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Developmental programming and reversibility in early life

Minglan Li

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Biomedical Science
The University of Auckland
March 2016
Outputs arising from this thesis

Published papers

Appendix I-XI:


Li M, Reynolds CM, Gray C, Vickers MH. Preweaning GH Treatment Normalizes Body Growth Trajectory and Reverses Metabolic Dysregulation in Adult Offspring After Maternal Undernutrition.. Endocrinology. 2015;156(9):3228-3238


Gray C, Li M, Reynolds CM, Vickers MH. Let-7 miRNA profiles are associated with the reversal of left ventricular hypertrophy and hypertension in adult male offspring from mothers undernourished during pregnancy following pre-weaning growth hormone treatment. *Endocrinology*. 2014; 155(12), 4808-4817.


**Conference oral presentations**


**Conference poster presentations**


Li M, Reynolds CM, Gray C, Vickers MH. Pre-weaning growth hormone treatment reverses programming effects in offspring following maternal undernutrition. First meeting of the DOHaD society of Australia and New Zealand, Perth, Australia, 8th -10th April, 2014.


Abstract

Background: The incidence of obesity and related metabolic disorders has become a major global health issue. A growing body of evidence suggests that early life nutritional adversity plays an important role in the development of long-term metabolic disorders. However, to date, the mechanisms underpinning the phenomenon is poorly understood. In addition, there is a growing interest in the field to explore potential intervention strategies to reverse the detrimental effects associated with a poor early life environment.

Aim: The primary aim of the work detailed in this thesis was to identify potential mechanisms in established rodent models of developmental programming of obesity and metabolic dysfunction and evaluate the effectiveness of targeted pharmacologic and nutrition intervention strategies.

Methods: The work described in this thesis investigated three animal models of altered maternal nutrition with corresponding interventions: 1) a maternal high fructose diet with taurine supplementation, whereby pregnant dams exposed to either chow or high fructose intake during pregnancy and lactation were supplemented either with or without taurine, 2) a maternal high fat:high fructose diet with taurine supplementation, whereby dams exposed to either chow or high fat:high fructose diet during pregnancy and lactation, and supplemented with or without taurine, and 3) maternal global caloric restriction with pre-weaning growth hormone (GH) treatment, in which dams were exposed to 50% caloric intake compared to control dams during pregnancy and offspring received GH treatment (2.5µg/g/day) daily during the pre-weaning period. The rational for taurine was based on limited evidence that taurine can ameliorate fructose and high fat diet induced insulin resistance in the non-pregnant state and has been shown to have protective effects on pancreatic development in rodent models of reduced maternal protein intake. However, taurine had yet to be examined as an intervention agent in the setting of a maternal obesogenic environment. GH was chosen due to known changes in the GH-insulin like growth factor axis in offspring born following a suboptimal early life nutritional environment but, as with taurine, had not been investigated in the setting of poor early life nutrition. The window of GH treatment was based on a key period of developmental plasticity where our group had shown previous efficacy of other interventions during this period such as leptin.

In all studies body weight and food intakes were recorded regularly in both mothers and offspring. Blood samples and tissue of interest for each independent experiment were collected
at the different key developmental time points. Importantly, both male and female offspring were examined to determine possible sexual-dimorphism in the offspring responsiveness to both the altered maternal dietary environment and the intervention.

**Results:** Excessive fructose intake during pregnancy and lactation induced impaired maternal insulin sensitivity, hepatic steatosis and low-grade inflammation, increased offspring susceptibility to impaired glucose metabolism and induced early onset of puberty in female offspring. Taurine supplementation reversed most of the metabolic dysfunction in mothers arising from a high fructose intake and showed beneficial effects on offspring health outcomes in a sex-specific manner.

A maternal high fat:high fructose diet led to profound maternal metabolic dysfunction and induced a number of adverse programming effects in offspring including an enhanced neonatal hepatic pro-inflammatory profile, impaired glucose metabolism and obesity in adulthood, altered food preference and early onset puberty. Taurine supplementation result in a number of protective effects in offspring in a sex-specific manner. Interestingly, despite showing some systemic beneficial effects taurine supplementation further impaired maternal hepatic lipid metabolism and inflammatory profile, which suggests a possible maternal trade-off to protect the offspring.

Maternal global undernutrition resulted in a typical metabolic syndrome phenotype in offspring with increased adiposity, low-grade inflammation, impaired insulin sensitivity, increased blood pressure and endothelial dysfunction. Manipulating GH-IGF axis in the pre-weaning period normalised offspring postnatal growth and adiposity, and protected adult male offspring against cardio-metabolic dysfunction.

**Conclusions:** Our findings further support the DOHaD hypothesis with evidence of adverse maternal and offspring health outcomes across a range of altered maternal nutritional environments. Both taurine and GH treatment during critical developmental windows showed great effectiveness in reducing long term adverse developmental programming effects in each respective cohort. Given that the directionality of effects was dependent upon prior maternal nutritional status and that sexually dimorphic responses to both altered maternal diet and intervention were observed, therapeutic approaches may need to be targeted to maximise potential efficacy. Our studies from a DOHaD perspective suggest promising strategies in combating the current global obesity and metabolic syndrome epidemic.
Acknowledgments

First of all, I would like to express my sincere gratitude to my supervisors Associate Professor Dr Mark Vickers and Dr Clare Reynolds of the Liggins Institute, who gave me the opportunity to explore research questions at a doctoral level, guided me patiently and provided me timely feedback throughout my project. All the knowledge I gained from them in the past four years are priceless treasures. I also acknowledge my first-year co-supervisor Associate Professor Dr Deborah Sloboda of McMaster University for providing me great support and encouragement when I first started the study, and my advisor Dr Clint Gray of the Liggins Institute who has been kindly providing valuable suggestions and help.

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Last but not the least, my partner Cameron Angus McLean supported me unconditionally at my most vulnerable and shared my joy over every delightful moment.
This thesis is dedicated to my great grandfather Dr Jintang Ma (30th April, 1902 - 15th January 1990) - a role model and true inspiration for my life.
# Table of contents

**OUTPUTS ARISING FROM THIS THESIS**.................................................................................................................. II

_PUBLISHED PAPERS_....................................................................................................................................... II

_CONFERENCE POSTER PRESENTATIONS_.............................................................................................................. III

**ABSTRACT**......................................................................................................................................................... V

**ACKNOWLEDGMENTS**.................................................................................................................................... VII

**DEDICATION**...................................................................................................................................................... VIII

**TABLE OF CONTENTS**.................................................................................................................................... IX

**LIST OF FIGURES**.......................................................................................................................................... XV

**LIST OF TABLES**........................................................................................................................................... XVII

**GLOSSARY**....................................................................................................................................................... XVIII

**CO-AUTHORSHIP FORMS**................................................................................................................................. XXI

**CHAPTER 1. INTRODUCTION**............................................................................................................................ 1

1.1 _DEVELOPMENTAL PROGRAMMING PHENOMENON: OBSERVATIONS FROM HUMAN COHORTS_ ................. 3

1.1.1 _Epidemiological studies of poor early life nutrition_................................................................................... 3

a) The Hertfordshire Cohort Study ....................................................................................................................... 3

b) Dutch Hunger Winter study (the Dutch Famine) ............................................................................................ 4

c) The Helsinki Birth Cohort ............................................................................................................................ 5

d) The Siege of Leningrad study ....................................................................................................................... 6

1.1.2 _Epidemiological studies related to maternal obesity and GDM or in a well-nourished population_ ...... 8

a) Southampton Women’s Survey cohort .......................................................................................................... 8

b) Pima Indian women study ............................................................................................................................ 9

c) Other cohorts ................................................................................................................................................. 10

1.1.3 _Summary of human epidemiological cohorts_........................................................................................ 11

1.2 _THE DEVELOPMENT OF THE HYPOTHESIS_............................................................................................. 12

1.3 _DEVELOPMENTAL PROGRAMMING: EVIDENCE FROM ANIMAL STUDIES_........................................ 15

1.3.1 _Rodent models of maternal obesogenic environment_......................................................................... 16

a) Single nutrient enriched diet ......................................................................................................................... 16

b) Mixed nutrient-induced high energy diet (cafeteria diet) ........................................................................... 17

1.3.2 _Rodent models of nutritional restriction in early life_ ............................................................................. 19

a) Global dietary restriction ................................................................................................................................. 19

b) Dietary protein restriction ............................................................................................................................ 20

c) Uterine artery ligation .................................................................................................................................. 22

d) Micronutrient deficiency ............................................................................................................................... 23

1.3.3 _Other animal models of developmental programming_ .................................................................... 24

a) Ovine models .................................................................................................................................................. 24

b) Non-human primates (NHP) .......................................................................................................................... 25
1.3.4 Mechanisms of developmental programming of metabolic syndrome ........................................ 26
  a) Leptin and the regulation of energy balance .............................................................................. 26
  b) Altered adiposity and inflammatory pathway ........................................................................... 28
  c) Pancreatic β-cell development ................................................................................................. 29
  d) Skeletal muscle and locomotor activity ..................................................................................... 30
1.3.5 Sexual dimorphism .................................................................................................................. 31
1.3.6 Summary of animal models of developmental programming .................................................. 32
1.4 Developmental programming in the liver .................................................................................. 34
  1.4.1 Developmental programming of liver .................................................................................... 35
    a) Evidence in maternal obesogenic dietary models ................................................................. 35
    b) Evidence from maternal nutrition insufficiency models ....................................................... 36
  1.4.2 Molecular mechanisms of hepatic programming .................................................................. 37
    a) Lipid accumulation ................................................................................................................ 37
    b) Mitochondrial dysfunction, oxidative and ER stress ............................................................. 38
    c) Pro-inflammatory cytokines ................................................................................................ 39
1.5 Interventions for developmental programming .......................................................................... 43
  1.5.1 Critical time frame for intervention ...................................................................................... 44
  1.5.2 Intervention strategies in early life ....................................................................................... 46
    a) Dietary supplementation ......................................................................................................... 46
    b) Pharmacologic approaches .................................................................................................... 47
    c) Exercise intervention ............................................................................................................. 48

Thesis objectives .......................................................................................................................... 50

Chapter 2. Methodology .................................................................................................................. 51

2.1 Study design ............................................................................................................................... 51
  2.1.1 Justification for use of animals in these studies ................................................................. 51
  2.1.2 Overall design of the animal experiments .......................................................................... 52
2.2 Generation of the animal experimental cohorts ....................................................................... 56
  2.2.1 Timed-mating .................................................................................................................... 56
  2.2.2 Phenotyping at birth ........................................................................................................... 56
  2.2.3 Weaning ............................................................................................................................. 57
  2.2.4 Evaluation of offspring puberty onset .................................................................................. 57
  2.2.5 Food challenge test ............................................................................................................ 57
  2.2.6 Oral glucose tolerance test (OGTT) .................................................................................... 57
  2.2.7 Evaluation of body composition ........................................................................................ 57
  2.2.8 Systolic blood pressure (SBP) measurement ....................................................................... 58
  2.2.9 Tissue collection ............................................................................................................... 58
  2.2.10 Pressure myograph ........................................................................................................... 59
  2.2.11 Maternal dietary manipulation with taurine supplementation .......................................... 61
  2.2.12 Maternal undernutrition with pre-weaning GH treatment .............................................. 63
2.3 Biochemical and molecular analysis ......................................................................................... 64
2.3.1 Plasma analysis .................................................................................................................. 64
2.3.2 H&E staining and morphological evaluation .................................................................. 65
2.3.3 Tissue disruption .............................................................................................................. 65
2.3.4 RNA extraction .................................................................................................................. 66
2.3.5 RNA quantity and quality evaluation ............................................................................... 67
2.3.6 cDNA synthesis ............................................................................................................... 68
2.3.7 Real time PCR ..................................................................................................................... 68
2.3.8 Ex vivo adipose tissue glucose uptake assay ................................................................. 71
2.3.9 Ex vivo adipose tissue culture ......................................................................................... 71
2.3.10 Isolation of stromal vascular fraction .......................................................................... 71
2.3.11 Protein analysis .............................................................................................................. 72
2.3.12 Statistical analysis ......................................................................................................... 73

CHAPTER 3. MATERNAL OBESOGENIC ENVIRONMENT (HIGH FRUCTOSE DIET): MATERNAL AND OFFSPRING OUTCOMES AND EFFECT OF TAURINE SUPPLEMENTATION ................................................................................. 74

3.1 Preface .................................................................................................................................. 74
3.2 Maternal and neonatal health outcomes .............................................................................. 75
  3.2.1 Introduction ...................................................................................................................... 77
  3.2.2 Methods .......................................................................................................................... 79
    3.2.2.1 Animal model ............................................................................................................ 79
    3.2.2.2 Plasma Analysis ....................................................................................................... 80
    3.2.2.3 Histological analysis ............................................................................................... 80
    3.2.2.4 Hepatic mRNA expression ..................................................................................... 80
    3.2.2.5 Statistical analysis ................................................................................................. 81
  3.2.3 Results .............................................................................................................................. 82
    3.2.3.1 Maternal and offspring weights ............................................................................ 82
    3.2.3.2 Maternal plasma profile ......................................................................................... 85
    3.2.3.3 Maternal hepatic morphology ............................................................................... 89
    3.2.3.4 Maternal hepatic gene expression ........................................................................ 90
    3.2.3.5 Neonatal plasma profile ....................................................................................... 93
    3.2.3.6 Neonatal hepatic inflammatory profile ............................................................... 94
  3.2.4 Discussion ......................................................................................................................... 96
3.3 Post-weaning offspring phenotype ...................................................................................... 101
  3.3.1 Methods .......................................................................................................................... 101
  3.3.2 Offspring post-weaning weight gain ............................................................................. 101
  3.3.3 Adult offspring adiposity ............................................................................................... 103
  3.3.4 Onset of puberty ............................................................................................................ 104
  3.3.5 Food challenge test ........................................................................................................ 105
  3.3.6 Adult offspring OGTT ................................................................................................... 107
  3.3.7 Adult offspring plasma leptin and insulin concentration ............................................. 108
3.4 Additional discussion .......................................................................................................... 109
  3.4.1 Discussion on post-weaning offspring ........................................................................ 109

XII
List of Figures

FIGURE 1.1 Total body fat mass (%) as quantified by dual energy X-ray absorptiometry (DEXA) in adult (day 150) male and female rat offspring of HF-fed mothers ................................................................. 17

FIGURE 1.2 Basic consequences of an altered maternal nutritional environment on the health and well-being of offspring...................................................................................................................... 33

FIGURE 1.3 Potential mechanisms underlying the developmental programming of NAFLD ................................................................. 41

FIGURE 1.4 Life course approach of NCD .................................................................................................................................................. 45

FIGURE 2.1 Experimental design utilised for the maternal high fructose diet study ................................................................. 53

FIGURE 2.2 Experimental model utilised for the maternal obesity diet study .......................................................................................... 54

FIGURE 2.3 Experimental model for maternal undernutrition with pre-weaning GH treatment .................................................. 55

FIGURE 3.1 Food and fluid intake during pregnancy and lactation .............................................................................................................................. 85

FIGURE 3.2 Maternal plasma lipid concentrations .................................................................................................................................... 87

FIGURE 3.3 Maternal HOMA-IR indices .................................................................................................................................................. 87

FIGURE 3.4 Maternal plasma proinflammatory cytokine profiles ............................................................................................................. 88

FIGURE 3.5 Maternal liver histology ...................................................................................................................................................... 89

FIGURE 3.6 Maternal hepatic gene expression of markers related to lipid and glucose metabolism ................................................. 91

FIGURE 3.7 Maternal hepatic gene expression of pro-inflammatory markers .......................................................................................... 92

FIGURE 3.8 Neonatal hepatic gene expression. ............................................................................................................................................. 95

FIGURE 3.9 Neonatal hepatic gene expression of markers related to IR and lipid metabolism .......................................................... 100

FIGURE 3.10 Post-weaning offspring growth curve ................................................................................................................................. 102

FIGURE 3.11 Adult offspring adiposity .................................................................................................................................................... 103

FIGURE 3.12 Age and weight at time of pubertal onset ............................................................................................................................ 105

FIGURE 3.13 Offspring body weight gain during food challenge test ...................................................................................................... 105

FIGURE 3.14 Offspring caloric intake during food challenge test ........................................................................................................... 106

FIGURE 3.15 Offspring OGTT ................................................................................................................................................................. 107

FIGURE 3.16 Offspring plasma leptin, insulin and HOMA-IR ....................................................................................................................... 108

FIGURE 4.1 Maternal body weight during pregnancy and lactation ................................................................................................................ 123

FIGURE 4.2 Maternal HOMA-IR, plasma homocysteine and glutamate concentrations ........................................................................... 126

FIGURE 4.3 Maternal liver histology ...................................................................................................................................................... 128

FIGURE 4.4 Maternal hepatic lipid and glucose metabolism related gene expression .................................................................................. 131

FIGURE 4.5 Maternal hepatic inflammatory gene expression ...................................................................................................................... 131

FIGURE 4.6 Neonatal liver inflammatory gene expression ........................................................................................................................ 134

FIGURE 4.7 Neonatal hepatic gene expression of markers related to IR or lipid metabolism .......................................................... 135

FIGURE 4.8 Post-weaning offspring growth curve ................................................................................................................................. 142

FIGURE 4.9 Post-weaning offspring food intake ........................................................................................................................................ 143

FIGURE 4.10 Offspring body composition ............................................................................................................................................... 144

FIGURE 4.11 Age and weight and puberty .......................................................................................................................................... 146

FIGURE 4.12 Offspring body weight gain during food challenge test .................................................................................................... 147

FIGURE 4.13 Offspring caloric intake during food challenge test ........................................................................................................... 148
List of Tables

TABLE 2.1 COMPOSITION OF THE STANDARD CHOW DIET (HARLAN TEKLAD GLOBAL DIET 2018) .................................................. 62
TABLE 2.2 COMPOSITION OF THE OBESOGENIC HIGH FAT:HIGH FRUCTOSE DIET (RESEARCH DIETS D03101602) .................. 62
TABLE 2.3 COMPOSITION OF THE HIGH FAT (HF) DIET (RESEARCH DIETS D12451) ................................................................. 63
TABLE 2.4 BIOCHEMICAL ASSAY INFORMATION ....................................................................................................................... 64
TABLE 2.5 NON-ALCOHOLIC STEATOHEPATITIS CLINICAL RESEARCH NETWORK SYSTEM FOR SCORING NAFLD ...................... 65
TABLE 2.6 PRIMER INFORMATION .............................................................................................................................................. 71
TABLE 3.1 MATERNAL AND NEONATAL WEIGHTS .......................................................................................................................... 83
TABLE 3.2 MATERNAL PLASMA PROFILE ...................................................................................................................................... 86
TABLE 3.3 MATERNAL LIVER NAFLD ACTIVITY SCORE (NAS) ..................................................................................................... 90
TABLE 3.4 NEONATAL PLASMA PROFILE ....................................................................................................................................... 93
TABLE 4.1 MATERNAL, NEONATAL AND WEANING WEIGHT DATA .................................................................................................. 124
TABLE 4.2 MATERNAL PLASMA PROFILE ...................................................................................................................................... 125
TABLE 4.3 MATERNAL LIVER SCORING ......................................................................................................................................... 127
TABLE 4.4 MATERNAL GENE EXPRESSION MAIN EFFECTS ........................................................................................................... 132
TABLE 4.5 NEONATAL PLASMA PROFILE ..................................................................................................................................... 133
TABLE 5.1 NEONATAL PHYSIOLOGICAL CHARACTERISTICS ......................................................................................................... 163
TABLE 5.2 ADULT OFFSPRING PHYSIOLOGICAL CHARACTERISTICS .............................................................................................. 167
TABLE 5.3 ADULT OFFSPRING PLASMA METABOLIC MARKERS .................................................................................................... 168
TABLE 5.4 PLASMA PRO-INFLAMMATORY CYTOKINES ..................................................................................................................... 180
### Glossary

#### Names of dietary treatment groups:

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>CON</td>
<td>control group fed chow diet <em>ad libitum</em></td>
</tr>
<tr>
<td>CT</td>
<td>control taurine group fed chow diet and taurine supplementation</td>
</tr>
<tr>
<td>F</td>
<td>high fructose diet group</td>
</tr>
<tr>
<td>FT</td>
<td>high fructose diet with taurine supplementation group</td>
</tr>
<tr>
<td>MO</td>
<td>maternal obesogenic diet group</td>
</tr>
<tr>
<td>MOT</td>
<td>maternal obesogenic diet with taurine supplementation group</td>
</tr>
<tr>
<td>UN</td>
<td>50% undernutrition</td>
</tr>
<tr>
<td>-S(-)</td>
<td>pre-weaning saline treatment</td>
</tr>
<tr>
<td>-GH(-)</td>
<td>pre-weaning GH treatment</td>
</tr>
<tr>
<td>-C</td>
<td>post-weaning chow diet</td>
</tr>
<tr>
<td>-HF</td>
<td>post-weaning high fat diet</td>
</tr>
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</table>

#### Other abbreviations:

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>11-β-HSD</td>
<td>11 β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>A230</td>
<td>absorbance at 230nm</td>
</tr>
<tr>
<td>A260</td>
<td>absorbance at 260nm</td>
</tr>
<tr>
<td>A280</td>
<td>absorbance at 280nm</td>
</tr>
<tr>
<td>ACC1</td>
<td>acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>Ach</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AKT</td>
<td>Akt kinase/protein kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>Arg1</td>
<td>arginase type 1</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BHB</td>
<td>β-hydroxybutyrate</td>
</tr>
<tr>
<td>BIP</td>
<td>binding of immunoglobulin protein</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>CBX</td>
<td>carbenoxolone</td>
</tr>
<tr>
<td>CD11</td>
<td>cluster of differentiation 11</td>
</tr>
<tr>
<td>CD36</td>
<td>cluster of differentiation 36</td>
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<tr>
<td>CD68</td>
<td>cluster of differentiation 68</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
</tr>
<tr>
<td>ChREBP</td>
<td>carbohydrate-responsive element-binding protein</td>
</tr>
<tr>
<td>CLA</td>
<td>conjugated linoleic acid</td>
</tr>
<tr>
<td>CPT1</td>
<td>carnitine palmitoyltransferase 1</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DAMPs</td>
<td>damage-associated molecular patterns</td>
</tr>
<tr>
<td>DEXA</td>
<td>dual energy x-ray absorptiometry</td>
</tr>
<tr>
<td>DGAC</td>
<td>Dietary Guidelines Advisory Committee</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
</tr>
<tr>
<td>DOHaD</td>
<td>developmental origins of health and disease</td>
</tr>
<tr>
<td>DPX</td>
<td>distrene plasticizer xylene</td>
</tr>
<tr>
<td>EDHFs</td>
<td>endothelium-derived hyperpolarizing factors</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPA</td>
<td>eicosapentaenoic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERO1-α</td>
<td>ER-associated oxidoreductin 1-α</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain complex</td>
</tr>
<tr>
<td>EX-4</td>
<td>Exendin-4</td>
</tr>
<tr>
<td>FASN</td>
<td>fatty acid synthase</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
</tbody>
</table>
SBP = systolic blood pressure
SCD1 = stearoyl-CoA desaturase-1
SIRT1 = silent mating type information regulation 2 homolog 1
SREBP1c = sterol regulatory element binding protein 1c
SVF = stromal vascular fraction
T2DM = type 2 diabetes
TGF-β = transforming growth factor-β
TLR4 = toll-like receptor 4
TNFR1 = tumor necrosis factor receptor 1
TNFα = tumor necrosis factor alpha
UN = undernutrition
β-ME = beta-mercaptoethanol
Co-Authorship Forms
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Chapter One Section 1.3 of this thesis are partially extracted from Minglan’s first author peer-reviewed article “Maternal obesity and developmental programming of metabolic disorders in offspring: evidence from animal models.” published in the Journal of Diabetes Research (formerly titled Experimental Diabetes Research) 2011; 2011:592408.

Nature of contribution by PhD candidate

Searching and reviewing literatures and writing the paper

Extent of contribution by PhD candidate (%)

60%

CO-AUTHORS

<table>
<thead>
<tr>
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<th>Nature of Contribution</th>
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<tr>
<td>Deborah M Sloboda</td>
<td>Critically evaluating the manuscript</td>
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<tr>
<td>Mark H Vickers</td>
<td>Critically evaluating and revising the manuscript</td>
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Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate’s contribution to this work, and the nature of the contribution of each of the co-authors; and
- in cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

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<tr>
<td>Deborah M Sloboda</td>
<td>[Signature]</td>
<td>April 25/15</td>
</tr>
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</table>
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Chapter One Section 1.4 of this thesis are partially extracted and modified from Minglan's first author peer-reviewed article "Developmental programming of non-alcoholic fatty liver disease (NAFLD): the effect of early life nutrition on susceptibility and disease severity in later life." published in BioMed Research International. 2015; Article ID 437107, in press.

**Nature of contribution by PhD candidate**

Searching and reviewing literatures, writing the paper, designed the figures, responding to reviewer's comments

**Extent of contribution by PhD candidate (%)**

70%

### CO-AUTHORS

<table>
<thead>
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<td>Stephanie Segovia</td>
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<td>Clint Gray</td>
<td>Critically evaluating the manuscript</td>
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<td>Mark H Vickers</td>
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### Certification by Co-Authors

The undersigned hereby certify that:

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- in cases where the PhD candidate was the lead author of the work that the candidate wrote the text.

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Chapter Three Section 3.2 of this thesis is an original version of Minglan's first author peer-reviewed article "Maternal taurine supplementation attenuates maternal fructose-induced metabolic and inflammatory dysregulation and partially reverses adverse metabolic programming in offspring" published on Journal of Nutritional Biochemistry. 2014; 26 (3), 267 – 276.

**Nature of contribution by PhD candidate**
Designing and conducting the experiments, analysing data, writing the paper

**Extent of contribution by PhD candidate (%)**
70%

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Chapter five Section 5.2 of this thesis is an original version of Minglan’s first author peer-reviewed article “Pre-weaning growth hormone treatment normalises body growth trajectory and reverses metabolic dysregulation in adult offspring following maternal undernutrition” which has been submitted and is under revision for the journal Endocrinology (EN-15-1041).

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Chapter Five Section 5.3 of this thesis are partially extracted and modified from Minglan's second author peer-reviewed article "Preweaning growth hormone treatment ameliorates adipose tissue insulin resistance and inflammation in adult male offspring following maternal undernutrition" published on the journal Endocrinology. 2013; 154(8), 2676-2686.

Nature of contribution by PhD candidate
Conducting the experiments, critically evaluating the manuscript

Extent of contribution by PhD candidate (%)
30%

CO-AUTHORS

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**Nature of contribution by PhD candidate**

- Conducting the experiments, critically evaluating the manuscript

**Extent of contribution by PhD candidate (%)**

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Clint Gray         |               | 23/4/15       
Clare Marie Reynolds | 29/4/15       
Mark H Vickers     | 29/4/15       

Chapter 1. Introduction

During the last century, the leading causes of morbidity and mortality for humans has transitioned from infectious disease to non-communicable diseases (NCDs), comprising mainly cardiovascular disease (CVD), cancer and diabetes (1). Worldwide, over 60% of mortality is due to NCDs, and in New Zealand, NCDs account for more than 80% of all deaths according to the most recent WHO data (2). The condition known as metabolic syndrome is characterised by the clustering of a number of different metabolic disorders including diabetes, obesity, hyperlipidaemia and hypertension all of which also contribute to the risk of CVD. The prevalence of metabolic syndrome has increased dramatically in Western societies over the last few decades and the trend is mirrored in developing nations that are transitioning to first-world economies.

Metabolic disorders represent an interaction between many factors, including genetic, physiological, behavioural, and environmental influences. However, the rates at which these diseases have increased suggest that environmental influences, rather than genetic causes, are fuelling the epidemic. The developmental origins of health and disease (DOHaD) hypothesis has highlighted the link between the peri-conceptual, fetal and early infant phases of life and the subsequent development of adult obesity and related metabolic disorders. The early life period whereby the essential biological processes such as embryogenesis, morphogenesis and organogenesis take place is a very sensitive window of developmental plasticity and as such is extremely vulnerable to environmental change (3). The DOHaD model speculates that the fetus makes predictive adaptations in response to intrauterine cues, resulting in permanent adjustments in homeostatic systems to aid immediate survival and improve success in an adverse postnatal environment. However, inappropriate interpretations of prenatal cues or changes to that immediate environment may result in a mismatch between prenatal predictions and postnatal reality. As a result, these adaptations, known as predictive adaptive responses (PARs), may ultimately be disadvantageous in postnatal life, leading to an increased risk of chronic NCDs in adulthood and/or the inheritance of risk factors and transgenerational transmission of disease risk.

Early epidemiological studies underpinning the DOHaD hypothesis suggested that fetal growth restriction is correlated with later disease, implying that fetal nutritional deprivation may be a strong programming stimulus. This led to the development of experimental animal models using controlled maternal calorie, protein or macronutrient deficiency during critical
periods of development. However, in many societies, maternal and postnatal nutrition are now either sufficient or excessive. As a result, excessive weight gain and/or obesity are the more common nutritional problems complicating pregnancy in developed countries. Thus, in view of the rising prevalence of obesity in pregnancy and the association with gestational diabetes (GDM), there is also now increasing interest in the detrimental influence of maternal obesity and excess maternal nutrition on the risk of disease in childhood and beyond (4). Interestingly, both ends of the maternal nutrition spectrum often elicit similar phenotypic outcomes in offspring with both maternal undernutrition and maternal obesity giving rise to increased adiposity and related metabolic disorders in offspring – the so-called “U” shaped response curve. Although the mechanisms are not fully defined, programming of later health and well-being was generally considered an irreversible change in developmental trajectory. It has now been shown that, at least in animal models, developmental programming of postnatal metabolic disorders is potentially reversible by nutritional or targeted therapeutic interventions during critical periods of development. Translation of the preclinical findings to the clinical setting therefore has the potential to have a major impact on reduction of obesity and NCD risk and provides an exciting opportunity for effective and lasting approaches to disease prevention rather than treating the disease once manifest.

Chapter One will therefore provide an overview of the original epidemiological findings by David Barker and colleagues which lay the foundation for the original DOHaD framework and clinical and experimental data which have provided empirical evidence to support the early life origins of health and disease hypothesis. Further, the experimental approaches that are commonly utilised will be discussed including key target tissues of interest that are a focus of mechanistic studies related to risk susceptibility. Finally, with a link between the early life environment and later disease risk established, the critical period of developmental plasticity is discussed in the context of potential strategies for intervention to prevent the later adverse sequelae associated with aberrant developmental programming.
1.1 Developmental programming phenomenon: observations from human cohorts

The first use of “programming” in the context of early life environmental influence on long term health outcomes can be traced back to early 1970s’, when German researchers used this term to describe the initial link between GDM and later risk for offspring diabetes mellitus (5). However, the concept of developmental programming (originally termed fetal programming) as a research framework was first generated and popularised by the British epidemiologist and physician Professor David Barker who described his findings on the association between CVD in middle age and low birth weight (6).

A wide range of data has now been amassed from epidemiological studies and clinical observations which show a range of early life exposures including undernutrition, obesity and stress from the pre-conceptual period through to early infancy can significantly increase the propensity towards development of late life metabolic and cardiovascular disease.

The interests of the field have expanded into intergenerational studies, microbiome studies, epigenetic profiling through to the potential role of paternal influences on developmental programming in offspring. In the following section the major findings from human cohorts on the association between altered early life nutrition and later life disease will be discussed.

1.1.1 Epidemiological studies of poor early life nutrition

a) The Hertfordshire Cohort Study

Early observation of geographic correlations between death rates from ischaemic heart disease and infant mortality rates in England and Wales indicated that poor birth conditions may increase the risk of CVD in later life (7). To test the hypothesis in a more robust setting, the historic record of the Hertfordshire Cohort was employed, and it provided perhaps the first evidence that laid the foundations for the developmental programming hypothesis. The Hertfordshire Cohort was originally established by a team of midwives and nurses led by Hertfordshire’s first ‘chief health visitor and lady inspector of midwives’ Ethel Margaret Burnside in 1911 with the aim of improving the health of children in Hertfordshire (8). Information regarding birth weight, development during infancy, feeding information and weight at age of 1 was recorded for all births in Hertfordshire from 1911 until 1948 (8). Using this record and national mortality register, Barker and colleagues identified mortality causes for 15000 men and women born in Hertfordshire between 1911 and 1930, and found that increased risk of death from CVD was related to low birth weight (9).
There are several significant findings from this early work. Firstly, it is the earliest large scale observation in the field that was based on an individual level rather than ecological level. This suggests that the relationship between low birth weight and increased risk of heart disease is more likely to be directly associated rather than be regulated via a communal ecological environment. Secondly, the relationship between a boy’s weight at one year old and death from ischemic heart disease is graded (9). Graded association is often an indicator of causal relationship in epidemiological studies (10). This highlighted, for the first time, the importance of the immediate postnatal environment.

Further ongoing projects were carried out to explore other causes of mortality in this cohort and morbidity outcomes in surviving participants. Later results on mortality outcomes largely agreed with previous findings and extended the death rate association with low birth weight to accidental falls in men, pneumonia, injury, diabetes, and musculoskeletal disease in women. Furthermore, clinical visits to surviving participants from the Hertfordshire cohort demonstrated that small size at birth and during infancy was associated with increased risk of developing coronary heart disease, type 2 diabetes (T2DM), the metabolic syndrome and insulin resistance, osteoporosis and sarcopenia in later life (8).

b) Dutch Hunger Winter study (the Dutch Famine)

The Dutch Hunger Winter study owns a unique place among all of the studies related to the developmental programming phenomenon. During the winter of 1944–1945 Nazi Germany blocked all food transport in occupied regions of the Netherlands in retaliation for a railroad strike to aid the Allies. People in the affected area, including pregnant women, had to live on weekly ration which was as little as 400-800 calories per day (11). Despite the war, the Dutch population was adequately nourished before and after the period of famine. The dramatic twist in nutrition supply created a unique “quasi-experimental” setting for research on the effect of early life nutritional deprivation during different developmental periods on long-term human development. Medical records detailing the place and date of birth allowed the exposure window for offspring to be identified in relation to specific stages of gestation. The famine was caused so rapidly in time and place that it equally affected all social classes, while social class of the Hertfordshire Cohort study has been regarded as a main confounding factor (12).

Extensive studies have been carried out on the long term health outcomes of exposed offspring affected by the Dutch Hunger Winter. This cohort demonstrates that individuals exposed to famine in early gestation have a higher rate (13) and earlier onset of coronary artery disease.
(14), more atherogenic dyslipidemia (15), neurological disorders (16) and increased prevalence of addiction (17) compared to non-exposed individuals. Those who had been exposed to famine in mid-gestation had an increased prevalence of obstructive airway disease (18). Exposure to famine in late or mid gestation was associated with impaired glucose tolerance (19) and insulin resistance (IR) (20). Of note, these observations were subject to the particular timeframe of the exposure, providing early evidence that the outcomes of developmental programming might be time sensitive and different organs and physiological systems might be particularly vulnerable in certain developmental stages.

Similar to that of the Hertfordshire Cohort Study, sex-specific outcomes were observed in the Dutch Hunger Winter cohort. Female participants seemed particularly affected as a result of famine exposure. Maternal undernutrition during early gestation was associated with obesity (increased body mass index (BMI) and waist circumference)) in 50 year old women but not in men (21). Women who were born during the famine had an almost five times increased risk of breast cancer (18). Acute undernutrition during the fetal period did not alter female fertility judged by reproductive performance, however, reduced the age of menopause within the normal range (22).

The Dutch Hunger Winter cohort still provides for ongoing analysis, particularly as regards intergenerational transmission of disease traits. Indeed, a recent study showed that the effects of in utero undernutrition may be transmitted across generations. Offspring of prenatally exposed fathers, but not mothers, were heavier and more obese than offspring of fathers and mothers who had not been exposed to famine (23). Offspring of mothers who exposed to famine in their first six months in utero were smaller at birth than offspring of non-exposed mothers (L.H. Lumey Decreased birthweights in infants after maternal in utero exposure). As per potential mechanisms, studies on the blood samples of 60 offspring born in the Dutch Hunger Winter and their unexposed same-sex siblings showed that the methylation of 6 out of 15 loci that are related to growth and metabolic disease were significantly different after famine exposure during the periconception period. These changes depend on the sex of the exposed individual as well as the gestational timing of the exposure (24, 25).

**c) The Helsinki Birth Cohort**

The Helsinki Birth Cohort study is a large follow-up study consisting of two birth cohorts and 15846 individuals born at the Helsinki University Central Hospital. The earlier cohort covered subjects born in 1924-1933, and the later cohort included the subjects born in 1934-1944 (26).
Because of the comprehensive system of registers in Finland, more detailed information compared to the previously outlined cohorts is available for the study. The original information regarding socio-economic factors of subjects in the Helsinki birth cohort was well-documented. Growth patterns of the subjects in childhood were also traceable - in the earlier cohort growth data from 7-15 years of age were recorded, and in the later cohort postnatal health and growth were evaluated on average 18 times from birth to 11 years old (27).

The Finnish data largely agreed with the findings from the Hertfordshire Cohort study, and extended the association to postnatal growth patterns. In the earlier cohort, boys who were thin at birth but whose weight caught up so that they had an equal or above average body mass from the age of 7 years had the highest death rates from coronary heart disease (28). In the later cohort, adults who had a coronary event had been small at birth and thin at two years of age and gained weight rapidly afterwards (29). This small at birth, followed by rapid catch up growth pattern was also associated with IR and hypertension in later life (29, 30). These findings suggested that the pattern of growth through childhood may modify the risk of cardio-metabolic diseases in relation to size at birth. It was speculated that poor prenatal nutrition followed by improved postnatal nutrition might be a key for the programming of cardio-metabolic diseases (29).

d) The Siege of Leningrad study

Another large scale famine study related to developmental programming is the Siege of Leningrad study, which investigated a more severe famine disaster during the Second World War compared to the Dutch Hunger Winter. The food supply was blocked for a total of 872 days from 8th September 1941 to 27th January 1944. In the first winter of the famine, the average daily ration for most of people in Leningrad contained only 300 calories with almost no protein (31).

