 2006 Oct;44(4):865-873.

95. Adams LA, Lymp JF, St SJ, Sanderson SO, Lindor KD, Feldstein A, et al. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. Gastroenterology 2005 Jul;129(1):113-121.

96. El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. Gastroenterology 2004 Feb;126(2):460-468.

97. Oben JA, Nikolopoulos A, Paulon E. Chapter: Non-Alcoholic Fatty Liver Disease.

98. Brunt EM. Nonalcoholic steatohepatitis: pathologic features and differential diagnosis. Semin Diagn Pathol 2005 Nov;22(4):330-338.

99. Kleiner DE, Brunt EM, Van NM, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005 Jun;41(6):1313-1321.

100. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology 1998 Apr;114(4):842-845.

101. Harrison SA, Di Bisceglie AM. Advances in the understanding and treatment of nonalcoholic fatty liver disease. Drugs 2003;63(22):2379-2394.

102. de Alwis NM, Day CP. Non-alcoholic fatty liver disease: the mist gradually clears. J Hepatol 2008;48 Suppl 1:S104-S112.

103. Angulo P. Nonalcoholic fatty liver disease. N Engl J Med 2002 Apr 18;346(16):1221-1231.

104. Sreekumar R, Rosado B, Rasmussen D, Charlton M. Hepatic gene expression in histologically progressive nonalcoholic steatohepatitis. Hepatology 2003 Jul;38(1):244-251.

105. Marchesini G, Brizi M, Morselli-Labate AM, Bianchi G, Bugianesi E, McCullough AJ, et al. Association of nonalcoholic fatty liver disease with insulin resistance. Am J Med 1999 Nov;107(5):450-455.

106. Garcia-Monzon C, Martin-Perez E, Iacono OL, Fernandez-Bermejo M, Majano PL, Apolinario A, et al. Characterization of pathogenic and prognostic factors of nonalcoholic steatohepatitis associated with obesity. J Hepatol 2000 Nov;33(5):716-724.

107. Letteron P, Fromenty B, Terris B, Degott C, Pessayre D. Acute and chronic hepatic steatosis lead to in vivo lipid peroxidation in mice. J Hepatol 1996 Feb;24(2):200-208.

108. Tilg H, Diehl AM. Cytokines in alcoholic and nonalcoholic steatohepatitis. N Engl J Med 2000 Nov 16;343(20):1467-1476.

109. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest 2004 Dec;114(12):1752-1761.

110. Radaelli T, Varastehpour A, Catalano P, Hauguel-de MS. Gestational diabetes induces placental genes for chronic stress and inflammatory pathways. Diabetes 2003 Dec;52(12):2951-2958.

111. Sen S, Simmons RA. Maternal antioxidant supplementation prevents adiposity in the offspring of Western diet-fed rats. Diabetes 2010 Dec;59(12):3058-3065.

112. Perez-Carreras M, Del HP, Martin MA, Rubio JC, Martin A, Castellano G, et al. Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. Hepatology 2003 Oct;38(4):999-1007.

113. Diehl AM. Hepatic complications of obesity. Gastroenterol Clin North Am 2005 Mar;34(1):45-61.

114. Li Z, Yang S, Lin H, Huang J, Watkins PA, Moser AB, et al. Probiotics and antibodies to TNF inhibit inflammatory activity and improve nonalcoholic fatty liver disease. Hepatology 2003 Feb;37(2):343-350.

115. Schmitt-Graff A, Kruger S, Bochard F, Gabbiani G, Denk H. Modulation of alpha smooth muscle actin and desmin expression in perisinusoidal cells of normal and diseased human livers. Am J Pathol 1991 May;138(5):1233-1242.

116. Gressner AM, Bachem MG. Cellular communications and cell-matrix interactions in the pathogenesis of fibroproliferative diseases: liver fibrosis as a paradigm. Ann Biol Clin (Paris) 1994;52(3):205-226.

117. Gressner AM, Bachem MG. Molecular mechanisms of liver fibrogenesis--a homage to the role of activated fat-storing cells. Digestion 1995;56(5):335-346.

118. Stefanovic B, Hellerbrand C, Holcik M, Briendl M, Aliebhaber S, Brenner DA. Posttranscriptional regulation of collagen alpha1(I) mRNA in hepatic stellate cells. Mol Cell Biol 1997 Sep;17(9):5201-5209.

119. Zhan YT, An W. Roles of liver innate immune cells in nonalcoholic fatty liver disease. World J Gastroenterol 2010 Oct 7;16(37):4652-4660.

120. Hines IN, Wheeler MD. Recent advances in alcoholic liver disease III. Role of the innate immune response in alcoholic hepatitis. Am J Physiol Gastrointest Liver Physiol 2004 Aug;287(2):G310-G314.

121. Li Z, Diehl AM. Innate immunity in the liver. Curr Opin Gastroenterol 2003 Nov;19(6):565-571.

122. Rivera CA, Adegboyega P, van RN, Tagalicud A, Allman M, Wallace M. Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. J Hepatol 2007 Oct;47(4):571-579.

123. Su GL. Lipopolysaccharides in liver injury: molecular mechanisms of Kupffer cell activation. Am J Physiol Gastrointest Liver Physiol 2002 Aug;283(2):G256-G265.

124. Swain MG. Natural killer T cells within the liver: conductors of the hepatic immune orchestra. Dig Dis 2010;28(1):7-13.

125. Yang L, Jhaveri R, Huang J, Qi Y, Diehl AM. Endoplasmic reticulum stress, hepatocyte CD1d and NKT cell abnormalities in murine fatty livers. Lab Invest 2007 Sep;87(9):927-937.

126. Guebre-Xabier M, Yang S, Lin HZ, Schwenk R, Krzych U, Diehl AM. Altered hepatic lymphocyte subpopulations in obesity-related murine fatty livers: potential mechanism for sensitization to liver damage. Hepatology 2000 Mar;31(3):633-640.

127. Kremer M, Thomas E, Milton RJ, Perry AW, van RN, Wheeler MD, et al. Kupffer cell and interleukin-12-dependent loss of natural killer T cells in hepatosteatosis. Hepatology 2010 Jan;51(1):130-141.

128. Elinav E, Abd-Elnabi A, Pappo O, Bernstein I, Klein A, Engelhardt D, et al. Suppression of hepatocellular carcinoma growth in mice via leptin, is associated with inhibition of tumor cell growth and natural killer cell activation. J Hepatol 2006 Mar;44(3):529-536.

129. Bahjat KS, Prell RA, Allen HE, Liu W, Lemmens EE, Leong ML, et al. Activation of immature hepatic NK cells as immunotherapy for liver metastatic disease. J Immunol 2007 Dec 1;179(11):7376-7384.

130. Lamas O, Martinez JA, Marti A. Energy restriction restores the impaired immune response in overweight (cafeteria) rats. J Nutr Biochem 2004 Jul;15(7):418-425.

131. Sun R, Gao B. Negative regulation of liver regeneration by innate immunity (natural killer cells/interferon-gamma). Gastroenterology 2004 Nov;127(5):1525-1539.

132. Jeong WI, Gao B. Innate immunity and alcoholic liver fibrosis. J Gastroenterol Hepatol 2008 Mar;23 Suppl 1:S112-S118.

133. Scribner KB, Pawlak DB, Ludwig DS. Hepatic steatosis and increased adiposity in mice consuming rapidly vs. slowly absorbed carbohydrate. Obesity (Silver Spring) 2007 Sep;15(9):2190-2199.

134. Schwarz JM, Linfoot P, Dare D, Aghajanian K. Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high-carbohydrate isoenergetic diets. Am J Clin Nutr 2003 Jan;77(1):43-50.

135. Fromenty B, Pessayre D. Inhibition of mitochondrial beta-oxidation as a mechanism of hepatotoxicity. Pharmacol Ther 1995;67(1):101-154.

136. Oben JA, Roskams T, Yang S, Lin H, Sinelli N, Li Z, et al. Norepinephrine induces hepatic fibrogenesis in leptin deficient ob/ob mice. Biochem Biophys Res Commun 2003 Aug 22;308(2):284-292.

137. Oben JA, Roskams T, Yang S, Lin H, Sinelli N, Torbenson M, et al. Hepatic fibrogenesis requires sympathetic neurotransmitters. Gut 2004 Mar;53(3):438-445.

138. Oben JA, Roskams T, Yang S, Lin H, Sinelli N, Li Z, et al. Norepinephrine induces hepatic fibrogenesis in leptin deficient ob/ob mice. Biochem Biophys Res Commun 2003 Aug 22;308(2):284-292.