A study conducted in 1997 investigated 549 subjects born in and around Leningrad at the time of the siege, of whom one third were exposed to severe intrauterine starvation. No effects of intrauterine undernutrition were found in terms glucose intolerance, dyslipidaemia, hypertension, or cardiovascular disease in adulthood (31). However, individuals who have been exposed to undernutrition did show endothelial dysfunction and a stronger influence of obesity on blood pressure.
Different postnatal environments may account for the inconsistencies in these studies. The duration of the famine for the subjects in the Leningrad siege study in fact lasted about 2-3 years after birth. Even after the famine people in Leningrad remained relatively poor. In the Netherlands, however, the food supplies were quickly restored after the war and the Netherlands quickly developed into a wealthy country. As discussed in the Helsinki birth cohort, poor prenatal nutrition followed by improved postnatal nutrition may play an important role in the development of later cardio-metabolic dysfunction. The fact that the Siege of Leningrad subjects lacked a conflicting postnatal environment may have been the reason that no profound programming effects were observed in some studies utilising this cohort (32).
1.1.2 Epidemiological studies related to maternal obesity and GDM or in a well-nourished population

Historic cohorts related to early life and poor nutrition provide much valuable information in understanding the relationship between early life insults and later life disease, however, in developed countries and many rapidly growing developing countries, maternal obesity and GDM are emerging problems. It has to be pointed out that maternal obesity and GDM are often accompanied by micronutrient deficiencies (33, 34). Interpretation of the results from maternal hyperglycaemia and obesity studies therefore need to be balanced with potential micronutrient deficiency.

a) Southampton Women's Survey cohort

Southampton Women's Survey cohort is a prospective cohort that was established by Cooper and Inskip from Medical Research Council Epidemiology Resource Centre in Southampton to investigate pre-pregnancy characteristics of young women living in the city and their subsequent pregnancy and offspring health outcomes (35). It is one of the largest prospective cohorts examined in modern society and was designed to explore the relationship between early life events including maternal pre-conception profile and offspring long-term health outcomes. 12579 women who were aged between 20–34 years were recruited from 1998 to 2002, of whom 3159 subsequently had singleton pregnancies (36). Participants’ information was collected before conception regardless of whether or not they were planning a pregnancy (35). The information obtained in the cohort covered a wide range of potential confounders (for example maternal smoking, maternal BMI, height) which have only been recognised in recent decades. This added strength to the analysis in this cohort. Since it has been established, Southampton Women's Survey cohort has generated an enormous amount of data to support the original work of David Barker and colleagues.

Recent studies from the cohort demonstrated that excessive maternal nutritional intake is strongly associated with childhood obesity. Offspring of women who gained excessive weight during pregnancy had significant greater body fat mass at birth and at the age of 6 than offspring of women whose weight gain was adequate (37). Further studies showed that maternal dietary glycemic index and glycemic load in early pregnancy, were positively associated with offspring fat mass at 4 and 6 years of age (38). This association remained significant and showed a graded relationship after adjustment for a number of confounders such as maternal pre-pregnancy BMI, maternal smoking, maternal and offspring height (38).
Besides carbohydrate, maternal n-6 polyunsaturated fatty acids (PUFA) concentrations, which are mainly derived from dietary plant oils, have shown a similar positive association with offspring weight and fat mass (39). These data provide robust evidence that imbalanced maternal food intake can have long term influence on offspring adiposity and related metabolic disorders.

b) Pima Indian women study

Pima Indians who reside in Gila River Indian community in southern Arizona USA have the world’s highest reported prevalence of T2DM (40). Since 1965 this community has participated in a longitudinal epidemiologic diabetes study. Oral glucose tolerance tests and measurements of height and weight were performed every two years for all residents whose age was above 5, and an additional oral glucose tolerance test was performed for pregnant women after 24 weeks gestation (41).

In the 1980s, a series of investigations by Pettitt and his colleagues pointed out that the intrauterine environment might be an important determinant of the development of diabetes and obesity in later life (42-45). Offspring of Pima Indian women with pre-existent T2DM and GDM were large for gestational age at birth, and, after approximately 5 years of age, were more obese and had higher glucose concentrations and incidence of diabetes than the offspring of pre-diabetic or non-diabetic women (46). Maternal diabetes was the strongest single risk factor for T2DM in Pima Indian adolescence (47). However, it was argued that the effects of in utero exposure to diabetes might be confounded by genetic factors - the women who developed diabetes at younger age may be more genetically susceptible to diabetes than those who have the onset later, therefore their offspring may inherit greater genetic susceptibility.

Further studies on siblings highlighted that the timing and environmental exposure to maternal diabetes per se is a strong contributing factor to later obesity and diabetes regardless of genetic legacy (48). Via analysis of siblings that were born not more than 3 years apart and at least one exposed and one unexposed to maternal diabetes from the same family, Dabelea et al. found that BMI and the risk of diabetes was significantly higher in siblings born after the mother developed diabetes than in those born before the mother’s diagnosis of diabetes (48). Moreover, there were no significant differences in risk of diabetes or BMI between offspring born before and after the father was diagnosed with diabetes (48). As these findings were observed in subjects that shared a similar genetic background, the increased risk in those
exposed to maternal diabetes can be likely attributed to the hyperglycaemic *in utero* environment.

**c) Other cohorts**

**The Western Australian pregnancy cohort (Raine) study**

The Raine cohort was a prospective randomized controlled trial established in Western Australia in 1989 that recruited pregnant women less than 18 gestational weeks to evaluate the effect of repeated ultrasounds during pregnancy. The majority of the participants was well-nourished. The children from this cohort were regularly followed-up. Results from this cohort showed an interesting U-shaped relationship between birth weight and several risk factors for metabolic syndrome at the age of 8 - both higher and lower birth weights are related to increased risk of obesity, diabetes, hypertension and dyslipidaemia (49). They also reported for the first time that both birth weight and weight gain in childhood are associated with age at menarche (50).

**Swedish cohort**

A mandatory national conscription examination for all Swedish men was carried out from 1995 to 2005. Researchers linked these data with the Swedish Multi-generation Register and Swedish Medical Birth Register to study the intrauterine effect of maternal diabetes mellitus on offspring BMI in early adulthood. In agreement with the observation from Pima Indian study, this study showed that *in utero* exposure to maternal diabetes during pregnancy was associated with greater BMI at the age of 18. The association was similar within-sibling and between-nonsibling independent of maternal early gestation BMI. BMI of men whose mothers had diabetes during their pregnancy was greater than in their brothers born before their mother was diagnosed with diabetes (51). This study confirmed the developmental programming effect of intrauterine hyperglycaemia in a large general population without high susceptibility of diabetes or obesity, and added value to the understanding of the DOHaD phenomenon.
1.1.3 Summary of human epidemiological cohorts

The above cohorts provide valuable information in understanding the developmental programming phenomenon. Below are key points that can be summarised for developmental programming in the setting of macronutrient malnutrition.

1. An altered early life nutritional environment, either restricted or excessive intrauterine nutrient supply, can predispose individuals to a number of NCDs such as CVDs, T2DM, obesity, pulmonary disease and cancer.

2. There is a “critical” period in early life when particular environmental modifications can lead to specific long term health outcomes - the timing and duration of exposure matters.

3. There is a “U”-shaped relationship between maternal malnutrition and obesogenic programming response in offspring – both ends of the maternal nutrition spectrum are associated with an obesogenic offspring phenotype – whether the mechanisms are similar remains to be fully elucidated.

4. In the setting of maternal undernutrition, an immediate postnatal rebound growth pattern, which includes being small and thin at birth and infancy and having rapid catch-up growth in childhood, may be a critical factor influencing the development of cardio-metabolic dysfunction in later life.

5. Sexual dimorphism is common in developmental programming phenomenon whereby male and female may have different long term outcomes after being exposed to a similar suboptimal early environment.

6. Epigenetic regulation (DNA methylation, histone modifications and altered miRNA profiles) play a role in the developmental programming although the exact mechanisms and longer term effects remain poorly defined with most data to date being associative.

7. Developmental programming should be viewed as a transgenerational phenomenon with some studies reporting effects through to the F2 generation following the initial environmental challenge (23).
1.2 The development of the hypothesis

Observations from human cohorts presented a strong association between early life environment and disease risks in later life. One major question for epidemiologists and fetal physicians is whether or not this association represents true causation. Although rising evidence indicates a causal relationship, it is not until we can fully explain the underlying mechanisms that solid conclusions can be drawn. Several hypotheses were proposed based on the existing evidence to lead the research in this field.

The “fetal origins” hypothesis proposed that alterations in fetal nutrition and endocrine status lead to fetal adaptations during critical development periods which can permanently change structure and function of tissue and physiological systems, hence increasing the individual’s susceptibility to cardiovascular, metabolic and endocrine disease in adult life (52-55). The initial concept of ‘fetal origins of adult disease’ was first put forward by Barker in 1990 inspired by the ground breaking findings showing the relationship between ischemic heart disease and low birth weight in the Hertfordshire Cohort and the Helsinki Birth Cohort (6, 9) (Section 1.1.1). It presented a new angle for viewing the origin of disease - as Barker wrote: “The old model of adult degenerative disease was based on the interaction between genes and an adverse environment in adult life. The new model that is developing will include programming by the environment in fetal and infant life” (6). The original proposal of the hypothesis emphasised that disproportionate fetal growth reflected by offspring size at birth and infancy has a key implication for health throughout life (52). It was explained that when materno-placental supply cannot meet fetal nutrient supply in middle to late gestation, the fetus would go through a range of adaptations including redistribution of blood flow, endocrine changes and alternation in body composition, and result in disproportionate growth – these structural and functional adaptations may be permanent and fundamental to the later cardio-metabolic disorder (53, 55).

Approximately parallel with the proposal of “fetal origins” hypothesis, Hales & Barker proposed the “thrifty phenotype hypothesis” with a specific focus on explaining the associations between low birth weight and adult T2DM (56). It was proposed that poor nutrition, particularly protein and amino acids deficiency, in fetal and early infant life imposes nutritional thrift on the growing individual - which leads to structural and functional changes in the β-cells of the pancreatic islets, and in turn increases the susceptibility for the development of T2DM and metabolic syndrome (56). This hypothesis was supported by maternal low protein diet rat models which showed that offspring born to pregnancy protein
restricted mothers had reduced β-cell proliferation, islet size and vascularization (57, 58). At the time, the thrifty phenotype hypothesis was very innovative and different from the traditional concept that predisposition to diabetes might arise from genetic selection (59). This provocative hypothesis inspired several subsequent animal studies which suggested that not only the pancreas but also other insulin sensitive tissues such as liver, muscle and adipose were shown to be permanently affected by poor maternal nutrition (60, 61). This lead to the further refinement of the content and precision of the thrifty phenotype hypothesis (62).

While stimulating a number of great research works in the field, both the “fetal origin” hypothesis and “thrifty phenotype hypothesis” gradually evolved with respect to evolutionary aspects, and speculated that the fetal adaptation to undernutrition might be a mechanism to favour short term survival as well as to guarantee success in a similar postnatal life, however, it can be detrimental if the postnatal environment differs from expectation (55, 62).

Upon the extension of the previous hypotheses, the ‘Predictive Adaptive Responses’ (PARs) hypothesis proposed by Gluckman and Hanson provided an alternative interpretation of the programming phenomenon as a set of developmental and evolutionary strategies (63). It was proposed that organisms (including humans) have the ability to make adaptive responses to environmental cues in early life in anticipation of the adult environment. The adaptation does not necessarily provide an immediate advantage, but acts via developmental plasticity to modify the phenotype so that it is matched to the environment that it is predicted to experience in later life. PARs can lead to disease or disadvantage when the later life environment is “mismatched” from prediction (63, 64). Although PARs occur across the full range of developmental environments not only in response to extreme environmental challenge, it still does not adequately explain the outcomes in offspring observed in the context of maternal obesity, for example – offspring from over-nourished mothers are likely to become obese regardless of their postnatal environment and it is well documented that obesity is detrimental to reproductive success. Thus, in this case, any programming consequences may not always help offspring to match future scenarios. The detrimental effect in offspring is not a consequence of a “mismatched” postnatal environment. This might be due to the fact that overnutrition is not a common event in human evolutionary history (65), perhaps we simply have not developed the ability to optimise for this modern condition and assimilate it into our genetic code.

The DOHaD paradigm is an expanded concept from the fetal origin hypothesis based on accumulating observations from a wider range of studies focused on early life events such as
undernutrition, overnutrition and other insults, as well as the timing of exposure - from pre-conception to immediate postnatal life. It proposes from a general perspective, that alterations in the environment during the critical period of life can affect the developing fetus in a number of aspects including organogenesis, cell differentiation and lipid and glucose metabolism - thereby altering the susceptibility to a range of NCDs in later life (66). It is not a strictly defined hypothesis, however, it covers most scenarios and key features that have been uncovered to date, and there remains room for the further development of potential mechanisms.
1.3 Developmental programming: evidence from animal studies

The initial observations from human cohorts and the development of related hypotheses has led to the development of a number of animal models of maternal nutritional manipulation to stimulate the suboptimal early life environment and provide empirical data to support the DOHaD framework. These studies, across a range of species, have provided important insights into the mechanisms underpinning developmental programming.

Animal studies play a key role in investigating the developmental programming phenomenon. Compared to human cohorts, animal studies can be conducted in a more controlled experimental environment. The variations of stress and experimental factors can be minimised by performing standardised procedures. While the majority of human studies only provide non-invasive data, animal experiments can easily provide a number of samples at desired time points to examine potential mechanisms. Moreover, laboratory animals have a shorter life span than humans which is particularly helpful as this allows for a life course approach and, for small animal models, lends itself ideally towards transgenerational studies. An obvious limitation of animal studies has been direct translation to humans. Animal work is essential to understand the mechanisms and critical windows of development, particularly in regards to the design of effective strategies for intervention in the clinical setting. Importantly, many of the pathways which are perturbed as a result of early life programming are consistent between animal models and human cohorts.

In this section, animal models of developmental programming in relation to maternal nutritional imbalance are reviewed, with a particular focus on potential mechanisms and key tissues/pathways of interest.

This section generated a published first author peer-reviewed article “Maternal obesity and developmental programming of metabolic disorders in offspring: evidence from animal models.” in the Journal of Diabetes Research (formerly titled Experimental Diabetes Research) in 2011 (Appendix I). This journal had an impact factor of 3.536 in 2013. Since publication, this review has been cited 72 times.
1.3.1 Rodent models of maternal obesogenic environment

Rodent models, particularly the outbred rat, are the most commonly utilised by investigators for studies related to developmental programming resultant from maternal diet induced-obesity. In general, two main approaches that have been utilised - a single nutrient enriched diet such as a high fat or a high sugar diet approach, and a mixed nutrient induced high energy diet also known as “junk food diet” or “cafeteria diet” approach which is designed to mimic a complex western dietary pattern (67-69). Over recent years, both approaches have been extensively studied and have provided important insights into disease development, particularly in relation to the development of the metabolic syndrome.

a) Single nutrient enriched diet

The most well studied single nutrient enriched diet in maternal obesogenic model is a purified high fat diet. The calories in the diet provided from fat range from 34-60% in different studies (70). It has to be noted that although a high fat diet can successfully induce maternal obesity in rodents, the overall caloric consumption remained the same as control as a result of self-regulation (71, 72). Therefore, a high fat diet model in the rodent is more of an obesogenic model rather than “overnutrition” model per se. The main outcomes in offspring following a maternal high fat diet closely resemble those seen in the human metabolic syndrome. These include obesity, insulin and leptin resistance (71, 73), hypertension(74-77), hepatic steatosis and non-alcoholic fatty liver disease (NAFLD) in offspring (76, 78, 79). Notably, even a mild maternal high fat (34%) diet has been shown to induce obesity, glucose intolerance and altered brain appetite regulation in offspring (80). Our own previous work has shown that a moderate maternal high fat (45% of kcals) diet can result in significant obesity and hyperinsulinaemia in male and female offspring, independent of the extent of pre-conceptional obesity (81) (Figure 1.1). Interestingly in this study, although the obesity displayed in offspring was similar between maternal pre-conceptional obesity group and maternal obesity induced during pregnancy and lactation group, the mechanisms may be different, particularly as regards changes in adipoinsular axis signalling pathways (82).
Figure 1.1 Total body fat mass (%) as quantified by dual energy x-ray absorptiometry (DEXA) in adult (day 150) male and female rat offspring of HF-fed mothers

CON = offspring of control pregnancies, MHF = offspring of mothers fed a HF diet from weaning and throughout pregnancy and lactation, PLHF mothers fed a control diet until conception and a HF diet throughout pregnancy and lactation. Note that a pre-conceptional HF diet did not confer an increased risk for obesity development over that of HF exposure during pregnancy and lactation alone. Data are means ± SEM, n = 10-11 per group, *P < 0.05 versus CON. Modified from Howie et al. (81).

There is growing interest in the role of high sugar (carbohydrate) diets in the developmental programming setting. Diets high in sucrose or fructose are used to establish models as both sucrose and fructose are common sweeteners in food industry and are considered obesogenic (83). It was reported by D’Alessandro et al. that offspring of dams fed a high-sucrose diet during pregnancy and lactation developed impaired lipid and glucose homeostasis in adulthood despite the fact they were fed a standard chow diet after weaning (84). In a mouse model of maternal high sucrose diet, hypertension and NAFLD were observed in adult offspring from high sucrose diet dams (85, 86). A previous study from our group has shown that a maternal high fructose diet can induce offspring leptin and insulin dysregulation in early postnatal life in a sex-specific manner (87). However, the mechanisms by which maternal high sugar diet predisposes offspring to adiposity are not yet well defined and need to be further investigated.

b) Mixed nutrient-induced high energy diet (cafeteria diet)

A mixed nutrient high energy diet (cafeteria diet) is another well cited animal model of a maternal obesogenic environment. This animal diet is a mix of foods typified in the human
setting such as highly processed snack foods and condensed milk, thus it is directly comparable to western dietary patterns in humans (88). Indeed, rats given cafeteria style food have been reported to increase their overall caloric and fat intake with unchanged protein intake compared with rats fed a control chow (89, 90). A cafeteria diet has been shown to induce cardiovascular and metabolic dysfunction in offspring similar to that observed in the model of high fat diet (67, 91-95). However, interpretation of such results can be difficult due to the wide variety of nutrients across diets and the potential interaction between different diet components. Furthermore, there is some evidence suggesting that specific components (e.g. dairy protein) utilised in the cafeteria diet may have deleterious effects in the rodent (96, 97). Recent work by Sampey et al. investigated the obesogenic and inflammatory consequences of a cafeteria diet compared to a lard-based 45% (of kcal) high fat diet in the rodent. Both diets resulted in increased adiposity and hepatosteatosis but cafeteria-fed rats displayed increased inflammation in white fat, brown fat and liver compared to high fat and control groups (98).
1.3.2 Rodent models of nutritional restriction in early life

Section 1.3.1 dealt with the maternal obesogenic environment – in this section we turn our focus to the other end of the nutrition spectrum – scenarios where there is an insufficient supply of nutrition during early development. There are a number of different models to mimic suboptimal nutrition during pregnancy and early life. We briefly review the most commonly utilised experimental models in the following sections.

a) Global dietary restriction

Global dietary restriction has been used extensively in many laboratories. It is directly relevant as a model to parallel conditions experienced in the famine cohorts (Section 1.1.1 b). Various degrees of dietary restriction have been applied, ranging from 30% reduction (mild) to 70% reduction (severe) of ad libitum food intake (99-103).

Mild maternal food restriction during pregnancy can induce developmental programming effects in adult offspring. Ozaki et al. used 30% reduction in maternal food intake from day 0-18 of pregnancy in the rat. They showed that even a moderate restriction in maternal intake can induce offspring growth retardation which was followed by a rapid postnatal catch-up growth. Although there were no signs of altered glucose homeostasis, offspring born from undernourished dams developed higher blood pressure in adulthood when compared to the control offspring. Interestingly, this observation has a sexually dimorphic effect with male offspring developing differences in blood pressure earlier than females (99). In a murine model, 30% food restriction applied in the latter half of gestation also induced offspring growth restriction, and led to a postnatal diet-induced obesity in adult life (100). In the latter study, glucose and insulin metabolism were both impaired as a result of maternal food restriction in male offspring at 17 weeks of age (100).

50% maternal food reduction is the most commonly utilised model for global dietary restriction. It was shown by Garofano et al. that 50% restriction of maternal ad libitum dietary intake in rats during late gestation induced offspring intrauterine growth restriction (IUGR) followed by catch-up growth (101). The accelerated postnatal growth may be attributed to increased food intake and reduced anorexigenic signaling responses (104). The growth retarded offspring had significantly reduced β-cell mass and insulin content of isolated islets at birth. Interestingly, after nursing by control mothers for the whole pre-weaning period, the
offspring born to dietary restricted mothers still displayed 25% reduction in total insulin content, 40% reduction in β-cell mass, although their body weight and pancreatic weight both recovered at weaning (101). 50% reduction of maternal *ad libitum* intake in later half of pregnancy led to decreased plasma insulin, increased glucose concentrations and endothelial dysfunction in adult rat offspring, however, with no effect on blood pressure or heart rate (105). The reason that the latter study failed to induce a programming effect on blood pressure is likely due to the window of undernutrition exposure, which was limited to latter half of pregnancy. Introducing the same level of maternal food restriction throughout gestation increased blood pressure in adult offspring in other studies (106). Interestingly, prevention of catch-up growth by maintaining the food restriction into lactation prevented the development of obesity and later metabolic and cardiovascular disorders (107).

An even more severe (70%) maternal food restriction model leads to permanent growth restriction in offspring. Woodall *et al.* showed that pups born to dams that only had 30% of control food intake had decreased body weight and placental weight at the end of pregnancy, and never caught up to the body weight of control offspring (102). This observation was reproduced in later studies conducted by Vickers *et al.* (103). Interestingly, at this “severe” degree of undernutrition, litter size from undernourished dams was not affected when compared to controls (102). Moreover, studies conducted by Vickers *et al.* showed that offspring of severely food restriction mothers were hyperphagic, hypertensive, and had markedly elevated fasting plasma insulin and leptin concentrations in adulthood (103). These effects were amplified markedly in the presence of a post-weaning high fat diet.

All the ranges of dietary restriction discussed above can induce fetal growth restriction with various programming effects on glucose metabolism and endothelial function, while only offspring from the moderate and 50% nutritional reduction models displayed catch-up growth. This is an interesting observation, as the majority of human growth restricted babies display catch-up growth with only severe cases remaining permanently growth retarded (108). This potentially helps the experimental design depending on whether or not the research focus is on extreme cases or more common scenarios with catch-up growth.

**b) Dietary protein restriction**

The maternal low-protein rodent model has been favoured particularly in the study of developmental programming of T2DM - the thrifty phenotype hypothesis suggests a key role for protein and amino acids supply in this setting (Section 1.2). Typically, in low protein
models, isocaloric diets with 50% reduction in dietary protein are commonly employed - although the exact composition of protein slightly varies across different laboratories (109).

A maternal low protein diet can predispose offspring to the development of IR and type 2 diabetes. The maternal low-protein rat model was first established by Snoeck et al. in 1990s via the administration of an isocaloric diet consisting of 8% protein to pregnant dams (57). The offspring of dams fed the low-protein diet had significantly decreased birth weights; however, placental weights remained unchanged when compared to control offspring born to the dams fed a 20% protein control diet (57). Reduced offspring pancreatic β-cell mass and proliferation, and impaired insulin secretion was observed as a result of a maternal low protein diet (57, 58). Although there is consistent evidence of impaired pancreatic β-cell function in offspring due to a maternal low protein diet, there are inconsistencies in observations related to insulin and glucose homeostasis in adult life. One study reported impaired glucose tolerance in females at a young age (9 weeks) (110), while one only observed the adverse outcomes in male offspring at very late stage in life (68 weeks) (111). The cause of the inconsistent observations may lie in the different percentages of protein restriction utilised (6% vs 8%) and exposure time frame (pre-pregnancy to birth vs pregnancy and lactation).

In addition to the development of IR and T2DM, a maternal low protein diet during pregnancy can also induce hypertension in offspring during adulthood (112-114) and the progressive decline of renal function (115). However, these observations are not consistent and differ across laboratories and may reflect differences in diet composition (109). Langley-Evans compared the effects of two commonly utilised low protein diet formulations in developmental programming models and found that only the low protein diet with relatively high methionine content (5%) induced hypertension (116). This finding suggests that the balance of protein with other nutrients can be a critical determinant of the long-term health effects of maternal low protein diets during pregnancy.

Despite some inconsistencies in experimental outcomes, maternal low protein models have provided important insights into the mechanisms underpinning developmental programming. However, it is still debatable whether or not these observations reflect a true maternally “undernourished” condition. It has been shown when fed different isocaloric foods, rodents regulate their intake of protein and carbohydrate toward a relatively well-defined intake target, and when offered diets with fixed protein to carbohydrate ratio, they regulated the intake of protein more strongly than with carbohydrate (117). This self-regulation system suggests that rodents are likely to passively over-consume fat and carbohydrate to meet protein
requirements. Indeed, this has been reported in a maternal low protein diet model by Bai et al. (118).

c) Uterine artery ligation

In addition to dietary manipulation models, uterine artery ligation is a well-characterized procedure to induce IUGR in experimental animals. The underlying mechanism is to mimic utero-placental insufficiency which is a common cause of IUGR in developed societies (119). Uterine artery ligation results in offspring exposed to acute hypoxia in addition to overall nutrient restriction.

The ligation procedure can be performed either bilateral or unilateral. In the bilateral ligation model, offspring from the ligated group are compared with controls born to mothers who underwent a sham operation. In a unilateral ligation model, the comparison is between offspring born from uterine horn on the ligated side and the unligated side in the same animal. The bilateral procedure has been preferred by most research groups as in the unilateral ligation model the unligated horn is likely to experience hyperperfusion which may lead to increased fetal growth with possible metabolic consequences (120).

The uterine artery ligation model can induce offspring IUGR with altered glucose metabolism. Simmons et al. performed bilateral uterine artery ligation in pregnant rats on day 19 of gestation. This procedure induced offspring IUGR with a prolonged catch-up growth, and eventually contributed to increased offspring adiposity. Despite becoming obese, these IUGR offspring also gradually developed exacerbated hyperglycaemia and hyperinsulinaemia (121). A later study by the same group suggested that the altered glucose metabolism may be due to permanently impaired liver insulin signaling as evidenced by decreased phosphorylation of insulin-signaling proteins in the IUGR rat (122). The uterine artery ligation model may be a good model to study glucose metabolism, however, it might not be suitable to study programming-induced lipid dysregulation. A further study by Nusken et al., reported that offspring from ligated and sham operated groups all developed dyslipidaemia at postnatal 15 weeks when compared to the non-surgical controls. This observation indicates the programming of lipid metabolism can be induced by sham operation alone - therefore the effect of uterine artery ligation on lipid metabolism cannot be distinguished and independently assessed in this setting (120).

In addition to the possible confounds related to sham operated controls, another limitation for the uterine artery ligation procedure compared to the nutritional manipulations is that the
Investigation window for uterine artery ligation is very limited. The operation can only be conducted in the last 2-4 days of pregnancy (123), therefore some programming effects that are subject to critical time frames other than late pregnancy cannot be explored.

d) Micronutrient deficiency

Micronutrient undernutrition during the critical period of early life is an important health issue. Short-term consequences of vitamin and mineral deficiencies during pregnancy including maternal anaemia and fetal malformation are fairly well studied (124). However, the long term impacts on health outcomes due to maternal micronutrient deficiency are less well understood. Various animal experiments suggest that a lack of vitamins A and D, folate (vitamin B9), iron, and zinc during pregnancy all play important roles in predisposing offspring to chronic diseases in later life. Vitamin A deficiency \textit{in utero} is associated with changes in fetal cardiovascular development (125), reduced nephron numbers in the kidney (126) and reduced \(\beta\)-cell area per islet and \(\beta\)-cell proliferation in the pancreas (127). Vitamin D deficiency in pregnancy may increase the risk of prenatal and early postnatal overweight in offspring (128). Iron and zinc deficiencies during gestation in rodents have been linked with increased blood pressure (129, 130), reduction in nephron numbers (131) and increased adiposity (132, 133) in offspring. Interestingly, folate supplementation during pregnancy in the low protein fed rat can restore maternal diet-induced endothelial dysfunction and hepatic hypomethylation in offspring (134, 135). Although the underlying mechanisms for all the above micronutrient effects are largely unknown, these studies highlight the importance of micronutrients in the developmental programming of chronic disease, particularly as regards impact of methyl donor deficiencies. Importantly, these phenomena should be taken into account when interpreting observations from macronutrient malnutrition studies into human setting - as macronutrient imbalance is often accompanied by micronutrient deficiency.
1.3.3 Other animal models of developmental programming

Rodent models are the most commonly utilised models for investigation of human disease due to their similarities in both disease phenotypes and genomic sequence compared with human (136). Additionally, rodents are genetically tractable and offer the affordances of genetic manipulation and trans-genetic experiments, providing for molecular targeting of specific pathways and genes (137). However, in addition to rodents, large animal models including the sheep have also been successfully used to test the DOHaD paradigm with consistent observations, which reassures that the phenomenon is comparative across species.

a) Ovine models

Sheep are the second most popular model utilised in the study of developmental programming. One major advantage of the sheep compared to rodents is that lambs are born at an equivalent level of maturity as humans, while rodents are born with an underdeveloped brain and endocrine system with significant maturation during the early postnatal period (138). Thus, the timing of treatment in sheep models is better translatable to human than rodents. However, one major downside for this model is that molecular details such as the genomic sequence are less well-characterised. Moreover, it is resource intensive to generate a sheep cohort compared to small animals particularly in surgically induced models.

There is strong evidence from ovine models that both maternal obesogenic and insufficient nutrition supply environments predispose offspring to altered growth and metabolic sequelaes; data that closely parallels that observed in the rodent models.

Maternal 50% food restriction during early to mid-gestation in the ewe has been shown to induce accelerated body weight gain, increased adiposity, and impaired glucose tolerance in male offspring (139). However, in another study by Gardner et al. 50% food restriction in early pregnancy induced glucose intolerance but with reduced adipose in both male and female offspring (140). This again, indicates that the timing of malnutrition exposure may affect different offspring outcomes. Increased blood pressure and endothelial dysfunction have been also observed in sheep offspring following maternal undernutrition (141, 142), which parallels the observations in rodent models.

As observed in the rodents, similar offspring metabolic dysfunction phenotypes are observed in models utilising a maternal obesogenic environment. Maternal obesity and increased nutrient intake before and during gestation in the ewe can result in altered growth, adiposity,
and glucose tolerance in adult offspring (143). In a study by Zhang *et al.*, fetuses from obese ewes exhibited markedly altered pancreatic development and insulin homeostasis in relation to different gestational stages compared with fetuses of ewes fed to requirement. In the effected offspring, there was a marked increase in pancreatic growth, β-cell proliferation, and insulin secretion in the first half of gestation, followed by a reduction in pancreatic growth and β-cell numbers in late gestation, resulting in reduced circulating insulin at term (144).

**b) Non-human primates (NHP)**

The extensive body of evidence in small animal and ovine models linking an altered maternal nutrition status to later disease risk in the offspring has been supported by studies, albeit limited, in the non-human primate.

A series of work on NHP by a group in Texas, USA has demonstrated that maternal nutrient restriction can lead to altered glucose and insulin metabolism in offspring. It was reported that offspring born to female baboon fed 70% of control nutrient supply have elevated plasma fasting glucose and fasting insulin concentrations, and increased IR and β-cell responsiveness as measured by hyperinsulinemic-euglycemic clamp (145). The gluconeogenic gene phosphoenolpyruvate carboxykinase 1 (PEPCK1) was increased at a gene expression and protein level in fetal hepatocytes in response to maternal nutrient reduction (146, 147). These findings are in line with low protein rodent models which have identified that upregulation of PEPCK expression can persist from early development to adulthood, resulting an increased hepatic glucose output (148).

In a maternal obesogenic environment, a maternal HF fat diet triggers lipotoxicity in fetal liver in the macaque (149) and predisposes the offspring to develop NAFLD in adulthood. This aligns closely with work in the rodent where a maternal high fat diet has been shown to lead to “developmental priming” of hepatic steatosis in offspring (78). Additionally, a NHP study by Grayson *et al.* showed that the offspring of mothers fed a HF diet pre-conceptionally and throughout pregnancy had smaller body weights in the early third trimester but displayed catch-up growth and increased adiposity in the postnatal period – the offspring also developed early-onset excess weight gain independent of postnatal diet (150).
1.3.4 Mechanisms of developmental programming of metabolic syndrome

The mechanisms underpinning programming of metabolic disorders in offspring in response to maternal nutritional insults are not well defined. The role of altered leptin regulation and changes in key genes involved in appetite control and energy balance in the hypothalamus have been implicated. Given the central role of obesity in the metabolic syndrome, dysfunctional adipose tissue development accompanied by chronic low-grade inflammation may represent another candidate mechanism. There is also evidence of altered pancreatic β-cell development and skeletal muscle metabolism.

a) Leptin and the regulation of energy balance

Leptin is a 167-amino acid peptide (151) secreted primarily by white adipose tissue. Leptin plays an important role in the central regulation of energy balance and the maintenance of body weight in mammals. Increased adiposity therefore enhances leptin secretion. Leptin interacts with leptin-sensitive neurons in the arcuate, ventromedial, and dorsomedial hypothalamic nuclei and regulates a number of neurotransmitters that are implicated in the central regulation of energy balance (152). Generally, increased leptin concentrations lead to negative energy balance (energy expenditure > food intake), while decreased leptin leads to positive energy balance (food intake > energy expenditure) (151). When the body fails to respond to leptin appropriately, a state of leptin resistance occurs and contributes to the development of obesity.

One of the most studied and consistent observations in offspring following maternal macronutrient manipulation is hyperphagia and altered energy intake (153). This is commonly observed in offspring from both obese mothers (93, 154, 155) and undernourished mothers (103, 156) independent of postnatal diet. As both ends of the nutrition spectrum can result in similar hyperphagic obese offspring phenotype, it is tempting to speculate that altered leptin regulation and energy balance may be a common pathway in the developmental programming of obesity.

There is significant evidence pointing to leptin resistance as a central mechanism in the developmental programming of obesity and dysregulation of energy balance. Early studies by Vickers et al. showed that adult rat offspring born to severely undernourished mothers (global undernutrition) have increased adiposity and hyperleptinaemia (103, 156). However, these results could not differentiate whether the later effects were mediated by early life programming of central leptin regulation conferring obesity risk or simply a later consequence
of programmed obesity that ultimately led to leptin dysregulation. Direct leptin challenge in offspring from maternal undernutrition and maternal obesity models provides further evidence of peripheral leptin resistance (93, 157). Krechowec et al. demonstrated that leptin treatment in adult rat offspring from undernourished dams failed to reduce food intake, and the effect of leptin treatment on weight loss was diminished in offspring of undernourished mothers (157). Furthermore, Kirk et al. reported that leptin administration failed to reduce food intake or decrease body weight at an early age (postnatal day 30) in offspring following maternal obesogenic diet (93). These findings are independent of offspring adiposity, which suggests that programmed alterations in leptin sensitivity may explain the increased susceptibility to obesity in developmental programming.

As noted above, leptin resistance has a noteworthy role in developmental programming of obesity and altered energy regulation, and furthermore, there is evidence that resistance to leptin may be programmed in early life. During the neonatal period in rodents, there is a sudden increase of plasma leptin concentrations that occurs around the second postnatal week (158). This leptin surge in neonates is independent of body fat mass and circulating concentrations fall rapidly after weaning. It has been demonstrated that this rise in leptin establishes the set-point for energy regulation by preceding the establishment of adult corticosterone, thyroxine, and estradiol concentrations, all of which are important energy metabolism hormones (158). Moreover, the development of the arcuate nucleus in the hypothalamus, the centre of appetite regulation, also relies on this leptin surge in early life. Bouret et al. demonstrated that the neural projection pathways from the arcuate nucleus are permanently damaged in leptin-deficient ob/ob mice and leptin treatment in neonate period, but not in adulthood, can rescue the development of these neurons (159). Delahaye et al. showed that maternal food restriction can lead to a dramatic decrease in the neonatal leptin surge (160). This reduction of leptin is accompanied by marked alterations of the hypothalamic proopiomelanocortin system - a key regulator in energy balance (160). Injections of exogenous leptin from postnatal day 3–13 to female rat pups from undernourished dams normalized the caloric intake, locomotor activity, body weight, and fat mass as well as insulin and leptin plasma concentrations in adult offspring (161). Interestingly, in response to maternal nutrient excess, the neonatal leptin surge is also altered (delayed and amplified) with evidence of changes in hypothalamic function involving the arcuate nucleus and paraventricular nucleus (93). It is therefore plausible to speculate that maternal malnutrition induced modification in leptin surge jeopardises the early establishment of hypothalamic energy regulating system, leading to alterations in leptin sensitivity which contribute to the increased susceptibility to obesity.
b) Altered adiposity and inflammatory pathway

Developmental programming of early life macronutrient imbalance can induce a number of metabolic complications including obesity. There is significant evidence that obesity is associated with a state of chronic low-grade inflammation and plays a central role in connecting the disorders that collectively contribute to metabolic syndrome (162).

In addition to energy storage, the adipose tissue serves as an endocrine organ and secretes a number of adipokines, these include leptin and a range of pro-inflammatory cytokines which have a major impact on energy metabolism and the development of IR (163). It was first reported by Hotamisligil et al. that adipose tissue constitutively produces tumor necrosis factor (TNF)α and TNFα expression is significantly increased in several animal models of obesity. Neutralizing systemic TNFα leads to the restoration of insulin sensitivity (164). Accumulating data over the past decade has revealed the roles of many inflammatory mediators linking obesity and IR. Particularly, activation of c-Jun N-terminal protein kinase (JNK) and inhibitor of nuclear factor κB kinase IκB kinase (IKK) by pro-inflammatory cytokines is known to directly inhibit insulin action, which links inflammation with the insulin signalling pathway (165). In obesity, the increase in pro-inflammatory cytokine production is primarily due to macrophage polarization and infiltration. Adipose tissue macrophages can span the spectrum from pro-inflammatory, M1-like cells to anti-inflammatory, M2-like macrophages. With obesity, the resident macrophages in the adipose switch from M2 to M1 phenotype (166). Moreover, increased fatty acid accumulation stresses the adipocyte and leads to secretion of TNFα and chemokines such as monocyte chemoattractant protein (MCP)-1 which recruit more macrophages. This forms a vicious cycle of pro-inflammatory cytokine production and contributes to the systemic IR. Given the central role of IR in the development of metabolic syndrome, obesity is of particular interest – however little is known about the role of adipose tissue related inflammation and pro-inflammatory mechanisms in the setting of developmental programming.

There are two main mechanisms through which obesity develops; an increase in the number of adipocytes (hyperplasia) or increased adipocyte size (hypertrophy), both of which have been reported in maternal malnutrition programming models. In male offspring of undernourished dams (50% global undernutrition), genes that promote terminal differentiation of adipocytes are upregulated at birth (167). During adulthood, these offspring have adipocyte hyperplasia, hypertrophy and increased lipogenic capacity (167). Similarly increased adipogenesis and lipogenesis in adipose tissue was found in fetal sheep exposed to increased maternal nutrition
(overfeeding) during late gestation (168). These findings indicate that adipocytes exposed to an altered early life nutrition environment have increased capacity to store and to produce lipids. Increased lipid storage in the adipocyte causes cellular stress which promotes instigation of inflammation and IR. Indeed, it has been recently reported that maternal high fat diet feeding in mice can lead to increased gene expression of several inflammatory markers in fetal adipose tissue accompanying with adipose tissue hypertrophy (169). However, more studies on offspring immune function in the setting of maternal nutrition-induced developmental programming are needed to understand the potential mechanisms.

c) Pancreatic β-cell development

Compromised development of the pancreatic β-cell leads to a predisposition for obesity and metabolic disease. It has been demonstrated that the size of fetal pancreatic β-cell mass is diminished in various models of maternal nutritional insufficiency (Section 1.3.2). However, the cellular and molecular mechanisms underlying this reduction in size may be different across the different models used. β-cells from fetuses following maternal protein restriction displayed decreased proliferation with a prolonged cell cycle and an increased apoptotic rate (170-172). Whereas with global food restriction during pregnancy, fetal β-cell mass reduction was not attributed to lower proliferation or increased apoptosis but because of altered islet neogenesis (101). Dumortier et al. directly compared the β-cell development between maternal low protein diet and 50% food restriction conditions. They found that decreased fetal β-cell neogenesis in the food restricted group was likely due to an elevated fetal corticosterone concentration in response to the limited calories in early pregnancy, and decreased β-cell mass in low protein group was related to reduced proliferation and vascularisation in pancreas in late pregnancy (173). Interestingly, supplementation with the amino acid taurine can normalise β-cell volume in the protein restriction model (172), suggesting that the combination of protein in the diet is critical for β-cell development.

Several studies have investigated β-cell development under conditions of excessive nutrition supply in early life. Mice born to dams chronically fed an obesogenic diet displayed elevated insulin concentration from young adulthood, and progressed to diabetes in an age related fashion (69). Ford et al. reported that fetal sheep pancreatic β-cell development was initially accelerated in mid-gestation (174), but quickly declined with increased apoptosis in the late gestation as a result of maternal obesity (144). In vivo glucose infusion of lean adult rats for 24 hours can lead to a rapid activation of neogenesis of new β-cells (175), which is known as “glucose hypersensitization” (176). However, prolonged exposure to elevated glucose
concentration leads to increased β-cell apoptosis (176). It is thus plausible to speculate that a maternal obesogenic diet may induce a chronic high glucose fetal environment which in turn may lead to altered fetal β-cell development, predisposing offspring to T2DM. In addition to glucose, fatty acids are also known to play a role in the development of β-cell dysfunction. Fatty acids have been shown to induce endoplasmic reticulum (ER) stress in normal adult β-cells and initiate apoptosis (177). Increased free fatty acid concentration has been evidenced in materno-fetal circulation as a result of maternal obesity (178), and these high free fatty acid concentrations may potentially contribute to β-cell apoptosis in the developing fetus via ER-mediated stress responses.

d) Skeletal muscle and locomotor activity

Several studies have examined changes in locomotor activity in the setting of maternal undernutrition. Consistent results demonstrate that offspring born to undernourished mothers displayed diminished locomotor activity than those born of ad libitum-fed mothers independent of the development of obesity (156, 179, 180). These changes in locomotor activity may reflect compromised skeletal muscle development. Skeletal muscle has less priority in nutrition deprivation during fetal development compared to organs like the brain, heart and liver, and is particularly vulnerable to nutrition availability (181). It has been evidenced in sheep that 50% food restriction in early pregnancy can lead to a reduction in fetal myogenesis (182). In adulthood, sheep born to undernourished ewes had reduced muscle fiber number with reduced glucose metabolism and fatty acid oxidation in skeletal muscle tissue (183), which lead to a predisposition to IR in skeletal muscle.

In models of maternal obesity, numerous studies have observed differences in muscle development in offspring of obese mothers in terms of muscle mass and insulin signalling. Reduced muscle mass has been reported in 3- and 6-month old male and female offspring from obese mice (69) and reduced muscle force was evident in the offspring of mothers fed a junk food diet (184). Work in sheep has shown that lambs born to obese mothers have impaired insulin signalling in muscle compared with control lambs, and this correlated with increased intramuscular triglycerides and higher expression of fatty acid transporters and peroxisome proliferator-activated receptor (PPAR)-γ (185). These results mirror that of similar studies reported in the rodent (186).

Together, these observations show that changes to skeletal muscle contribute to the development of offspring metabolic disorder in settings of maternal nutritional imbalance.
1.3.5 Sexual dimorphism

Sexual dimorphism is frequently observed in developmental programming models, with molecular and phenotypic outcomes of suboptimal in utero conditions appearing more prominent in male offspring (187). A number of direct comparisons between male and female offspring subjected to the same early life stimuli have been made in rodent models (99, 188-190). While some phenotypic observations agree between males and females, it appears that male rat offspring are more susceptible to developmentally programmed hypertension than female offspring in fetal growth restriction models (188-190). In line with the prominent male hypertensive outcomes, studies have found greater impairment of renal development in male than in female rat offspring following suboptimal early life environment (190, 191). In addition to hypertension, male offspring also seem more susceptible to maternal obesogenic diet-induced liver steatosis. Strakovsky et al. found that feeding a high fat diet to an obesity resistant strain of rats (Crl:OR(CD) from Charles River Laboratories) during pregnancy led to a significant increase in hepatic triglycerides in male but not female neonates with a sex-specific change in the anti-oxidative system (192). In another maternal obesogenic diet model, high fat feeding during early life programmed hepatic steatosis and IR in male offspring, whereas female offspring were protected (193). Nevertheless, developmental programming effects are not always consistently more marked in male offspring. Impaired glucose tolerance and IR have been observed in both male and female rat offspring in various growth restricted models (103, 111, 121, 194). In a maternal high fructose diet study by our group, a more pronounced dysregulation of glucose metabolism was found in female offspring in fetal and neonatal period (87). Although the underlying mechanisms of sexual dimorphism are largely unknown, it was speculated that differences in patterns of development, differences in timing of development and the influence of steroid hormone exposure in utero and postnatally are major factors counted for such sex-specific responses (187). It must be noted however, that a large number of studies still do not examine sex-specific effects – this can be the result of logistical issues or the use of males in cases when behavioural outcomes are assessed to avoid the potential confounds of estrus.
1.3.6 Summary of animal models of developmental programming

1. Various animal models across different species, despite differences in experimental conditions (timing, duration and nature of exposure), have linked both excessive and insufficient maternal nutritional environments with a metabolic syndrome-like phenotype in offspring.

2. Although the molecular mechanisms remain largely unknown, there is speculation that common mechanistic pathways may underpin the developmental programming phenomena cross a range of maternal nutritional intakes.

3. Sexual dimorphism is common in developmental programming, although the molecular and other mechanisms that contribute to this phenomenon remain largely unexplored. Understanding the basis for the observed sexual dimorphism will be important for translating animal studies to human as responsiveness to interventions for example, may be dependent upon both sex and the model species used.

Figure 1.2 below summarises the basic consequences of altered maternal nutritional environment on the health and well-being of offspring. This figure is adopted from Minglan Li’s published review. (Appendix I)
Figure 1.2 Basic consequences of an altered maternal nutritional environment on the health and well-being of offspring
1.4 Developmental programming in the liver

The liver is the largest solid organ in the body with a central role in metabolic homeostasis. The main functions of the liver include the metabolism of dietary compounds, regulation of blood glucose concentrations, production of bioactive proteins (including insulin-like growth factors (IGFs) and clotting factors), bile synthesis, and biotransformation of xenobiotics and endogenous by-products of metabolism (195).

Early studies in the context of developmental programming mainly focused on the contribution of the pancreas to offspring metabolic dysfunction (i.e., thrifty phenotype). However, growing evidence suggests that the liver is also a key determinant of metabolic abnormalities (196). In particular, NAFLD which has been recognized as both a cause and a consequence of metabolic syndrome (196), and has been recently highlighted as a primary phenotypic feature in several developmental programming models.

NAFLD is a clinical term which refers to excess fat (> 5% weight or volume) deposition in the liver in the absence of excessive alcohol intake. NAFLD frequently presents in patients with metabolic syndrome, and it is a strong predictor of all the metabolic disorders characterising metabolic syndrome such as IR and lipid dysregulation - independent of overall body adiposity (197, 198). In recent developmental programming models, both early life obesity and insufficient nutrition status increases the susceptibility and severity of NAFLD in offspring (78, 149, 199-204).

In this section we summarise the evidence of developmental programming effects on liver from recent animal studies, and discuss the possible mechanisms underlying the development of hepatic dysfunction. This section has generated a review “Developmental programming of non-alcoholic fatty liver disease (NAFLD): the effect of early life nutrition on susceptibility and disease severity in later life.” published in the journal BioMed Research International in 2015 (Appendix II). The journal has an impact factor of 2.706 in 2013.
1.4.1 Developmental programming of liver

There have been several clinic trials and epidemiological cohorts providing evidence that an altered early life nutritional status is associated with an increased risk of NAFLD in offspring (205-209)(Appendix II). However, due to the scope of this thesis, we limit discussion to animal models in the following section.

a) Evidence in maternal obesogenic dietary models

Different experimental animal models using a range of dietary approaches have provided detailed evidence linking maternal obesogenic environments and the development of NAFLD in offspring. It is established in both NHP and rodents that a chronic maternal high fat diet can lead to a NAFLD phenotype in offspring - independent of maternal and offspring obesity (78, 149). In the NHP, chronic consumption of a HF diet prior to and during pregnancy, independent of maternal obesity, led to fetal liver steatosis which persisted into the juvenile period (149). Of note, changing the maternal diet to a low fat diet in subsequent pregnancy improved offspring outcome. This observation highlights the idea that diet during pregnancy has a significant role in the programming of offspring hepatic fat deposition (149). Similar findings to NHP models were observed in rodent offspring born to dams chronically fed a high fat diet, with a maternal high fat diet inducing hepatic steatosis in offspring fed a standard chow diet after weaning (78).

A number of studies have shown that maternal obesogenic diets also increase the severity of NAFLD in programmed offspring. When a post-weaning high fat diet is used to provide a susceptible postnatal environment for NAFLD, offspring born to obesogenic diet fed dams exhibit evidence of hepatocyte injury and hepatic inflammation which indicates the severe form of NAFLD - nonalcoholic steatohepatitis (NASH) in early adulthood, while offspring born to the normal diet dams only developed simple hepatic steatosis (78, 79, 85, 199-201, 210). These observations suggest that maternal obesogenic diet increases the offspring’s vulnerability to steatohepatitis, which is particularly interesting as less than 10% of human NAFLD cases progress to steatohepatitis and there is no liable risk factor to distinguish susceptibility. The findings in animal models may therefore support a role for changes in the early life nutritional environment as a candidate for risk evaluation of NASH.

In contrast to exposure to a postnatal high fat diet, Kruse et al. demonstrated that offspring following maternal overnutrition in pregnancy and lactation had increased susceptibility to NAFLD development, despite consuming a normal chow diet for 23 weeks after weaning.
This finding emphasises that the pregnancy and lactation period are the critical windows for programming susceptibility to NAFLD and highlights the irreversibility of such effects in later life, which is consistent with the developmental programming models of other metabolic conditions (107).

In addition to a maternal obesogenic diet, pre-existing maternal metabolic dysfunction such as IR can also contributes to offspring NAFLD. Thorn et al. compared juvenile NHP born to females chronically exposed to a high fat diet where mothers either displayed normal insulin sensitivity or IR. Offspring from insulin resistant females, but not insulin sensitive females, developed significant hepatic steatosis despite consuming a healthy diet after weaning and in the absence of obesity (212).

In summary, a maternal obesogenic diet and IR status is related to the increased susceptibility and severity of NAFLD in offspring later life. This programming effect is sensitive during pregnancy and lactation and likely irreversible in later life.

b) Evidence from maternal nutrition insufficiency models

The effect of maternal nutritional insufficiency on offspring susceptibility to liver steatosis is less well studied - perhaps because it was considered as a secondary outcome of increased offspring adiposity. However, recent evidence suggests that maternal poor nutrition supply may directly increase offspring hepatic lipogenesis before the development of obesity. Moderate to severe dietary protein restriction during pregnancy and lactation in rats leads to offspring hepatic steatosis in late adulthood without a paralleled increase in adiposity (202, 203). In sheep, aged lean female offspring born to mothers that received global nutrient restriction in the first half of gestation showed significantly increased hepatic lipid accumulation (204). Moreover, a study by Yamada et al. showed that hepatic fat deposition occurs in fetuses exposed to maternal undernutrition, as early as embryonic day 20, prior to the development of offspring adiposity (213). Therefore, it is possible to speculate that growth restriction induced susceptibility to NAFLD is at least partially independent of development of obesity and probably primed in utero.
1.4.2 Molecular mechanisms of hepatic programming

a) Lipid accumulation

Lipid accumulation occurs when lipid uptake and synthesis exceeds hepatocyte oxidative capacity. The dominant source of fat which accumulates in the liver originates from serum free fatty acids (214), and is mainly via lipolysis in white adipose tissue (215). However, this is not the case during fetal life as WAT only starts to develop in the middle of the third trimester in human and NHP and after birth in the rodent (216, 217). Fat accumulation in fetal liver may thus originate directly from maternal lipid transfer because it has been shown in NHP that maternal obesogenic diets induce fetal hepatic steatosis and that fetal and maternal plasma glycerol concentrations are strongly correlated (149). This early lipid accumulation in liver may represent a “very first hit” of lipotoxicity during initial organ development.

In animal studies, offspring de novo lipogenesis can be increased during early adulthood as a result of a maternal obesogenic diet. These various studies have reported increased expression of hepatic transcription factor sterol regulatory element binding protein 1c (SREBP1c) and its co-activators and downstream lipogenic targets: PPARs, fatty acid synthase (FASN), stearoyl-CoA desaturase-1 (SCD1) and acetyl-CoA carboxylase (ACC1) in adult offspring exposed to obesogenic diets in utero (79, 200, 201, 212, 218). The proposed causes for SREBP1c activation include altered offspring insulin signalling and PUFA metabolism (95, 200, 219, 220). Apart from lipogenesis, the role of hepatic fatty acid β-oxidation is not consistent across animal studies. Some find no change in the key enzyme for fatty acid oxidation - carnitine palmitoyltransferase 1 (CPT1) (78, 199), while one study observed persistent decreases in CPT1A gene expression from late gestation to weaning (201). The synthesis and secretion of VLDL in adult offspring appears to be enhanced by a maternal obesogenic diet (95, 201), which is likely to be a result rather than a cause of hepatic lipid accumulation. Of note, in the maternal obesogenic environment, several genes that are involved in lipid metabolism showed epigenetic modification in adult offspring. Epigenetic modification is considered as a key regulatory mechanism in developmental programming (221). Liver X receptor-α (LXRα), which is an important mediator for SREBP1c (222), displayed decreased histone methylation after three generations of HF diet consumption in rats, providing a possible explanation for the intergenerational programming effects observed [41]. In a mouse model, alterations in DNA CpG methylation in PPARα, FASN and insulin-induced gene protein (Insig) was observed in NAFLD offspring with perinatal exposure to western diet [36]. Overall, these findings suggest that maternal lipid dysregulation and de novo lipogenesis have major effects on the
developmental programming of offspring hepatic steatosis in the maternal obesogenic setting, with epigenetic modification representing a potential mechanism.

Upon exposure to an undernourished in utero environment, increased activation of de novo lipogenesis is observed in parallel with the occurrence of fatty liver in rat offspring, with upregulation of hepatic carbohydrate-responsive element-binding protein (ChREBP) and SREBP1c expression at both transcriptional and protein levels (203, 213, 223). Glucocorticoid exposure is proposed to play a role in the programming of offspring lipogenesis in this setting. It has been demonstrated that maternal protein restriction can lead to reduction of 11 β-hydroxysteroid dehydrogenase 2 (11-β-HSD2) in the placenta and subsequently increases fetal exposure to maternal glucocorticoids (224). Inhibiting glucocorticoid synthesis reversed the suppressive effect of low-protein diet on offspring hepatic SREBP-1c expression (225). The role of glucocorticoids in NAFLD programming is also supported by Drake et al., who showed that prenatal dexamethasone treatment can increase rat offspring susceptibility to fatty liver without promoting adiposity (226). However, in vitro experiments showed a contrary effect of glucocorticoids on the expression of SREBP1c, suggesting other factors may be involved in the glucocorticoid effect in vivo (225).

b) Mitochondrial dysfunction, oxidative and ER stress

Mitochondria are important organelles for energy generation and are the primary site for fatty acid β-oxidation (227). The relationship between mitochondrial dysfunction and fatty liver disease has been reviewed extensively by others (228, 229). In brief, mitochondrial fatty acid oxidation increases to adapt to excessive hepatic fat accumulation (230) and in turn leads to a rise in oxidative products – reactive oxygen species (ROS). Most mitochondrial ROS are detoxified to residual molecules through mitochondrial respiratory chain (MRC) activity. However, increased mitochondrial oxidation can progressively induce a vicious cycle including reduction in MRC activity, overproduction of ROS and damage to mitochondrial DNA. The imbalanced state that favours ROS production over antioxidant defence is defined as oxidative stress. Oxidative stress with excessive mitochondrial ROS has been shown to participate in cell death, inflammation and fibrosis (231), and therefore may play an important role in the progression of liver disease. In maternal obesogenic diet models, mitochondrial dysfunction and oxidative stress is observed in rodent offspring, reflected by reduced MRC key components- mitochondrial electron transport chain complex (ETC) I, II/III, and IV activity (78), uncoupling MCR activity (232), decreased liver mitochondrial DNA copy number (193) and reduced concentrations of antioxidant enzymes (232, 233). Nonetheless, the
mechanism through which maternal factors elicit these changes into the next generation remains mostly unclear. It has been shown by Igosheva et al. that diet-induced maternal obesity prior to conception is associated with altered mitochondrial function in mouse oocytes and zygotes (234). Since mitochondria are maternally inherited, it is possible to speculate that the mitochondrial dysfunction in offspring might be a combination of inheritance of predisposed maternal mitochondria and exposure to suboptimal early life environment.

The ER is the organelle for lipid and protein synthesis and export. Emerging evidence suggests that ER stress is associated with de novo lipogenesis, mitochondrial dysfunction, oxidative stress, inflammation, and cell death. Details of these interactions have been reviewed elsewhere (235). Disturbance in ER homeostasis (ER stress) has been shown to contribute to both steatosis and the progression to NASH (236, 237). In the intergenerational obesogenic diet study, ER stress markers (Binding of immunoglobulin protein BIP, C/EBP homologous protein CHOP, ER-associated oxidoreductin 1-α ERO1-α, eukaryotic translation initiation factor 2a eIF2a) were progressively increased, indicating an intergenerational accumulation of ER stress in these animals (218). Epigenetic modification on the ERO1-α promoter provides a possible explanation for this observation (218).

c) Pro-inflammatory cytokines

It has been well established that inflammatory mediators have a pivotal role in the progression from NAFLD to NASH (238). Gene expression as well as serum concentrations of the pro-inflammatory cytokine TNFα is positively correlated to the severity of steatohepatitis (239, 240). Interestingly, reduction of TNFα using metformin improves steatosis in leptin deficient ob/ob mice (241). Free fatty acid accumulation promotes a pro-inflammatory status via activation of the toll-like receptor 4 (TLR4) signalling pathway, which culminates in nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation (242). Chronic low-level hepatic activation of NFκB further contributes to hepatic production of TNFα, interleukin-1β (IL-1β) and IL-6, and local and systemic IR (243). Moreover, in the progression of NAFLD, damaged hepatocytes release a group of molecules, which are recognized as damage-associated molecular patterns (DAMPs) (244). DAMPs serve as danger signals activating specialized hepatic macrophages - Kupffer cells and in turns promote pro-inflammatory processes (245). When steatosis occurs, the liver is more susceptible to injury from pro-inflammatory cytokine stimulation, resulting in progression from NAFLD to NASH. Although the mechanisms underpinning this progression remain unclear, NASH is
characterized by hepatocellular degeneration and infiltration of immune and inflammatory cells, which can advance to fibrosis and cirrhosis.

In maternal obesity models, offspring born to obese dams have significantly reduced natural killer T (NKT) cell populations, and increased expression of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, IL-18, and TNFα (78, 85, 210, 246). It was reported by Ashino et al. that male offspring from dams fed a HF diet display IR, hepatic steatosis and activation of JNK (246). Pruis et al. demonstrated in mice that exposure to a maternal western diet during pregnancy and/or lactation primed NAFLD in adult male offspring (200). Early life exposure to a western-style diet during pregnancy and lactation resulted in hepatic cholesterol/triglyceride accumulation, upregulated de novo lipid synthesis and increased expression of inflammatory mediators and macrophage markers including TNFα, transforming growth factor-β (TGF-β), MCP-1 and cluster of differentiation 11 (CD11) (200). Thorn et al. demonstrated in NHP that in utero exposure to HF diet-induced IR resulted in a programmed increase in hepatic triglycerides and upregulation of hepatic de novo lipid synthesis and inflammatory pathways, despite post-weaning consumption of a healthy chow diet (212). Additionally, even though these offspring did not display obesity or IR, they had both classical and alternatively activated hepatic macrophages and NKT cells, suggesting that maternal IR programs dysregulation in the juvenile hepatic immune system, which may represent an irreversible “first hit” of NAFLD. Muralidarane et al. demonstrated in mice that maternal obesity in combination with post-weaning consumption of an obesogenic diet induces NAFLD, accompanied by alterations in innate immune function (210). Kupffer cells activation, ROS production in response to lipopolysaccharide (LPS) and hepatic inflammatory cytokines IL-12 and IL-18 were increased in offspring exposed to maternal obesity. Taken together, these findings suggest that maternal obesity predisposes offspring to development of NAFLD through alterations in the innate immune system. However, as obesity, IR and NAFLD commonly occur together in humans and are all linked with inflammatory processes, disentangling the specific pathways involved in the developmental programming of NAFLD remains a challenge.

Overall, the mechanisms involved in the developmental programming of NAFLD are multifactorial. At a molecular level, de novo lipogenesis, primed mitochondrial and ER dysfunction and the activation of inflammatory response are the main pathways that are most likely to have long lasting adaptations under different early life environments. Potential mechanisms that contribute to the developmental programming of NAFLD are summarised in Figure 1.3.
Figure 1.3 Potential mechanisms underlying the developmental programming of NAFLD
(1) Maternal obesity and high fat diet induced mitochondrial dysfunctional may be programmed in the fetus; (2) maternal circulating lipids are shuttled to the fetal liver contributing to mitochondrial oxidative stress, this is characterised by reduced MRC activity, overproduction of ROS and mitochondrial DNA damage. Increased concentrations of TAG and FFA contribute to ER stress which can induce additional oxidative stress, increase de novo lipogenesis, and activate inflammatory responses via JNK/NFκB pathway. Lipid toxicity can active inflammation via TLR4 signalling pathway in Kupffer cells and hepatocytes, the former is a major source of proinflammatory cytokines such as TNFα, IL-1β and IL-6. Chronic low-level hepatic NFκB activation further contributes to local and systemic IR, which in turn influences de novo lipogenesis. (3) Maternal undernutrition can reduce 11-β-HSD in the placenta and therefore increase fetal exposure to maternal glucocorticoids. Increased glucocorticoids can lead to fetal de novo lipogenesis. Markers that indicate ER stress and de novo lipogenesis can be modified by early life epigenetic mechanism which may represent a path for intergenerational transmission of disease risk. Abbreviations: MRC: mitochondrial respiratory chain; ROS: reactive oxygen species; TAG: triglyceride; FFA: free fatty acid; ER: endoplasmic reticulum; SREBP: sterol regulatory element binding protein; JNK: c-Jun N-terminal kinase; IKK: IκB kinase; NFκB: nuclear factor kappaB; AP-1: activator protein 1; TLR4: Toll-like receptor 4; LXRα: Liver X receptor-α; PPARs peroxisome proliferator-activated receptors; ChREBP: carbohydrate-responsive element-binding protein; FASN: fatty acid synthase; SCD1: stearoyl-CoA desaturase-1; ACC1: acetyl-CoA carboxylase; 11-β-HSD: 11 β-hydroxysteroid dehydrogenase.
1.5 Interventions for developmental programming

As mentioned in the beginning of the chapter, NCDs are the world’s biggest killers – in both developing and developed countries (2). The substantial socioeconomic cost of NCDs has become a concern – not only because of the human cost of so many deaths attributable to potentially preventable causes, but also because the economic burden on national and global health care is significant (247). Mitigation strategies to combat human chronic health problems should consider the early life environment as a potential key target, and a developmental programming perspective can inform such strategies.

In this section, we discuss the importance of timing for intervention and summarise several potential intervention strategies that show promise for translation to a human setting.
1.5.1 Critical time frame for intervention

Developmental programming is a life-course phenomenon. Sub-optimal environments in early life can modify offspring development, and these early changes are often very subtle - impaired health outcomes only show up with aging or after exposure to conflicting postnatal life conditions. When considering interventions to combat negative offspring programming outcomes, there are two broad approaches that can be applied: (1) to treat the adverse outcomes when they start to appear at later stage of life, or (2) to conduct the intervention in early life to prevent the adverse outcome from occurring. There are a number of reasons however that suggest that the earlier the intervention the greater the long term benefits.

While a number of animal studies have attempted to reverse or ameliorate developmental programming effects by a range of different intervention paradigms during adulthood, some have added unwanted side-effects. For example, work in the rodent has shown that treatment of adult offspring with growth hormone (GH) can resolve several aspects of the metabolic phenotype such as hypertension and obesity in developmentally programmed offspring, but simultaneously exacerbated the hyperinsulinemia (248).

Godfrey et al. proposed that the earlier the intervention during the life course the greater the impact on later life health and well-being of offspring (249) (Figure 1.4). The development of NCD is quite unlike the medical model in infectious disease in which an individual is healthy until they contract the pathogen; risk of NCDs is cumulative throughout the life course. Early life environmental conditions can influence, and largely determine the response of the individual to later life conditions. Because developmental plasticity generally declines during aging, the individual becomes less effective in adapting new insults, and may begin to gradually accumulate adverse effects that lead to chronic disease (249). If the nature of developmental plasticity provides the possibility that an adverse early life environment can lead to increased susceptibility to disease in later life, this suggests that an appropriate intervention in the same time frame as any unfavourable conditions may reverse the programming changes.

Many developmental programming effects on organ and tissue structure are irreversible. For example, muscle fiber number is established before birth; after birth, muscle fiber size can be increased by hypertrophy, but muscle fibre numbers remain fixed (181). Thus, factors affecting fetal muscle development can lead to permanent, irreversible changes in muscle structure and its growth potential (250). Similarly, kidney nephron number is also set during
the early development, and once reduced in a suboptimal maternal condition cannot be reversed in later life (251). These fundamental structural changes in tissue and organ development are determined in the early life period, reinforcing the suggestion that only intervention during these early periods can prevent any detrimental changes from becoming permanent.

Figure 1.4 Life course approach of NCD

Adopted from Godfrey et al. with permission (249).
1.5.2 Intervention strategies in early life

Ideally, elimination of any adverse environment prior to pregnancy would be the most effective prevention. However, in many cases it is very hard to modify pre-existing conditions prior to pregnancy, and not all pregnancies are carefully planned (252). Intervention in pregnancy and immediate postnatal life may however present a practical opportunity to minimize any potential offspring predispositions when unfavourable maternal conditions can not be removed or prevented prior to or during the pregnancy. The following section summarises some key studies that have investigated potential intervention strategies from three categories: dietary supplementations, pharmacologic approaches and exercise intervention.

a) Dietary supplementation

Taurine

Taurine is a sulfonic amino acid which plays a role in glucose metabolism (253, 254). It was first reported by Cherif et al. that taurine supplementation during pregnancy restored the islets insulin secretion to a normal level in offspring following maternal low protein diet (255). Later studies demonstrated that the beneficial effect of taurine on pancreatic islets is attributed to normalisation of β-cells apoptosis (172), normalisation of islet vasculogenesis (256), reversal of the vulnerability of islet to pro-inflammatory cytokines (257, 258) and prevention of mitochondrial dysfunction in the β-cells (259). Interestingly, a recent study showed that maternal taurine supplementation tended to improve hepatic insulin sensitivity in a similar maternal protein restriction model, suggesting that the beneficial effect of taurine on glucose metabolism is not limited to pancreas development but also may apply to liver (260).

N−3 PUFAs

Long-chain n−3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are commonly found in fish oils. High consumption of n−3 PUFAs has been shown to decrease the production of inflammatory cytokines and give rise to a number of anti-inflammatory mediators (261). Given the role of inflammatory pathway in the programming of metabolic disorders (section 1.3.4-b), n−3 PUFAs represent a promising candidate to prevent adverse offspring health outcomes caused by altered inflammatory responses. Heerwagen et al. reported that increasing n−3 PUFAs via viral transfection reduced maternal obesity associated inflammation and prevented obesity, adipose tissue inflammation, hepatic lipid accumulation
and overall IR in adult mice offspring (262). Nevertheless, this increase in n−3 PUFAs was achieved by transgenic modification and the level of n−3 PUFAs is so high that it is hardly comparable with an oral supplementation of n−3 PUFAs in human (263). Whether a reasonable level of n−3 PUFAs supplementation can achieve a similar reversal effect in the developmental programming of metabolic disease needs further investigation.

**Folic acid**

There is evidence that alterations in gene transcription, particularly DNA methylation, play an important role in the offspring phenotype changes in the setting of developmental programming. Folic acid is an essential component for the synthesis of common methyl donor required for the maintenance of methylation patterns in DNA (264). It has been shown that folic acid deficiency is associated with deleterious effects of programming on offspring (section 1.3.2-d). Maternal folic acid supplementation in the rat following protein restriction prevents epigenetic modification of hepatic gene expression in the offspring that related to carbohydrate and lipid metabolism (135), and this effect persists to adulthood (265). This finding suggests that folic acid may compensate protein deficiency-induced hypomethylation, however, it is not clear whether the reversal of hypomethylation in this setting improves metabolic outcome in offspring. Additionally, there is evidence in both human cohort and rodent models showing that folic acid supplementation in pregnancy can lead to an allergic asthma phenotype in offspring (266, 267). Findings to date therefore support the view that maternal folic acid supplementation induces persistent changes in a number of phenotypic outcomes in the offspring. However, the number of studies is limited and insufficient to indicate a need to change current recommendations for folic acid intake during pregnancy. Nevertheless, such effects should be investigated thoroughly in order to support firm conclusions about the risk of unanticipated long-term negative effects of maternal folic acid supplementation in humans.

**b) Pharmacologic approaches**

**Exendin-4 (EX-4)**

Work by Simmons *et al.* has shown that treatment of neonatal rats with the glucagon-like peptide (GLP)-1 analog EX-4 reversed the adverse consequences of being born growth restricted, and prevented the development of diabetes in adulthood (268, 269). Neonatal EX-4 treatment can prevent reduction in β-cell mass that is observed in IUGR rats via restoring β-cell proliferation and islet vascularisation (268, 269). It has been shown that EX-4 can
increase histone acetylase activity and reverses chromatin modifications that silence genes involved in β-cell differentiation in the IUGR rat (270). Similar beneficial effects of EX-4 have also been shown in an IUGR sheep model with significant reduction in fat accumulation and normalised β-cell function during the treatment period(271). There is no adverse effect of EX-4 observed in animals born to control mothers.

**Leptin**

Leptin treatment to neonatal female rats born to undernourished mothers prevented the development of metabolic compromise in adulthood (161). The complete normalization of the programmed metabolic phenotype by neonatal leptin treatment implied that leptin can reverse prenatal adaptations resulting from fetal undernutrition. Additionally, the effects were specific to low birth weight animals, whilst leptin had no effect in animals born to control mothers (272). However, the effects of leptin were sex-specific and also reliant on the level of postnatal nutrition (161, 272). Recent independent studies using a similar leptin treatment model have shown that early leptin intervention reverses perturbed energy balance regulating hypothalamic neuropeptides in the pre- and postnatal calorie-restricted female rat offspring (273). Importantly, the effect of leptin in reversing the metabolic sequelae associated with IUGR has also been replicated in another model species with work in the pig showing efficacy of neonatal leptin treatment reversing some of the metabolic disturbances in offspring (274).

c) **Exercise intervention**

There is recent interest in the field to investigate physical activity and exercise as an intervention option during pregnancy to reduce developmental programming. Traditionally, no level of exercise during pregnancy was considered to be beneficial in improving perinatal outcome and it was not suggested to pregnant women with medical complications (275). However, this opinion has changed over the recent 10 years with growing evidence suggesting a possible role for exercise in the prevention and management of GDM (276, 277). If maternal obesogenic environment can be improved by exercise, it is possible that offspring long term health can be benefit from enhanced maternal condition. Nathanielsz et al. reported that maternal exercise prior to and during pregnancy in a maternal obesity rat model normalised plasma leptin concentration in male offspring at a young age (postnatal day 35) and prevented obesity in later life (postnatal day 650) (278). Late-onset exercise in female offspring has been shown to ameliorate the detrimental metabolic impact of maternal obesity in the rat (279). Exercise has also been reported to improve metabolic outcomes following IUGR (280).
although more work is required to understand the effect of different exercise modalities, duration and timing of exercise interventions. Nonetheless, more detailed measurements regarding offspring metabolic function are needed to evaluate the effectiveness of exercise as an intervention approach.
Thesis Objectives:

Previous work by our research group has comprehensively established that several distinct maternal nutritional insults during pregnancy and/or lactation can have major impacts on the long term health and wellbeing of offspring. In addition there is a large body of evidence by our group and others demonstrating that early life interventions, either pharmaceutical or nutritional, can ameliorate these detrimental effects. However, to date, the mechanisms underpinning both the developmental programming phenotype and the observed efficacy of intervention strategies remains poorly described. Therefore this thesis encompassed two broad aims:

1) Identify potential mechanisms in established rodent models of developmental programming of obesity and metabolic dysfunction. This will include models at both ends of the maternal nutritional spectrum with models of both maternal undernutrition (global undernutrition) and maternal obesity (high-fat and/or fructose induced obesity).

2) Examine the potential of early-life intervention strategies to reverse programming in adult male and female offspring. We will firstly outline the impact of taurine supplementation during pregnancy in a maternal obesogenic model. The final study will examine the potential of pre-weaning growth hormone treatment as a strategy to combat the early life growth and related metabolic and cardiovascular disorders observed in offspring following maternal undernutrition.

Given the effects of programming on cardio-metabolic health, the studies outlined in this thesis will focus on systemic measures of IR and cardiovascular dysfunction with in-depth analysis of peripheral metabolic organs (liver and adipose tissue) along with cardiac and vascular function.
Chapter 2. Methodology

2.1 Study design

2.1.1 Justification for use of animals in these studies

Established rat models were used in experiments outlined in this thesis. Given the longitudinal nature of the studies outlined, the short life-span, similar placental structure and metabolic systems make rats an ideal model. All animal experiments were designed and performed following the guiding principles of laboratory animal experiments - the “Three Rs”: Replacement, Reduction and Refinement (281, 282).

**Replacement** refers to efforts to find alternatives to animal use. In this study, replacement of animals with other surrogates is not possible. This project requires the use of whole animal physiology and encompasses the quantification of metabolic outcomes that can only be assessed in animals due to the defined maternal manipulations and the need for tissue-specific gene expression analysis. Particularly, we aim to test therapeutic interventions to normalise adverse programming effects. It is essential to obtain data from animal trials to estimate the effectiveness and eliminate the potential risk before translation to the clinical setting can be implemented.

**Reduction** refers to efforts to minimize the number of animals used during the experiment, and prevention of unnecessary replication of previous experiments. Animal numbers for the experiments detailed in this thesis were estimated based on power equations derived from previous animal cohorts in our laboratory and n-requirements to attained expected differences. A 10% non-pregnancy rate and a percentage of non-viable litters (e.g. litter size < 5) were allowed based on previous cohort studies. It is important to note that each litter represents a single biological replicate so experimental power was based on maternal group size. In the maternal obesogenic diet cohort, the control groups were shared across the two sub-cohorts to minimize number of animals used. These studies focused on intervention strategies and had not been undertaken previously so there is no replication of previous experiments. All groups in the study design were essential to obtain a balanced factorial experimental design. However, where possible, we have minimised the number of animals required through the use of shared control groups.
Refinement refers to efforts to make experimental design as efficient as possible in order to minimize the suffering of each animal subject and thereby minimise potential experimental confounders. All animals were maintained in professional animal facility with a registered veterinary surgeon available at all times. Animals have free access to water throughout all experiments. Maternal undernutrition was induced at a moderate level, and all other groups were fed *ad libitum*. All researchers were appropriately trained in each procedure.

All animal experiments were approved by the Animal Ethics Committee at University of Auckland.

2.1.2 Overall design of the animal experiments

This thesis comprises three experiments corresponding to Chapter 3, 4 and 5 respectively. All experiments were based on a fully balanced factorial experimental design.
1. The first experiment (detailed in Chapter 3) uses a high fructose diet during pregnancy and lactation to establish a maternal obesogenic environment. Taurine supplementation is applied at the parallel time as an intervention to combat the adverse programming effect. Figure 2.1 is an overall outline of the experimental design.