139. Diehl AM. Lessons from animal models of NASH. Hepatol Res 2005 Oct;33(2):138-144.

140. Hirose A, Ono M, Saibara T, Nozaki Y, Masuda K, Yoshioka A, et al. Angiotensin II type 1 receptor blocker inhibits fibrosis in rat nonalcoholic steatohepatitis. Hepatology 2007 Jun;45(6):1375-1381.

141. Rinella ME, Elias MS, Smolak RR, Fu T, Borensztajn J, Green RM. Mechanisms of hepatic steatosis in mice fed a lipogenic methionine choline-deficient diet. J Lipid Res 2008 May;49(5):1068-1076.

142. Guo F, Jen KL. High-fat feeding during pregnancy and lactation affects offspring metabolism in rats. Physiol Behav 1995 Apr;57(4):681-686.

143. Oben JA, Mouralidarane A, Samuelsson AM, Matthews PJ, Morgan ML, McKee C, et al. Maternal obesity during pregnancy and lactation programs the development of offspring non-alcoholic fatty liver disease in mice. J Hepatol 2010 Jun;52(6):913-920.

144. Bouanane S, Merzouk H, Benkalfat NB, Soulimane N, Merzouk SA, Gresti J, et al. Hepatic and very low-density lipoprotein fatty acids in obese offspring of overfed dams. Metabolism 2010 Dec;59(12):1701-1709.

145. Shankar K, Kang P, Harrell A, Zhong Y, Marecki JC, Ronis MJ, et al. Maternal overweight programs insulin and adiponectin signaling in the offspring. Endocrinology 2010 Jun;151(6):2577-2589.

146. McCurdy CE, Bishop JM, Williams SM, Grayson BE, Smith MS, Friedman JE, et al. Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates. J Clin Invest 2009 Feb;119(2):323-335.

147. Bayol SA, Simbi BH, Fowkes RC, Stickland NC. A maternal "junk food" diet in pregnancy and lactation promotes nonalcoholic Fatty liver disease in rat offspring. Endocrinology 2010 Apr;151(4):1451-1461.

148. Aagaard-Tillery KM, Grove K, Bishop J, Ke X, Fu Q, McKnight R, et al. Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome. J Mol Endocrinol 2008 Aug;41(2):91-102.

149. Godfrey KM, Sheppard A, Gluckman PD, Lillycrop KA, Burdge GC, McLean C, et al. Epigenetic Gene Promoter Methylation at Birth Is Associated With Child's Later Adiposity. Diabetes 2011 Apr 6.

150. Lu L, Mamiya T, Lu P, Niwa M, Mouri A, Zou LB, et al. The long-lasting effects of cross-fostering on the emotional behavior in ICR mice. Behav Brain Res 2009 Mar 2;198(1):172-178.

151. Hager R, Cheverud JM, Wolf JB. Change in maternal environment induced by cross-fostering alters genetic and epigenetic effects on complex traits in mice. Proc Biol Sci 2009 Aug 22;276(1669):2949-2954.

152. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001 Dec;25(4):402-408.

153. FOLCH J, LEES M, SLOANE STANLEY GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957 May;226(1):497-509.

154. Mouralidarane A, Suleyman N, Soeda J, Oben JA. Practical management of the increasing burden of non-alcoholic fatty liver disease. 1 ed. Frontline Gastroenterology, 2010. 149-155.

155. Targher G, Bertolini L, Padovani R, Rodella S, Tessari R, Zenari L, et al. Prevalence of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2 diabetic patients. Diabetes Care 2007 May;30(5):1212-1218.

156. Ogden CL, Yanovski SZ, Carroll MD, Flegal KM. The epidemiology of obesity. Gastroenterology 2007 May;132(6):2087-2102.

157. Barker DJ. The fetal and infant origins of disease. Eur J Clin Invest 1995 Jul;25(7):457-463.

158. Ravelli AC, van Der Meulen JH, Osmond C, Barker DJ, Bleker OP. Obesity at the age of 50 y in men and women exposed to famine prenatally. Am J Clin Nutr 1999 Nov;70(5):811-816.

159. Yajnik C. Interactions of perturbations in intrauterine growth and growth during childhood on the risk of adult-onset disease. Proc Nutr Soc 2000 May;59(2):257-265.

160. Basso O. Birth weight is forever. Epidemiology 2008 Mar;19(2):204-205.

161. Catalano PM. Obesity and pregnancy--the propagation of a viscous cycle? J Clin Endocrinol Metab 2003 Aug;88(8):3505-3506.

162. Catalano PM, Kirwan JP, Haugel-de MS, King J. Gestational diabetes and insulin resistance: role in short- and long-term implications for mother and fetus. J Nutr 2003 May;133(5 Suppl 2):1674S-1683S.

163. Oken E, Gillman MW. Fetal origins of obesity. Obes Res 2003 Apr;11(4):496-506.

164. Napoli C, D'Armiento FP, Mancini FP, Postiglione A, Witztum JL, Palumbo G, et al. Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. Intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions. J Clin Invest 1997 Dec 1;100(11):2680-2690.

165. Campbell DM, Hall MH, Barker DJ, Cross J, Shiell AW, Godfrey KM. Diet in pregnancy and the offspring's blood pressure 40 years later. Br J Obstet Gynaecol 1996 Mar;103(3):273-280.

166. Shiell AW, Campbell DM, Hall MH, Barker DJ. Diet in late pregnancy and glucose-insulin metabolism of the offspring 40 years later. BJOG 2000 Jul;107(7):890-895.

167. Armitage JA, Taylor PD, Poston L. Experimental models of developmental programming: consequences of exposure to an energy rich diet during development. J Physiol 2005 May 15;565(Pt 1):3-8.

168. Guo F, Jen KL. High-fat feeding during pregnancy and lactation affects offspring metabolism in rats. Physiol Behav 1995 Apr;57(4):681-686.

169. Samuelsson AM, Matthews PA, Argenton M, Christie MR, McConnell JM, Jansen EH, et al. Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. Hypertension 2008 Feb;51(2):383-392.

170. Taylor PD, Khan IY, Hanson MA, Poston L. Impaired EDHF-mediated vasodilatation in adult offspring of rats exposed to a fat-rich diet in pregnancy. J Physiol 2004 Aug 1;558(Pt 3):943-951.

171. Morris MJ. Cardiovascular and metabolic effects of obesity. Clin Exp Pharmacol Physiol 2008 Apr;35(4):416-419.

172. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology 1998 Apr;114(4):842-845.

173. Nieto-Vazquez I, Fernandez-Veledo S, Kramer DK, Vila-Bedmar R, Garcia-Guerra L, Lorenzo M. Insulin resistance associated to obesity: the link TNF-alpha. Arch Physiol Biochem 2008 Jul;114(3):183-194.

174. Tilg H, Diehl AM. Cytokines in alcoholic and nonalcoholic steatohepatitis. N Engl J Med 2000 Nov 16;343(20):1467-1476.

175. Kallwitz ER, Kumar M, Aggarwal R, Berger R, Layden-Almer J, Gupta N, et al. Ethnicity and nonalcoholic fatty liver disease in an obesity clinic: the impact of triglycerides. Dig Dis Sci 2008 May;53(5):1358-1363.

176. Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, et al. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. Hepatology 2004 Dec;40(6):1387-1395.

177. Sanchez J, Oliver P, Miralles O, Ceresi E, Pico C, Palou A. Leptin orally supplied to neonate rats is directly uptaken by the immature stomach and may regulate short-term feeding. Endocrinology 2005 Jun;146(6):2575-2582.

178. Ahima RS, Hileman SM. Postnatal regulation of hypothalamic neuropeptide expression by leptin: implications for energy balance and body weight regulation. Regul Pept 2000 Aug 25;92(1-3):1-7.

179. Kirk SL, Samuelsson AM, Argenton M, Dhonye H, Kalamatianos T, Poston L, et al. Maternal obesity induced by diet in rats permanently influences central processes regulating food intake in offspring. PLoS One 2009;4(6):e5870.

180. Bayol SA, Simbi BH, Fowkes RC, Stickland NC. A maternal "junk food" diet in pregnancy and lactation promotes nonalcoholic Fatty liver disease in rat offspring. Endocrinology 2010 Apr;151(4):1451-1461.

181. Bouanane S, Merzouk H, Benkalfat NB, Soulimane N, Merzouk SA, Gresti J, et al. Hepatic and very low-density lipoprotein fatty acids in obese offspring of overfed dams. Metabolism 2010 Dec;59(12):1701-1709.

182. Mouralidarane A. Developmental programming in the pathogenesis of non-alcoholic fatty liver disease. In: Taylor PD, Samuelsson AM, Morgan ML, McKee C, Poston L, Oben JA, eds. 58 ed. 2008. 408A-505A.

183. Patel MS, Srinivasan M. Metabolic programming in the immediate postnatal life. Ann Nutr Metab 2011;58 Suppl 2:18-28.

184. Brunt EM. Nonalcoholic steatohepatitis: pathologic features and differential diagnosis. Semin Diagn Pathol 2005 Nov;22(4):330-338.

185. Caldwell SH, Swerdlow RH, Khan EM, Iezzoni JC, Hespenheide EE, Parks JK, et al. Mitochondrial abnormalities in non-alcoholic steatohepatitis. J Hepatol 1999 Sep;31(3):430-434.

186. Bachem MG, Melchior R, Gressner AM. The role of thrombocytes in liver fibrogenesis: effects of platelet lysate and thrombocyte-derived growth factors on the mitogenic activity and glycosaminoglycan synthesis of cultured rat liver fat storing cells. J Clin Chem Clin Biochem 1989 Sep;27(9):555-565.

187. Oben JA, Diehl AM. Sympathetic nervous system regulation of liver repair. Anat Rec A Discov Mol Cell Evol Biol 2004 Sep;280(1):874-883.

188. Saxena NK, Ikeda K, Rockey DC, Friedman SL, Anania FA. Leptin in hepatic fibrosis: evidence for increased collagen production in stellate cells and lean littermates of ob/ob mice. Hepatology 2002 Apr;35(4):762-771.

189. Vickers MH, Reddy S, Ikenasio BA, Breier BH. Dysregulation of the adipoinsular axis -- a mechanism for the pathogenesis of hyperleptinemia and adipogenic diabetes induced by fetal programming. J Endocrinol 2001 Aug;170(2):323-332.

190. Commins SP, Marsh DJ, Thomas SA, Watson PM, Padgett MA, Palmiter R, et al. Norepinephrine is required for leptin effects on gene expression in brown and white adipose tissue. Endocrinology 1999 Oct;140(10):4772-4778.

191. Khan I, Dekou V, Hanson M, Poston L, Taylor P. Predictive adaptive responses to maternal high-fat diet prevent endothelial dysfunction but not hypertension in adult rat offspring. Circulation 2004 Aug 31;110(9):1097-1102.

192. Anstee QM, Goldin RD. Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. Int J Exp Pathol 2006 Feb;87(1):1-16.

193. Hager R, Cheverud JM, Wolf JB. Change in maternal environment induced by cross-fostering alters genetic and epigenetic effects on complex traits in mice. Proc Biol Sci 2009 Aug 22;276(1669):2949-2954.

194. Robert S, Martineau GP. Effects of repeated cross-fosterings on preweaning behavior and growth performance of piglets and on maternal behavior of sows. J Anim Sci 2001 Jan;79(1):88-93.

195. Zorn AM. Liver development. 2008.

196. Shankar K, Harrell A, Liu X, Gilchrist JM, Ronis MJ, Badger TM. Maternal obesity at conception programs obesity in the offspring. Am J Physiol Regul Integr Comp Physiol 2008 Feb;294(2):R528-R538.

197. Hubscher SG. Histological assessment of non-alcoholic fatty liver disease. Histopathology 2006 Nov;49(5):450-465.

198. Souza-Mello V, Mandarim-de-Lacerda CA, Aguila MB. Hepatic structural alteration in adult programmed offspring (severe maternal protein restriction) is aggravated by post-weaning high-fat diet. Br J Nutr 2007 Dec;98(6):1159-1169.

199. Angulo P. Nonalcoholic fatty liver disease. N Engl J Med 2002 Apr 18;346(16):1221-1231.

200. Day CP, Saksena S. Non-alcoholic steatohepatitis: definitions and pathogenesis. J Gastroenterol Hepatol 2002 Dec;17 Suppl 3:S377-S384.

201. Hua J, Ma X, Webb T, Potter JJ, Oelke M, Li Z. Dietary fatty acids modulate antigen presentation to hepatic NKT cells in nonalcoholic fatty liver disease. J Lipid Res 2010 Jul;51(7):1696-1703.

202. Rivera CA, Adegboyega P, van RN, Tagalicud A, Allman M, Wallace M. Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. J Hepatol 2007 Oct;47(4):571-579.

203. Zhan YT, An W. Roles of liver innate immune cells in nonalcoholic fatty liver disease. World J Gastroenterol 2010 Oct 7;16(37):4652-4660.

204. Asanuma T, Ono M, Kubota K, Hirose A, Hayashi Y, Saibara T, et al. Super paramagnetic iron oxide MRI shows defective Kupffer cell uptake function in non-alcoholic fatty liver disease. Gut 2010 Feb;59(2):258-266.

205. Wheeler MD. Endotoxin and Kupffer cell activation in alcoholic liver disease. Alcohol Res Health 2003;27(4):300-306.

206. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology 1998 Apr;114(4):842-845.

207. Baffy G. Kupffer cells in non-alcoholic fatty liver disease: the emerging view. J Hepatol 2009 Jul;51(1):212-223.

208. Doherty DG, O'Farrelly C. Innate and adaptive lymphoid cells in the human liver. Immunol Rev 2000 Apr;174:5-20.

209. Su GL. Lipopolysaccharides in liver injury: molecular mechanisms of Kupffer cell activation. Am J Physiol Gastrointest Liver Physiol 2002 Aug;283(2):G256-G265.

210. Maemura K, Zheng Q, Wada T, Ozaki M, Takao S, Aikou T, et al. Reactive oxygen species are essential mediators in antigen presentation by Kupffer cells. Immunol Cell Biol 2005 Aug;83(4):336-343.

211. Wei Y, Clark SE, Morris EM, Thyfault JP, Uptergrove GM, Whaley-Connell AT, et al. Angiotensin II-induced non-alcoholic fatty liver disease is mediated by oxidative stress in transgenic TG(mRen2)27(Ren2) rats. J Hepatol 2008 Sep;49(3):417-428.

212. Maina V, Sutti S, Locatelli I, Vidali M, Mombello C, Bozzola C, et al. Bias in macrophage activation pattern influences non-alcoholic steatohepatitis (NASH) in mice. Clin Sci (Lond) 2012 Jun;122(11):545-553.

213. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 2012 Feb 9;482(7384):179-185.

214. Becher B, Blain M, Giacomini PS, Antel JP. Inhibition of Th1 polarization by soluble TNF receptor is dependent on antigen-presenting cell-derived IL-12. J Immunol 1999 Jan 15;162(2):684-688.

215. Asanuma T, Ono M, Kubota K, Hirose A, Hayashi Y, Saibara T, et al. Super paramagnetic iron oxide MRI shows defective Kupffer cell uptake function in non-alcoholic fatty liver disease. Gut 2010 Feb;59(2):258-266.

216. Neyrinck AM, Cani PD, Dewulf EM, De BF, Bindels LB, Delzenne NM. Critical role of Kupffer cells in the management of diet-induced diabetes and obesity. Biochem Biophys Res Commun 2009 Jul 31;385(3):351-356.

217. Li Z, Soloski MJ, Diehl AM. Dietary factors alter hepatic innate immune system in mice with nonalcoholic fatty liver disease. Hepatology 2005 Oct;42(4):880-885.

218. Ichi I, Nakahara K, Kiso K, Kojo S. Effect of dietary cholesterol and high fat on ceramide concentration in rat tissues. Nutrition 2007 Jul;23(7-8):570-574.

219. Cho H, Zhao X, Hatori M, Yu RT, Barish GD, Lam MT, et al. Regulation of circadian behaviour and metabolism by REV-ERB-alpha and REV-ERB-beta. Nature 2012 Mar 29.

220. Schibler U, Ripperger J, Brown SA. Peripheral circadian oscillators in mammals: time and food. J Biol Rhythms 2003 Jun;18(3):250-260.

221. Hastings MH, Reddy AB, Maywood ES. A clockwork web: circadian timing in brain and periphery, in health and disease. Nat Rev Neurosci 2003 Aug;4(8):649-661.