![Figure 2.1 Experimental design utilised for the maternal high fructose diet study](image-url)

**Figure 2.1 Experimental design utilised for the maternal high fructose diet study**
2. The second experiment (detailed in Chapter 4) investigates the effect of taurine supplementation in a maternal obesogenic environment induced by an obesogenic diet which is high in both fructose and fat (Figure 2.2). For logistical issues, the second cohort study detailed in Figure 2.2 was undertaken at the same time as the first cohort (Figure 2.1) and thus the control groups, handled identically across the two experimental paradigms, were shared across the two experimental models.

Figure 2.2 Experimental model utilised for the maternal obesogenic diet study
3. The third experiment (detailed in Chapter 5) investigates the effects of pre-weaning GH treatment in offspring following maternal global food restriction during pregnancy. (Figure 2.3)

**Figure 2.3** Experimental model for maternal undernutrition with pre-weaning GH treatment
2.2 Generation of the animal experimental cohorts

Standard conditions as regards animal husbandry, housing and research personnel involvement were utilised for all three experimental protocols unless otherwise stated. All animals used for breeding were acquired from the Vernon Janson Animal Unit, the University of Auckland, at a weaning age and housed until mating age to allow familiarisation and acclimatisation with the research staff and handling procedures involved.

2.2.1 Timed-mating

Timed matings were conducted at approximately 100 days of age when animals had reached full sexual maturity. Females were probed via an EC40 estrus cycle monitor (FST 22500-1 Rat, Fine Science Tools, Canada) between 12pm - 2pm daily to check for proestrus. A reading of 4.0 or higher indicated the female to be eligible for mating. This technique is based on electrical impedance of the epithelial cell layer. The vaginal mucosal layer is significantly higher in the proestrus stage than in the other stages of the estrus cycle, which results in a higher impedance.

Eligible females were placed into a clean cage overnight with a selected male breeder. In the morning the male was removed from the cage and mating was confirmed by the presence of spermatozoa under the light microscopy following a vaginal lavage. All dams were then housed as singletons upon confirmation of mating and it counted as day one of pregnancy.

Mating was staggered over a two week period to ensure later cull time points and sample collections could be managed logistically and culls could completed within a consistent and maximal timeframe between 9am and 12pm.

2.2.2 Phenotyping at birth

Onset of delivery was checked from 21 day of pregnancy. After confirming delivery was completed, litter size, offspring sex, offspring body weight and nose to anus (NA) lengths were recorded. Offspring sex was determined based on the anogenital distance, which is larger in male pups compared to female pups.

On postnatal day 2, each litter was reduced to 8 pups (4 male and 4 female) to standardise nutrition until weaning. Randomly excluded pups were killed by decapitation.
2.2.3 Weaning

At postnatal day 22, all offspring were weaned and housed 2 per cage with their same sex sibling. Offspring body weights were measured every three days thereafter.

2.2.4 Evaluation of offspring puberty onset

From postnatal day 24 (P24) onwards female offspring were examined daily by the same research staff for vaginal opening (VO). On the day when VO was observed age and body weight was recorded. From postnatal 35 (P35) male offspring were examined daily for balanopreputial separation. All pubertal observations were performed by the same research investigator between 0900 and 0930.

2.2.5 Food challenge test

A food challenge test was performed in offspring at postnatal day 130 to assess food preference and body weight responsiveness to obesogenic diets. 200g of the standard chow diet and 200g of the obesogenic high fructose high fat diet (detailed composition in section 2.2.12) was placed in each cage each day for a period of 9 days. Animals had free choice to select which diet they preferred. Body weight and food intakes were recorded daily during this period.

2.2.6 Oral glucose tolerance test (OGTT)

An OGTT was performed at postnatal day 143 in offspring. Rats were fasted overnight in clean cages. Rats were gavaged with a glucose solution (2g/kg body weight) and repeated tail blood samples were taken at 0, 5, 10, 20, 30, 60, 90 and 120min. Blood glucose concentrations at each time point were determined using a glucose meter (Optium, Abbott Laboratories, New Zealand). Tail snip was performed once for each animal to reduce the stress as sequential blood can be obtained from the same wound. The first drop of blood was discarded, and the second drop of blood was used for glucose measurement.

2.2.7 Evaluation of body composition

Animal body composition was assessed using DEXA at postnatal day 143. Inhalational anaesthesia was performed to achieve satisfactory sedation during the scan. Animals were anaesthetised in clear perspex chambers with 4% isoflurane as induction. Once anaesthetised,
rats were transferred and maintained under light anaesthesia (isofluorane 2.5%) using a nose-cone device. After scanning, rats were returned to their home cage. Our group has undertaken hundreds of DEXA scans and there is no effect of scanning on animal body weights or food intakes and recovery from anaesthesia is rapid.

2.2.8 Systolic blood pressure (SBP) measurement

At postnatal day 140, SBP was measured in male offspring by tail cuff plethysmography according to the manufacturer’s instructions (Model 179 with an automatic cuff inflation pump (NW20), IITC, Life Science, Woodland Hills, CA). Rats were restrained in a clear plastic tube in a pre-warmed room (25–28 °C) and allowed 10-15 minutes adjustment before the measurement. The tail cuff was then placed and inflated to 240 mmHg. Pulses were recorded during deflation at a rate of 3 mmHg/s. SBP was determined by the reappearance of a pulse. Final SBP was taken from the average of three indistinct recordings per animal. The coefficient of variation for repeated measurements was <5%.

2.2.9 Tissue collection

Neonatal samples were collected on postnatal day 2. Pups were killed by decapitation. Trunk blood was collected into heparinised capillary tubes and stored on ice at all times. Blood glucose and β-hydroxybutyrate (BHB) concentrations were measured from trunk blood samples using a glucose meter (Optium, Abbott Laboratories). Neonatal liver was collected and snap frozen in liquid nitrogen. Neonatal brain was weighed during the sample collection.

Tissue collection in adult animals for each experiment was undertaken over a two week staggered period. This is important to standardise collection of tissues to a defined time window (between 9am and midday) in order to avoid daily fluctuations in hormones and expression of some metabolically important genes. Importantly, all females were staged for estrus cycle prior to cull to minimise the potential interference from periodic sex hormone change. Estrus was identified by observation of clustered cornified cells from a vaginal smear. Animals in estrus were killed on the next day. On each cull day, experimental groups were rotated to avoid time-of-day effects on sample collection.

Animals were fasted overnight, and killed by decapitation following anaesthesia with sodium pentobarbitone (60mg/kg intraperitoneal injection (IP)). Blood glucose and BHB were measured from tail blood samples using a glucose meter as described above. Trunk blood was collected into heparinised vacutainers and immediately stored on ice until later processing and
supernatant collection. Two pieces of 5mm thick sections from the left lobe of the liver were collected, one was snap frozen and stored at -80°C, the other one was fixed in 4% paraformaldehyde. Since evidence suggests potential for differential gene expression across different lobes of the liver, the same region of liver was isolated from all samples (283). Heart, kidney, retroperitoneal fat pad, adrenal, spleen and brain were dissected, weighed then snap frozen and fixed in 10% neutral buffered formalin. The mesenteric 3rd order arteries were collected for resistance artery function evaluation using a pressure myograph. Gonadal adipose tissue was collected from male offspring for ex vivo processing.

Blood samples were centrifuged immediately after cull at 2500rpm for 20 minutes at 4°C. Plasma supernatants were aliquoted and stored at -20°C until subsequent analysis – multiple aliquots were taken to avoid potential effects of freeze-thaw cycles and stored at -20°C.

Note – not all tissues collected are presented in data form in this thesis – from an ethical point of view as many tissues were collected and banked as was logistically possible for potential use by other researchers in independent studies.

2.2.10 Pressure myograph

Resistance vessel function was assessed using pressure myograph during the tissue collection period (Clint’s plos one paper).

Mounting vessel in single vessel chamber

Third order mesenteric vessels were isolated from the mesenteric vascular bed using a dissecting microscope and placed in physiological salt solution (PSS) (119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 24 mM NaHCO3, 1.18 mM KH2PO4, 1.2 mM MgSO4, 0.01 mM EDTA, 5.5 mM glucose). Vessel segments were then mounted on two glass microcannulaes in the single vessel chamber from a pressure myograph system (Living System, Burlington, VT, USA.). Once vessels were mounted, nylon thread was used to secure both ends of the vessel and the distance between the cannulaes was adjusted without stretching the vessel. The vessel chamber was set to 36°C with a temperature tolerance of 1°C. Vessels were then flushed with PSS to remove intraluminal blood and debris. The vessel chamber unit was then mounted on the stage over an inverted microscope attached to a video camera.

Vessel integrity assessment
Intraluminal pressure was gradually elevated from 10 to 100 mmHg using PSS gassed with a mixture of 95% O2 and 5% CO2. The distance of the cannulas was adjusted when necessary. The viability of the vessel was assessed with five, 1 min washes with PSS and pre-constriction with phenylephrine (PE) (concentration equal to 80% of maximal response; pEC80). Vessels failing to reproduce consistent constriction (pEC80) were considered non-viable and substituted with freshly excised tissue.

**Assessment of vessel function**

Intraluminal pressure was increased by 10 mmHG from 10 to 90 mmHg with 10 mmHg to construct pressure-diameter curves. External vessel diameters were measured at each pressure point. Vessel function assessment was conducted following a 30 mins equilibration period in PSS or following a cessation of basal vascular activity, whichever was sooner. Cumulative constriction response curves were constructed for the α1-adrenoceptor agonist PE (1 nM to 100 µM) and cumulative relaxation response curves were constructed with endothelium-dependant vasodilator acetylcholine (ACh; 0.1 nM to 1 mM). Changes in diameter at each concentration were compared to initial vessel diameter as % constriction, and then normalised as % maximum constriction or relaxation respectively.
2.2.11 Maternal dietary manipulation with taurine supplementation

In Experiments 1 and 2, dams were randomly assigned into one of 6 nutritional groups:

- **Control group (CON):** fed standard laboratory chow diet (2018; Harlan Teklad Global Diets, Oxford, UK) (Table 2.1).

- **High fructose diet group (F):** standard chow diet *ad libitum* supplemented with fructose solution in water. This was designed to provide 20% calories from fructose. A fructose solution of fixed volume was freshly made on a daily basis according to the previous day consumption. Crystalline fructose was used to make the fructose solution. All animals also received water *ad libitum* via a second drinking bottle.

- **Obesogenic diet group (MO):** fed a high fructose high fat diet (D03101602, Research Diets, USA). It provided 45% calories from fat and 26% from fructose. (Table 2.2)

and 3 corresponding taurine treatment groups (CT, FT, MOT).

Maternal diets were maintained throughout gestation and lactation. Taurine supplementation was given in drinking water to the respective treatment groups. Concentration of taurine in CT and MOT group was 1.5% w/v. Because the consumption of fructose solution affects water intake, taurine concentrations in the FT group were adjusted where necessary according to the previous day water intake. This was essential to ensure that the taurine dose was equivalent across CT, FT and MOT groups. Maternal body weight, food intake (by measuring the weight remaining from food pre-weighed the previous day) and water intake were measured daily during the gestation and lactation.

At weaning (day 21), all offspring were weaned onto the standard chow diet.
<table>
<thead>
<tr>
<th>Harlan Teklad Global Diet 2018</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories from protein</td>
</tr>
<tr>
<td>Calories from fat</td>
</tr>
<tr>
<td>Calories from carbohydrate</td>
</tr>
<tr>
<td>Energy Density (kcal/g)</td>
</tr>
<tr>
<td>Macronutrients:</td>
</tr>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>Fat</td>
</tr>
<tr>
<td>Carbohydrate</td>
</tr>
<tr>
<td>Crude fibre</td>
</tr>
<tr>
<td>Neutral detergent fibre</td>
</tr>
<tr>
<td>Ash</td>
</tr>
</tbody>
</table>

Table 2.1 Composition of the standard chow diet (Harlan Teklad Global Diet 2018)

<table>
<thead>
<tr>
<th>Research diet D03101602</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories from protein</td>
</tr>
<tr>
<td>Calories from fat</td>
</tr>
<tr>
<td>Calories from carbohydrate</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Energy Density (kcal/g)</td>
</tr>
<tr>
<td>Macronutrients:</td>
</tr>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>Fat</td>
</tr>
<tr>
<td>Carbohydrate</td>
</tr>
</tbody>
</table>

Table 2.2 Composition of the obesogenic high fat:high fructose diet (Research Diets D03101602)
2.2.12 Maternal undernutrition with pre-weaning GH treatment

Dams were randomly assigned into either the control (CON) or undernutrition (UN) group from day one of pregnancy. CON mothers were fed standard chow diet *ad libitum* throughout pregnancy. UN mothers were given 50% of *ad libitum* intake diet based on the daily measurement from CON group from the previous day. After birth, food supply was immediately restored to *ad libitum* in UN mothers throughout lactation. Maternal body weight and food intake were measured daily during the gestation and lactation.

From neonatal day 3 to day 13, pups were injected either with saline (CON-S and UN-S) or GH (recombinant bovine GH) at a dose of 2.5μg/g/day (CON-GH and UN-GH) delivered between 0800h and 0830h. The injection site is the skinfold at the nape of the neck with an injection volume of 50μl using a fine gauge (29G) diabetic syringe.

At weaning, offspring were weaned on either chow diet or a high fat (HF) diet. (Table 2.3) Diets were fed *ad libitum* until end of the study.

<table>
<thead>
<tr>
<th>Research Diet D12451</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories from protein</td>
</tr>
<tr>
<td>Calories from fat</td>
</tr>
<tr>
<td>Calories from carbohydrate</td>
</tr>
<tr>
<td>Energy Density (kcal/g)</td>
</tr>
</tbody>
</table>

**Table 2.3 Composition of the high fat (HF) diet (Research Diets D12451)**
2.3 Biochemical and molecular analysis

2.3.1 Plasma analysis

Commercially sourced assay kits were used for a number of plasma markers. Rat specific kits were utilised where available. Each kit was performed as per manufacturer’s provided protocol. In general, plasma samples were thawed on ice and centrifuged for 3 minutes at 2500rpm at 4°C prior to performing the kits to remove fibrous clots which are common in rodent plasma. When multiple plates were needed for one marker, samples were performed in a randomised order which was generated via an Excel database to avoid time-of-day effects and inter-assay effects. All intra- and inter-assay coefficients of variation were < 5%.

Plasma taurine and glutamate concentrations were analysed using a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan).

Manufacturers’ information regarding each analyte measured is summarised in the following table. (Table 2.4)

<table>
<thead>
<tr>
<th>Name</th>
<th>Assay type</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>ELISA</td>
<td>BioAssay Systems, USA</td>
</tr>
<tr>
<td>Insulin</td>
<td>ELISA</td>
<td>CrystalChem, USA</td>
</tr>
<tr>
<td>Leptin</td>
<td>ELISA</td>
<td>CrystalChem, USA</td>
</tr>
<tr>
<td>IL-1β</td>
<td>ELISA</td>
<td>Quantikine; R&amp;D Systems Europe, UK</td>
</tr>
<tr>
<td>IL-6</td>
<td>ELISA</td>
<td>Quantikine; R&amp;D Systems Europe, UK</td>
</tr>
<tr>
<td>TNFα</td>
<td>ELISA</td>
<td>Quantikine; R&amp;D Systems Europe, UK</td>
</tr>
<tr>
<td>IGF-1</td>
<td>ELISA</td>
<td>Mediagnost, Germany</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>ELISA</td>
<td>CrystalChem, USA</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>ELISA</td>
<td>CrystalChem, USA</td>
</tr>
<tr>
<td>Ghrelin (unacylated)</td>
<td>ELISA</td>
<td>Cayman, USA</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>Immunoassay</td>
<td>Abbott AxSYM system, USA</td>
</tr>
<tr>
<td>Uric acid</td>
<td>Fluorescence-based assay</td>
<td>Cayman, USA</td>
</tr>
</tbody>
</table>

Table 2.4 Biochemical assay information
2.3.2 H&E staining and morphological evaluation

Fixed liver tissue was embedded in paraffin and cross-sections were prepared (5µM) (Leica RM 2135 rotary microtome, Leica Instruments, Germany). Haematoxylin and Eosin (H&E) staining was applied for overall morphological analysis. In brief, each slide was deparaffinised in xylene and stained with haematoxylin Gills 2 and Eosin Y solution. 1% lithium carbonate was used as a bluing reagent. Sections were then mounted using distrene plasticizer xylene (DPX) mounting medium (BioLab ltd, New Zealand) and observed under a light microscope (Nikon 800, Tokyo, Japan). Representative images were taken (Nikon FDX-35, Tokyo, Japan) and processed with NIS Elements-D software (Nikon, Tokyo, Japan).

Scoring of steatosis, lobular inflammation, hepatocyte ballooning and overall NAS score (NAFLD Activity Score) was performed by a blinded observer using the methodology adapted from Kleiner et al. (284). The scoring system comprised four primary features evaluated semi-quantitatively: steatosis grade (0–3), lobular inflammation (0–3), hepatocellular ballooning (0–2) and NAS score (unweighted sum of steatosis, lobular inflammation, and hepatocellular ballooning scores) (Table 2.5). Lobular inflammation was counted in 20× fields. At least 5 fields were examined for each animal.

<table>
<thead>
<tr>
<th>Steatosis grade</th>
<th>Lobular inflammation</th>
<th>Hepatocyte ballooning</th>
</tr>
</thead>
<tbody>
<tr>
<td>0: &lt; 5%</td>
<td>0: None</td>
<td>0: None</td>
</tr>
<tr>
<td>1: 5%-33%</td>
<td>1: &lt; 2</td>
<td>1: Few ballooned cells</td>
</tr>
<tr>
<td>2: 34%-66%</td>
<td>2: 2-4</td>
<td>2: Many ballooned cells</td>
</tr>
<tr>
<td>3: &gt; 66%</td>
<td>3: &gt; 4</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.5 Non-alcoholic steatohepatitis Clinical Research Network system for scoring NAFLD

Derived from Kleiner et al. (284)

2.3.3 Tissue disruption

All adult liver samples which were collected from the left lobe of the liver were ground into powder manually using a mortar and pestle. Neonatal liver samples, due to limited size, were collected whole and disrupted using SPEX Freezer/Mill 6750 Cryogenic Grinder (SPEX SamplePrep, LLC, Stanmore, UK) following the manufacturer’s protocol. The Cryogenic Grinder was used for liver because it can achieve a more homogeneous disruption for larger pieces of tissue.
All samples remained chilled in liquid nitrogen during the period of tissue disruption. All equipment that physically contacted tissue was cleaned and freshly autoclaved before use, and was wiped several times with isopropyl alcohol between samples to avoid any cross-contamination.

Disrupted tissue powder was aliquoted into pre-cooled tubes and placed on the dry ice at all times before storage at -80°C.

2.3.4 RNA extraction

Two methods were used for RNA extraction across the different experimental cohorts.

1) Spin column-based RNA extraction was used initially because it was the standard method of RNA extraction in the group at the time.

2) Magnetic particle based RNA extraction using a magnetic particle processor instrument was used in the third experiment because the number of samples that needed to be processed in this experiment is large (n = 248) and this method is partially automated, the quality of product is relatively more consistent than manual methods. The magnetic particle processor instrument was installed after the samples in the first two experiments had been processed, therefore this method could only be applied in the last experiment.

Spin column-based RNA extraction

RNA from disrupted liver tissue was extracted by using spin column-based method with commercial sourced RNeasy Mini Kit from Qiagen (Hilden, Germany).

Disrupted liver powder was homogenized in beta-mercaptoethanol (β-ME) buffer by passing lysate 15-20 times through a 20-gauge needle fitted to an RNase-free syringe. Lysate was centrifuged for 3 min at maximum speed (≥10,000 rpm) at 4°C. Supernatant was then transferred to the RNeasy Mini spin column. The isolation and collection of RNA nucleotide sequences was performed in accordance with the manufacturer’s protocol.

The mechanism of spin column-based RNA extraction is to pass lysate through a silica gel membrane that can bind nucleic acid at an optimal PH and salt condition. After purification RNA can be eluted from the membrane by RNase free water. It is important to ensure that the amount of tissue in the lysate does not exceed the capacity of the column membrane. Pilot
extraction experiments were carried out to determine the optimal amount of tissue to use per column.

**Magnetic particle based RNA extraction**

KingFisher Total RNA Kit was commercially sourced from Thermo Scientific (Vantaa, Finland) for this method. RNA extraction was conducted using KingFisher Duo magnetic particle processor instrument (Thermo Scientific, Vantaa, Finland) following the manufacturer’s protocol.

Disrupted liver powder was homogenized in the provided lysis buffer in TissueLyser (Qiagen, Hilden, Germany). Lysate was centrifuged for 3 min at maximum speed ($\geq 10,000$ rpm) at $4^\circ$ C. Supernatants were then transferred to a 96 deep well plate with the provided washing buffer and beads loaded in advance.

The mechanism of magnetic particle-based RNA extraction is to let RNA bind to the functionalised surface of magnetic particles and removal of unwanted content by washing particles in different washing buffer.

**2.3.5 RNA quantity and quality evaluation**

**Nanodrop**

The concentration of all RNA samples was obtained by Nanodrop ND-1000 by calculating from spectrophotometric absorbance at 260nm ($A_{260}$). In this study, RNA extracted from liver generally is enriched and therefore has a high concentration (~2000 ng/µl). Therefore a 1:5 dilution was routinely prepared at the time of extraction for Nanodrop measurement.

The ratio of absorbance at 260nm and 280nm ($A_{280}$) was used as the primary criteria to assess the purity of RNA. A ratio of $\sim 2.0$ is generally accepted as “pure”. The ratio of absorbance at 260nm and 230nm ($A_{230}$) was used as a secondary measure of nucleic acid purity. The $A_{260}/A_{230}$ values for “pure” nucleic acid are often higher than the $A_{260}/A_{280}$. In this study, an $A_{260}/A_{280}$ ratio between 1.9-2.1 and $A_{260}/A_{230}$ 1.9-2.2 was accepted as appropriate for further experimental analysis.

**Agilent's 2100 Bioanalyzer**
RNA quality control was also carried out by using Agilent's 2100 Bioanalyzer in selected samples following manufacturer’s instruction. Agilent's 2100 Bioanalyzer uses a chip to perform capillary electrophoresis and uses a fluorescent dye that binds to RNA to determine RNA concentration and integrity. A RNA Integrity Number (RIN) ranged from 1 to 10 was calculated by Agilent's 2100 Bioanalyzer software to indicate the integrity of RNA - 1 means the most degraded and 10 suggests the most intact.

Due to the large amount of samples, only selected samples underwent analysis on the Bioanalyzer. The selection method is as follows: RNA extraction was conducted on at least 10 samples at once. The samples extracted at the same time were counted as a batch. Three samples per batch were randomly selected to perform electrophoretic assays on Agilent's 2100 Bioanalyzer. All samples tested with Bioanalyzer had RIN above 8 which is indicative of high quality RNA.

2.3.6 cDNA synthesis

Total RNA was reverse transcribed into cDNA using SuperScript VILO cDNA Synthesis Kit (InvitrogenTM; Life Technologies Corporation, California, USA). 2 μg total RNA was mixed with water, 2 μl 10× SuperScript Enzyme Mix and 4 μl 5× VILO Reaction Mix (supplied in kit) to make up 20 μl reaction volume in a PCR tube.

The reaction was incubated in an Eppendorf 5331 MasterCycler Gradient Thermal Cycler as following steps:

1<sup>st</sup> step: 25°C for 10 minutes,

2<sup>nd</sup> step: 42°C for 60 minutes,

3<sup>rd</sup> step: 85°C for 5 minutes then hold at 4°C

Reverse transcribed cDNA was stored at -20°C for quantitative polymerase chain reaction (PCR).

2.3.7 Real time PCR

Two methods were used to conduct real time PCR: SYBR Green dye and TaqMan™ Assays. This mix of methods was in accordance with standard laboratory protocols at the time of each study as detailed above.
In the maternal high fructose diet with taurine experiment (Study 1) and all studies in Study 3, real time PCR was conducted using TaqMan™ Assays.

It should be noted that although the experiments outlined in Chapters 3 and 4 shared the control groups, the gene expression of these animals was conducted twice under each experiment independently using the same method with the respective treatment groups.

**SYBR Green method**

Real time PCR was performed using SYBR green I master in the LightCycler 480 System (Roche Diagnostics; Auckland, New Zealand). The relative amounts of genes were quantitated using standard curve method and normalized to the geometric mean of cyclophilin A and β-actin expression. There is no significant difference in the expression of reference genes among groups. Ct values of reference genes are at medium value for the target tissue, ranging between 19-25.

**TaqMan™ method**

Real time PCR for gene expression was performed using TaqMan™ Assays in the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). To control for between-sample variability, mRNA expression was normalized to the geometric mean of cyclophilin A and hypoxanthine phosphoribosyltransferase (HPRT) for each sample by subtracting the Ct of controls from the Ct for the gene of interest producing a Ct value. The ΔCt for each treatment sample was compared to the mean ΔCt for control samples using the relative quantification 2^{-ΔΔCt} method to determine fold-change (285).

All assays (both SYBR Green and TaqMan™) were validated for amplification efficiencies before experiments were carried out. This was done by performing a series dilution standard curve for each candidate gene. The CT value of each dilution point (Y) was plotted against log of the dilution factor (X) and a linear standard curve was generated by Excel. The slope of the standard curve was used to calculate the amplification efficiency (E) using flowing formula:

\[
E = 10^{-\frac{1}{\text{slope}}} - 1
\]

The linearity of the dilution points, combined with the precision of pipetting replicates, affect the correlation coefficient (denoted as the R^2 value of accompanied standard curve). A value of 0.99 or higher is generally considered acceptable.
All primers used in this thesis have amplification efficiencies close to 1 (range from 0.991 to 1.026) and good correlation coefficients ($R^2 > 0.99$).

Primers used in the study are listed in the following table.

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<th>Gene</th>
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<th>Sequence/ Catalogue no./ Identification no.</th>
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<td>QT00183218</td>
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<tr>
<td>Fructokinase</td>
<td>QuantiTect Primer Assay (NM_031855)</td>
<td>QT00186305</td>
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<tr>
<td>SIRT1</td>
<td>QuantiTect Primer Assay (NM_001107627, XM_001080493, XM_228146)</td>
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<tr>
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<tr>
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</tbody>
</table>

Table 2-6 Primer information

2.3.8 *Ex vivo* adipose tissue glucose uptake assay

Freshly isolated gonadal adipose explants were placed in 24-well plates (100 mg tissue/well) in PBS+0.2% BSA before stimulation ± insulin (100 nM) for 30 minutes. [3H]glucose (0.1 mM 2-deoxyglucose, 0.5 μCi/mL [3H]deoxyglucose) was added for 45 minutes. Tissue was washed, lysed in radioimmunoprecipitation assay buffer (RIPA), and homogenized using Tissuelyser. Deoxyglucose uptake was measured by liquid scintillation counting. Results are presented as CPMA or fold increase in insulin-stimulated glucose transport relative to basal concentrations.

2.3.9 *Ex vivo* adipose tissue culture

Freshly isolated gonadal adipose tissue was isolated from chow and HF-fed animals from CS, CGH, UN, and UNGH groups. Adipose explants were placed in 24-well plates (100 mg tissue well) with 1 mL of complete media (DMEM, 10% fetal bovine serum, and 1% penicillin/streptomycin) for 24 hours. Media were harvested and stored at -80°C until analysis. Cytokine secretion (TNFα, IL-6, and IL-1β) was analyzed by enzyme-linked immunosorbent assay (ELISA) (Quantikine kits; R&D Systems Europe, Abingdon, UK).

2.3.10 Isolation of stromal vascular fraction

Adipose tissue was collagenase (2 mg/mL) digested for 60 minutes at 37°C in a water bath. Cell suspensions were filtered and washed with PBS followed by centrifugation and resuspension in PBS/2% BSA. Cells were stained with fluorescently labelled antibodies; CD68-fluorescein isothiocyanate and CD11C-AF647/PE (AbD Serotec). Unstained, single stains, and fluorescence minus one were used for setting compensations and gates. Flow cytometry was performed on a Becton Dickinson LSRII flow cytometer (BD Biosciences Ltd.,...
Palo Alto, California) and analyzed using FacsDiva Software. In a separate experiment stromal vascular fraction (SVF) cells were cultured (200,000 cells/mL) and cytokine secretion (TNFα, IL-6, and IL-1β) was analyzed by ELISA.

2.3.11 Protein analysis

Protein was harvested from adipose tissue by homogenizing in RIPA buffer (50 mg/mL) containing complete protease inhibitors (Roche Ltd). Protein concentration was quantified by Bradford assay (Bio-Rad Laboratories, Inc., Hercules, California). Equal concentrations of lysate (25 μg) were used to determine transcription factor activity of NFκBp65 and PPARγ (TransAm assay; Active Motif, Carlsbad, California). Levels of tyrosine-phosphorylated insulin receptor substrate (IRS)-1, total AKT, and phosphorylated AKT were measured in protein lysates using a Pathscan ELISA platform (Cell Signaling Technology, Danvers, Massachusetts).
2.4 Statistical analysis

All statistical analyses in this thesis were conducted using SAS software (SAS Institute, Cary, NC, USA). Appropriate analysis methods were chosen based on the research questions, design of the experiment, nature of the variables and distribution of the data sets.

Most experiments in this thesis are of a balanced 2×2 factorial design, therefore data were analysis by two way Analysis of Variance (ANOVA) if they meet the assumptions re parametric testing. Data that failed to meet the criteria required for parametric analysis were transformed to achieve normal distribution and equal variance. Where appropriate, post-hoc analysis were performed (Holm-Sidak method) to determine which groups were significantly different from each other.

For repeated measures data including body weights, food intakes and OGTT, repeated by repeated analysis of variance were conducted, with maternal diet and intervention as main factors and time as a repeated measures factor.

For non-continuous data (such as analysis of maternal liver scoring data and the proportion of neonatal deaths per litter), a non-parametric Wilcoxon test rank sum followed by Bonferonni correction was used.

For frequency data (e.g. the frequency of dams having neonatal deaths), Fisher’s exact test was utilised.

All data are shown as means ± SEM unless otherwise stated. A p-value of < 0.05 was accepted as statistically significant.
Chapter 3. Maternal obesogenic environment (high fructose diet): Maternal and offspring outcomes and effect of taurine supplementation

3.1 Preface

The following chapter contains an unaltered published original research article “Maternal taurine supplementation attenuates maternal fructose-induced metabolic and inflammatory dysregulation and partially reverses adverse metabolic programming in offspring” (Appendix III). The article was published in the Journal of Nutritional Biochemistry in 2014. The Journal of Nutritional Biochemistry is one of the leading journals in nutritional sciences, which presents experimental nutrition research related to biochemistry, molecular biology, toxicology, or physiology. The journal had an impact factor of 4.686 in 2013.

The consumption of sugar, particularly added sweeteners such as fructose and sucrose, is currently a topical and much debated public health issue. The most recent Report of the Dietary Guidelines Advisory Committee (DGAC) on the Dietary Guidelines for Americans suggested a maximal intake level of 25% or less of total energy in adult from added sugars (286). A cross national analysis has suggested that soft drink consumption is significantly linked to overweight, obesity and diabetes worldwide (287). Numerous animal experiments have demonstrated that high fructose intake is obesogenic and can lead to IR and NAFLD (288-290). However, some have argued that these experimental models utilised fructose at supraphysiological levels (up to 80% of dietary intake) and thus not relevant to the human setting (291).

Under the principal of developmental programming, it is necessary to evaluate the potential effect of fructose consumption on the long term offspring development. More importantly, if there is any adverse effect from maternal fructose consumption, can the effect be treated by given taurine supplementation- an amino acid known to reduce fructose induced metabolic dysfunction in non-pregnancy models? Further, to be translatable, the level of fructose utilised in any experimental model needs to represent a meaningful level relevant to normal intake conditions. Excessive derivation of calories from fructose will lead to secondary insults including protein deficiency which themselves will elicit programming type effects independent of fructose.
3.2 Maternal and neonatal health outcomes

Maternal taurine supplementation attenuates maternal fructose-induced metabolic and inflammatory dysregulation and partially reverses adverse metabolic programming in offspring

Li M\textsuperscript{1}, Reynolds CM\textsuperscript{1}, Sloboda DM\textsuperscript{1,2}, Gray C\textsuperscript{1}, Vickers MH\textsuperscript{1}.

\textsuperscript{1}Liggins Institute and Gravida: National Centre for Growth and Development, University of Auckland, Auckland, New Zealand.

\textsuperscript{2}Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada.

Abstract

Excessive fructose consumption is associated with insulin resistance and non-alcoholic fatty liver disease (NAFLD), and high fructose intake during pregnancy can lead to compromised fetal development in the rat. Evidence suggests the amino acid taurine can ameliorate fructose-induced IR and NAFLD in non-pregnant animals. This study investigated the efficacy of taurine supplementation on maternal fructose-induced metabolic dysfunction and neonatal health. Time-mated Wistar rats were randomised to four groups during pregnancy and lactation: 1) control diet (CON); 2) CON supplemented with 1.5% taurine in drinking water (CT); 3) CON supplemented with fructose solution (F); 4) F supplemented with taurine (FT). Maternal and neonatal weights, plasma cytokines and hepatic gene expression were analysed. Maternal hyperinsulinemia, increased HOMA-IR indices, and elevated proinflammatory cytokines were observed in F group and normalised in FT group. Maternal fructose-induced hepatic steatosis accompanied with increased liver weight was ameliorated with taurine supplementation. Maternal hepatic SREBP-1c and FASN expression was significantly increased in the F group compared to CON, CT and FT groups. Neonatal hepatic PEPCK expression was increased in male F neonates compared to CON, CT and FT groups, and was increased in female F and FT neonates compared to CON and CT. IL-1β expression was decreased in male CT and FT neonates compared to other male groups. Hepatic TNFR1 was lower in male FT group than F group. These results demonstrate that maternal taurine supplementation can partially reverse fructose-induced maternal metabolic dysfunction and may ameliorate adverse developmental programming effects in offspring in a sex-specific manner.
Keywords: developmental programming; taurine; fructose; inflammation; fatty liver disease; maternal nutrition; animal models
3.2.1 Introduction

Over the last few decades the use of fructose as a food additive to sweeten beverages and other processed foods has increased globally (292) and correlates closely with the rise in obesity, metabolic syndrome and type 2 diabetes (293). Given the potential of excessive fructose consumption to induce adiposity and metabolic dysfunction, increased availability of products containing fructose has the potential to fuel the global obesity epidemic (83, 294, 295). Animal studies have shown that high fructose diets can lead to a series of metabolic disorders including IR, hypertension and non-alcoholic fatty liver disease (NAFLD) (288, 296). Research from our group and others has shown that fructose consumption during pregnancy and lactation leads to hyperinsulinemia, hyperglycemia and hepatic steatosis in dams (87, 297, 298). However, there are few studies that examine the impact of intervention strategies to combat the detrimental effects of excessive fructose intake on the health and well-being of pregnant mothers and their offspring.

Growing evidence from both human and animal studies suggests that the development of metabolic disorders during pregnancy is strongly associated with adverse effects on the long term health of offspring (299, 300). As proposed by the developmental origins of health and disease (DOHaD) paradigm, altered maternal nutrition during critical periods of developmental plasticity can alter offspring development, which in turn can lead to an increased risk of obesity and metabolic dysfunction in later life (301). We have previously shown that high fructose consumption during pregnancy can lead to changes in placental growth, hyperglycemia and hyperleptinemia in female offspring (87). Of importance, the fructose load in our experimental model is designed to provide the mother only 20% of total calories thus highlighting that even at relatively low concentrations fructose can induce significant metabolic abnormalities in offspring. Further work by Rodriguez et al. (302) demonstrated that maternal fructose intake can alter fetal leptin signalling. It is also important to delineate between the different forms of fructose. Most studies to date have utilised high fructose corn syrup (HFCS) which is a commonly used sweetener in food and beverages. However, the terms HFCS and fructose are often, and incorrectly, used interchangeably. While pure crystalline fructose, as used in our previous work (87) and in the present study contains fructose alone, the most widely used variety of HFCS contains approximately 55% fructose and the rest as glucose.

Taurine (2-aminoethanesulfonic acid) is an amino acid which is produced endogenously in humans and rodents (303). Several studies have demonstrated that taurine supplementation has
potential as a regulator of insulin secretion and promotes insulin sensitivity (304, 305). Furthermore, taurine can ameliorate fructose-induced hyperglycaemia, hypertension, and hepatic steatosis in non-pregnant rat models (306, 307). In addition to its proximal effects, taurine supplementation to pregnant rats fed a low protein diet has been shown to normalise pancreatic islet development and glucose and insulin homeostasis in offspring (172, 255, 258). These beneficial effects on glucose metabolism persist into adult life (260). We have also shown recently that maternal taurine supplementation can modify maternal and offspring markers related to hepatic inflammation and lipid metabolism in the setting of maternal obesity (308).

The current study aims to further evaluate the adverse consequences of excessive fructose consumption during pregnancy and lactation, and to determine whether maternal taurine supplementation can reverse the metabolic disorders induced by fructose in dams and neonates.
3.2.2 Methods

3.2.2.1 Animal model

Details on the protocol utilised for fructose and taurine supplementation have been published previously by our group (87, 308). Virgin Wistar rats were time mated at 100 days of age using an estrous cycle monitor (EC-40, Fine Science Tools, San Francisco, USA) and mating was confirmed by the presence of spermatozoa following a vaginal lavage. Animals were then housed as singletons and randomly allocated to one of four nutritional groups: control group (CON) fed a chow diet (Diet 2018, Harlan Teklad, Blackthorn, Bicester, UK) (n = 9); control taurine group (CT) fed chow diet with additional 1.5% w/v taurine supplementation in drinking water (n = 7); maternal high fructose diet group (F) fed chow diet with an additional fructose solution which was designed to provide 20% calories from fructose (n = 8); maternal high fructose diet and taurine group (FT) fed chow diet with additional fructose solution and taurine supplementation in drinking water (n = 8). All cages had two water bottles which were placed in the same position throughout the trial. In the F and FT groups, one of the water bottles contained a fixed volume (70 ml) of fructose and, based on measured chow intake, aimed to provide an additional 20% of total daily calories from fructose. All maternal diets were maintained throughout pregnancy and lactation. As fructose consumption can potentially affect water intake (87), taurine concentrations in the FT group were adjusted where necessary according to the previous days water intake. This was essential to ensure that the taurine dose was equivalent across both CT and FT groups. All dams had ad libitum access to chow diet and water throughout pregnancy and lactation.