222. Reppert SM, Weaver DR. Coordination of circadian timing in mammals. Nature 2002 Aug 29;418(6901):935-941.

223. Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, et al. Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science 2000 Sep 29;289(5488):2344-2347.

224. Le MN, Damiola F, Tronche F, Schutz G, Schibler U. Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators. EMBO J 2001 Dec 17;20(24):7128-7136.

225. Reddy AB, Maywood ES, Karp NA, King VM, Inoue Y, Gonzalez FJ, et al. Glucocorticoid signaling synchronizes the liver circadian transcriptome. Hepatology 2007 Jun;45(6):1478-1488.

226. Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, et al. Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 2002 May 3;109(3):307-320.

227. Yang X, Downes M, Yu RT, Bookout AL, He W, Straume M, et al. Nuclear receptor expression links the circadian clock to metabolism. Cell 2006 Aug 25;126(4):801-810.

228. Zvonic S, Ptitsyn AA, Conrad SA, Scott LK, Floyd ZE, Kilroy G, et al. Characterization of peripheral circadian clocks in adipose tissues. Diabetes 2006 Apr;55(4):962-970.

229. Kim W, Woo JS, Kim W. Disrupted circadian rhythm in night shift workers: what can we do? Int J Cardiol 2012 Feb 9;154(3):369-370.

230. Monsees GM, Kraft P, Hankinson SE, Hunter DJ, Schernhammer ES. Circadian genes and breast cancer susceptibility in rotating shift workers. Int J Cancer 2012 Apr 2.

231. Portaluppi F, Tiseo R, Smolensky MH, Hermida RC, Ayala DE, Fabbian F. Circadian rhythms and cardiovascular health. Sleep Med Rev 2012 Apr;16(2):151-166.

232. Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, et al. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 2002 Jul 26;110(2):251-260.

233. Huang W, Ramsey KM, Marcheva B, Bass J. Circadian rhythms, sleep, and metabolism. J Clin Invest 2011 Jun;121(6):2133-2141.

234. Rudic RD, McNamara P, Curtis AM, Boston RC, Panda S, Hogenesch JB, et al. BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. PLoS Biol 2004 Nov;2(11):e377.

235. Shimba S, Ishii N, Ohta Y, Ohno T, Watabe Y, Hayashi M, et al. Brain and muscle Arnt-like protein-1 (BMAL1), a component of the molecular clock, regulates adipogenesis. Proc Natl Acad Sci U S A 2005 Aug 23;102(34):12071-12076.

236. Zhang EE, Liu Y, Dentin R, Pongsawakul PY, Liu AC, Hirota T, et al. Cryptochrome mediates circadian regulation of cAMP signaling and hepatic gluconeogenesis. Nat Med 2010 Oct;16(10):1152-1156.

237. Marquez S, Crespo P, Carlini V, Garbarino-Pico E, Baler R, Caputto BL, et al. The metabolism of phospholipids oscillates rhythmically in cultures of fibroblasts and is regulated by the clock protein PERIOD 1. FASEB J 2004 Mar;18(3):519-521.

238. Grimaldi B, Bellet MM, Katada S, Astarita G, Hirayama J, Amin RH, et al. PER2 controls lipid metabolism by direct regulation of PPARgamma. Cell Metab 2010 Nov 3;12(5):509-520.

239. Englund A, Kovanen L, Saarikoski ST, Haukka J, Reunanen A, Aromaa A, et al. NPAS2 and PER2 are linked to risk factors of the metabolic syndrome. J Circadian Rhythms 2009;7:5.

240. Le MG, Claudel T, Gatfield D, Schaad O, Kornmann B, Sasso GL, et al. REV-ERBalpha participates in circadian SREBP signaling and bile acid homeostasis. PLoS Biol 2009 Sep;7(9):e1000181.

241. Vgontzas AN, Papanicolaou DA, Bixler EO, Lotsikas A, Zachman K, Kales A, et al. Circadian interleukin-6 secretion and quantity and depth of sleep. J Clin Endocrinol Metab 1999 Aug;84(8):2603-2607.

242. Bedogni G, Miglioli L, Masutti F, Tiribelli C, Marchesini G, Bellentani S. Prevalence of and risk factors for nonalcoholic fatty liver disease: the Dionysos nutrition and liver study. Hepatology 2005 Jul;42(1):44-52.

243. Fan JG, Farrell GC. Epidemiology of non-alcoholic fatty liver disease in China. J Hepatol 2009 Jan;50(1):204-210.

244. Vickers MH. Developmental programming and adult obesity: the role of leptin. Curr Opin Endocrinol Diabetes Obes 2007 Feb;14(1):17-22.

245. Buckley AJ, Keseru B, Briody J, Thompson M, Ozanne SE, Thompson CH. Altered body composition and metabolism in the male offspring of high fat-fed rats. Metabolism 2005 Apr;54(4):500-507.

246. Martin-Gronert MS, Ozanne SE. Mechanisms linking suboptimal early nutrition and increased risk of type 2 diabetes and obesity. J Nutr 2010 Mar;140(3):662-666.

247. Kammoun HL, Chabanon H, Hainault I, Luquet S, Magnan C, Koike T, et al. GRP78 expression inhibits insulin and ER stress-induced SREBP-1c activation and reduces hepatic steatosis in mice. J Clin Invest 2009 May;119(5):1201-1215.

248. Gregor MF, Yang L, Fabbrini E, Mohammed BS, Eagon JC, Hotamisligil GS, et al. Endoplasmic reticulum stress is reduced in tissues of obese subjects after weight loss. Diabetes 2009 Mar;58(3):693-700.

249. Li J, Huang J, Li JS, Chen H, Huang K, Zheng L. Accumulation of endoplasmic reticulum stress and lipogenesis in the liver through generational effects of high fat diets. J Hepatol 2012 Apr;56(4):900-907.

250. Engel PJ, Smith R, Brinsmead MW, Bowe SJ, Clifton VL. Male sex and pre-existing diabetes are independent risk factors for stillbirth. Aust N Z J Obstet Gynaecol 2008 Aug;48(4):375-383.

251. Vatten LJ, Skjaerven R. Offspring sex and pregnancy outcome by length of gestation. Early Hum Dev 2004 Jan;76(1):47-54.

252. McCormick CM, Smythe JW, Sharma S, Meaney MJ. Sex-specific effects of prenatal stress on hypothalamic-pituitary-adrenal responses to stress and brain glucocorticoid receptor density in adult rats. Brain Res Dev Brain Res 1995 Jan 14;84(1):55-61.

253. Gotz F, Dorner G, Malz U, Rohde W, Stahl F, Poppe I, et al. Short- and long-term effects of perinatal interleukin-1 beta-application in rats. Neuroendocrinology 1993 Sep;58(3):344-351.

254. Grigore D, Ojeda NB, Alexander BT. Sex differences in the fetal programming of hypertension. Gend Med 2008;5 Suppl A:S121-S132.

255. Kistner A, Celsi G, Vanpee M, Jacobson SH. Increased blood pressure but normal renal function in adult women born preterm. Pediatr Nephrol 2000 Dec;15(3-4):215-220.

256. Ward AM, Moore VM, Steptoe A, Cockington RA, Robinson JS, Phillips DI. Size at birth and cardiovascular responses to psychological stressors: evidence for prenatal programming in women. J Hypertens 2004 Dec;22(12):2295-2301.

257. Nobili V, Pinzani M. Paediatric non-alcoholic fatty liver disease. Gut 2010 May;59(5):561-564.

258. Compare D, Coccoli P, Rocco A, Nardone OM, De MS, Carteni M, et al. Gut-liver axis: The impact of gut microbiota on non alcoholic fatty liver disease. Nutr Metab Cardiovasc Dis 2012 Apr 28.

259. Abu-Shanab A, Quigley EM. The role of the gut microbiota in nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol 2010 Dec;7(12):691-701.

260. Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A 2004 Nov 2;101(44):15718-15723.

261. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature 2006 Dec 21;444(7122):1022-1023.

262. Korkman N. Selection with regard to sex difference of body weight in mice. 43 ed. 1957. 665-678.

9 Appendix

9 Appendix

9.1 Gender Differences in Offspring NAFLD programmed by Maternal Obesity

9.1.1 Introduction

Emerging evidence suggests that male and female offspring differentially adapt to developmental insults/stimuli such as maternal obesity, influencing disease states. Sex specific differences in offspring NAFLD programmed by maternal obesity is unknown. Given the collective use and analysis of both male and female offspring in Chapter 7, it is important to ascertain any gender influenced bias in assayed markers.