Two distinct time points were investigated. Firstly, effects of maternal taurine supplementation on neonatal outcomes were examined on the day after birth, and secondly the effects of taurine on maternal metabolic and inflammatory profiles were investigated at the end of the lactation period. Maternal body weight, food and fluid intake were recorded daily. At the time of birth, litter size, sex ratio and birth weight were documented. On postnatal; day 2, any neonatal deaths were recorded and litter size was adjusted to 8 pups per litter (4 males and 4 females). Neonatal (non-fasting) plasma and liver samples were collected from randomly chosen pups following decapitation. At the end of lactation, dams were fasted overnight and killed by decapitation following anaesthesia with sodium pentobarbitone (60mg/kg IP). Maternal body composition was evaluated by dual energy X-ray absorptiometry using dedicated small animal software (DEXA, Lunar Prodigy, Madison, WI, USA). Maternal and neonatal blood glucose
and β-hydroxybutyrate (BHB) were measured from tail blood samples using a glucose meter (Optium, Abbott Laboratories) at the time of cull.

Some baseline physiologic and plasma data from the CON and CT groups utilised in this study have been reported elsewhere in a parallel project examining maternal obesity (308). Ethical approval was obtained from the Animal Ethics Committee at the University of Auckland (Ethical Approval R888).

3.2.2.2 Plasma Analysis

Plasma insulin and leptin (CrystalChem, USA), tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 (Quantikine; R&D Systems Europe, Abingdon, UK) were measured by rat specific ELISA. Plasma uric acid and fructose were measured using commercially sourced assay kits (Cayman Chemical, Ann Arbor, MI, USA; EFRU-100, BioAssay Systems, Hayward, CA). Homocysteine (HcY) was measured via immunoassay (Abbott AxSYM system). Maternal plasma taurine concentrations were analysed using a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan). The homeostasis model assessment of IR (HOMA-IR) was calculated as: Fasting glucose (mmol/l) × fasting insulin (mU/l)/22.5 (309).

3.2.2.3 Histological analysis

5mm thick sections from the left lobe of maternal livers were fixed in 4% paraformaldehyde and embedded in paraffin, followed by microtome cross sectioning (Leica RM 2135 rotary microtome, Leica Instruments, Germany). Haematoxylin and Eosin (H&E) staining was applied for overall morphological analysis. Sections were mounted using distrene plasticizer xylene (DPX) mounting medium (BioLab ltd, New Zealand) and observed under the light microscope (Nikon 800, Tokyo, Japan). Representative images were taken (Nikon FDX-35, Tokyo, Japan) and processed with NIS Elements-D software (Nikon, Tokyo, Japan). Scoring of steatosis, lobular inflammation, hepatocyte ballooning and overall NAS score (NAFLD Activity Score) was performed by a blinded observer using methodology described previously (308).

3.2.2.4 Hepatic mRNA expression

Total RNA isolation was conducted using RNeasy® mini kit (QIAGEN, Hilden, Germany). Maternal hepatic RNA was extracted from the left lobe of liver and neonatal RNA was
extracted from the homogenized whole liver. cDNA was synthesized from 2µg of RNA using SuperScript® VILO™ cDNA Synthesis Kit (InvitrogenTM; Life Technologies Corporation, California, USA). Real time PCR analysis was carried out by using PreDeveloped TaqMan® Assay Reagent Kits in the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). To control for inter-sample variability, mRNA levels were normalized to two housekeeper genes: cyclophilinA and hypoxanthine phosphoribosyltransferase (HPRT) by subtracting the geometric mean Ct of house keepers from the Ct for the gene of interest producing a ΔCt value. The ΔCt for each treatment sample was compared to the mean ΔCt for control samples using the relative quantification 2-(ΔΔCt) method to determine fold-change (310).

3.2.2.5 Statistical analysis

Data analysis was completed using two way analysis of variance (ANOVA) with maternal diet and taurine as factors except when otherwise stated. Data that failed to meet the criteria required for parametric analysis were transformed to achieve normal distribution and equal variance. Where appropriate, post-hoc analysis were performed (Holm-Sidak method) to determine which groups were significantly different from each other. A repeated measures ANOVA was used to analyse maternal food/caloric and fluid intakes. A non-parametric Wilcoxon test rank sum followed by Bonferonni correction was used to analyse maternal liver scoring data and the proportion of neonatal deaths per litter. The frequency of dams having neonatal death was analysed via Fisher’s exact test. All data are shown as means ± SEM. A p-value of < 0.05 was accepted as statistically significant. All analysis was conducted using SAS software (SAS Institute, Cary, NC, USA).
3.2.3 Results

3.2.3.1 Maternal and offspring weights

Maternal fructose consumption significantly increased maternal body weight in F and FT groups by the end of lactation (Table 3.1). Total caloric intake was slightly but significantly increased in the F and FT groups compared to CON and CT during pregnancy but was not different during lactation (Figure 3.1a). Chow intake was slightly but significantly decreased in the F and FT groups compared to CON and CT groups throughout pregnancy and lactation (Figure 3.1b). Total fluid intake was slightly but significantly increased in the F and FT groups compared to CON and CT during pregnancy but was not different during the lactation period (Figure 3.1c). Maternal liver weight (relative to body weight) was significantly increased in the F group and this increase was significantly reversed by taurine supplementation in FT group (Table 3.1). Maternal total fat and fat:lean ratio were not different between the groups (Table 3.1). Neonatal sex ratio and litter size were not different across groups (data not shown). Neonatal weights were not different across any of the female groups but were reduced overall by taurine supplementation in male neonates (Table 3.1). Interestingly, the proportion of neonatal deaths per litter was significantly increased in CT and F groups, as was the frequency of dams having neonatal death. The neonatal death proportion and frequency was normalized in FT group (the proportion of neonatal death per litter: CON: 1.4%, CT: 7.1%, F:6.8% , FT: 0%; CON vs F P < 0.05, F vs FT P < 0.05; the frequency of dams having neonatal death (yes:no): CON 1:8, CT 3:4, F 5:3, FT 0:8: CON vs F P < 0.05, F vs FT P < 0.05).
<table>
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<th>FT</th>
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<th>Taurine</th>
<th>Interaction</th>
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<td>Maternal weight (g)</td>
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<td>298.7±8.1(^A)</td>
<td>319.4±9.9(^B)</td>
<td>328.8±10(^B)</td>
<td><strong>F=7.88</strong></td>
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<td>F=0.52 P=0.4771</td>
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<td>3.78±0.11(^a)</td>
<td>3.79±0.11(^a)</td>
<td>4.49±0.12(^b)</td>
<td>3.99±0.08(^a)</td>
<td><strong>F=17.47</strong></td>
<td><strong>P=0.0003</strong></td>
<td>F=5.52 P=0.0264</td>
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<td>Maternal total fat (%)</td>
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<td>F=0.0 P=0.9782</td>
<td>F=1.46 P=0.2368</td>
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<td>0.167±0.04</td>
<td>0.143±0.02</td>
<td>0.116±0.01</td>
<td>F=0.77 P=0.3873</td>
<td>F=0.01 P=0.9141</td>
<td>F=1.54 P=0.2253</td>
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<td>Birthweight (male, g)</td>
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<td>6.34±0.13(^B)</td>
<td>5.8±0.17(^A)</td>
<td>6.26±0.13(^B)</td>
<td>6.20±0.1(^A)</td>
<td><strong>F=4.39</strong></td>
<td><strong>P=0.0406</strong></td>
<td>F=2.95 P=0.0913</td>
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<td>Birthweight (female, g)</td>
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<td>6.33±0.15</td>
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</table>

**Table 3.1 Maternal and neonatal weights**
Values are presented as means ± SEM, n = 7-9 per group. Bold font indicates effect P value <0.05 via two-way ANOVA. Upper case letter (A, B) superscripts indicate comparison procedures were conducted between all groups fed fructose diet and all groups fed CON diet. Lower case letter superscripts (a,b,c) indicate multiple comparison procedures were conducted for diet and taurine interaction. Groups that do not share the same letter are significantly different from each other (P < 0.05).
(a) Pregnancy vs. Lactation:
- CON
- CT
- F
- FT

Food eaten (g) vs. Day

(b) Pregnancy vs. Lactation:
- CON
- CT
- F
- FT

Fluid intake (ml) vs. Day

(c) Pregnancy vs. Lactation:
- CON
- CT
- F
- FT
Figure 3.1 Food and fluid intake during pregnancy and lactation

(a) Total caloric intake (chow intake (CON and CT groups) plus additional calories derived from fructose (F and FT groups) during pregnancy and lactation; (b) Food intake — total chow intake (grams) during pregnancy and lactation and (c) total fluid intake during pregnancy and intake (grams) during pregnancy and lactation. Data are means ± SEM with 7–9 dams per group. * denotes P < 0.05 when CON and CT compared with F and FT.

3.2.3.2 Maternal plasma profile

Maternal plasma taurine was significantly increased in the CT and FT groups (Table 3.2). Maternal plasma triglyceride, cholesterol, lipase and free fatty acid were significantly increased by fructose consumption in the F and FT groups (Figure 3.2). HcY concentrations were significantly increased in response to fructose intake in F and FT dams (Table 3.2). Hyperinsulinemia (Table 3.2) and increased HOMA-IR indices (Figure 3.3) were observed in the F group, and were normalised by taurine supplementation in the FT group. Maternal plasma TNFα and IL-1β was significantly increased in F group compared to CON, CT and FT groups, while IL-6 concentrations were not affected by either fructose or taurine supplementation (Figure 3.4). There were no differences between groups in maternal plasma fasting fructose, glucose, BHB, uric acid or leptin at the end of lactation (Table 3.2).
<table>
<thead>
<tr>
<th>Groups</th>
<th>Effect</th>
<th>CON</th>
<th>CT</th>
<th>F</th>
<th>FT</th>
<th>Diet</th>
<th>Taurine</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose (µmol/l)</td>
<td></td>
<td>231.6±14.1</td>
<td>259.3±42</td>
<td>283.9±38</td>
<td>239.1±37.1</td>
<td>F=0.24</td>
<td>P=0.6305</td>
<td>F=1.20 P=0.2831</td>
</tr>
<tr>
<td>Taurine (µmol/l)</td>
<td></td>
<td>328±24.5\textsuperscript{A1}</td>
<td>436±40\textsuperscript{B1}</td>
<td>291±15.6\textsuperscript{A1}</td>
<td>428±15.4\textsuperscript{B1}</td>
<td>F=0.75</td>
<td>P=0.3947</td>
<td>F=22.02 \textsuperscript{P&lt;0.0001} P=0.3 P=0.5877</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td></td>
<td>6.11±0.49</td>
<td>7.0±0.40</td>
<td>7.9±0.78</td>
<td>7.11±0.36</td>
<td>F=3.40</td>
<td>P=0.076</td>
<td>F=2.94 P=0.0978</td>
</tr>
<tr>
<td>BHB (mmol/l)</td>
<td></td>
<td>1.09±0.13</td>
<td>0.91±0.11</td>
<td>0.77±0.06</td>
<td>0.8±0.08</td>
<td>F=3.18</td>
<td>P=0.0856</td>
<td>F=0.22 P=0.6448</td>
</tr>
<tr>
<td>Homocysteine (µM)</td>
<td></td>
<td>5.11±0.19\textsuperscript{A}</td>
<td>5.81±0.44\textsuperscript{A}</td>
<td>6.36±0.48\textsuperscript{B}</td>
<td>6.86±0.18\textsuperscript{B}</td>
<td>F=1.57</td>
<td>P=0.2218</td>
<td>F=3.18 P=0.0862</td>
</tr>
<tr>
<td>Uric acid (µM)</td>
<td></td>
<td>2.98±0.45</td>
<td>3.8±0.59</td>
<td>3.65±0.9</td>
<td>2.61±0.69</td>
<td>F=1.57</td>
<td>P=0.2218</td>
<td>F=0.92 P=0.3473</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td></td>
<td>1.42±0.13</td>
<td>1.61±0.18</td>
<td>1.68±0.08</td>
<td>1.42±0.1</td>
<td>F=0.07</td>
<td>P=0.7866</td>
<td>F=0.08 P=0.0844</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td></td>
<td>1.25±0.14\textsuperscript{a}</td>
<td>1.73±0.38\textsuperscript{a}</td>
<td>2.16±0.43\textsuperscript{b}</td>
<td>1.21±0.18\textsuperscript{a}</td>
<td>F=0.35</td>
<td>P=0.56</td>
<td>F=0.63 P=0.4348</td>
</tr>
</tbody>
</table>

Table 3.2 Maternal plasma profile

Values are presented as mean ± SEM, n = 7-9 per group. Bold font indicates effect P value <0.05 via two-way ANOVA, main effects were not indicated when significant interaction was determined. Comparisons between groups for each significant effect where applicable are denoted by 2 sets of superscripts. Upper case letter (A, B) superscripts indicate comparison procedures were conducted between all groups fed fructose diet and all groups fed CON diet. Upper case letter (A1, B1) superscripts indicate comparison procedures were conducted between groups with taurine supplementation and groups without taurine supplementation. Lower case letter superscripts (a,b,c) indicate multiple comparison procedures were conducted for diet and taurine interaction. Groups that do not share the same letter are significantly different from each other (P < 0.05).
Figure 3.2 Maternal plasma lipid concentrations

Values are presented as mean ± SEM, n = 7–9 per group. Comparisons between groups for each effect where significant are indicated by the bar, P < 0.05.

Figure 3.3 Maternal HOMA-IR indices
Values are presented as mean ± SEM, n = 7–9 per group. Comparisons between groups for each effect where significant are indicated by the bar, P < 0.05. HOMA-IR indices = Fasting glucose (mmol/l) × fasting insulin (mU/l)/22.5 (307)

Figure 3.4 Maternal plasma proinflammatory cytokine profiles

Values are presented as mean ± SEM, n = 7–9 per group. Comparisons between groups for each effect where significant are indicated by the bar, P < 0.05.
3.2.3.3 Maternal hepatic morphology

Macrovesicular and microvesicular steatosis were observed in F group (Figure 3.5). Scores related to steatosis and overall NAS score were significantly increased in F group compared to CON (Table 3.3). Maternal taurine supplementation reversed steatosis and overall NAS score in the FT group (Table 3.3).

![Figure 3.5 Maternal liver histology](image)

H&E staining, 40× magnification, representative micrographs from each experimental group: (A) CON group, (B) CT group, (C) F group, (D) FT group. n = 7–9 per group. Two sections per animal were examined under 5×, 20× and 40× magnification, respectively. Representative images were taken under 40× magnification. At least 10 random fields per animal were evaluated for NAS grading. Significant microvesicular steatosis characterized by small intracytoplasmic fat vacuoles was observed in the F group along with sparse macrovesicular steatosis characterized by round-shaped empty space between hepatocytes. CON, CT and FT groups showed normal liver morphology.
<table>
<thead>
<tr>
<th>Groups</th>
<th>CON</th>
<th>CT</th>
<th>F</th>
<th>FT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steatosis</td>
<td>0.11±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.14±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.29±0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.25±0.16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lobular inflammation</td>
<td>0.11±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0±0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.14±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.25±0.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hepatocyte ballooning</td>
<td>0±0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0±0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0±0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.13±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NAS score</td>
<td>0.22±0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.14±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.43±0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.63±0.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Table 3.3 Maternal liver NAFLD Activity Score (NAS)**

Values are presented as means ± SEM, n = 7-9 per group. Pairwise comparisons between groups were conducted via Wilcoxon rank sum test with Bonferroni correction, groups that did not share the same letter are significantly different from each other (P < 0.05).

**3.2.3.4 Maternal hepatic gene expression**

To further assess hepatic steatosis, we assessed markers of lipogenesis and inflammatory responsiveness. Hepatic sterol regulatory element-binding protein-1c (SREBP-1c), fatty acid synthase (FASN) and fructokinase expression was significantly increased in the F group when compared to the CON, CT and FT groups (Figure 3.6 A, B, C). Lipoprotein lipase (LPL) and CD36 was significantly increased in CT and FT groups compared to CON and F groups (Figure 3.6 D, E). Phosphoenolpyruvate carboxykinase (PEPCK), peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α) and peroxisome proliferator-activated receptor alpha (PPARα) expression was decreased by fructose consumption in F and FT groups compared to CON and CT (Figure 3.6 F, G, H). Hepatic IL-1β expression was down-regulated in fructose fed groups compared to CON and CT (Figure 3.7 B). No effect from either taurine or fructose was observed on maternal hepatic TNFα, IL6 or TLR4 expression (Figure 3.7 A, C, D).
Figure 3.6 Maternal hepatic gene expression of markers related to lipid and glucose metabolism
Values are presented as mean ± SEM, n = 7–9 per group. Comparisons between groups for each effect where significant are indicated by the bar, P < 0.05.

Figure 3.7 Maternal hepatic gene expression of pro-inflammatory markers

Values are presented as mean ± SEM, n = 7–9 per group. Comparisons between groups for each effect where significant are indicated by the bar, P < 0.05.
3.2.3.5 Neonatal plasma profile

Decreased blood glucose concentrations were observed in male neonates from F and FT groups when compared to CON and CT, but not in females (Table 3.4). In female offspring, plasma BHB concentrations were significantly increased in F and FT group compared to CON and CT (Table 3.4), while in male offspring plasma BHB concentrations were increased in F compared to CON and normalised in FT group (Table 3.4). No significant effect of either taurine or fructose was detected for neonatal plasma leptin and insulin concentrations (Table 3.4).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Main Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
</tr>
<tr>
<td>Males</td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.79±0.23B</td>
</tr>
<tr>
<td>BHB (mmol/l)</td>
<td>2.05±0.08b</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>1.18±0.24</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.7±0.21</td>
</tr>
<tr>
<td>Females</td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.41±0.18</td>
</tr>
<tr>
<td>BHB (mmol/l)</td>
<td>2.15±0.13A</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>1.5±0.3</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.81±0.47</td>
</tr>
</tbody>
</table>

Table 3.4 Neonatal plasma profile

Values are presented as mean ± SEM, n = 5-9 per group. Bold font indicates effect P value <0.05 via two-way ANOVA, main effects were not indicated when significant interaction was determined. Comparisons between groups for each effect where significant are denoted by 3 sets of superscripts. Upper case letter (A, B) superscripts indicate comparison procedures were conducted between all groups fed MO diet and all groups fed CON diet. Upper case letter (A1, B1) superscripts indicate comparison procedures were conducted between groups with taurine supplementation and groups without taurine supplementation. Lower case letter superscripts
(a,b,c) indicate multiple comparison procedures were conducted for diet and taurine interaction. Groups that do not share the same letter are significantly different from each other (P < 0.05).

### 3.2.3.6 Neonatal hepatic inflammatory profile

Neonatal hepatic PEPCK expression was increased in female F and FT neonates compared to CON and CT (Figure 3.8 A). In male offspring, PEPCK expression was increased in F neonates compared to CON and was normalised by maternal taurine supplementation in FT groups (Figure 3.8 A). IL-1β expression was decreased in male CT and FT neonates compared to CON and F groups (Figure 3.8 B). Hepatic TNFR1 was lower in male FT group than F group (Figure 3.8 C). No difference was observed in TNFR1 and IL-1β expression between groups in female offspring (Figure 3.8 B, C).
Figure 3.8 Neonatal hepatic gene expression.

Values are presented as mean ± SEM, n = 7–9 per group. Comparisons between groups for each effect where significant are indicated by the bar, P < 0.05.
3.2.4 Discussion

This study demonstrated significant metabolic and proinflammatory alterations in the response of dams and neonates to maternal high fructose consumption. Excessive fructose consumption during pregnancy and lactation led to increased maternal weight gain, elevated homocysteine concentrations, reduced insulin sensitivity, increased systemic proinflammatory cytokine concentrations and hepatic steatosis. Maternal taurine supplementation reversed indices associated with IR, proinflammatory plasma profiles and maternal hepatic steatosis. A maternal high fructose diet also resulted in sex-specific neonatal hypoglycemia and elevated plasma BHB concentrations which were partially reversed following taurine supplementation. Taken together, taurine supplementation has broad effects on fructose-induced maternal and neonatal health outcomes. There were small but significant changes in food and fluid intakes during pregnancy and lactation in fructose supplemented dams but these are consistent with our previous observations (308) and were not affected by taurine supplementation.

Importantly, the present study demonstrated the potential of maternal taurine supplementation as a therapeutic agent for amelioration of fructose-induced IR during pregnancy. IR is a condition whereby the body or specific target organs fail to respond appropriately to normal insulin concentrations, which often results in compensatory hyperinsulinemia (311). IR, along with obesity, type 2 diabetes, hypertension and NAFLD, represent a series of disorders which comprise the metabolic syndrome (311, 312). While IR is a normal physiologic state which gradually develops during pregnancy, excessive IR during pregnancy is strongly associated with gestational diabetes and preeclampsia (313, 314), both of which can lead to severe adverse pregnancy outcomes and negative long term effects on both mothers and offspring (315, 316). Studies by others have shown that taurine supplementation can attenuate IR in spontaneously diabetic rats and fructose-induced hyperglycemic rodent models (305, 317, 318). Our study confirms these published findings and for the first time extends them to a pregnancy setting. The mechanisms underpinning taurine-mediated reversal of IR remain largely unknown, and although not examined in the current study, others have hypothesised that the effect might be due to taurine’s antioxidative properties and/or by regulating glucose-stimulated insulin secretion via Ca^{2+} mobilisation (317, 319).

Links between obesity, IR and immune function have provided the basis for the emerging field of immunometabolism (320). This current study has demonstrated significant systemic proinflammatory cytokine concentrations in fructose-fed dams which are reversed following taurine supplementation. There is significant evidence that IL-1β and TNFα are master
regulators of immunometabolism, initiating inflammatory cascades which culminate in metabolic dysfunction (321). Studies in rodent models have demonstrated that blockade of IL-1β, TNFα and indeed their signaling pathways, can stem high-fat diet induced IR and diabetes (322, 323). Taurine has been shown to downregulate systemic inflammation in acute trauma, sepsis and other immune deficient conditions in non-pregnant subjects (324). Furthermore, the chlorinated form of taurine inhibits the production of proinflammatory mediators in innate immune cells via the NFκB signaling pathway (325-327). It is therefore possible that the beneficial effects on maternal metabolic parameters may be attributed to the anti-inflammatory actions of taurine.

Hepatic steatosis is a primary feature of NAFLD and can be attributed to increased de novo lipogenesis, increased lipid uptake, or reduced fatty acid utilization (328). There is now a substantial amount of evidence that fructose can induce hepatic steatosis via fructokinase-mediated fructose phosphorylation (329, 330). The present study demonstrates that fructose-fed mothers have increased expression of SREBP-1c which is responsible for de novo lipogenesis and its downstream target FASN (298, 331). While expression of genes associated with lipid uptake such as LPL and CD36 (332) were unchanged by fructose consumption, hepatic fatty acid β-oxidation related markers such as PPARα and PGC-1α (333) were downregulated. Taurine-induced resolution of maternal hepatic steatosis is likely due to act through lipogenic pathways as indicated by the changes in SREBP-1c signaling pathway which is key to the regulation of lipid homeostasis (334) and the reduction in fructokinase. Moreover, our previous study showed that taurine supplementation further exacerbated hepatic steatosis induced by an obesogenic diet high in fat and fructose (308), indicating that the taurine effect on the hepatic lipid metabolism might be conditional to the presence of additional dietary fat content.

The perinatal period is a critical window for plasticity in a DOHaD context (301). Adverse early life events can lead to offspring long term health problems. Several studies have shown that intervention during this critical period can have potentially long term benefit for the offspring (161, 172, 258, 335, 336). The present study shows that taurine supplementation reversed fructose-induced male offspring hepatic PEPCK, which potentially prevented maternal programming initiated predisposition to dysregulated glucose metabolism. PEPCK is a rate-controlling enzyme for gluconeogenesis and over-expression of hepatic PEPCK is associated with impaired glucose tolerance (337). Low protein models of developmental programming have indicated that upregulation of PEPCK expression can persist from early development to adulthood, resulting an impaired hepatic glucose output (148). It is therefore
possible that fructose-programmed early life PEPCK over-expression may increase the risk of dysregulated glucose metabolism in later life and taurine supplementation may protect against this. We also looked at the expression of other genes associated with IR and lipid metabolism in the neonates (Figure 3.9); however there were no significant changes observed which is unsurprising given the age and lack of metabolic challenge in these animals.

Neonatal hepatic proinflammatory cytokine expression was downregulated following taurine supplementation in male offspring. It has been reported that IL-1β knockout mice display attenuated hepatic steatosis and inflammation when exposed to alcohol and high cholesterol diet (338). Decreased neonatal hepatic IL-1β and TNFR1 expression in our study suggests that maternal taurine supplementation may offset the progress of liver injury mediated by inflammation in offspring when exposed to a later negative environment. Comparable results were observed in a previous study by our group in an obesogenic diet background, which found that taurine supplementation reversed maternal obesogenic diet-induced neonatal hepatic proinflammatory cytokine over-expression (308).

In this study, excessive fructose intake increased the proportion and frequency of neonatal death. Previous work has indicated that a high-fructose high-salt diet during pregnancy can reduce litter size (339). While a possible explanation for this observation is not yet clear, we did observe moderately elevated concentrations of circulating ketone bodies in neonates as a result of excessive maternal fructose intake. The blood concentration of ketone body is extremely sensitive to physiological alterations, any change in blood ketone body concentration (either decrease or increase) is likely to indicate a pathologic process (340). The most common cause of hyperketonemia during infancy is in response to fasting (341). Therefore, increased plasma BHB might indicate a starving status which might lead to an increased incidence of neonatal death. With taurine supplementation, no neonatal death was observed in fructose fed dams. However, in direct contrast to the fructose supplemented mothers, we observed that taurine supplementation in control pregnancy increased neonatal mortality. This finding has been reported in our previous study which utilised the same control groups (308). Due to the limited sample size and sex-specific effect of taurine, our observation is preliminary. However, possible taurine toxicity in the setting of normal pregnancy outcomes should be further investigated.

Taken together, the present data suggests that maternal taurine supplementation can normalise fructose-induced maternal metabolic dysfunction and may ameliorate the adverse developmental programming effects in offspring. This study extends previous findings on the
effects of taurine to treat fructose-induced metabolic dysfunction into a pregnancy setting. Moreover, it provides some evidence that dietary supplementation with taurine may present a non-invasive intervention strategy to prevent adverse programming effects in at-risk maternal environments where excessive fructose intake may play a significant role. However, given the increased neonatal mortality in control pregnancies supplemented with taurine, these effects are clearly dependent upon maternal nutritional background and further work is required to understand the differential outcomes between control and compromised pregnancies on health outcomes in offspring.
Figure 3.9 Neonatal hepatic gene expression of markers related to IR and lipid metabolism.

Values are presented as mean ± SEM, n = 7–9 per group.
3.3 Post-weaning offspring phenotype

The paper in Section 3.2 dealt with maternal and neonatal outcomes. In this section we present additional results from the above study related to outcomes in post-weaning offspring.

3.3.1 Methods

Following weaning, all offspring were fed a standard chow diet until postnatal day 150. Pubertal onset was checked from postnatal day 24 onwards for female offspring and from postnatal day 35 for male offspring. A food challenge test was performed at postnatal day 130 for 10-12 animals per group for 9 days. Animals were provided with 200g chow diet and 200g MO diet daily; animals had free choice to select which diet they preferred. Body weight and food intakes were recorded daily during this period. At postnatal day 143, 6 animals per group underwent an OGTT and repeated tail blood samples were taken at 0, 5, 10, 20, 30, 60, 90 and 120min. Offspring were culled at postnatal day 150. Fasting plasma insulin and leptin (CrystalChem, USA) were measured by rat specific ELISA. Single measured results were analysed by two-way ANOVA with maternal diet and taurine supplementation as main factors. Repeated measured data such as body weight, food intakes and OGTT results were assessed by repeated analysis of variance. All data in section 3.3 met the assumptions of parametric test (normality and homogeneity of variance). Detailed procedures are described in Chapter 2.

3.3.2 Offspring post-weaning weight gain

From weaning to postnatal day 150, there were no significant differences in growth trajectories between any groups in either male or female offspring (Figure 3.10).
Figure 3.10 Post-weaning offspring growth curve

A) Male and (B) female offspring body weights from weaning to postnatal day 150. Data are means ± SEM, n = 22-34 per group.
3.3.3 Adult offspring adiposity

There was no significant difference in retroperitoneal fat percentage (per body weight) in either male or female adult offspring (Figure 3-11 A, B).

Figure 3.11 Adult offspring adiposity

A) Male offspring retro fat percentage (per body weight); B) female offspring retro fat percentage (per body weight). Data are means ± SEM, n = 22-34 per group.
3.3.4 Onset of puberty

In male offspring, age at puberty onset was significantly lowered in CT, F and FT groups when compared with CON (Figure 3.12 A, C). Male offspring from CT, F and FT groups displayed significant lower body weight compared to CON offspring when entering puberty (Figure 3.12 E). In female offspring, pubertal onset was significantly brought forward in CT and F groups compared to CON and FT offspring (Figure 3.12 B, D). Weight at time of puberty was significantly reduced in offspring of CON and F mothers compared to those of CON and FT dams (Figure 3.12 F).
Figure 3.12 Age and weight at time of pubertal onset

A) % of male offspring which had entered puberty; B) % of female offspring which had entered puberty; C) average age of puberty onset in male offspring; D) average age of puberty onset in female offspring; E) weight of male offspring at onset of puberty; F) weight of female offspring at onset of puberty. Data are means ± SEM, n = 22-34 per group. Lower case letter superscripts (a,b,c) indicate multiple comparison procedures were conducted for diet and taurine interaction. Groups that do not share the same letter are significantly different from each other (P < 0.05).

3.3.5 Food challenge test

During a 9 day diet challenge, male offspring weight gain did not differ among the four groups (Figure 3-13 A). However, female offspring from the CT group had significantly less body weight gain compared to CON, F and FT groups, with a significant effect of maternal diet × taurine supplementation interaction observed (Figure 3-13 B). There was no significant difference in total calorie intake during the MO diet challenge period in either male or female offspring among all groups (Figure 3-14 A, B). However, the percentage of calories derived from MO diet was significantly reduced in CT and FT groups compared with CON and F groups in both male and female offspring (Figure 3-14 C, D).

Figure 3.13 Offspring body weight gain during food challenge test.

A) Male offspring body weight gain during the food challenge period; B) female offspring body weight gain during food challenge test. Data are means ± SEM, n = 10-12 per group. Lower case letter superscripts (a,b,c) indicate multiple comparison procedures were conducted for diet and taurine interaction. Groups that do not share the same letter are significantly different from each other (P < 0.05).
Figure 3.14 Offspring caloric intake during food challenge test

A) Male offspring body weight gain during the food challenge period; B) female offspring body weight gain during food challenge test. Data are means ± SEM, n = 10-12 per group. Upper case letter (A1, B1) superscripts indicate comparison procedures were conducted between groups with taurine supplementation and groups without taurine supplementation. Groups that do not share the same letter are significantly different from each other (P < 0.05).
3.3.6 Adult offspring OGTT

OGTT results revealed that the blood glucose concentrations in all groups for both sexes peaked at the 20 min sampling point after oral glucose loading and then decreased. There was no significant difference in tail blood glucose concentration at any time point of OGTT or area under the curve (AUC) of OGTT (Figure 3.15 A, B, C, D).

![Graphs showing OGTT results for male and female offspring](image)

**Figure 3.15 Offspring OGTT**

A) Male offspring tail glucose following an OGTT; B) female offspring tail glucose following an OGTT; C) male offspring area under the curve (AUC) following an OGTT; D) female offspring AUC following an OGTT. Data are means ± SEM, n = 6 per group.
3.3.7 Adult offspring plasma leptin and insulin concentration

We observed no significant differences in plasma insulin or leptin concentration in either male or female offspring between groups (Figure 3.15 A-F).

Figure 3.16 Offspring plasma leptin, insulin and HOMA-IR

A) Male offspring plasma insulin concentrations; B) female offspring plasma insulin concentrations; C) male offspring HOMA-IR index; D) male offspring HOMA-IR index. HOMA-IR index = Fasting glucose (mmol/l) × fasting insulin (mU/l)/22.5. Data are means ± SEM, n = 22-34 per group.
3.4 Additional discussion

3.4.1 Discussion on post-weaning offspring

Early life nutrition status influences reproductive development. In mammals, one of the most important periods of reproductive development is the attainment of puberty - a transition period whereby individuals progress from a juvenile to an adult state of reproductive maturity. The timing of puberty onset is controlled by a collection of complex interactions between genetic and environmental factors, with the latter providing fine tuning to maximize reproductive potential to fit the prevailing or predicted environment (342). It has been shown in human that prepubertal body composition can affect the progression of puberty, with higher body fat mass resulting in earlier attainment of pubertal stages (50, 343, 344). Sloboda et al. reported in rats that a maternal HF diet exposure can lead to early pubertal onset in offspring (345). Interestingly, improving maternal lipid profile with the anti-inflammatory lipid conjugated linoleic acid (CLA) reversed early pubertal onset in rodent offspring that were exposed to a maternal HF diet (346). In the present study, maternal excessive fructose consumption led to increased maternal plasma lipid concentrations and lowered the age of first puberty onset in both male and female offspring. Although there is no significant change in adiposity in adult offspring, we speculate that increased lipid supply during pregnancy and lactation might prime an early reproductive maturation in the offspring.

Our study showed that maternal taurine supplementation reversed early pubertal onset in female but not male offspring born to high fructose fed dams. We speculate that this reversal effect of taurine may be via leptin acting on the central reproductive axis. The initiation of puberty in mammals needs an increase in pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus (347). GnRH enters the pituitary and induces the synthesis and release of the pituitary gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH), which act on target cells in the testes and ovaries to induce the development of sperm and ovum, as well as the secretion of steroid hormones (348). Growing evidence has pointed out that the amplified GnRH neuronal network activity can be affected by leptin via interneuronal pathways (349-352). Administration of leptin restores fertility in leptin deficient ob/ob mice (353) and led to early onset of puberty in normal mice (354). It was proposed that puberty is metabolically gated (occurring only when there is sufficient energy storage) and leptin may serve as a signal of nutritional state that permits the activation of reproductive axis (351, 355). In the present study, taurine supplementation displayed a trend towards reversing increased plasma leptin concentrations in female neonatal offspring exposed to maternal high
fructose consumption \((P = 0.069, \text{Table 3.4})\), which indicates a possible modification of leptin regulation by taurine in the early life period. Interestingly, plasma leptin concentrations showed a trend towards reduction in taurine supplemented control neonates (Table 3.4) which coincidently echoes the early pubertal onset in the offspring from the same groups. Nonetheless, further investigation - particularly on prepubertal leptin regulation and hypothalamic GnRH neuronal network - is needed to uncover the effect of taurine supplementation on reproductive maturity.

Another interesting observation in the present study is the altered food preference in adult offspring due to maternal taurine supplementation. Offspring born to mothers with taurine supplementation during pregnancy and lactation show less preference to a high fructose high fat diet than offspring born to mothers without taurine consumption regardless maternal diets. There is evidence suggesting that the drive to consume high fat and/or high sugar food is beyond the need to satisfy hunger \((356, 357)\). The consumption of high fat and/or high sugar food can induce the synthesis and secretion of opioids and dopamine and thus activate the central reward system in a manner similar to alcohol and drugs \((358-362)\), and taurine has shown the potential to interact with the central reward system \((363, 364)\). Taurine is the second most abundant cerebral amino acid with multiple cellular functions including a central role as a neurotransmitter \((365)\). Administration of taurine in rats via intraperitoneal injection \((363)\) or local perfusion \((364)\) can increase dopamine concentrations in the ventral tegmental area which is the main region of central reward system. It has been proposed that the perinatal period, particularly from pregnancy to weaning in rodents, is the critical window for the development of the central reward system \((366)\). Although we lack direct evidence, it is plausible to speculate that taurine supplementation during the critical window of the brain development might involve long term modifications in the central reward system and lead to the altered food preference we observe. Nonetheless, the food challenge test in our study was only conducted for 9 days, it is not clear whether this preference change is stable and persistent, and if so, whether this preference change can lead to long term metabolic consequences.

Although we observed reduced hepatic PEPCK expression in newborns from mothers fed high fructose diet, we did not find evidence of impaired glucose metabolism in adult offspring. This might be because the offspring in our study were not metabolically challenged as they were fed chow diet after weaning. However, given the association between PEPCK and glucose metabolism (see discussion in Section 3.2.4), we cannot rule out that these animals may have increased susceptibility to glucose metabolic disorder. In fact, in many cases in developmental
programming, only when exposed to a susceptible postnatal environment, offspring fail to respond to the insult adequately and thus display a disease phenotype. Further investigation regarding potential molecular level modifications and environment interactions are essential to understand whether adult disease risk is increased in offspring.

3.4.2 Contribution and significance

Firstly, we report the first data on the effects of excessive fructose intake on maternal health during pregnancy in an animal model. Most reported studies on maternal obesogenic developmental programming models mainly focus on offspring outcomes and lack a thorough examination of maternal metabolic conditions. Excessive fructose intake has been studied in many non-pregnant animal models with detrimental effects on glucose and lipid metabolism (305, 317, 318). Pregnancy is accompanied by many physiological changes including those related to glucose and lipid metabolism, yet few studies have looked at the fructose intake in this context. We provide initial data on maternal physiological changes following high fructose consumption during pregnancy and lactation.

An important feature of this study is that the level of fructose in the experimental condition was designed to provide 20% of total calories - which is lower than DGAC Dietary Guidelines suggested maximum level of sugars (25%) (Section 3.1). This study is the first to identify the adverse effect of fructose consumption during pregnancy and lactation at an “accepted” high level in a rodent model. Our findings suggest that the guidelines for fructose consumption, particularly during pregnancy, may need to be re-evaluated due to the maternal obesogenic phenotype, pro-inflammatory status, IR, hepatic steatosis, and offspring effects we observed (Section 3.2 and 3.3).

Secondly, we also demonstrate that taurine supplementation can ameliorate most of the detrimental effects in dams and offspring caused by excessive fructose consumption during pregnancy and lactation, and this is the first study to our knowledge to demonstrate such an effect in the context of pregnancy. Our study provides some evidence that dietary supplementation with taurine can be used as a non-invasive intervention strategy to prevent adverse programming effects in at-risk maternal environments where excessive fructose intake may play a significant role.
3.4.3 Limitations

One limitation of this study is the use of pure crystalline fructose – a deliberate choice was made to investigate purely the effects of a single sugar – however this may not reflect a typical human situation. In a human setting, beverages and food products often use HFCS – which contains an approximately equal mixture of fructose and glucose (367). The immediate metabolic response to fructose and glucose intake is different however - dietary fructose consumption can cause significant increase in plasma triglyceride concentration, whereas glucose does not. Furthermore, glucose consumption increases blood glucose concentrations and insulin secretion while fructose does not (368, 369). Given the different metabolic features of fructose and glucose, additional pure fructose consumption in our study may not precisely reflect the use of HFCS in human setting.

Excessive fructose consumption is known to contribute to increased blood pressure in animal models (370, 371). However, in order to limit stress during pregnancy, we did not measure blood pressure in the dams. Therefore, we cannot fully exclude the possibility that these dams might be hypertensive and the programming effects in offspring maybe partially resultant from blood pressure-mediated effects. Nonetheless, we speculate that dams are unlikely to have hypertension in the present study because the amount of fructose consumption given is less than half of the level reported to induce hypertension (370, 371), and the offspring in the present study didn’t display features of IUGR - a common complication of hypertension during pregnancy (372).

In order to investigate long term effects in adult offspring, we sampled neonates rather than late gestation fetuses to evaluate early stage programming effects in offspring. However, it is known that glucose and lipid metabolism undergoes many dynamic changes in response to milk intake immediately after birth (373-375). It is conceivable that neonates from each litter might be at slightly different stages of milk ingestion during the sample collection, depending on their exact birth times. This may explain the variation in the hepatic gene expression of markers related to glucose and lipid metabolism in the neonates that we observe in our study (Section 3.2.4 Figure 3.8). Interpreting neonatal gene expression data in this study should consider such effects.

3.4.4 Future direction

Despite the reversal effect of taurine on maternal and neonatal health outcomes in a high fructose consumption setting, there is limited evidence of altered long term health outcomes in
adult offspring. Given the observation on food preference change, we are interested in investigating several aspects that affect food ingestion such as gut microbiome profile, taste receptors on taste buds and hypothalamic neural pathways.

We see the timing of pubertal onset is changed as a result of maternal fructose intake. This suggests that the reproductive function in these offspring may be altered, and it would be of interest to investigate male testis and female ovary function in future studies.
Chapter 4. Maternal obesogenic environment (high fructose high fat diet): Maternal and offspring outcomes and effect of taurine supplementation

4.1 Preface

This section contains an unaltered published research article “Effects of taurine supplementation on hepatic markers of inflammation and lipid metabolism in mothers and offspring in the setting of maternal obesity” (Appendix IV). The article was published in the journal PLoS ONE in 2013. PLoS ONE is a peer-reviewed, open-access journal for original research from all disciplines within science and medicine. The journal had an impact factor: 3.534 in 2013. Since published, the article has been cited 17 times.

In the previous section, we showed that excessive fructose consumption during pregnancy can lead to detrimental health outcomes in mothers and potentially predispose offspring to steatohepatitis and T2DM in a sex-specific manner. We also showed that taurine supplementation can reverse negative effects associated with fructose in mothers and can have beneficial effect on offspring health outcomes. Together, these observations provide valuable information contributing to the understanding of high fructose diets. However, Western diets are more complex than overconsumption of fructose. It is not clear how maternal and offspring health outcomes would be affected when exposed to a complex diet containing a mixture of high fructose and high fat contents. More importantly, can taurine supplementation still confer beneficial effects to mothers and offspring in the complex obesogenic environment typical of Western diets.
4.2 Maternal and neonatal health outcomes

Effects of Taurine Supplementation on Hepatic Markers of Inflammation and Lipid Metabolism in Mothers and Offspring in the Setting of Maternal Obesity

Li M1, Reynolds CM1, Sloboda DM1,2, Gray C1, Vickers MH1.

1Liggins Institute and Gravida: National Centre for Growth and Development, University of Auckland, Auckland, New Zealand.

2Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada.

Abstract

Maternal obesity is associated with obesity and metabolic disorders in offspring. However, intervention strategies to reverse or ameliorate the effects of maternal obesity on offspring health are limited. Following maternal undernutrition, taurine supplementation can improve outcomes in offspring, possibly via effects on glucose homeostasis and insulin secretion. The effects of taurine in mediating inflammatory processes as a protective mechanism have not been investigated. Further, the efficacy of taurine supplementation in the setting of maternal obesity is not known. Using a model of maternal obesity, we examined the effects of maternal taurine supplementation on outcomes related to inflammation and lipid metabolism in mothers and neonates. Time-mated Wistar rats were randomised to either: 1) control: control diet during pregnancy and lactation (CON); 2) CON supplemented with 1.5% taurine in drinking water (CT); 3) maternal obesogenic diet (high fat, high fructose) during pregnancy and lactation (MO); or 4) MO supplemented with taurine (MOT). Maternal and neonatal weights, plasma cytokines and hepatic gene expression were analysed. A MO diet resulted in maternal hyperinsulinemia and hyperleptinemia and increased plasma glucose, glutamate and TNFα concentrations. Taurine normalised maternal plasma TNFα and glutamate concentrations in MOT animals. Both MO and MOT mothers displayed evidence of fatty liver accompanied by alterations in key markers of hepatic lipid metabolism. MO neonates displayed a pro-inflammatory hepatic profile which was partially rescued in MOT offspring. Conversely, a pro-inflammatory phenotype was observed in MOT mothers suggesting a possible maternal trade-off to protect the neonate. Despite protective effects of taurine in MOT offspring, neonatal mortality was increased in CT neonates, indicating possible adverse effects of taurine in the setting of normal pregnancy. These data suggest that maternal taurine supplementation
may ameliorate the adverse effects observed in offspring following a maternal obesogenic diet but these effects are dependent upon prior maternal nutritional background.
4.2.1 Introduction

Obesity and overweight during pregnancy has become a major emerging issue for maternal and neonatal health over the past decade (376, 377). Periconceptional and gestational obesity are associated with IR and low-grade inflammation which increases the incidence of gestational diabetes, preeclampsia, miscarriage, and neonatal mortality and the long-term risk of developing metabolic syndrome (314, 378, 379). A recent clinical study highlighted the relationship between intrahepatic fat and IR in women with previous gestational diabetes (GDM) (380), indicating mild hepatic steatosis in postpartum women may contribute to IR-related metabolic dysfunction.

In addition to metabolic disorders and adverse pregnancy outcomes, maternal obesity has been shown to impact the long term health of the offspring (381). The developmental origins of health and disease (DOHaD) paradigm proposes that insults such as poor maternal nutrition during critical windows of development, can lead to an increased propensity in offspring to develop obesity and related metabolic and cardiovascular disorders in later life (301). Both human studies (382, 383) and animal models (69, 81) clearly show a link between maternal obesity and heightened risk of metabolic disorders in offspring, yet relatively little is known about the mechanisms involved. Therefore, broad lifestyle recommendations remain the most common preventative strategies (384).

A number of studies have reported the effectiveness of taurine (2-aminoethanesulfonic acid) in treating IR (306, 385, 386). Taurine is a sulphonic amino acid derived from methionine and cysteine metabolism and is found ubiquitously in all mammalian tissues. The synthesis and metabolism of taurine has known species-specific differences although taurine can be synthesised in vivo in both the human and rodent (303). Taurine is involved in bile acid synthesis, osmoregulation, modulation of neurotransmitters, glucose homeostasis and insulin secretion (304, 387). Reports suggest that taurine supplementation can enhance insulin sensitivity through modification of insulin signaling enzymes in fructose-fed rats (388). Furthermore, maternal taurine supplementation to low protein mothers has been documented to normalise pancreatic islet development in offspring with normalisation of glucose and insulin homeostasis in later life (172, 255, 258). These beneficial effects on glucose metabolism have been shown to persist into adult life (260). Although the effects of maternal taurine supplementation as relates to improved glucose homeostasis and beta-cell function in offspring have been well documented, the direct effects of taurine supplementation on the mother are not well documented. Further, taurine has been proposed to play a role in
mediating inflammatory processes but this has yet to be examined as a potential mechanism by which maternal taurine supplementation leads to protective effects in the offspring. Recent work by Lin et al. has shown that taurine can improve obesity-induced inflammatory responses and modulates the unbalanced phenotype of adipose tissue macrophages (389). Obesity is characterised by a state of low grade inflammation and maternal obesity is well established to lead to obesity and related metabolic disorders in offspring (70, 81). In this context, the efficacy of maternal taurine supplementation as an intervention in the setting of maternal obesity has yet to be investigated. Since most studies in the area of developmental programming focus on offspring outcomes, very little attention is paid to the direct effects on maternal health and wellbeing. The current study therefore investigated the effect of taurine supplementation to pregnant and lactating dams fed either a control or obesogenic diet on both maternal and offspring metabolic and hepatic inflammatory profiles.
4.2.2 Methods

4.2.2.1 Animal Model

Ethics statement

All procedures described were approved by the Animal Ethics Committee at the University of Auckland (Approval R888).

Virgin Wistar rats were time mated at 100 days of age using an estrous cycle monitor (EC-40, Fine Science Tools, San Francisco, USA). Day 1 of pregnancy was determined by the presence of spermatozoa after a vaginal lavage. Pregnant rats were then housed individually with free access to food and water and maintained at 25°C and a 12 h light: 12 h darkness cycle. Animals were randomly assigned to one of four nutritional groups: control group (CON) fed a standard chow diet (Diet 2018, 24% calories from protein, 18% from fat, 58% from carbohydrate, Harlan Teklad, Blackthorn, Bicester, UK) ad libitum throughout pregnancy and lactation (n = 9); control taurine group (CT) fed standard chow diet with additional 1.5% w/v taurine supplementation in drinking water (255, 258) (n = 7); maternal obesogenic diet group (MO) fed a high-fat high-fructose diet (20% calories from protein, 45% from fat, 35% from carbohydrate (including 26% from fructose); Diet D03101602, Research Diets, NJ, USA; n = 8); maternal obesogenic diet and taurine group (MOT) fed the obesogenic diet with additional 1.5% w/v taurine supplementation in drinking water (n = 8).

Two discrete time points were investigated. Firstly, effects of maternal taurine supplementation on neonatal outcomes and secondly the direct effects of taurine on maternal lipid and inflammatory profiles at the end of the lactation period. Maternal body weight, food and fluid intake were recorded daily. After birth, litter size was adjusted to 8 pups per litter (post-weaning offspring were utilised in an independent study). Neonatal plasma and liver samples were collected from randomly chosen excluded pups following decapitation. Litter size, sex ratio and birth weight were recorded at the time of birth. At the end of lactation, dams were fasted overnight and killed by decapitation following anaesthesia with sodium pentobarbitone (60 mg/kg IP). Maternal body composition was measured by dual energy X-ray absorptiometry using dedicated small animal software (DEXA, Lunar Prodigy, Madison, WI, USA). Maternal and neonatal blood glucose and β-hydroxybutyrate (BHB) were measured from tail blood samples using a glucose meter (Optium, Abbott Laboratories) at the time of cull.
4.2.2.2 Plasma Analysis

ELISA kits were used to measure plasma insulin and leptin (CrystalChem, USA), tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 (Quantikine ELISA; R&D Systems Europe, Abingdon, UK). Plasma uric acid was measured using a commercially sourced assay kit (Cayman Chemical, Ann Arbor, MI, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as: Fasting glucose (mmol/l)×fasting insulin (mU/l)/22.5 (309). Maternal plasma glutamate and taurine were analysed using a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan). Homocysteine (HcY) was measured by commercial immunoassay (Abbott AxSYM system). Limited plasma sample precluded measurement of taurine, glutamate or HcY in neonatal samples.

4.2.2.3 Hepatic mRNA Expression

Total RNA was isolated from liver tissue using RNeasy® mini kit (QIAGEN, Hilden, Germany) and cDNA synthesized from 2 µg of RNA by using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen™; Life Technologies Corporation, California, USA). Real-time PCR analysis for maternal hepatic sterol regulatory element-binding protein-1c (SREBP-1c), peroxisome proliferator-activated receptor alpha (PPARα), lipoprotein lipase (LPL), silent mating type information regulation 2 homolog 1 (SIRT1), fructokinase and phosphoenolpyruvate carboxykinase (PEPCK) expression was performed using LightCycler®480 SYBR green I master (Roche Diagnostics; Auckland, New Zealand). The relative amounts of genes were quantitated using standard curve and normalized to the geometric mean of cyclophilin A and β-actin expression. Real time PCR analysis for maternal hepatic fatty acid synthase (FASN), CD36, TNFα, IL-1β, IL-1R1 and all neonatal samples was carried out by using PreDeveloped TaqMan® Assay Reagent Kits in the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). To control for between-sample variability, mRNA levels were normalized to the geometric mean of cyclophilinA and hypoxanthine phosphoribosyltransferase (HPRT) for each sample by subtracting the Ct of controls from the Ct for the gene of interest producing a Ct value. The ΔCt for each treatment sample was compared to the mean ΔCt for control samples using the relative quantification 2-(ΔΔCt) method to determine fold-change (310).

4.2.2.4 Histological Analysis

5 mm thick representative sections from the left lobe of maternal livers were fixed in paraformaldehyde and paraffin embedded. Cross sections were prepared using Leica RM 2135
rotary microtome (Leica Instruments, Nussloch, Germany). Haematoxylin and Eosin (H&E) staining was conducted for general histology. Sections were mounted using distrene plasticizer xylene (DPX) mounting medium (BioLab ltd, New Zealand) and analysed under light microscope (Nikon 800, Tokyo, Japan) and images taken (Nikon FDX-35, Tokyo, Japan) and processed with NIS Elements-D software (Nikon, Tokyo, Japan). Scoring of steatosis, lobular inflammation, hepatocyte ballooning and overall NAS score (NAFLD Activity Score) was undertaken by a blinded observer using the methodology of Kleiner et al. (284). The scoring system comprised four primary features evaluated semi-quantitatively: steatosis (0–3), lobular inflammation (0–3), hepatocellular ballooning (0–2) and NAS score (unweighted sum of steatosis, lobular inflammation, and hepatocellular ballooning scores).

4.2.2.5 Statistical Analysis

Data analysis was completed using factorial analysis of variance (ANOVA) using SigmaStat software (Systat Software, San Jose, Ca, USA). For the maternal data, maternal diet and taurine were used as factors. For neonatal data, maternal diet, taurine and were used as factors. Where appropriate, post-hoc analyses were performed (Holm-Sidak method) to determine which groups were significantly different from each other. Maternal body weight data was analysed using repeated measures analysis. Maternal liver histology data were analysed via non-parametric methods (Wilcoxon rank sum test followed by Bonferonni correction). Data that failed to meet the criteria required for parametric analysis (normal distribution and equal variance) were transformed where necessary. All data are shown as means ± SEM. A p-value of <0.05 was accepted as statistically significant.
4.2.3 Results

4.2.3.1 Maternal and Offspring Weights

A maternal obesogenic diet resulted in an overall increase in maternal body weights in both MO and MOT groups (Figure 4.1) during pregnancy compared to CON and CT groups and was statistically significant from day 8 to day 18 gestation. Of note there was a small decrease in maternal body weights in CT dams compared to CON which was not evident in the MOT group and this was reflected in a significant maternal diet taurine statistical interaction ($P < 0.0001$). In the immediate post-partum period, MO and MOT dams remained significantly heavier than CON and CT mothers but there were no differences in maternal body weights between groups for the remainder of the lactation period (Figure 4.1). Birth weights were significantly reduced in female MO and MOT offspring compared to CON and CT groups (Table 4.1) but were not different between male groups. Interestingly, maternal taurine supplementation significantly increased neonatal mortality in the CT group (CON 1.4±0.1%; CT 7.1±4.0%, $P < 0.05$), without significant difference between CON, MO and MOT groups. Litter size and sex ratios were not affected by maternal diet or taurine supplementation (data not shown). Total maternal fat mass was significantly increased in MO but not in MOT animals when compared with CON at the end of lactation (Table 4.1). An effect of maternal diet was observed on relative maternal liver weight, increasing significantly in MO and MOT groups compared to CON (Table 4.1). Weaning weight was significantly increased in MO offspring compared to CON. There was a significant maternal diet x taurine interaction ($P = 0.01$) in males whereby CT body weights were higher than CON but the reverse holding true in MO groups with MO weights being higher than MOT. Weaning (postnatal day 22) weights were not significantly different between any of the female offspring groups (Table 4.1).
Figure 4.1 Maternal body weight during pregnancy and lactation

Body weights were measured daily throughout pregnancy and gestation. Data are means ± SEM, n = 7–9 per group. * denotes P < 0.05 when CON and CT compared with MO and MOT.
Table 4.1 Maternal, neonatal and weaning weight data

Values are presented as means ± SEM, n = 7-9 per group for maternal data, minimum 20 per group for birth and weaning weights. Bold font indicates effect P value < 0.05 via two-way ANOVA. Upper case letter (A, B) superscripts indicate comparison procedures were conducted between all groups fed MO diet and all groups fed CON diet. Lower case letter superscripts (a, b) indicate multiple comparison procedures were conducted for diet and taurine interaction. Groups that do not share the same letter are significantly different from each other (P < 0.05).

4.2.3.2 Maternal Plasma Profile

Maternal plasma taurine was increased in CT and MOT groups compared to CON and MO groups (Table 4.2). Maternal fasting glucose was increased while BHB concentrations decreased in MO and MOT groups compared to CON and CT groups and there was no effect of taurine (Table 4.2). Maternal plasma uric acid was significantly decreased in MOT compared to MO groups (Table 4.2). MO and MOT groups displayed significant hyperinsulinemia and increased HOMA-IR indices when compared to CON and CT groups (Table 4.2 and Figure 4.2 A). Hyperleptinemia was observed in MO but not MOT groups although there was no significant overall effect of taurine supplementation. TNFα was significantly increased in MO compared to CON, CT and MOT groups. Plasma IL-1β and IL-6 concentrations were not affected by diet or taurine supplementation (Table 4.2). Maternal plasma homocysteine (HcY) concentrations were significantly increased in response to the
maternal obesogenic diet in MO and MOT dams (Figure 4.2 B). Plasma glutamate concentrations were significantly increased in MO dams compared to all other groups (Figure 4.2 C, maternal diet and taurine interaction $P < 0.05$).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Effect</th>
<th>Taurine</th>
<th>Interaction</th>
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</thead>
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<tr>
<td>Taurine (umol/l)</td>
<td>Diet</td>
<td>F=0.00304</td>
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<tr>
<td>MO</td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>F=10.186</td>
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Table 4.2 Maternal plasma profile

Values are presented as mean ± SEM, n = 7-9 per group. Bold font indicates effect P value <0.05 via two-way ANOVA, main effects were not indicated when a significant interaction was determined. Comparisons between groups for each significant effect where applicable are denoted by 3 sets of superscripts. Upper case letter (A, B) superscripts indicate comparison procedures were conducted between all groups fed MO diet and all groups fed CON diet. Upper case letter (A1, B1) superscripts indicate comparison procedures were conducted between groups with taurine supplementation and groups without taurine supplementation. Lower case letter superscripts (a, b, c) indicate multiple comparison procedures were conducted for diet and taurine interaction. Groups that do not share the same letter are significantly different from each other (P < 0.05).
Figure 4.2 Maternal HOMA-IR, plasma homocysteine and glutamate concentrations

A) Maternal HOMA-IR values; B) maternal plasma homocysteine (HcY); C) maternal plasma glutamate concentrations. Data are means ± SEM, n = 7-9 per group. Upper case letter (A, B) superscripts indicate comparison procedures were conducted between all groups fed MO diet and all groups fed CON diet. Lower case letter superscripts (a, b, c) indicate multiple comparison procedures were conducted for diet and taurine interaction. Groups that do not share the same letter are significantly different from each other (P < 0.05).
4.2.3.3 Maternal Hepatic Morphology

Given the changes observed in maternal liver weights in response to diet as shown in Table 4.1, analysis of the histological features associated with NAFLD was undertaken using the method of Kleiner et al. (284). Scores related to steatosis, lobular inflammation, hepatocyte ballooning and overall NAS score were significantly increased in MO and MOT groups compared to CON and CT groups. There was no significant effect of maternal taurine supplementation on any of the markers analysed (Table 4.3 and Figure 4.3).

<table>
<thead>
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<th>CT</th>
<th>MO</th>
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<td>4.63±0.53a</td>
<td>3.86±0.34a</td>
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**Table 4.3 Maternal liver scoring**

Values are presented as means ± SEM, n = 7-9 per group. Pairwise comparisons between groups were conducted via Wilcoxon rank sum test with Bonferroni correction, groups that did not share the same letter are significantly different from each other (P < 0.05).
Figure 4.3 Maternal liver histology

Left column, H&E, 10× magnification; right column 40× magnification. CON: control, n = 9; CT: control with taurine, n = 7; MO: maternal obesogenic diet, n = 8; MOT: MO maternal obesogenic diet with taurine, n = 7. Arrows indicate ballooned hepatocytes.
4.2.3.4 Maternal Hepatic Gene Expression

To further assess the fatty liver phenotype observed via hepatic morphology, we conducted gene expression analysis for genes related to lipogenesis and inflammatory responsiveness. Hepatic SREBP-1c expression was significantly increased in the MO and MOT groups when compared to the CON and CT groups (Figure 4.4 A). FASN expression was increased in both MO and MOT groups compared to CON and CT (Figure 4.4 B). PPARα was significantly down-regulated in MO and MOT groups compared to CON and CT groups (Figure 4.4 C). LPL was significantly increased in MO and MOT groups compared to CON and CT and increased in CT versus CON groups (Figure 4.4 D). Fructokinase expression was significantly reduced in MOT group compared to CON, CT and MO groups (Figure 4.4 E). CD36 expression was significantly increased in CT and MOT groups when compared to CON and MO groups (Figure 4.4 F). SIRT1 expression was increased in CT versus CON and MOT groups (Figure 4.4 G). PEPCK was significantly reduced in both MO and MOT groups compared to CON and CT (Figure 4.4 H). Hepatic TNFα, IL-1β and IL-1R1 expression was significantly increased in MOT animals compared to CON, CT and MO groups (Figures 4.5 A-C). No effect was observed on maternal hepatic TNFR1 expression (Figure 4.5 D). Overall main effect statistical data are provided in Table 4.4.
Figure 4.4 Maternal hepatic lipid and glucose metabolism related gene expression

Values are presented as mean ± SEM, n = 7-9 per group. Bold font indicates effect P value <0.05 via two-way ANOVA, main effects were not indicated when significant interaction was determined. Comparisons between groups for each effect where significant are denoted by 3 sets of superscripts. Upper case letter (A, B) superscripts indicate comparison procedures were conducted between all groups fed MO diet and all groups fed CON diet. Upper case letter (A1, B1) superscripts indicate comparison procedures were conducted between groups with taurine supplementation and groups without taurine supplementation. Lower case letter superscripts (a, b, c) indicate multiple comparison procedures were conducted for diet and taurine interaction. Groups that do not share the same letter are significantly different from each other (P < 0.05).

Figure 4.5 Maternal hepatic inflammatory gene expression.

A) TNFα; B) IL-1β; C) IL-1R1; D) TNFR1. Lower case letter superscripts (a, b, c) indicate multiple comparison procedures were conducted for diet and taurine interaction. Groups that do not share
the same letter are significantly different from each other ($P < 0.05$). Data are means ± SEM, $n = 7–9$ per group.

<table>
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<th>Interaction</th>
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<td>F=6.737</td>
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<td></td>
<td>P=0.015</td>
<td>P=0.269</td>
<td>P=0.153</td>
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<td>LPL</td>
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<td>SIRT1</td>
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Table 4.4 Maternal gene expression main effects

$N = 7-9$ per group. SREPB1, sterol regulatory element-binding protein-1c; FASN, fatty acid synthase; PEPCK, phosphoenolpyruvate carboxykinase; LPL, lipoprotein lipase; PPAR-α, peroxisome proliferator-activated receptor alpha; SIRT1, silent mating type information regulation 2 homolog 1; CD36, cluster of differentiation 36; IL, interleukin.; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor 1.
4.2.3.5 Neonatal Plasma and Physiology Profile

There were no significant effects of maternal diet or taurine supplementation on neonatal plasma insulin or leptin concentrations although there was a trend toward reduced leptin concentrations in MO and MOT offspring versus controls (P = 0.075 and P = 0.07 for males and females respectively, Table 4.5). Plasma BHB concentrations were significantly reduced in CT, MO and MOT offspring compared to CON and there was a significant diet and taurine interaction in both male and female neonates (CON>CT but MO<MOT) (Table 4.5). Female offspring relative liver weights were significantly increased in MO and MOT compared to CON and CT groups (CON 3.9±0.1%; CT 3.8±0.2%; MO 4.0±0.1%; MOT 4.2±0.1%). Interestingly, male offspring relative liver weights did not differ among groups (data not shown).

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<td>Insulin (ng/ml)</td>
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<td>BHB (mmol/l)</td>
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<td>Glucose (mmol/l)</td>
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<td>BHB (mmol/l)</td>
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Table 4.5 Neonatal plasma profile

Values are presented as mean ± SEM, n = 7-9 per group. Bold font indicates effect P value <0.05 via two-way ANOVA, main effects were not indicated when significant interaction was determined. Comparisons between groups for each effect where significant are denoted by 3 sets of superscripts. Upper case letter (A, B) superscripts indicate comparison procedures were conducted between all groups fed MO diet and all groups fed CON diet. Upper case letter (A1, B1)
superscripts indicate comparison procedures were conducted between groups with taurine supplementation and groups without taurine supplementation. Lower case letter superscripts (a, b, c) indicate multiple comparison procedures were conducted for diet and taurine interaction. Groups that do not share the same letter are significantly different from each other (P < 0.05).

4.2.3.6 Neonatal Hepatic Inflammatory Profile

IL-1R1 expression in male and female neonates was significantly increased in MO offspring compared to all other groups (Figure 4.6 A). Hepatic IL-1β expression was increased in female MO neonates compared to all other female groups. In male neonates, IL-1β was decreased in CT versus all other groups (Figure 4.6 B). TNFR1 expression was decreased in MOT female neonates compared to the MO group (Figure 4.6 C). There were no significant differences in TNFR1 between the male neonatal groups. There were no differences in neonatal hepatic TNFα expression (Figure 4.6 D). There were no significant differences in markers related to IR or lipid metabolism (Figure 4.7).

![Graphs showing neonatal liver inflammatory gene expression](image)

Figure 4.6 Neonatal liver inflammatory gene expression

Neonatal hepatic gene expression of A) IL-1R1; B) IL-1β; C) TNFR1 and D) TNFα. Lower case letter superscripts (a, b, c) indicate multiple comparison procedures were conducted for diet and
taurine interaction. Groups that do not share the same letter are significantly different from each other (P < 0.05). Data are means ± SEM, n = 7-9 per group.

Figure 4.7 Neonatal hepatic gene expression of markers related to IR or lipid metabolism
Data are means ± SEM, n = 7-9 per group.

4.2.4 Discussion

The present study demonstrated significant metabolic and inflammatory changes in dams and neonates in response to a maternal high-fat:high-fructose diet. Dams fed the obesogenic diet displayed an adverse metabolic phenotype which included increased weight gain, hyperglycaemia, hyperhomocysteinemia, IR, and evidence of hepatic steatosis and inflammation. Maternal taurine supplementation significantly attenuated the proinflammatory plasma profile and plasma glutamate induced by obesogenic diet. Despite these beneficial systemic effects, maternal hepatic lipid metabolism and inflammatory profile were further impaired in response to taurine supplementation. Conversely, the neonatal hepatic immunophenotype, which was worsened by maternal obesogenic diet, was normalised by maternal taurine supplementation. However, it must be noted that the neonatal and maternal time-points represent independent observations and causative associations can only be speculated upon. Nonetheless, even as independent observations, both the maternal and neonatal data point to a marked effect of taurine supplementation on modifying maternal and neonatal outcomes in the setting of a maternal obesogenic diet.

This study demonstrated that maternal obesogenic diet induced significant hepatic steatosis in dams. Hepatic lipogenic gene expression was altered by the obesogenic diet. Increased SREBP-1c, FASN, LPL and decreased PPARα expression was observed in MO dams. There are three possible main pathways by which these altered gene expression may contribute to hepatic steatosis. Firstly, SREBP-1c is an important transcriptional regulator of fatty acid synthesis and regulates the expression of key enzymes such as acetyl coenzyme-A carboxylase-1 (ACC1), Stearoyl-CoA desaturase-1 (SCD1) and FASN (331, 390). SREBP-1c expression is insulin sensitive (334), and overexpression can induce de novo lipogenesis via upregulation of FASN (331, 391) potentially contributing to lipotoxicity. Secondly, PPARα is a mediator of hepatic fatty acid β-oxidation (333). As a member of the nuclear hormone receptor superfamily, PPARα can be attenuated by insulin in hepatocytes (392). In our model, elevated circulating insulin may contribute to the downregulation of hepatic PPARα and associated reduction in BHB concentrations indicating reduced fatty acid utilization, a hypothesis supported by others (393). Thirdly, upregulated LPL expression may contribute to hepatic steatosis by increasing intracellular free fatty acid accumulation through hydrolysis of lipoprotein triglyceride (332, 394). Interestingly, it has been shown that fructose can induce hepatic steatosis via fructokinase-mediated fructose phosphorylation (329, 330). However, in
In the current study, fructokinase was downregulated in high-fat high-fructose diet group, suggesting high-fat, rather than high-fructose component of the maternal diet represented the predominant contributor to the maternal hepatic steatosis.

Contrary to obesity studies in non-pregnant animals (307, 395, 396), maternal diet-induced hepatic steatosis was not reversed, but further aggravated by taurine supplementation. The steatosis observed in the present study represents an instance of non-alcoholic fatty liver disease (NAFLD) which is a chronic condition which is characterized by two distinct phases (397). Firstly, hyperglycemia and IR contribute to ectopic fat deposition in the liver contributing to intracellular lipid accumulation in hepatocytes increasing oxidative stress and proinflammatory cytokine production which initiate the second phase characterized by hepatocyte apoptosis and progressive fibrosis. In the current study, exacerbated hepatic steatosis in MOT group was evidenced by an elevated hepatic immunophenotype which included upregulated TNFα and IL-1β expression. It is well established, that TNFα and IL-1β expression is correlated with the severity of steatohepatitis (239, 240, 398). Increasing free fatty acid accumulation promotes this proinflammatory phenotype through activation of the TLR4 signaling pathway which culminates in NFκB activation (242). In our study, in response to taurine supplementation, maternal hepatic CD36 expression was markedly increased. CD36 is a long chain fatty acid transporter (399) which can directly bind free fatty acid from plasma (332). Increased fatty acid uptake via CD36, together with potential de novo lipogenesis resulting from increased FASN expression in MOT mothers may contribute to intracellular hepatic fatty acid accumulation. Therefore, this ectopic fatty acid deposition may exacerbate inflammatory processes triggering NAFLD in taurine supplemented high-fat fed mothers.

Although hepatic proinflammatory profile deteriorated in the MOT group, circulating TNFα was significantly reduced. Maternal taurine supplementation has been previously shown to down-regulate systemic inflammation in acute trauma, sepsis and other immune deficient conditions in non-pregnant subjects (324). Adipose tissue represents a major contributor to circulating TNFα concentrations (163). In the present study, we observed reduced plasma TNFα concentrations in response to maternal taurine supplementation concomitant with reduced adiposity in the MOT mothers which is in agreement with previous observations whereby weight loss is associated with improvements in inflammatory markers (400).

We noted a reduction in maternal weight gain in taurine supplemented control and MO dams in the last stage of gestation. This was not reflected in an overall change in birth weights or a change in maternal water or food intake. There is evidence for a role of taurine in reducing fat
mass in the rodent (401, 402) and in the present study we observed a reduction in fat mass in MOT dams but this was at the time of lactation; whether taurine had an effect on modifying maternal body composition in late pregnancy is not known.

Given the link between high fat diets and changes in glutamate metabolism (403), we also examine circulating glutamate concentrations in the lactating dams. Glutamate at high concentrations is well known to be toxic to the central nervous system (404), retinal neurons (405) and pancreatic islets (406). As the fetal blood brain barrier is relatively permeable, even a slight increase in circulating glutamate has been shown to overstimulate neurons in the arcuate nucleus which can lead to metabolic dysregulation in later life (407). Given the ability of glutamate to cross the placenta (408), it is possible that the elevated maternal glutamate concentrations observed in the present study may lead to significant adverse effects on later metabolic function in offspring. Taurine supplementation normalized circulating glutamate concentrations; previous studies have demonstrated that tissue specific levels of glutamate can be decreased by taurine supplementation. This may be due to taurine-mediated alterations in calcium influx via cysteine/glutamate antiport systems (409-411) and may be a mechanism by which beneficial effects of taurine are exerted in the present study but warrants further investigation. Of note in the present study was the sex-specific effect of maternal taurine on outcomes in the neonate. We have recently reported on sex-specific differences in placental weights in a model of maternal fructose intake (87) and it is possible that the sex-specific effects observed in offspring in the current study may in part be mediated by alterations in, for example, glutamate transfer across the placenta. In a recent paper by Tang et al. in the setting of a maternal low protein diet, it was shown that maternal taurine supplementation led to a reduction in insulin sensitivity in female but not male offspring although the mechanisms underpinning this sexual dimorphism are not clear (260).

Offspring hepatic inflammatory profile was significantly rescued as a result of maternal taurine supplementation. Data from the present study indicates that maternal obesogenic diet had adverse effects on neonatal hepatic inflammatory profiles. Increased expression of IL-1β and TNFα receptors in MO offspring suggest that maternal developmental programming induces a predisposition to hepatic inflammatory responses which may contribute to long term risk of hepatic IR, steatosis and fibrosis. Notably, a recent study by Chiappini et al. demonstrated the importance of IL-1R1 overexpression in response to early development of obesity-induced NAFLD (412). Furthermore, in other studies, IL1-R1 and IL-1β knockout mice display attenuated hepatic steatosis and inflammation when exposed to alcohol and high cholesterol diet (338, 413). This suggests that reversal of IL-1R1 and TNFR1 in neonates from
the MOT group may contribute to an overall improvement of long term metabolic health. We also examined parameters relating to IR and lipid metabolism (Figure 4.7); however these were unaffected in neonates which is unsurprising given the age and lack of metabolic challenge in these animals. Nevertheless, inflammation is a key pathological factor which we have shown can exacerbate hepatic steatosis. Therefore, the finding that maternal taurine supplementation reversed maternal diet-induced inflammatory receptor overexpression in neonates would suggest that taurine is having a protective effect on offspring liver inflammatory response and may confer protection against fatty liver disease in later life.

Additionally, we observed that in the control pregnancies, taurine supplementation increased neonatal mortality, despite no such an effect in the maternal obesogenic groups. Animal studies have demonstrated that taurine can prevent mortality in STD-induced diabetic adult rats (414). However, there are limited data on possible adverse effects of taurine in normal pregnancies. Earlier work by Boujendar et al. reported adverse effects of taurine supplementation in offspring of control pregnancies as reflected in fetal hypoglycaemia and decreased pancreatic and postnatal body weights (172). We did not observe any significant changes in neonatal glucose concentrations in the present study but there was a significant reduction in birthweight in female but not male offspring of taurine supplemented dams. Taurine supplementation in vivo has been reported to protect islets in offspring from low protein fed dams from cytokine toxicity, but increase islet sensitivity to cytokines and impaired pancreatic development in control animals (172, 258). Therefore, possible taurine toxicity in the setting of normal pregnancy outcomes should be further investigated.

In conclusion, a maternal obesogenic diet-induced postpartum impaired insulin sensitivity, hepatic steatosis and contributed to a programmed neonatal hepatic inflammatory profile. Maternal taurine supplementation exacerbated maternal hepatic steatosis yet benefited the circulating proinflammatory profile in dams and reversed the detrimental neonatal hepatic inflammatory cytokine receptor expression. Our findings suggest that maternal taurine supplementation may protect the offspring of obese mothers against developmentally programmed NAFLD despite worsening maternal postpartum fatty liver disease. While a few other studies investigated reversing the programming effects in offspring of undernutrition models (161, 172, 255, 258, 272, 335), our study first reports the reversing effect in a model of maternal obesity. Together, these studies indicate that the early period of life may be a critical window for reversing programming effects. However, while taurine supplementation during pregnancy may modify developmental programming of metabolic dysfunction in offspring,
adverse maternal effects in normal pregnancies warrant caution and must be further investigated.
4.3 Post-weaning offspring phenotype

The paper in section 4.2 focused on maternal and neonatal outcomes. In this section, additional results on post-weaning offspring health outcomes from the above study are presented.

4.3.1 Methods

All offspring were fed a standard chow diet after weaning until postnatal day 150. Puberty onset was evaluated from postnatal day 28 onwards for female offspring and from postnatal day 30 for male offspring. A food challenge test was performed at postnatal day 130 for 10-12 animals per group for 9 days, with free access to 200g chow diet and 200g MO diet daily. Offspring body weight and food intakes were recorded daily during this period. At postnatal day 143, an OGTT was performed for 6 animals per group and repeated tail blood samples were taken at 0, 5, 10, 20, 30, 60, 90 and 120 min. Offspring were culled at postnatal day 150. Plasma insulin and leptin (CrystalChem, USA) concentrations were measured by rat specific ELISA. Single measured results were analysed by two-way ANOVA with maternal diet and taurine supplementation as main factors. Repeated measures data such as body weight, food intakes and OGTT results were assessed by repeated analysis of variance. All data in section 4.3 met the assumptions of parametric test (normality and homogeneity of variance). Detailed procedures are described in chapter 2.