9.1.2 Methods

Female C57BL6/J mice were fed standard laboratory or obesogenic chow 7 weeks prior to mating, throughout gestation and lactation. Litters were standardised to 6 pups and weaned on to either a standard or obesogenic diet. At 6 months post-partum, male and female offspring were analysed for biochemical evidence of liver injury and fibrogenesis. All data was analysed using two-way ANOVA; $p < 0.05$ was considered statistically significant.

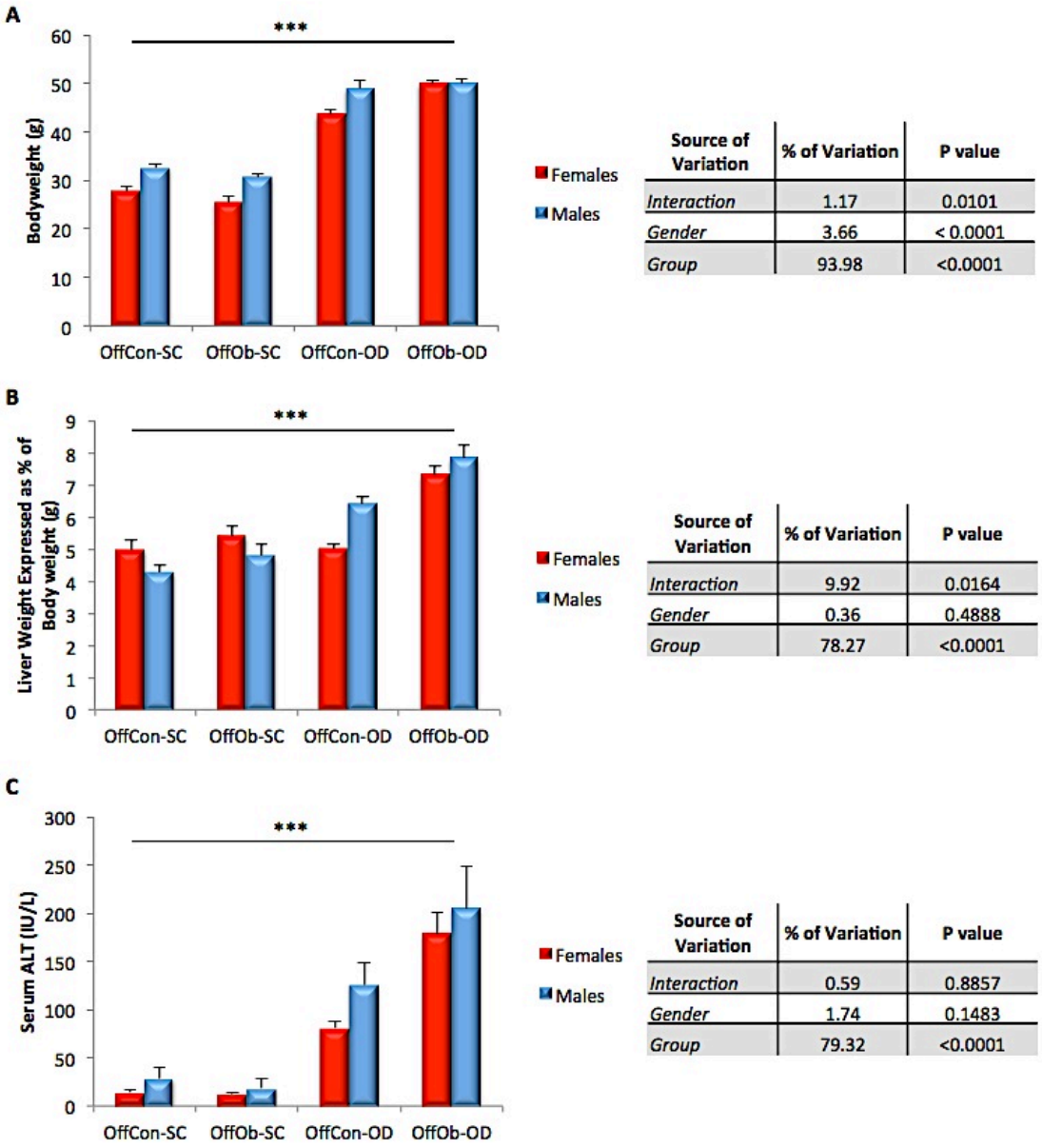
9.1.3 Results

As previously reported in preceding chapters, exposure to maternal obesity and/or an obesogenic diet post-weaning induces offspring adiposity,

dysmetabolism and NAFLD. These findings are consistently reproduced in preliminary analysis of both male and female offspring.

9.1.3.1 Exposure to maternal obesity and a post-weaning obesogenic diet similarly increases body and liver weights in male and female offspring

Both body and liver weights were increased in male and female offspring exposed to maternal obesity and fed a post-weaning obesogenic diet. There was an independent effect of gender on offspring body weight as revealed by two-way ANOVA analysis. No statistically significant gender specific difference was observed for offspring liver weight, however (Figure 9.1).



Source of Variation	% of Variation	P value
Interaction	1.17	0.0101
Gender	3.66	< 0.0001
Group	93.98	<0.0001

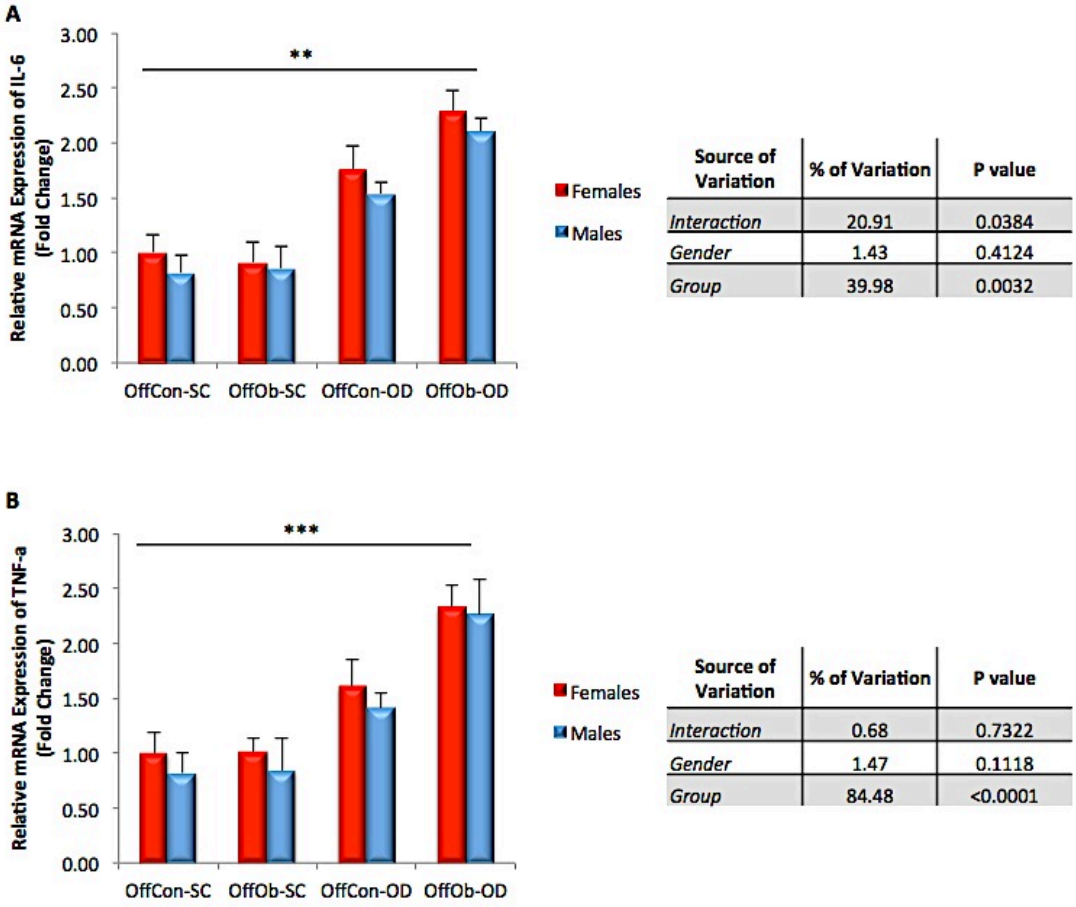
Source of Variation	% of Variation	P value
Interaction	9.92	0.0164
Gender	0.36	0.4888
Group	78.27	<0.0001

Source of Variation	% of Variation	P value
Interaction	0.59	0.8857
Gender	1.74	0.1483
Group	79.32	<0.0001

Figure 9.1: Male and Female Offspring Body and Liver Weights: (A) Body weight and (B) Liver Weight Expressed as % of Body Weight. Offspring of lean (OffCon) and obese (OffOb) weaned on to standard chow (SC) or obesogenic diet (OD). n = 5/group; values shown are mean ± SEM; two-way ANOVA.