4.3.2 Offspring post-weaning weight gain and food intake

Male offspring exposed to maternal obesogenic diet in MO and MOT groups had significantly increased body weights from postnatal day 112 onwards compared to offspring from maternal chow diet groups (CON and CT) with a significant maternal diet effect dependant on time (Figure 4.8 A; time×maternal diet effect P < 0.05). Female offspring in the CT group had significantly reduced body weight gain from postnatal day 136 onwards compared to offspring from MO and MOT groups with a significant taurine and maternal diet interaction effect dependant on time (Figure 4.8 B; time×taurine×maternal diet effect P < 0.05). Male offspring in MO and MOT group had significantly increased total caloric intakes from postnatal day 51 onwards compared to offspring in CON and CT groups with a significant maternal diet effect dependant on time (Figure 4.9 A; time×maternal diet effect P < 0.05). Female offspring in MO and MOT group had significantly increased total caloric intakes from postnatal day 51 until postnatal day 106 compared to offspring in CON and CT groups with a significant maternal diet effect dependant on time (Figure 4.9 B; time×maternal diet effect P < 0.05).
Figure 4.8 Post-weaning offspring growth curve

A) Male and B) female offspring growth curve of body weight from weaning to postnatal day 150. Data are means ± SEM, n = 22-34 per group. * denotes P < 0.05 when MO and MOT compared...
with CON and CT during a certain period, \# denotes P < 0.05 when CT compared with MO and MOT during a certain period.

Figure 4.9 Post-weaning offspring food intake

A) Male and B) female offspring food intake from weaning until postnatal day 130. Data are means ± SEM, n = 22-34 per group. * denotes P < 0.05 when MO and MOT compared with CON and CT during a certain period.
4.3.3 Adult offspring adiposity

Male offspring retroperitoneal fat percentage (per body weight) was significantly increased in MO and MOT groups compared with CON and CT groups (Figure 4.10 A). Retroperitoneal fat percentage was significantly lower in MOT males than MO males (Figure 4.10 A). There was a significant interaction effect between maternal diet and taurine supplementation (Figure 4.10 A). Female offspring retroperitoneal fat percentage were significantly increased in MO and MOT groups compared with CON and CT groups with a significant effect of MO diet (Figure 4.10 B).

Figure 4.10 Offspring body composition

A) Male offspring retro fat percentage (per body weight); B) female offspring retro fat percentage (per body weight). Data are means ± SEM, n = 22-34 per group. Lower case letter superscripts (a, b, c) indicate multiple comparison procedures were conducted for diet and taurine interaction. Upper case letter (A, B) superscripts indicate comparison procedures were conducted between all groups fed MO diet and all groups fed CON diet. Groups that do not share the same letter are significantly different from each other (P < 0.05).
4.3.4 Offspring onset of puberty

Age of puberty onset in male offspring was significantly lower in CT, MO and MOT groups when compared with CON (Figure 4.11 A, C). Male offspring from CT, MO and MOT groups displayed significant lower body weight compared to CON offspring when entering puberty (Figure 4.11 E). Male offspring from MO and MOT groups had significant lower body weight compared to CT group when entering puberty (Figure 4.11 E). In female offspring, age of pubertal onset was significantly lower in CT and MO groups compared to CON offspring (Figure 4.11 B, D). There was no significant difference in mean age of puberty onset between female MO and MOT offspring or between CON and MOT offspring (Figure 4.11 B, D). Weight at time of entering puberty was significantly reduced in offspring from CT, MO and MOT groups compared with CON group (Figure 4.11 F).
Figure 4.11 Age and weight and puberty

A) % of male offspring which had entered puberty; B) % of female offspring which had entered puberty; C) average age of puberty onset in male offspring; D) average age of puberty onset in female offspring; E) weight of male offspring at onset of puberty; F) weight of female offspring at onset of puberty. Data are means ± SEM, n = 22-34 per group. Lower case letter superscripts (a, b, c) indicate multiple comparison procedures were conducted for diet and taurine interaction. Groups that do not share the same letter are significantly different from each other (P < 0.05).
4.3.5 Food challenge test

During a 9 day MO diet challenge, male MO offspring had significantly increased body weight gain compared with CON, MO and MOT offspring, with significant maternal diet and taurine effects dependant on time (Figure 4.12 A). Female offspring from CON and CT groups had significantly less body weight gain compared to MO and MOT groups, with a significant effect of maternal diet depend on time (Figure 4.12 B). Male offspring from MO and MOT groups had significantly increased total caloric intake compared to CON and CT groups during the food challenge period with a significant maternal diet effect (Figure 4.13 A). Taurine supplementation had a trend of reducing total caloric intake in male offspring (Figure 4.13 A, Taurine P = 0.065). The percentage of calories derived from MO diet was significantly increased in MO and MOT male offspring compared with those in CON and CT groups with a significant maternal diet effect (Figure 4.13 C). In female offspring, total caloric intake was significantly increased in MO and MOT groups compared to CON and CT groups, with a significant maternal diet effect (Figure 4.13 B). The percentage of calories derived from MO diet in female CT offspring was significantly reduced compared to CON, MO and MOT groups, with significant maternal diet and taurine effects (Figure 4.13 D).

![Figure 4.12 Offspring body weight gain during food challenge test](image)

**A)** Male offspring body weight gain during the food challenge period; **B)** female offspring body weight gain during food challenge test. Data are means ± SEM, n = 10-12 per group. # denotes P < 0.05 when MO compared with CON, CT and MOT during a certain period, * denotes P < 0.05 when MO and MOT compared with CON and CT during a certain period.
Figure 4.13 Offspring caloric intake during food challenge test

A) Male offspring body weight gain during the food challenge period; B) female offspring body weight gain during food challenge test. Data are means ± SEM, n = 10-12 per group. Upper case letter (A, B) superscripts indicate comparison procedures were conducted between all groups fed MO diet and all groups fed CON diet. Lower case letter superscripts (a, b, c) indicate multiple comparison procedures were conducted between four groups. Groups that do not share the same letter are significantly different from each other (P < 0.05).

4.3.6 Adult offspring OGTT test

OGTT results revealed that the blood glucose concentrations in all groups for both sexes peaked at 20 min after oral glucose loading and then decreased. In male offspring, tail glucose concentrations were significantly elevated in MO group compared to CON and MOT groups at 5, 10, 20, 30 and 60 minutes during OGTT, with a significant interaction effect between maternal diet and taurine supplementation dependant on time (Figure 4.14 A). Area under the
curve (AUC) of OGTT was significantly increased in MO males compared with CON males ((Figure 4.14 C). There was a trend of reduction of AUC in MOT male compared to MO male (Figure 4.14 C MO vs MOT P=0.08). In female offspring, tail glucose concentrations were significantly increased in MO and MOT groups compared to CON and CT groups at 10, 20, 30, 60, 90 and 120 minutes during OGTT, with a significant effect of maternal diet depend on time (Figure 4.14 B). AUC of OGTT was significantly increased in MO and MOT females than CON and CT females (Figure 4.14 D).

Figure 4.14 Offspring OGTT results

A) Male offspring tail glucose OGTT; B) female offspring tail glucose OGTT; C) male offspring area under the curve (AUC) of OGTT; D) female offspring AUC of OGTT. Data are means ± SEM, n = 6 per group. # denotes P < 0.05 when MO compared with CON, CT and MOT during a certain period, * denotes P < 0.05 when MO and MOT compared with CON and CT during a certain period. Upper case letter (A, B) superscripts indicate comparison procedures were conducted between all groups fed MO diet and all groups fed CON diet. Lower case letter superscripts (a, b, c) indicate multiple comparison procedures were conducted between four groups. Groups that do not share the same letter are significantly different from each other (P < 0.05).
4.3.7 Adult offspring plasma leptin and insulin concentrations

Male offspring plasma leptin and insulin concentrations and HOMA-IR index were significantly increased in MO and MOT groups compared to CON and CT with a significant maternal diet effect (Figure 4.15 A, C, E). There was no significant difference in female offspring plasma leptin and insulin concentrations and HOMA-IR index (Figure 4.15 B, D, F).

![Bar charts showing plasma leptin, insulin, and HOMA-IR comparisons across diet groups A, B, C, D, E, F.](image)

**Figure 4.15 Offspring plasma leptin, insulin and HOMA-IR**

A) Male offspring plasma insulin concentrations; B) female offspring plasma insulin concentrations; C) male offspring HOMA-IR index; D) male offspring HOMA-IR index. HOMA-IR index = Fasting glucose (mmol/l) × fasting insulin (mU/l)/22.5. Data are means ± SEM, n = 22-34 per group.
4.4 Additional discussion

4.4.1 Discussion on post-weaning offspring

Excessive weight gain is a common programming effect in offspring exposed to maternal obesity (69, 71, 73, 81). In this study, we observed that a maternal MO diet that is high in fat and fructose has a time dependent effect on male offspring postnatal growth - leading to increased body weight gain and adiposity during adulthood. Mammalian body weight is generally controlled by the balance of energy expenditure and food intake (Section 1.3.4). A previous study from our group showed that offspring exposed to maternal HF diet had increased postnatal body weight gain, however, their total caloric intake remained unchanged in the adult life (81). In this study, we evidenced increased total daily caloric intake in offspring exposed to maternal MO diet, suggesting that a diet high in fat and fructose may have changed the offspring’s ability to maintain energy balance, perhaps by modifying mechanisms that control caloric intake. This finding is consistent with other studies that showed increased offspring caloric intake as a result of a maternal cafeteria diet (high in fat and sugars) (69, 91, 155). Interestingly, in a study directly comparing high fat diet and cafeteria diet, only rats fed a cafeteria diet displayed voluntary hyperphagia (98). Taken together, we further speculate that the type or combination of high energy components in a maternal diet is important for offspring programming outcome.

There are two potential mechanisms that may contribute to the hyperphagia observed in our study. One possible mechanism is early exposure to high glutamate concentrations as a result of maternal MO diet as discussed in Section 4.2. However, given the fact hyperphagia was not completely reversed with normalised maternal glutamate concentrations in MOT group, we speculate that additional factors, particularly leptin, may be more important players in programming hyperphagia. Leptin is a key regulator of energy balance and plays an important role in programming offspring obesity (Section 1.3.4). Genetic deletion of leptin leads to profound hyperphagia and obesity in mice (415), and leptin replacement can effectively reverse this altered physiology (416, 417). When the body fails to respond to leptin appropriately, a state of leptin resistance occurs and contributes to the development of obesity (418). Kirk et al. reported that offspring, following maternal obesogenic diet, can develop leptin resistance independent of adiposity at an early age (postnatal day 30) (93). The present study showed that male offspring born to MO diet fed dams were heavier and had increased plasma leptin concentrations compared with controls. Although we did not conduct direct
leptin challenge tests, we speculate that maternal MO diet-induced hyperphagia and weight gain in males may be due to alternations in offspring leptin tolerance.

In the present study, both male and female offspring from MO diet fed dams exhibited an increased preference for MO diet, increased caloric intake and a sequential increase in body weight gain during the food challenge period. Several studies have previously demonstrated that preference for high energy diets (cafeteria) can be programmed in early life by maternal high energy diets (154, 419). Perinatal exposure to a cafeteria diet (including biscuits, marshmallows, cheese, doughnuts, chocolate etc.) can lead to a persisting exacerbated preference for fatty, sugary and salty foods (154). Our observation is in line with other studies and suggests that high fat high fructose diets have similar programming effects on offspring appetite with cafeteria diets (154, 419). Interestingly, the body weight gain observed in females from mothers fed a MO diet only occurred during the food challenge period, but not in the chow only feeding environment – this is despite the fact that female offspring also showed a long period of increased chow intake. This observation suggests that high energy food preference plays an important role in the development of excessive weight gain.

The present study showed that maternal taurine supplementation had a protective effect on male offspring end-point adiposity and normalised body weight gain in male offspring during the food challenge period. This protective effect may be partially achieved via modification of caloric intake, as we observed a trend of taurine reducing total caloric intake. However, this trend towards an overall reduction in caloric intake alone cannot fully explain the reduced weight gain in male offspring - taurine has a trend to reduce caloric intake in both CT and MOT groups, but only MOT group displayed reduced weight gain. More interestingly, adiposity is normally correlated with plasma leptin concentrations (Section 1.3.4). In the present study, we observed reversed adiposity in MOT males without a companying reversal in plasma leptin. We therefore speculate that taurine supplementation in combination with specific diets may differentially affect pathways (other than caloric intake or leptin) that are involved in energy metabolism.

Impaired pancreatic β-cell development is another common phenotype observed in the offspring following maternal obesity and is known to contribute to offspring obesity and metabolic disease (Section 1.3.4). Although we didn’t directly measure the pancreatic β-cell function, we observed compromised glucose metabolism in offspring as a result of maternal MO diet, suggesting impairment in pancreatic function. β-cells reside in the islets of Langerhans in pancreas and are responsible for insulin storage and release. Ford et al. reported
that maternal obesity in sheep accelerated fetal pancreatic β-cell premature development and reduced β-cell number in late gestation (144, 174). A study by Oben et al. found that a maternal cafeteria diet was associated with offspring pancreatic fibrogenesis in mice (420). Although there is lack of direct evidence in developmental programming models, it is known that both high glucose and high free fatty acid concentrations can contribute to the pancreatic β-cell dysfunction. Long-term (> 7days) exposure to elevated glucose concentration induces β-cell apoptosis (176). Increased free fatty acids can initiate β-cells apoptosis via ER stress, mitochondrial dysfunction and TLR signalling induced inflammation (177, 421, 422). In our study, we observed increased maternal glucose and lipid concentrations, which might potentially impair offspring β-cell development.

Taurine supplementation rescued glucose tolerance in male offspring in the present study. It has been shown in a maternal low protein model, taurine supplementation restored offspring pancreatic islet development with normalisation of glucose and insulin homeostasis in later life (172, 255, 258, 260). To our knowledge, we are the first to report similar taurine protective effects on offspring glucose metabolism in a maternal obesity model. Taken together, our observations further support the idea that the pancreas is a common target organ for developmental programming and that taurine might have beneficial effects on pancreatic development under suboptimal maternal nutrition.

Maternal obesity is associated with offspring early puberty onset (50, 343, 344). Similar to the study reported in Chapter 3, we observed precocious puberty in offspring born to mothers fed MO diet. Taurine was able to rescue early puberty onset in female offspring born to mothers fed high fructose diet (Chapter 3) but not in those born to mothers fed a high fat and high fructose (MO) diet. It seems that the effect of taurine in reversing precocious puberty is dependent on the type of maternal nutritional insults. As discussed previously (Section 3.4), growing evidence suggests that leptin plays a key role in gating puberty onset. In the present study, there was no significant effect of taurine on plasma leptin concentrations in neonates born to obese mothers (Table 4.5), which may explain the ineffectiveness of taurine supplementation on the timing of puberty onset in offspring in this study.

In this study sexual dimorphism was observed in the post-weaning offspring in the form of programming effects being more profound in male than female offspring. As previously discussed (Section 1.3.5) sexual dimorphism is commonly observed in developmental programming. While the causal mechanisms of such dimorphism are still largely unexplained,
the fact that we observed such effects again in this study underscores the need to always investigate both sexes in studies of this nature.

### 4.4.2 Contribution and significance

To our knowledge, we are the first to identify the effects of a perinatal high fat high fructose diet on maternal and offspring health outcomes. A number of results in our study including increased maternal inflammation, offspring hyperphagia and increased preference for high energy diets are in agreement with observations in a maternal cafeteria diet model (69, 91, 98, 154, 155, 419). However, a cafeteria diet is a complex mix of various nutrients, which makes it difficult to interpret results due to the potential complex interactions between different dietary components (Section 1.3.1.b). We suggest that a high fat high fructose diet in many cases can act as a relatively simplified model to mimic an unhealthy human Western diet scenario during pregnancy, which only emphasises the effect of a high fat and high sugar combination.

One significant novel finding in this study is exacerbated maternal liver steatosis as a result of taurine supplementation in mothers fed MO diet. This observation is surprising and different from the study in chapter 3, where taurine supplementation normalised high fructose diet induced maternal liver steatosis. It thus leads to the speculation that taurine supplementation may have detrimental effect on the liver depending on the fat content in the diet during pregnancy. When translated to a human setting, caution regarding taurine consumption might be needed for pregnant women who consume a high fat diet, as currently there is no clear recommendation on taurine intake during pregnancy.

Despite the maternal liver steatosis, taurine supplementation in our study showed a number of positive effects on maternal and male offspring health outcomes. To our knowledge, we are the first to report the beneficial effect of taurine on offspring glucose metabolism in a maternal obesity models and we are the first to report the beneficial effect of taurine in reversing maternal overall inflammatory status. Further study is required to understand the molecular mechanisms of taurine effects on maternal obesity-induced fetal programming.

### 4.4.3 Limitations

Hyperinsulinemic-euglycemic clamp are the gold standards for assessing insulin action in vivo. In this study, we used HOMA-IR index to evaluate IR as it is less invasive and less time-consuming. While fasting glucose and insulin concentrations are measured at a single time
point, nonetheless, the estimate of IR obtained by homeostasis model assessment has a good correlation with estimate obtained by use of the euglycaemic clamp (309). We also attempted to conduct islet isolation to assess β-cell function ex vivo, however, it was not successful.

We observed that increased adiposity in adult females born to obese mothers was reversed by maternal taurine supplementation; however, this change in adiposity was not reflected in plasma leptin concentrations. The inconsistency might be due to the different sample size and the timing of the measurements. Therefore, with limited data we cannot conclusively conclude the effect of taurine in reversing female offspring adiposity.

Similar to the study in chapter 3, we did not identify whether the dams were hypertensive due to the concern on reducing stress during pregnancy, and we sampled neonates rather than late gestation fetuses to assess early stage programming effects. Albeit low possibility, we cannot fully exclude maternal hypertension as a cofounder in our model and interpretation of neonatal data should consider the potential variations caused by milk ingestion.

4.4.4 Future directions

As we observed interesting food intake and preference changes in adult offspring, central control systems that potentially regulate these changes is of interest in the future study. Hypothalamic arcuate nucleus and the ventral tegmental area are the primary candidates for investigation. The arcuate nucleus of the hypothalamus is an integrated centre for feeding regulation (423), and the ventral tegmental area is a reward centre that is related to high energy food preference (424). Both of the areas can be affected by insulin and leptin concentrations, and lead to alternations in food intake (425, 426). Additionally, the early development of the arcuate nucleus is sensitive to glutamate concentrations - maternal administration of glutamate can lead to permanent damage to fetal arcuate nucleus and sequential offspring appetite dysregulation (407). Given the observation that maternal plasma glutamate concentrations and offspring plasma insulin and leptin concentrations are altered in this study, investigation of the arcuate nucleus and the ventral tegmental area may provide an in-depth view on the mechanisms of food intake modification in this study.

It has been demonstrated by others that offspring born to obese mothers have increased susceptibility to NAFLD in adulthood. Further investigations on adult offspring liver samples from the present study at a morphological and molecular level are necessary. Given the observation that hepatic pro-inflammatory cytokine gene expression is altered in neonates, it is worth looking at the hepatic pro-inflammatory and fibrogenic profiles in adult offspring.
Moreover, *de novo* lipogenesis, mitochondrial function and ER stress are also interesting pathways to consider in the future (Section 1.4).

Growing evidence has demonstrated that maternal obesity can induce macrophage accumulation and inflammation in the placenta (427, 428), which may potentially contribute to compromised development in offspring. In the present study, we observed a significant reversal effect of taurine on maternal overall inflammatory status. It will be meaningful to study the placenta inflammatory signals network in the same setting. Moreover, there is evidence suggesting that taurine transport in placental trophoblast cells is important for regulation of cell differentiation and survival (429). Further study on the placenta function under taurine supplementation condition may help to uncover more potential mechanisms of taurine beneficial effects on the offspring.
Chapter 5. Maternal undernutrition with pre-weaning GH treatment study

5.1 Preface

In addition to maternal obesity, undernutrition during pregnancy is also a well-known maternal nutritional insult that is associated with increased susceptibility to obesity and metabolic syndrome in offspring. Although less common than maternal obesity and GDM in Western countries, IUGR remains one of the main challenges in maternity care given the limited antenatal diagnostic accuracy (430). From a DOHaD prospective, IUGR offspring are likely predisposed to a number of metabolic disorders, which in the future may contribute to the global obesity and metabolic syndrome epidemic. Therefore, it is important to look for intervention strategies to reverse the programming outcomes in the immediate postnatal life if the condition of growth restriction failed to be removed.

The section 5.2 contains an original research article “Pre-weaning growth hormone treatment normalises body growth trajectory and reverses metabolic dysregulation in adult offspring following maternal undernutrition”. (Appendix V).

The section 5.3 contains data from two published articles “Preweaning growth hormone treatment ameliorates adipose tissue insulin resistance and inflammation in adult male offspring following maternal undernutrition” (Appendix VI); and “Pre-weaning growth hormone treatment reverses hypertension and endothelial dysfunction in adult male offspring of mothers undernourished during pregnancy” (Appendix VII). Minglan Li is the second author of these two papers. The adipose tissue culture experiments and histology evaluation were conducted by Dr Clare Reynolds, and the myograph experiments were carried out by Dr Clint Gray. As primary tissue culture and vessel myograph experiments are time consuming and need to be conducted at the time of cull, only male offspring were investigated. Minglan Li was taught the techniques afterwards. The use of data in this thesis was permitted by Dr Clare Reynolds and Dr Clint Gray.
5.2 Pre-weaning growth hormone treatment normalises body growth trajectory

Pre-weaning growth hormone treatment normalises body growth trajectory and reverses metabolic dysregulation in adult offspring following maternal undernutrition

Minglan Li, Clare M Reynolds, Clint Gray, Mark H Vickers

1Liggins Institute and Gravida: National Centre for Growth and Development, University of Auckland, Auckland, New Zealand

Abstract

Maternal undernutrition (UN) results in growth disorders and metabolic dysfunction in offspring and aberrant postnatal development is linked to changes in the GH-IGF axis. Although dysregulation of the GH-IGF axis in offspring is a known consequence of maternal undernutrition, little is known about the effects of GH treatment during the early period of developmental plasticity on later health and well-being in offspring following maternal UN. The present study investigated the effect of pre-weaning GH treatment on growth, glucose metabolism and the GH-IGF axis in adult male and female offspring following maternal UN. Female Sprague Dawley rats were fed either a chow diet ad libitum (CON) or 50% of ad libitum (UN) throughout pregnancy. From postnatal day 3, CON and UN pups received either saline (CON-S and UN-S) or GH (2.5µg/g/day, CON-GH and UN-GH) daily throughout lactation via subcutaneous injection. At weaning, 2 male and female offspring were randomly selected from each litter and fed a standard chow diet for the remainder of the study. Pre-weaning GH treatment normalised maternal UN-induced alterations in post-weaning growth trajectory and concomitant adiposity in offspring. Plasma leptin concentrations were increased in UN-S offspring and normalised in the UN-GH group. Hepatic growth hormone receptor (GHR) expression was significantly elevated in UN-S offspring and normalised with GH treatment. Hepatic IGFBP-2 gene expression and plasma IGF-1:IGFBP-3 ratio was reduced in UN-S offspring and elevated with GH treatment. Our findings suggest that GH treatment during a critical developmental window prevents maternal UN-induced changes in postnatal growth patterns and related adiposity, providing evidence that manipulation of the GH-IGF-1 axis in early development may represent a promising strategy to prevent adverse developmental programming effects in later life.
5.2.1 Introduction

Alterations in the early life maternal nutritional environment are associated with long term adverse health outcomes in offspring (13, 431, 432). It has been well established in both human and experimental studies that maternal undernutrition (UN) leads to an increased risk of obesity and metabolic and cardiovascular dysfunction in offspring in later life (13, 103, 431). Although the underlying mechanisms are not fully defined, developmental plasticity and fetal adaptations to an environment of poor nutrition lead to an altered developmental trajectory and accompanying changes in organogenesis and metabolic homeostasis (3). The predictive adaptive response theory proposes that in utero undernutrition adjusts fetal metabolic set-points to match the predicted environment to ensure survival, however, if the subsequent postnatal environment is nutritionally enriched, these early adjustments become incongruous and can initiate rapid postnatal growth, contributing to metabolic dysfunction in later life (301).

GH and IGF-1 are key regulators of somatic growth. Dysregulation in the GH-IGF-1 axis plays an important role in developmental programming of metabolic dysfunction and related growth disorders arising as a consequence of early life undernutrition (433). Offspring from UN mothers in experimental animal models and human infants with intrauterine growth restriction (IUGR) display decreased plasma IGF-1 concentrations at birth (102, 434). Conversely, adult offspring exposed to famine during fetal development have elevated plasma IGF-1 concentrations along with increased body weight (435). Manipulating the GH-IGF-1 axis via treatment with either GH or IGF-1 in adult offspring following maternal UN has shown efficacy in improving programmed metabolic and cardiovascular outcomes related to hypertension, hyperinsulinemia, hyperleptinemia and adiposity (248, 436). However, both GH and IGF treatment showed adverse effects in either glucose metabolism or body weight gain (248, 436), in part associated with the diabetogenic effects of GH treatment in adults.

Recent evidence suggests that intervention strategies to reverse adverse developmental programming effects should be introduced early in life as the effect size is greater the earlier the intervention (249). IUGR infants display a phenotype characterised by obesity and IR as early as 2-6 years of age (437, 438). In low birth weight babies alterations in cardiac structure is observed in late childhood (439). These observations indicate that in order to prevent the first onset of pathological changes the intervention strategies need to occur early. Furthermore, animal studies by our group and others have shown the effectiveness of early life intervention in reversing developmental programming effects. Studies utilising leptin and the GLP-1 analog Exendin-4 treatment during the neonatal period have demonstrated the effectiveness of
the pre-weaning window to treat maternal UN mediated developmental programming, yet the effects of leptin were sex-dependent and also reliant on the level of postnatal nutrition (161, 268, 272). Given the degree of developmental plasticity present in early life, early intervention may indeed be more opportunistic and effective compared to current strategies which focus on lifestyle modification in later life (249).

Using a well-established model of moderate maternal UN, we have recently shown that pre-weaning GH treatment can normalise metabolic and cardiovascular dysfunction in offspring characterised by hypertension, endothelial dysfunction, inflammation and adipocyte-derived changes in insulin sensitivity (335, 336, 440). However, to date these studies have only investigated effects in male offspring and sexually dimorphic responses to GH treatment on growth and related metabolic outcomes have yet to be examined in relation to the GH-IGF axis. The present study therefore investigated the impact of pre-weaning GH treatment on postnatal growth, glucose metabolism and hepatic GH-IGF axis regulation in both male and female offspring.

5.2.2 Materials and Methods

Animal model

The animal model of maternal UN has been described in detail elsewhere (107, 440). Virgin female Sprague Dawley rats were time mated at 100 days of age using an estrous cycle monitor (EC-40, Fine Science Tools, San Francisco, USA). Mating was confirmed by the presence of spermatozoa following vaginal lavage. Animals were then housed as singletons and randomly assigned into either a control (CON, n = 19) or undernutrition (UN, n = 15) group. CON mothers were fed a standard chow diet (Diet 2018; Harlan Teklan, Oxon) ad libitum throughout pregnancy and lactation. UN mothers were given 50% of ad libitum intake diet based on the daily measurement from CON group during pregnancy and had ad libitum access to chow following birth. At the time of birth, litter size, sex and birth weight were recorded. On postnatal day 2 litter size was adjusted to 8 pups per litter (4 males and 4 females) to standardise nutrition until weaning (day 21). Neonatal liver and brain weight were measured from randomly excluded animals. Neonatal trunk blood glucose and β-hydroxybutyrate (BHB) was measured at the time of cull using a glucose/ketone meter (Optium Xceed; Abbott Laboratories, USA). From postnatal day 3, CON and UN neonates received either saline (CON-S and UN-S) or GH (bGH; Cyanamid; 2.5 μg/g, CON-GH and UN-GH) daily (0900h) throughout lactation by subcutaneous injection in the nape of the neck. Treatments were performed on a by-litter basis to avoid within litter treatment effects and potential effects of GH treatment on neonatal behaviour in non-GH treated offspring. At weaning, 4 offspring (2
males and 2 females) were randomly selected from each litter and fed the chow diet ad libitum for the remainder of the study (day 150). All animals had free access to water and were maintained in the same environment at 22°C with 12-hour light, 12-hour dark cycle throughout the study. Maternal and offspring body weights and food intakes were measured regularly. On postnatal day 150, offspring were fasted overnight and killed by decapitation following anaesthesia with sodium pentobarbitone (60 mg/kg IP). Trunk blood was collected in heparinized vacutainers and plasma supernatant stored at -20°C until later analysis. Retroperitoneal fat, spleen and liver weights were recorded. Liver was snap frozen in liquid nitrogen and stored at −80°C for later gene expression analysis. All animal work was approved by the Animal Ethics Committee of the University of Auckland (Ethical Approval R888).

**Plasma Analysis**

Fasting plasma insulin, leptin, insulin-like growth factor binding protein (IGFBP)-2, IGFBP-3 (Crystal Chem, USA), unacylated ghrelin (Cayman, USA) and IGF-1 (Mediagnost, Germany) were measured by rat-specific ELISA. Plasma glucose concentrations were analysed using a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as: fasting plasma glucose (mmol/l) × fasting plasma insulin (mU/l)/22.5 (309).

**Hepatic mRNA expression**

Total RNA isolation was conducted using the RNeasy® mini kit (QIAGEN, Germany). cDNA was synthesized from 2µg of RNA using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen™; Life Technologies Corporation, USA). Real time PCR analysis was carried out using PreDeveloped TaqMan® Assay Reagent Kits in the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). To control for inter-sample variability, mRNA levels were normalized to two housekeeper genes: cyclophilinA and HPRT by subtracting the geometric mean Ct of house keepers from the Ct for the gene of interest producing a ΔCt value. The ΔCt for each treatment sample was compared to the mean ΔCt for control samples using the relative quantification 2-(ΔΔCt) method to determine fold-change (285).

**Statistical analysis**

Neonatal data were analysed two-way factorial ANOVA with maternal diet and sex as factors. Adult offspring data analysis was completed using two-way factorial ANOVA with maternal diet and pre-weaning GH treatment as factors. Food intake and body weight data were analysed by repeated measures ANOVA. Data that failed to meet the criteria required for parametric analysis were transformed to achieve normal distribution and equal variance. Post-hoc analysis was performed where appropriate (Holm-Sidak multiple comparisons procedure)
to determine which groups were significantly different from each other. All data are shown as means ± SEM unless otherwise stated. A p-value of < 0.05 was accepted as statistically significant. All analysis was conducted using SAS software (SAS Institute, Cary, NC, USA).
5.2.3 Results

Maternal UN-induced neonatal growth restriction

Male and female offspring of UN mothers were growth restricted with a significant reduction in birth weight and nose-to-anus (NA) length when compared to CON offspring (Table 5.1). Body weights and NA lengths were reduced in female neonates compared to males (Table 5.1). The growth restriction in UN offspring was asymmetrical with significantly decreased liver weight and liver:body weight ratio, but increased brain:body and brain:liver weight ratio indicative of brain sparing that typically accompanies fetal growth restriction and resultant haemodynamic changes in blood flow (Table 5.1). There were no significant differences in neonatal blood glucose or BHB concentrations between UN and CON offspring at birth (Table 5.1). Male:female sex ratio and mortality rates did not differ between groups (data not shown).

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
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<tbody>
<tr>
<td></td>
<td>CON</td>
<td>UN</td>
</tr>
<tr>
<td>Birth weight (g)</td>
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<td>5.40±0.05b</td>
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<tr>
<td>NA (mm)</td>
<td>46.99±0.16a</td>
<td>44.06±0.24b</td>
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<tr>
<td>Glucose (mmol/l)</td>
<td>5.61±0.15</td>
<td>5.23±0.16</td>
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<tr>
<td>BHB (mmol/l)</td>
<td>2.49±0.12</td>
<td>2.46±0.14</td>
</tr>
<tr>
<td>Brain (g)</td>
<td>0.260±0.004</td>
<td>0.265±0.005</td>
</tr>
<tr>
<td>Brain: body ratio (%)</td>
<td>3.90±0.04b</td>
<td>4.34±0.05a</td>
</tr>
<tr>
<td>Liver (g)</td>
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<td>0.23±0.01b</td>
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<td>3.72±0.08b</td>
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<tr>
<td>Brain: liver ratio (%)</td>
<td>0.89±0.02b</td>
<td>1.19±0.03e</td>
</tr>
</tbody>
</table>

Table 5.1 Neonatal physiological characteristics

Data are presented as mean ± SEM. For birth weight and NA data, male offspring CON n = 145, UN n = 105, female offspring CON n = 135, UN n = 112; for the remaining data, male offspring CON n = 39, UN n = 32, female offspring CON n = 33, UN n = 37. Data was analysed via one-way ANOVA with maternal diet as factor and litter as a covariate. Groups that do not share the same letter are significantly different from each other (p <0.05).
Pre-weaning GH treatment prevented maternal UN-induced catch-up growth and adiposity

The direct effects of GH on neonatal weight gain in males have been reported elsewhere (335). In brief, both male and female offspring in CON and UN groups had significantly increased weight gain in a response to GH administration. Interestingly, the weight gain response induced by GH treatment was delayed in UN groups compared to CON in both sexes but was similar to that of controls by the end of treatment (Figure 5.1 A, B). Post-weaning body weights were significantly higher in male and female offspring from UN-S compared to CON-S, CON-GH and UN-GH groups (Figure 5.1 C, D). This was reflected in increased adiposity in the UN-S group with retroperitoneal fat pad weight body weight increased in both male and female UN-S offspring when compared to CON-S and CON-GH groups (Table 5.2). The increased retroperitoneal fat pad weight and retroperitoneal fat to body weight ratio observed in UN-S offspring was normalised in male UN-GH offspring. For female offspring, absolute retroperitoneal fat weight was significantly reduced in UN-GH compared to UN-S, with a strong trend towards a reduction in retroperitoneal fat:body ratio with GH treatment (p=0.058, Table 5.2). The changes in body weight and increased adiposity in UN-S offspring were reflected in significant overall increases in caloric intake in UN-S males and females compared to all other groups (Figure 5.2 A, B). Final body lengths (nose-anus) were not different between any of the groups (Table 5.2). Absolute liver weight and ratio to body weight was significantly increased in male UN-S offspring compared to CON-S and CON-GH, and was normalised in the UN-GH group (Table 5.2). In female offspring, pre-weaning GH treatment significantly reduced absolute liver weight in the UN-GH group when compared to UN-S group, but there was no difference in the liver:body weight ratio (Table 5.2). Absolute spleen weight in UN-S male offspring was significantly increased when compared to CON-S and UN-GH groups (Table 5.2). However, spleen:body weight ratio was significantly reduced in UN-GH group compared to CON-S, CON-GH and UN-S groups (Table 5.2). There were no changes in spleen weight or its ratio observed in female offspring (Table 5.2). Male UN-S offspring had significantly elevated rectal temperature compared to CON-S, CON-GH and UN-GH groups, while in females, rectal temperatures were unchanged across groups (Table 5.2).
Figure 5.1 Offspring growth trajectory

A and B: delta change between saline and GH treated CON and UN neonates. C and D: offspring post-weaning growth curves. Data are presented as mean ± SEM; n = 12-20 per group.*denotes P < 0.05 when compared to CON-S; #denotes P < 0.05 when compares to UN-GH.
Figure 5.2 Offspring post-weaning caloric intake

Data are presented as mean ± SEM; Males n = 12-20 per group, females n = 14-19 per group. *denotes P <0.05 when compared to CON-S; #denotes P <0.05 when compared to UN-GH.
### Groups

<table>
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<th>CON-S</th>
<th>CON-GH</th>
<th>UN-S</th>
<th>UN-GH</th>
<th>Maternal diet</th>
<th>Treatment</th>
<th>Interaction</th>
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<td>36.71±0.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.4±0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.88±0.21&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>P &lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Retro fat (g)</td>
<td>18.04±1.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.90±1.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.22±3.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.11±1.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Retro fat: BW (%)</td>
<td>2.73±0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.54±0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.46±0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.80±0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>16.72±0.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.46±0.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.61±2.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.18±0.48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P &lt; 0.05</td>
<td>NS</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Liver: BW (%)</td>
<td>2.58±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.68±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.99±0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.67±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P &lt; 0.05</td>
<td>NS</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>0.94±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.00±0.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.11±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.89±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NS</td>
<td>NS</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Spleen: BW (%)</td>
<td>0.146±0.004&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.156±0.005&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.154±0.007&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.138±0.005&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NS</td>
<td>NS</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rect. temp (ºC)</td>
<td>37.03±0.24</td>
<td>36.85±0.16</td>
<td>36.98±0.16</td>
<td>36.64±0.19</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Retro fat (g)</td>
<td>5.97±0.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.4±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.54±0.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.41±0.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P &lt; 0.05</td>
<td>P=0.058</td>
<td>NS</td>
</tr>
<tr>
<td>Retro fat: BW (%)</td>
<td>1.74±0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.64±0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.38±0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.93±0.16&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>P &lt; 0.05</td>
<td>P=0.051</td>
<td>NS</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>9.75±0.52&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.01±0.28&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>10.49±0.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.71±0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NS</td>
<td>P &lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Liver: BW (%)</td>
<td>2.89±0.13</td>
<td>2.76±0.08</td>
<td>2.93±0.18</td>
<td>2.69±0.06</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>0.62±0.02</td>
<td>0.63±0.02</td>
<td>0.65±0.03</td>
<td>0.58±0.03</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Spleen: BW (%)</td>
<td>0.185±0.005</td>
<td>0.192±0.006</td>
<td>0.184±0.007</td>
<td>0.176±0.006</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 5.2 Adult offspring physiological characteristics

Data are presented as mean ± SEM. Male offspring, n = 12-20 per group; Female offspring, n = 14-19 per group. Data was analysed via two-way factorial ANOVA with maternal diet and neonatal GH treatment as factors. Groups that do not share the same letter are significantly different from each other (p < 0.05). NS denotes not significant; BW = body weight.
Pre-weaning GH treatment altered maternal UN-induced changes in markers of glucose metabolism in offspring in a sex-specific manner

In male offspring, significant effects of maternal diet on plasma glucose and insulin were observed, with an overall effect of maternal UN on increasing fasting plasma glucose and insulin in these animals (Table 5.3). However, post-hoc comparison did not show any significant differences on glucose or insulin between individual groups in either male or female offspring (Table 5.3). To evaluate insulin resistance (IR), the HOMA-IR index was calculated from fasting plasma glucose and insulin concentrations. In male offspring, the HOMA-IR index was significantly increased as a result of maternal UN in UN-S and UN-GH groups compared to CON-S and CON-GH with no effect of GH treatment observed (Table 5.3). In female offspring, the HOMA-IR index was significantly increased in the UN-S group compared to CON-S and normalised in the UN-GH group with a significant maternal diet × GH treatment effect (Table 5.3). In agreement with the observed changes in retroperitoneal fat mass, plasma leptin was significantly increased in both male and female UN-S offspring compared to CON-S and was normalised with pre-weaning GH treatment (Figure 5.3 A, B). There were no differences in plasma unacylated ghrelin or BHB concentrations in either male or female adult offspring (Table 5.3).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Effect</th>
<th>Maternal diet</th>
<th>Treatment</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON-S</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>8.65±0.33</td>
<td>9.00±0.26</td>
<td>9.39±0.24</td>
<td>9.74±0.22</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>2.24±0.27</td>
<td>2.92±0.41</td>
<td>3.09±0.49</td>
<td>3.12±0.4</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.88±0.14</td>
<td>0.98±0.11</td>
<td>1.31±0.21</td>
<td>1.37±0.16</td>
</tr>
<tr>
<td>Ghrelin (ng/ml)</td>
<td>270±40.2</td>
<td>311±31</td>
<td>279±27</td>
<td>319±48</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>7.84±0.17</td>
<td>7.76±0.21</td>
<td>8.61±0.39</td>
<td>7.48±0.21</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>2.49±0.25</td>
<td>1.76±0.24</td>
<td>3.53±0.74</td>
<td>2.66±0.46</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.88±0.10</td>
<td>0.63±0.10</td>
<td>1.44±0.23</td>
<td>0.88±0.16</td>
</tr>
<tr>
<td>Ghrelin (ng/ml)</td>
<td>503±68</td>
<td>630±96</td>
<td>384±65</td>
<td>577±103</td>
</tr>
</tbody>
</table>

Table 5.3 Adult offspring plasma metabolic markers

Data are presented as mean ± SEM. Male offspring, n = 12-20 per group; Female offspring, n = 14-19 per group. Data was analysed via two-way factorial ANOVA with maternal diet and neonatal treatment as factors. Groups that do not share the same letter are significantly different from each other (p < 0.05). NS denotes not significant.
Figure 5.3 Offspring plasma leptin concentration

A: male offspring, n = 12-20 per group; B: female offspring, n = 14-19 per group. Data are presented as mean ± SEM. Groups that do not share the same letter are significantly different from each other (p < 0.05). NS denotes not significant.