9.1.3.2 Exposure to maternal obesity and a post-weaning obesogenic diet similarly induces liver injury in male and female offspring

Serum ALT and relative gene expression of the pro-inflammatory cytokines, IL-6 and TNF-α were most profoundly increased in offspring exposed to maternal obesity and fed an obesogenic diet post-weaning. No statistically significant gender specific difference was observed (Figure 9.2).



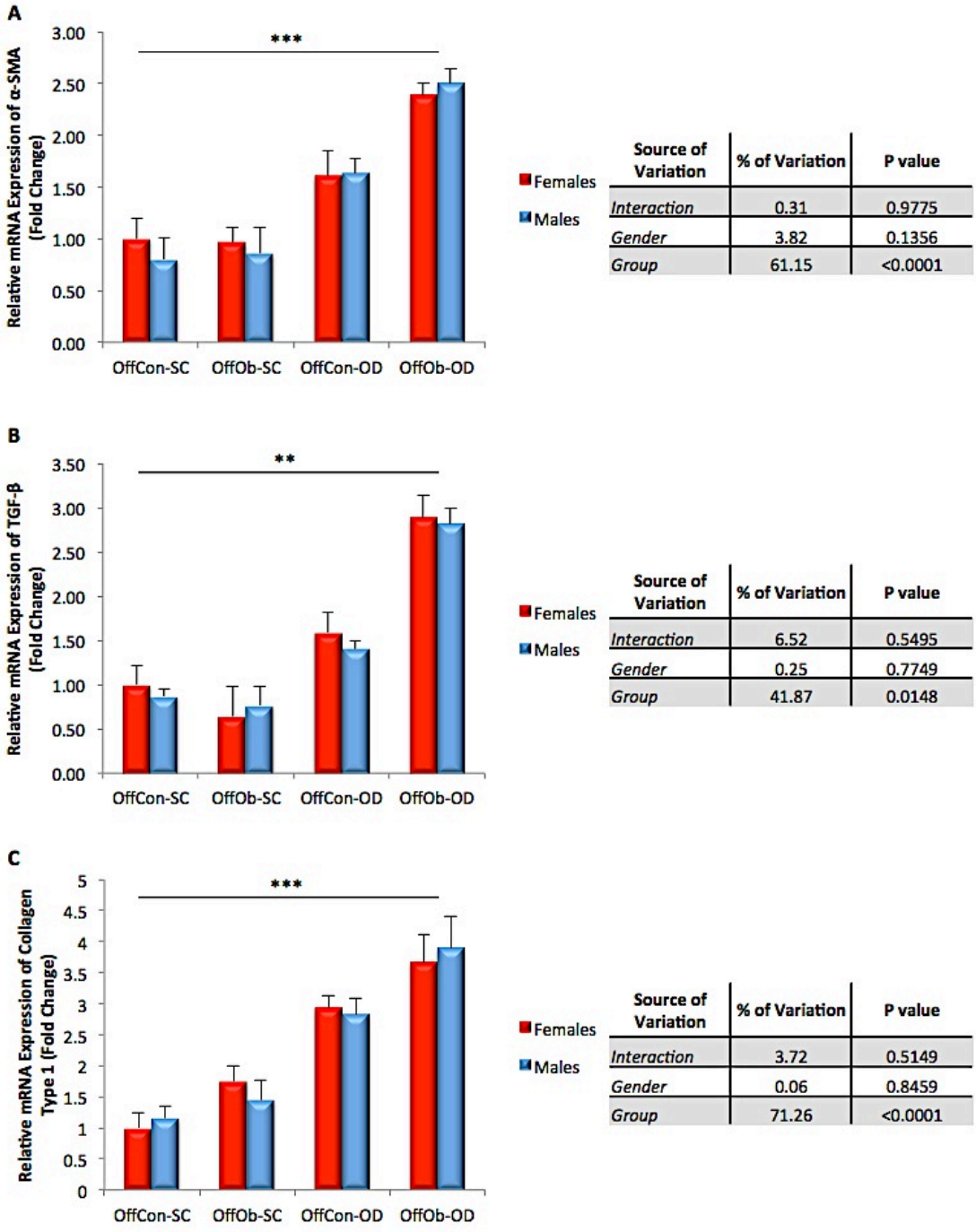
Source of Variation	% of Variation	P value
Interaction	20.91	0.0384
Gender	1.43	0.4124
Group	39.98	0.0032

Source of Variation	% of Variation	P value
Interaction	0.68	0.7322
Gender	1.47	0.1118
Group	84.48	<0.0001

Figure 9.2: Biochemical Evidence of Liver Injury in Male and Female Offspring: (A) Serum ALT, (B) IL-6 mRNA and (C) TNF-α mRNA. Offspring of lean (OffCon) and obese (OffOb) weaned on to standard chow (SC) or obesogenic diet (OD). n = 5/group; values shown are mean ± SEM; two-way ANOVA.

9.1.3.3 Exposure to maternal obesity and a post-weaning obesogenic diet similarly induces liver fibrogenesis in male and female offspring

The relative gene expression of the hepatic fibrogenic markers, α-SMA, TGF-β and collagen were profoundly up-regulated in offspring exposed to maternal obesity and a post-weaning obesogenic diet. No statistically significantly gender specific difference was observed for these fibrogenic markers (Figure 9.3).



Source of Variation	% of Variation	P value
Interaction	0.31	0.9775
Gender	3.82	0.1356
Group	61.15	<0.0001

Source of Variation	% of Variation	P value
Interaction	6.52	0.5495
Gender	0.25	0.7749
Group	41.87	0.0148

Source of Variation	% of Variation	P value
Interaction	3.72	0.5149
Gender	0.06	0.8459
Group	71.26	<0.0001

Figure 9.3: Biochemical Evidence of Liver Fibrogenesis in Male and Female Offspring: (A) α-SMA mRNA (B) TGF-β mRNA and (C) collagen mRNA. Offspring of lean (OffCon) and obese (OffOb) weaned on to standard chow (SC) or

obesogenic diet (OD). n = 5/group; values shown are mean ± SEM; two-way ANOVA.

9.1.4 Discussion

Both male and female offspring exposed to maternal obesity and fed a hyper-calorific diet post-weaning develop NAFLD in adulthood. Importantly, there were no independent effects of gender on such development as evidenced by similar gene expression of liver injury and fibrogenic markers and confirmed by two-way ANOVA analysis. Of note, an independent effect of gender was observed for offspring bodyweight which corroborates early reports of sex specific differences in murine body weight (262). Therefore, combining the use of male and female offspring for investigation of NAFLD programmed by maternal obesity does not introduce any confounding effects. Additionally, it is shown here for the first time that there are no sex specific differences in offspring NAFLD programmed by maternal obesity.

9.2 Supplementary Histology

9.2.1 Representative H&E Sections

These representative sections are from offspring aged 3, 6 and 12 months. As observed throughout the thesis, offspring exposed to maternal obesity in the context of a post-weaning hyper-calorific diet display profound hepatosteatosis Figures 9.4 – 9.6).

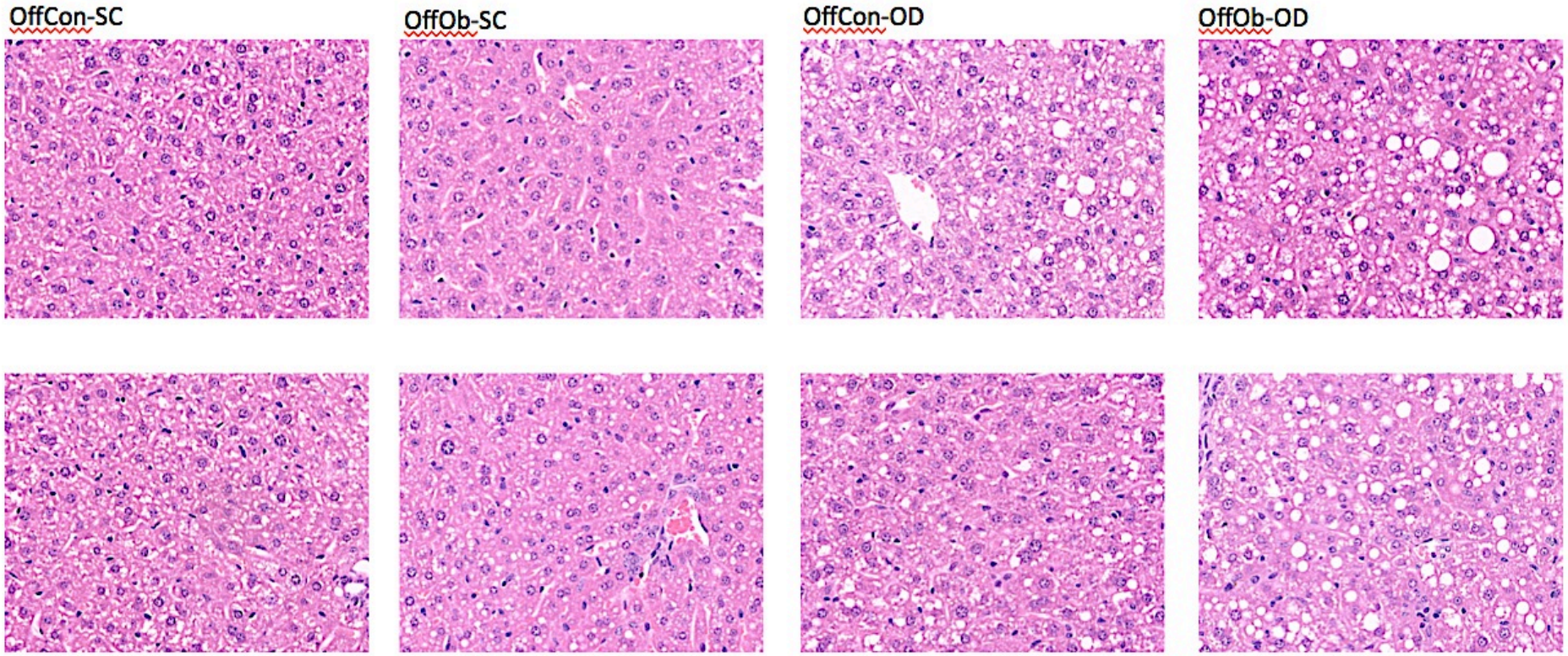


Figure 9.4 Representative H&E Sections from Offspring aged 3 Months. Exposure to maternal obesity in the context of a post-weaning obesogenic diet induced steatosis at 3 months. Offspring born to lean (OffCon) or obese dams (OffOb) weaned on to either standard chow (SC) or an obesogenic diet (OD).

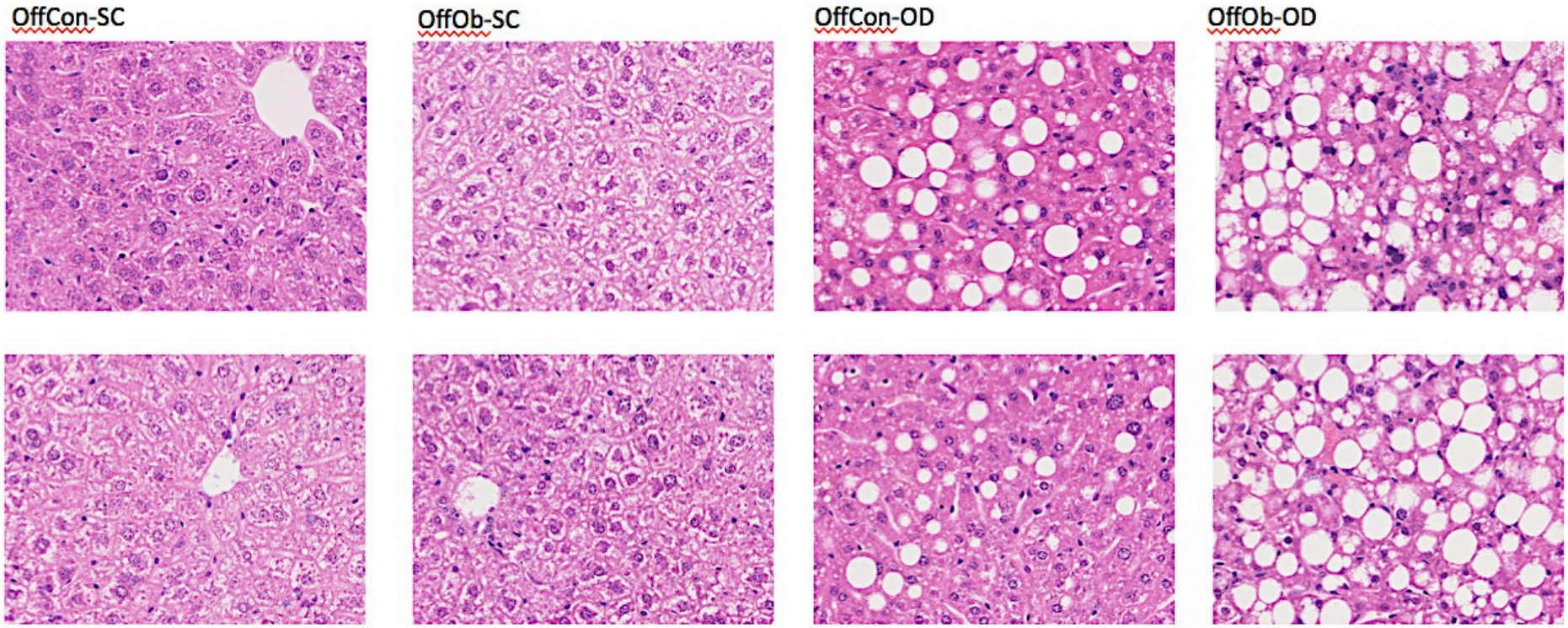


Figure 9.5 Representative H&E Sections from Offspring aged 6 Months. Exposure to maternal obesity in the context of a post-weaning obesogenic diet induced profound steatosis at 6 months. Offspring born to lean (OffCon) or obese dams (OffOb) weaned on to either standard chow (SC) or an obesogenic diet (OD).

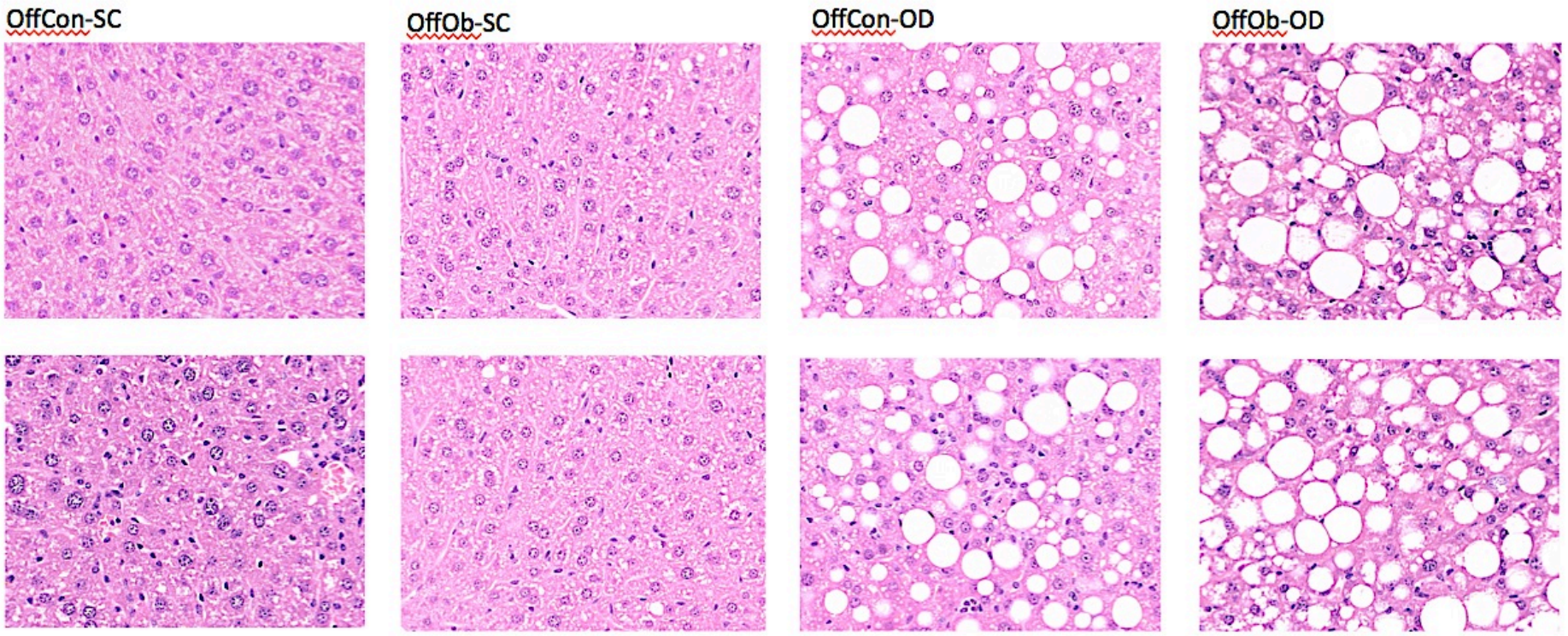


Figure 9.6 Representative H&E Sections from Offspring aged 12 Months. Exposure to maternal obesity in the context of a post-weaning obesogenic diet induced profound steatosis at 12 months. Offspring born to lean (OffCon) or obese dams (OffOb) weaned on to either standard chow (SC) or an obesogenic diet (OD).

9.2.2 Representative Masson's Trichrome Sections

These representative sections are from offspring aged 6 months. These sections are displayed at high (x40) and low (x10) magnification to permit clear visibility of peri-cellular fibrosis in offspring exposed to maternal obesity in the context of a post-weaning hyper-calorific diet (Figures 9.7 – 9.8).

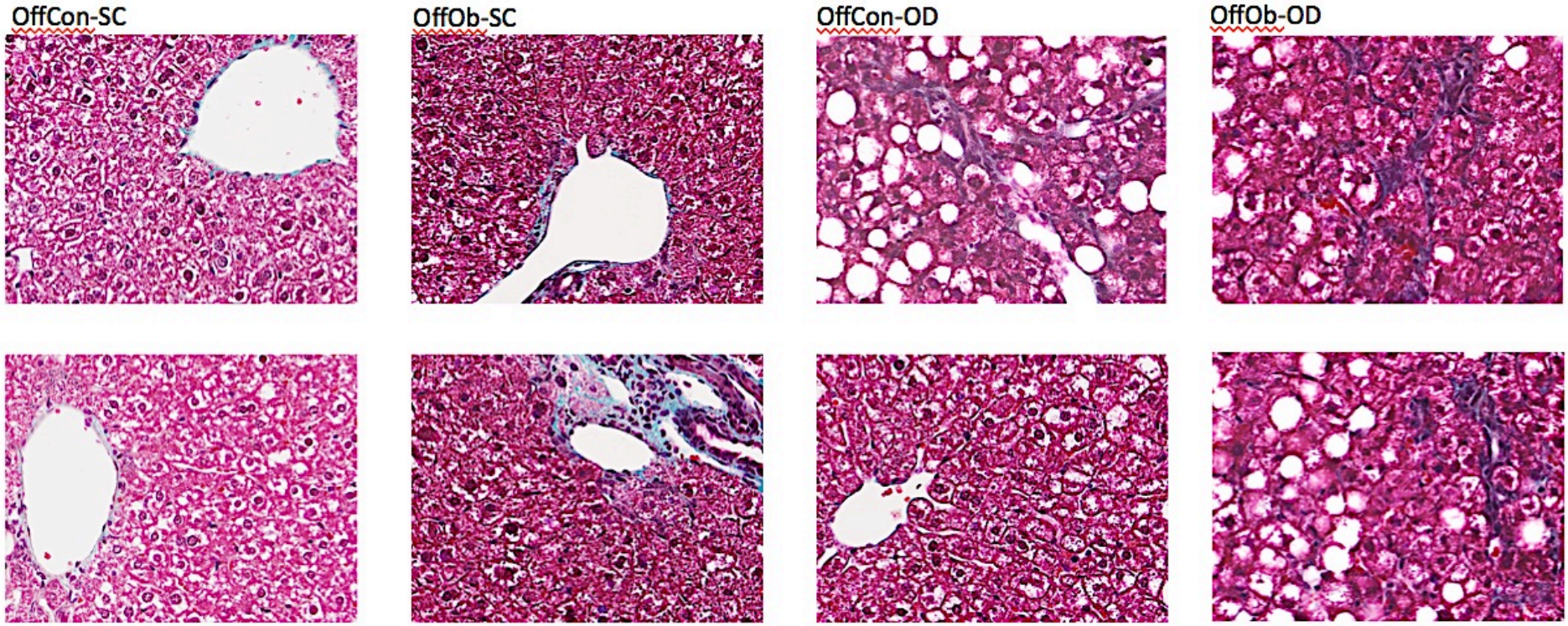


Figure 9.7 Representative Masson's Trichrome Sections x40 from Offspring aged 6 Months. Exposure to maternal obesity in the context of a post-weaning obesogenic diet induced liver fibrosis at 6 months. Offspring born to lean (OffCon) or obese dams (OffOb) weaned on to either standard chow (SC) or an obesogenic diet (OD).

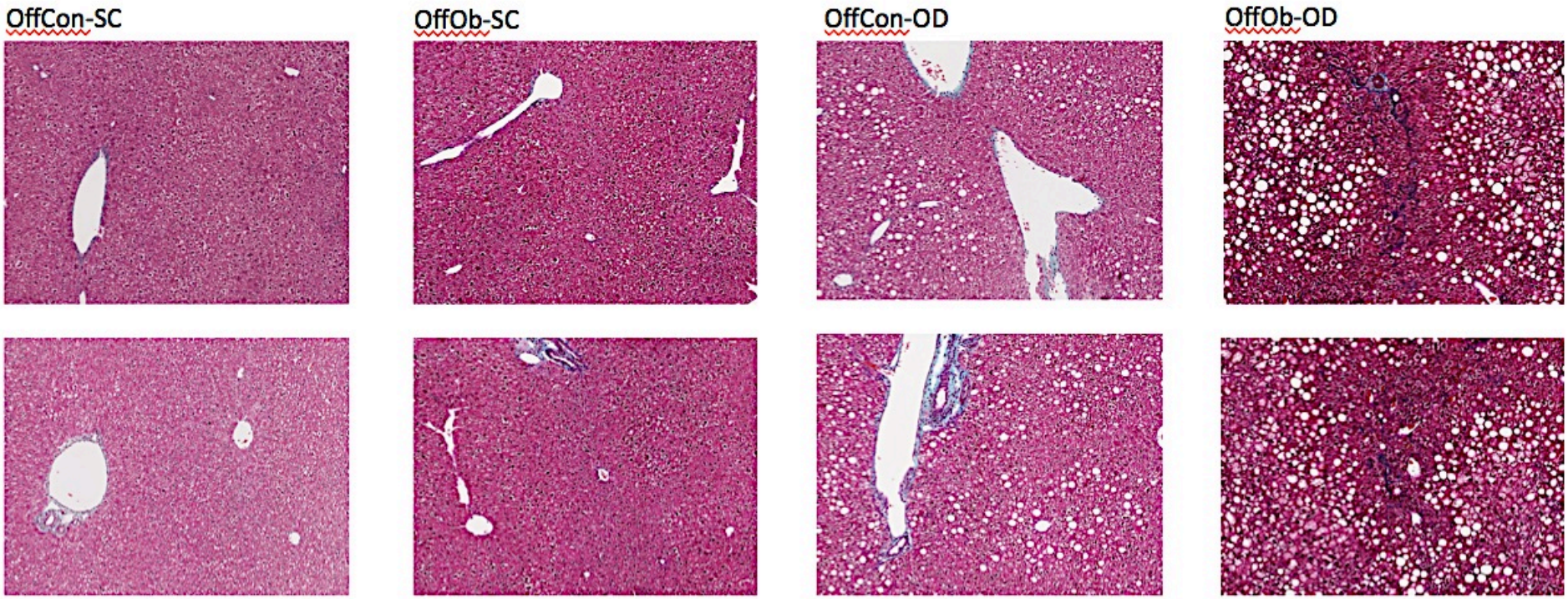


Figure 9.8 Representative Masson's Trichrome Sections x10 from Offspring aged 6 Months. Exposure to maternal obesity in the context of a post-weaning obesogenic diet induced liver fibrosis at 6 months. Offspring born to lean (OffCon) or obese dams (OffOb) weaned on to either standard chow (SC) or an obesogenic diet (OD).