Effect of pre-weaning GH treatment on maternal UN-induced changes in the GH-IGF axis

In male offspring, plasma IGF-1 concentrations remained similar across all groups, while plasma IGFBP-3 concentrations were significantly decreased in CON-GH, UN-S and UN-GH groups compared to CON-S, with a significant effect of maternal diet × pre-weaning GH treatment interaction observed (Figure 5.4 A, B). Of note, the plasma IGF-1:IGFBP-3 ratio was significantly increased in CON-GH and UN-S male offspring compared to CON-S and UN-GH groups. A significant interaction between maternal diet and pre-weaning GH treatment reflects GH-induced reduction of plasma IGF-1:IGFBP-3 ratio dependant on maternal UN (Figure 5.4 C). There was no significant difference observed in male offspring plasma IGFBP-2 concentrations (Figure 5.4 D). In female offspring, plasma IGF-1 was slightly but significantly increased in UN-S group compared to all other groups with a significant interaction between maternal diet and pre-weaning GH treatment (Figure 5.5 A). Female plasma IGFBP-3 concentrations were reduced in the UN-GH group compared to CON-S, CON-GH and UN-S with significant effect of both maternal UN and GH treatment (Figure 5.5 B). Consistent with male offspring, female offspring IGF-1:IGFBP-3 ratio was
significantly increased as a result of maternal UN, and was normalised by GH treatment (Figure 5.5 C). In contrast to the data in males, pre-weaning GH treatment increased plasma IGFBP-2 concentrations in female CON-GH and UN-GH offspring compared to controls (Figure 5.5 D).

Figure 5.4 Male offspring plasma IGF-1 and IGFBP-2 and IGFBP-3 concentrations
A, B, C, D: male offspring plasma IGF-1, IGFBP-3, IGF-1:IGFBP-3 ratio and IGFBP-2, n = 12-20 per group; E, F, G, H: female offspring plasma IGF-1, IGFBP-3, IGF-1:IGFBP-3 ratio and IGFBP-2, n = 14-19 per group. Data are presented as mean ± SEM. Groups that do not share the same letter are significantly different from each other (p < 0.05). NS denotes not significant.
Figure 5.5 Offspring plasma IGF-1 and IGFBP-2 and IGFBP-3 concentrations

A, B, C, D: male offspring plasma IGF-1, IGFBP-3, IGF-1:IGFBP-3 ratio and IGFBP-2, n = 12-20 per group; E, F, G, H: female offspring plasma IGF-1, IGFBP-3, IGF-1:IGFBP-3 ratio and IGFBP-2, n = 14-19 per group. Data are presented as mean ± SEM. Groups that do not share the same letter are significantly different from each other (p < 0.05). NS denotes not significant.
Hepatic GHR expression was increased in both male and female UN-S offspring compared to all other groups (Figure 5.6 A, B). A significant maternal diet × pre-weaning GH treatment interaction on hepatic GHR was observed in both males and females, indicating that the pre-weaning GH effect on reducing GHR is dependent on the level of maternal nutrition (Figure 5.6 A, B). Hepatic IGFBP-2 expression was reduced in both male and female UN-S offspring compared to CON-S groups, and was increased in both male and female UN-GH groups compared to UN-S (Figure 5.6 A, B). Similar to hepatic GHR, a significant effect of maternal diet × pre-weaning treatment interaction on hepatic IGFBP-2 expression was also observed in both male and female offspring (Figure 5.6 A, B). Furthermore, in male offspring a significant overall effect of maternal UN on down-regulation of hepatic IGFBP-1 expression was observed (Figure 5.6 A). However, post-hoc comparisons did not show any significant differences in hepatic IGFBP-1 expression between individual groups (Figure 5.6 A). There were no differences in hepatic IGF-1, insulin receptor (INSR) or IGFBP-3 gene expression in male offspring (Figure 5.6 A). In female offspring, hepatic IGF-1 gene expression was up-regulated in UN-S groups compared to other female groups, which is consistent with plasma IGF-1 concentration (Figure 5.6 B). A significant maternal diet × pre-weaning treatment interaction in IGF-1 expression was observed (Figure 5.6 B). Hepatic INSR was increased in the female UN-S group compared to CON-S and CON-GH groups (Figure 5.6 B). There were no significant differences in hepatic IGFBP-1 and IGFBP-3 expression in female offspring (Figure 5.6 B).
Figure 5.6 Hepatic gene expression markers in adult offspring related to GH-IGF-1 axis function

A: male offspring, n = 12-20 per group; B: female offspring, n = 14-19 per group. Data are presented as mean ± SEM. Groups that do not share the same letter are significantly different from each other (p < 0.05).
5.2.4 Discussion

The present study utilised a model of asymmetrical growth restriction in the rat to examine the effects of pre-weaning GH treatment on postnatal growth and hepatic and circulating markers related to GH-IGF-1 axis function in adult offspring. We report, for the first time, that pre-weaning GH treatment reversed maternal UN-induced changes in postnatal growth trajectory with paralleled increases in adiposity in both male and female offspring. Asymmetrical growth restriction in humans is characterised by reduced neonatal birth weight and increased relative brain:liver ratio (441), and accounts for up to 70% of IUGR cases (442). In the current study, growth restricted neonates demonstrate “brain sparing” as a result of maternal UN thereby paralleling the IUGR growth restriction pattern which is often observed in clinical practice (55). Infants and young children who have experienced early growth restriction due to poor nutrition are likely to achieve a higher than average growth rate than their age matched peers (443). There is evidence however, that this phenomenon of catch-up growth has a strong link with increased risk of adiposity, metabolic syndrome and cardiovascular disease in later life (444, 445). Previous studies by our group and others have shown in animal models that the absence of catch-up growth due to prolonged maternal undernutrition in the early postnatal period prevents later offspring adiposity and metabolic disorders (107, 446, 447). However, limiting postnatal nutrition in IUGR is unlikely to be a viable intervention due to well-known adverse effects on brain development and life span (448, 449). Additionally, we have shown that administration of GH during the pre-weaning period in UN offspring can lead to rapid immediate neonatal growth compared to UN-S animals, but rescues offspring weight gain and adiposity in the long term. This suggests that it is the hormonal modification rather than the catch-up growth phenomenon itself that leads to the longer term adverse offspring health outcomes.

The effect of pre-weaning GH treatment on normalising maternal UN-induced postnatal growth trajectory may be achieved via remodulation of GH-IGF-1 axis. IGF-1 is the key growth factor that stimulates somatic growth (433). Among the identified IGFBPs, IGFBP-3 binds up to 80% of circulating IGF-1 and is the most abundant IGF binding protein. Given its potent bioactive effects plasma IGF-1 is heavily regulated by binding to IGFBPs thereby altering its bioavailability. The plasma IGF-1:IGFBP-3 ratio reflects an estimate of free and bioactive IGF-1 (450). In the current study, maternal UN-induced increases in the plasma IGF-1:IGFBP-3 ratio were reversed by pre-weaning GH treatment in both male and female offspring, mirroring the change in postnatal growth trajectory. Interestingly, liver size was increased in adult UN offspring with overall effects of GH in decreasing liver weights. This
may reflect hepatic steatosis in UN offspring as has been observed in other rodent models of IUGR (451) but histological grading was not undertaken in the present study. The responsiveness of liver to GH-induced IGF-1 production is dependent on the expression of GHR (452). In present study, hepatic GHR expression was increased by maternal UN in both male and female adult offspring, and was normalised by pre-weaning GH treatment. This indicates a potential role for GHR in the programming of UN-altered growth trajectory and a potential mechanism for reversal by early life GH intervention. The physiological maturation and regulation of hepatic GHR in early life is still not fully understood. Early work in rodent models of GH-deficiency suggested that GH exerted no physiological effects during early postnatal development in the rodent. GH-deficient mice had normal birth weights and grow normally for the first two weeks of life suggestive of GH-independent growth e.g. the lit/lit mouse (453). However, work by Garcia-Aragon et al. showed the existence of GHR mRNA as early as embryonic day 12 (454) with hepatic GHR expression levels low during the neonatal period and then increasing until a peak around postnatal week 5 (455). There are conflicting data with respect to sexual differentiation of hepatic GHR expression with some showing higher numbers of GHR in females compared to males and others showing no differences (456). While hepatic tissue GHR expression is very low during the perinatal period (457), fetal hepatocytes have been shown to be responsive to exogenous GH (458). Given rapid changes in weight in response to GH treatment, it is clear that rodent neonates express the functional GHR. It has been reported in both human and animal studies that growth restriction is associated with decreased neonatal IGF-1 concentrations (459-462). Although not tested in the current study, we speculate that differential weight gain in response to GH treatment may be a direct consequence of IGF-1 correction in growth restricted offspring during the pre-weaning period. However, further studies are required to validate this observation including the potential role of GH and IGF-1 on changes in nutrient uptake and substrate partitioning (463).

This study also indicates a role for IGFBP-2 in the developmental programming of metabolic disorders as suggested previously by our group and others (464, 465). Alterations in IGFBP-2 expression as a result of maternal UN is consistent with our previous studies in Wistar rats (464). Although IGFBP-2 is the second most abundant IGF binding protein, its physiological role remains poorly understood. IGFBP-2 knockout mice do not show overt phenotypic modifications, however this may reflect compensation by other IGFBPs (466). Genetic overexpression studies in mice suggest that high doses of IGFBP-2 can increase insulin sensitivity (465, 467). In the current study, plasma IGFBP-2 expression was elevated in adult female offspring in response to pre-weaning GH treatment. This may partially contribute to the improvement of HOMA-IR in UN-GH female offspring. Leptin was recently reported to
regulate IGFBP-2, and adenoviral overexpression of IGFBP-2 ameliorates diabetic symptoms in many models of hyperglycaemia (465). However, the mechanism by which leptin mediates these effects remains ill-defined and recent work by Neumann et al. showed that physiological concentrations of IGFBP2 were neither sufficient to mimic nor required for the physiological action of leptin (467). In the present study we observed increased plasma leptin and reduced hepatic IGFBP-2 expression in UN-S animals compared to control and GH treated animals, suggesting that modification of IGFBP-2 in this study might be due to peripheral leptin resistance. Indeed, increased hyperphagia and concomitant adiposity in UN-S offspring supports this hypothesis. Of note, in contrast to females, despite changes in hepatic IGFBP-2 expression in UN-S male offspring compared to controls, this was not paralleled by any changes in circulating plasma IGFBP-2 concentrations. Work by others has suggested a discordant relationship between IGFBP-2 mRNA and protein levels in response to fasting (468). In the present study, changes in plasma IGFBP-2 may therefore also reflect a sexually dimorphic response to fasting as described previously (469).

Additionally, we observed an unexpected increase in basal rectal temperature in adult UN male offspring which was normalised with pre-weaning GH treatment. It has been shown in other experimental models that that catch-up growth can lead to elevated metabolic rate in adulthood (470). Metabolic rate is the major contributor of body temperature in mammals (471). Our observation may suggest potential re-programming of the basal metabolic rate in these animals. Moreover, GHR null mice which exhibit a lower core body temperature display increased longevity (472). Several studies on other transgenic mice and Ames dwarf mice also suggest that low body temperature is related to prolonged life span (473, 474). Given the reversal effect on hepatic GHR and core body temperature by GH treatment in maternal UN male offspring observed in our current study, we speculate that pre-weaning GH treatment might have a beneficial effect on longevity in these animals.

Overall, our results demonstrate that pre-weaning GH treatment can reverse maternal UN induced postnatal growth and adiposity in both male and female offspring. This suggests that GH-IGF-1 axis can be remodelled during early critical developmental windows. Evidence of adverse long term programming effects due to rapid growth following fetal growth restriction creates a dilemma in clinical practice for postnatal care in IUGR infants with rapid postnatal growth (475-477). In these instances neonatal rapid short term growth represents a trade-off in terms of long term health. Controlling catch-up growth by restraining nutrition intake can be risky, and may introduce even more detrimental outcomes from unintentional side effects of long term undernutrition (448, 449). Of importance, we have shown that pre-weaning GH treatment has no detrimental effects on glucose-insulin sensitivity in adulthood. Previous
studies using GH treatment in adult offspring subjected to programming via maternal UN demonstrated decreased adiposity and hypertension at the expense of reduced insulin sensitivity (248) due to the direct diabetogenic effects of GH. Clinical studies, albeit limited, have suggested that GH treatment in children aged 7 to 13 may result in a higher than expected incidence of type 2 diabetes mellitus in later life but this may reflect an acceleration of the disorder in predisposed individuals (478).

Our findings demonstrate that pre-weaning GH treatment can prevent UN-induced rapid growth trajectories in a normal postnatal dietary environment. Together with our previous observations demonstrating the efficacy of pre-weaning GH treatment in reversing cardiovascular abnormalities and adipocyte IR arising in offspring following maternal UN (335, 336, 440, 479), the current data suggests that early life GH treatment may represent a promising broad strategy to prevent long term adverse developmental programming effects in growth restricted offspring.
5.3 Pre-weaning growth hormone treatment protects adult male offspring against cardio-metabolic dysfunction

In the previous section, we present the data on overall growth trajectories, food intake and GH-IGF-1 axis related markers in both male and female offspring. In this section, we take a further look at programming effects on key targets in the development of metabolic syndromes, including adipose tissue and the cardiovascular system. Due to the cohort size and the time-consuming nature of the procedures utilised in the study, we only investigated male offspring in this section.

5.3.1 Programming effects on adipose tissue

5.3.1.1 Methods

Offspring born to UN mothers received either saline or GH treatment from postnatal day 3 to day 21 as described previously (Section 5.2). After weaning offspring in CON-S, CON-GH, UN-S, and UN-GH groups were further divided into dietary groups, either standard diet or HF diet. The experimental design is presented in Figure 5.7. At postnatal day 150, all offspring were culled and freshly isolated gonadal adipose tissue was used for adipose tissue glucose uptake assay, ex vivo adipose tissue culture and isolation of stromal vascular fraction (SVF). Adipose tissue culture media was harvested and cytokine secretion (TNFα, IL-6, and IL-1β) analysed by ELISA. RNA and protein were extracted from gonadal adipose tissue for real-time PCR and protein analysis. Retroperitoneal adipose tissue was fixed in 10% formalin and paraffin embedded. Sections were stained for H&E using standard procedures. A minimum of 6 litters were used per treatment group. Data were analysed by three-way ANOVA with maternal diet, GH treatment and postnatal diet as main factors. All data in section 5.3 met the assumptions of parametric test (normality and homogeneity of variance). Detailed procedures are described in Chapter 2.
Figure 5.7 Experimental design for section 5.3.1
5.3.1.2 GH treated UN offspring are protected against maternal UN-induced systematic inflammation

Plasma concentrations of TNFα, IL-1β and IL-6 were significantly increased in UN-S-C compared to CON-S-C and UN-GH-C groups (Table 5.4).

<table>
<thead>
<tr>
<th></th>
<th>TNFα (pg/mL)</th>
<th>IL-1β (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON-S-C</td>
<td>14.48 ± 3.26</td>
<td>60.69 ± 11.7</td>
<td>82.14 ± 4.12</td>
</tr>
<tr>
<td>CON-S-HF</td>
<td>12.08 ± 3.26</td>
<td>120.6 ± 21.2</td>
<td>107.22 ± 8.7</td>
</tr>
<tr>
<td>CON-GH-C</td>
<td>23.79 ± 4.04</td>
<td>103.1 ± 12.3</td>
<td>149.6 ± 15.4</td>
</tr>
<tr>
<td>CON-GH-HF</td>
<td>19.33 ± 3.65</td>
<td>115.9 ± 14.6</td>
<td>142.7 ± 17.7</td>
</tr>
<tr>
<td>UN-S-C</td>
<td>25.93 ± 4.15</td>
<td>205.9 ± 30.2</td>
<td>131.6 ± 14.2</td>
</tr>
<tr>
<td>UN-S-HF</td>
<td>30.72 ± 5.71</td>
<td>63.91 ± 14.2</td>
<td>70.7 ± 6.35</td>
</tr>
<tr>
<td>UN-GH-C</td>
<td>2.56 ± 0.61</td>
<td>50.12 ± 20.1</td>
<td>62.64 ± 4.6</td>
</tr>
<tr>
<td>UN-GH-HF</td>
<td>6.34 ± 1.3</td>
<td>76.61 ± 25.2</td>
<td>58.01 ± 5.08</td>
</tr>
<tr>
<td>Maternal diet (MD)</td>
<td>NS</td>
<td>NS</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>GH treatment (GH)</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Postnatal diet (PND)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MD×GH</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>MD×PND</td>
<td>NS</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>GH×PND</td>
<td>NS</td>
<td>P &lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>MD×GH×PND</td>
<td>NS</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

Table 5.4 Plasma pro-inflammatory cytokines

Italics indicates the effects of each factors and interactions computed from three-way ANOVA analysis and to distinguish from descriptive results.

5.3.1.3 GH treatment prevents maternal UN-induced adipocyte hypertrophy in offspring

Pronounced adipocyte hypertrophy was observed in the offspring of chow-fed UN animals evidenced by significantly increased adipocyte area (Figure 5.8). This hypertrophic phenotype was not present in CON-S-C, CON-GH-C, or UN-GH-C groups. Furthermore, adipocyte size was significantly reduced in CON-GH-C, or UN-GH-C groups (Figure 5.8).
Figure 5.8 Adipocyte tissue hematoxylin and eosin staining

A) Representative adipose tissue images are shown. B) Adipocyte area was measured using ImageJ software (n = 6 per group). Adipocytes (150–200 per slide) were assessed. Values are presented as mean ± SEM. ** denotes P < 0.01 compared to CON-S-C; +++ denotes P < 0.001 compared to UN-S-C.
5.3.1.3 UN-induced adipose tissue IR is reversed following neonatal GH treatment

Both UN-S-C and UN-S-HF offspring had significantly decreased insulin-stimulated \[^{[3H]}\text{glucose uptake compared to all other groups (Figure 5.9 A). p-AKT/total AKT levels were significantly reduced in UN-S-C group compared to CON-S-C, CON-GH-C and UN-GH-C groups (Figure 5.9 B). Gene expression of GLUT4, IRS1, PTEN and INSR were also significantly reduced in UN-S-C group compared to CON-S-C group (Figure 5.10 A). However only INSR gene expression was significantly reversed in UN-GH-C compared to UN-S-C (Figure 5.10 A). Despite similar IRS1 gene expression, \[^{32P}\text{IRS1 was significantly increased in both CON-GH-C and UN-GH-C groups compared to CON-S-C group (Figure 5.10 B).}}\]
A) Fold increase in $[^{3}H]$glucose levels in response to insulin- over non-insulin-stimulated samples ($n = 12–20$). B) Fold increase over basal levels of total and phosphorylated AKT ratio ($n = 6$). Values are presented as mean ± SEM. * denotes $P < 0.05$, ** denotes $P < 0.01$ compared to CON-S-C; + denotes $P < 0.05$ compared to UN-S-C.

**Figure 5.10 Adipose tissue glucose metabolism related markers**

A) Adipose tissue mRNA expression of GLUT4, IRS1, PTEN, and INSR ($n = 8$). B) Tyrosine phosphorylated IRS-1 in adipose tissue. Values are presented as mean ± SEM ($n = 6$). * denotes $P < 0.05$, ** denotes $P < 0.01$ compared to CON-S-C; + denotes $P < 0.05$, +++ denotes $P < 0.001$ compared to UN-S-C.
5.3.1.4 GH treatment ameliorates maternal UN heightened AT inflammatory profile

TNFα and IL-1β secretion was significantly enhanced in chow and HF fed UN-S adipose tissue explants compared to corresponding CON-S groups (Figure 5.11 A&B). TNFα concentrations were rescued by GH treatment in chow and HF fed UN-GH groups (Figure 5.11 A). There was no difference in IL-6 secretion between groups (data not shown). TNFα, IL-1β and IL-1R1 mRNA expression was significantly enhanced in UN-S groups compared to CON-S, CON-GH and UN-GH groups. However there was no difference in TNFR1 expression between groups (Figure 5.11 C-G).

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Figure 5.11 Adipose Tissue Inflammatory Profile
A) TNFα and B) IL-1β concentration in cultured adipose tissue explants (100 mg/mL) (n = 12–20). Adipose tissue mRNA expression of C) TNFα, D) IL-1β, E) TNFR1 and F) IL-1R1 (n = 8). Values are presented as mean ± SEM. * denotes P < 0.05; ** denotes P < 0.01 compared to CON-S; *** denotes P < 0.001 compared to CON-S; +, P < 0.05 compared to UN-S, +++ denotes P < 0.001 compared to UN-S.

5.3.1.5 UNS enhances adipose tissue immune cell infiltration and immunophenotype

Gene expression of MCP-1, a potent chemokine, was significantly increased in UN-S offspring compared to CON-S (Figure 5.12 A). Gene expression of macrophage infiltration markers CD68 (general macrophage marker) and CD11c (M1 pro-inflammatory macrophage marker) was enhanced in UN-S compared to the CON-S group (Figure 5.12 A). Gene expression of the M2 anti-inflammatory macrophage marker arginase-1 was significantly increased in UN-GH compared to CON-S, CON-GH and UN-S groups (Figure 5.12 A).

There was significantly increased infiltration of M1 macrophages (CD68+CD11c+) into adipose tissue in both chow and HF fed UN-S offspring compared to CON-S, CON-GH and UN-GH (Figure 5.12 B). Secretion of TNFα and IL-1β from isolated SVF was significantly increased in UN-S compared to CON-S, CON-GH and UN-GH groups, suggesting that infiltrating macrophages have an enhanced pro-inflammatory immunophenotype (Figure 5.12 C&D). IL-10 concentrations trended towards increase in UN-GH compared to CON-S, CON-GH and UN-S although statistical significance was not reached (Figure 5.12 E).
Figure 5.12 Adipose Tissue Macrophage Infiltration

A) Adipose tissue mRNA expression of CD68, CD11c, Arg1, MCR1, and MCP1 (n = 8). B) Percentage of M1 macrophages in total SVF. SVF cells were labelled with CD68 and CD11c. Double-positive cells (CD68^+CD11c^+) were classified as M1 macrophages (n = 12–20). SVF cells (200,000 cells/mL) were cultured for 24 hours and media was analysed for (C) TNFα, (D) IL-1β, and (E) IL-10 secretion (n = 12–20). Values are presented as mean ± SEM. * denotes P < 0.05, ** denotes P < 0.01 compared to CON-S; + denotes P < 0.05, ++ denotes P < 0.01 compared to UN-S; ## denotes P < 0.01 compared to chow.
5.3.2 Discussion on pre-weaning GH treatment ameliorates programming effects on adipose tissue

In line with the alteration in adiposity and postnatal growth trajectories observed in previous experiments (Section 5.2), the study in section 5.3.1 demonstrates that moderate maternal UN during pregnancy resulted in offspring adipocyte hypertrophy, decreased adipose tissue insulin sensitivity and an enhanced adipose tissue proinflammatory profile. Interestingly, pre-weaning exposure to GH reversed maternal UN-induced adipose tissue dysfunction without any obvious adverse effects on adipose tissue biology, insulin sensitivity or inflammatory profile in control GH offspring.

In the present study, we observed a number of alterations in adipose tissue insulin signalling pathways. The main mechanism that governs adipose tissue up-regulation of insulin-sensitive glucose uptake, the phosphatidylinositol-3-kinase/AKT signalling pathway, results in translocation of GLUT4 from intracellular storage vesicles to the plasma membrane (480). This pathway is initiated by binding of insulin to its receptor INSR and subsequent activation of IRS proteins. This process is negatively regulated by PTEN - an inhibitor of AKT phosphorylation (481). In this study, increased PTEN expression in UN offspring may reduce AKT phosphorylation with subsequent adverse effects on adipocyte insulin-stimulated glucose uptake via GLUT4. Interestingly GH-treated UN offspring did not display these adverse effects and demonstrated a normalization of insulin sensitivity. In addition, reduced insulin-dependent glucose uptake into adipose tissue in UN-S animals was accompanied by decreased INSR and IRS-1 gene expression. While there was a degree of rescue observed in the UNGH group, significance was only attained with INSR expression. However, IRS-1 tyrosine phosphorylation was significantly enhanced by pre-weaning GH treatment which may have also contributed to the increased adipose tissue glucose uptake observed in UNGH animals.

As discussed in Section 1.3.4, obesity is associated with a state of chronic low-grade inflammation characterized by activation of inflammatory signalling pathways culminating in abnormal cytokine secretion which drives IR and can have a major impact on energy metabolism (165, 166). The current study revealed increased plasma IL-6, IL-1β and TNFα concentrations as a result of maternal UN. Each of these cytokines are well characterised mediators of both local and systemic IR (164, 322, 482) and represent an important link between adipose tissue and the immune system. While several studies have reported the impact of maternal UN on offspring global inflammation (483-485), local adipose tissue inflammation in adult offspring has not been comprehensively investigated despite convincing
evidence that UN leads to deleterious alterations in glucose and lipid homeostasis, significant adipose expansion and visceral adiposity (107, 167).

Given the negative effects of maternal UN on offspring adipose tissue insulin sensitivity, it is reasonable to test if these effects were accompanied with increased adipose tissue inflammation. Indeed, culture of adipose explants from offspring following maternal UN demonstrated significantly increased secretion and gene expression of TNFα and IL-1β accompanied with enhanced IL-1R1 gene expression compared to control and GH treated animals. It was reported that IL-1β secretion in adipose explants from obese IR patients had a 10-fold increase compared to controls (486). Studies in IL-1R1 knockout mice demonstrated significantly improved adipose insulin sensitivity regardless of diet (322). To our knowledge, we are the first to demonstrate significant alterations in expression and regulation of the IL-1R1 pathway in the context of maternal UN-induced obesity and metabolic dysfunction. It is therefore plausible to speculate that maternal UN induced adipose tissue inflammation and associated metabolic dysregulation may, at least in part, be mediated through the IL-1R1 pathway.

In an obese setting, the increase in pro-inflammatory cytokine production is primarily due to macrophage polarization and infiltration. Adipose tissue macrophages can span the spectrum from the pro-inflammatory, M1-like cells (CD68⁺CD11c⁺) to anti-inflammatory, M2-like (CD68⁺CD11c⁻) macrophages. Lean adipose is associated with the presence of M2 macrophages, however an obese phenotype can cause a switch resulting in macrophage polarization towards an M1 phenotype (166). In our study there was significantly increased M1 adipose tissue macrophages as well as enhanced gene expression of adipose tissue CD68 and CD11c as a result of maternal UN, suggesting macrophage infiltration in these offspring. These observations agree with the increased secretion of IL-1β and TNFα from cultured SVF cells in UNS animals. Interestingly, macrophage infiltration and pro-inflammatory cytokine production in our study were normalised by pre-weaning GH treatment. Another study has shown that GH treatment can alter the immunogenicity of macrophage populations in an IL-1β dependent manner (487), which may account for the normalization of macrophage infiltration and inflammation seen in the adipose tissue of UN-GH animals.
5.3.3 Programming effects on endothelial function

5.3.2.1 Methods

Offspring born to UN mothers received either saline or GH treatment from postnatal day 3 to day 21 as described previously (Section 5.2). At postnatal day 140, SBP was measured in post-weaning chow fed male offspring before cull. The mesenteric 3rd order arteries were collected for resistance artery function evaluation using a pressure myograph. In this study, a few blocking substances were chosen to investigate 3 main pathways responsible for endothelium-dependent relaxation - nitric oxide (NO), prostaglandin I2 (PGI2) and non-NO/prostaglandin endothelium-derived hyperpolarizing factors (EDHF) pathway. The non-specific NO synthase inhibitor L-NG-Nitroarginine Methyl Ester (L-NAME, 100 µM) and the selective inhibitor of soluble guanylyl cyclase 1H-[1,2, 4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 5 µM) were used to block NO production and soluble guanylate cyclase activity. Indomethacin (INDO, 10 µM) was used to investigate the contribution of vasodilators PGI2 derived from the cyclooxygenase pathway. The role of gap junctions and EDHFs activity were investigated using the putative gap junction inhibitor carbenoxolone (CBX, 100 µM) and ATP-type Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel blocker apamin (30 µM) and intermediate-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel blocker TRAM-34 (1 µM). Both apamin and TRAM-34 in the presence of L-NAME (100 µM), ODQ (5 µM) and INDO (10 µM) were analysed to ensure that relaxation to Ach in the presence of L-NAME and INDO was a EDHF\textsuperscript{s} response independent of potassium channels. All concentrations of test substances were chosen based on previously reported results (488).

A minimum of 6 litters were used per treatment group. SBP data were analysed by two-way ANOVA with maternal diet and GH treatment as main factors. Vessel diameter/pressure relationships were analysed by linear regression. Concentration-relaxation curves were constructed using Prism software (GraphPad Software Inc., USA.). Detailed procedures are described in Chapter 2.
5.3.2.2 GH treatment protects against maternal UN induced high blood pressure

SBP at day 140 was significantly increased in UN-S male offspring when compared to CON-S and CON-GH offspring and normalised in UN-GH animals (Figure 5.13). Pre-weaning growth hormone treatment did not have an effect on SBP in CON-GH offspring when compared to CON-S animals.

![Figure 5.13 Systolic blood pressure (SBP) at postnatal day 140 in male offspring](image)

* denotes $P < 0.001$ for UN-S compared to all other groups. Values are presented as mean ± SEM, $n = 10$ per group.

5.3.2.3 Mesenteric vessel responsiveness to PE and ACh

**Mesenteric vessel responsiveness to PE**

PE induced a dose-dependent vasoconstriction in all groups. Mesenteric vessel responsiveness to PE was significantly reduced in UN-S offspring compared to CON-S and CON-GH, and normalised in UN-GH offspring (Figure 5.14 A). Pre-weaning GH treatment did not have an effect on constriction in CON-GH when compared to CON-S group (Figure 5.14 A).

**Mesenteric vessel responsiveness to ACh**

ACh produced a dose-dependent vessel dilatation in all groups. Mesenteric vessel responsiveness to ACh was significantly reduced in UN-S compared to CON-S and CON-GH,
and pre-weaning GH treatment normalised the responsiveness to Ach in UN-GH group (Figure 5.14 B). The maximum relaxation response was reduced on average 10% in UN-S animals compared to other groups (UN-S 85.3±0.41, CON-S, 93.4±0.9, CON-GH 95.1±0.8, UN-GH 94.5±1.4). No differences were observed between CON-S and CON-GH vessels in response to Ach (Figure 5.14 B).

**Vessel diameter-pressure relationship**

A pressure-dependant vasodilatation was observed in all vessels. Third order mesenteric vessels from UN-S male offspring displayed an impaired dilation response to increased pressure when compared to CON-S, CON-GH and UN-GH offspring (Figure 5.14 C). Pre-weaning GH treatment did not have an effect on the diameter-pressure relationship in CON-GH offspring vessels when compared with CON-S.

*Figure 5.14 Mesenteric vessel responsiveness to PE and Ach*
A) Mesenteric vessel responsiveness following phenylephrine (PE) treatment, measured as % change from initial resting diameter and normalised as % maximum constriction in CON-S, CON-GH, UN-S and UN-GH adult male offspring. * denotes P < 0.001 for overall effect of UN-S versus all other groups. 

B) Mesenteric vessel responsiveness following cumulative additions of vasodilator acetycholine (ACh) and expressed as % change from initial resting diameter after pre-constriction with PE (10 µM). * denotes P < 0.001 for overall effect of UN-S versus all other groups. 

C) Mesenteric vessel myogenic responsiveness to pressure, measured as % change from initial vessel diameter at 10 mmHg. * denotes P < 0.001 for UN-S versus all other groups. Values are presented as mean ± SEM.

5.3.2.4 Mesenteric vessel relaxation to ACh in the presence of endothelium-dependent mediators

ACh-induced relaxation in vessels incubated with L-NAME and ODQ

In the presence of L-NAME (100µM) and ODQ (5µM), there was a dose-dependent ACh-induced relaxation in all vessels (Figure 5.15). Ach-induced relaxation was significantly reduced in UN-S mesenteric vessels compared to UN-GH vessel responsiveness (Figure 5.15). The maximum relaxation response was reduced by average 11% in UN-S group compared with CON-S and CON-GH groups (% maximum response: UN-S 27.2±1.0, CON-S 38.2±1.07, CON-GH 38.9±0.78, UN-GH 29±1.82). There was a significant intermediary effect of pre-weaning GH treatment in the UN-GH group compared to UN-S group (Figure 5.15).

Figure 5.15 ACh-induced relaxation in vessels incubated with L-NAME and ODQ

Mesenteric vessel responsiveness following cumulative addition of vasodilator ACh measured as % change from initial resting diameter after pre-constriction with PE (10 µM) in the presence of L-NAME (100 µM) and ODQ (5 µM). Values are presented as mean ± SEM. * denotes P < 0.001 for
UN-S versus CON-S and CON-GH; ** denotes P < 0.001 for overall difference between UN-GH and CON-S and CON-GH.

**ACh-induced relaxation in vessels incubated with CBX**

In the presence of CBX (100μM), Ach induced vasodilatation was reduced in all groups. Ach-induced vasodilatation was significantly reduced in UN-S offspring compared to CON-S, and was completely normalised by pre-weaning GH treatment in UN-GH group (% maximum response: UN-S 52.5±0.47, UN-GH 66±1.10, CON-S 68.9±1.23, CON-GH 66.4±1.42, Figure 5.16 A). CON-S and CON-GH were not different from each other (Figure 5.16 A).

**ACh-induced relaxation in vessels incubated with CBX and L-NAME**

In the presence of CBX (100 μM), and L-NAME (100 μM), ACh induced vasodilatation was observed in all groups. Vessel responsiveness to Ach was significantly reduced in UN-S group compared to CON-S, CON-GH groups (Figure 5.16 B). UN-GH vessel responsiveness was significantly reduced after -6Log [ACh] M (1 μM) compared to CON-S and CON-GH, however, significantly improved when compared to UN-S (Figure 5.16 B). CON-S and CON-GH were not different from each other (Figure 5.16 B).

**ACh-induced relaxation in vessels incubated with TRAM-34 and Apamin**

In the presence of TRAM-34 (1 μM) and Apamin (30 μM), ACh induced vasodilatation was observed in all groups. UN-S vessel responsiveness to ACh was significantly reduced when compared to CON-S and CON-GH offspring and normalised in UN-GH male offspring (Figure 5.16 C). Pre-weaning GH treatment did not have an effect on the vessel responsiveness in CON-GH offspring when compared to CON-S offspring (Figure 5.16 C).

**ACh-induced relaxation in vessels incubated with TRAM-34, Apamin, L-NAME and INDO**

The combination of TRAM-34 (1 μM) and Apamin (30 μM) in the presence of L-NAME (100 μM) and INDO (10 μM) induced a significant reduction in Ach-induced vasodilatation in all groups. Pre-weaning growth hormone treatment improved vessel responsiveness at –4 Log (100 μM) and -3 Log [ACh] M (1 mM) in UN-GH offspring vessels when compared to UN-S vessels (Figure 5.16 D). Vasodilatory response did not differ between CON-S and CON-GH groups (Figure 5.16 D).
ACh-induced relaxation in vessels incubated with INDO and L-NAME

In the presence of indomethacin (10 μM), Ach induced vasodilatation was reduced in all groups and ACh dose-dependent relaxation was not different between groups (Figure 5.16 E). In the presence of L-NAME (100 μM) and INDO (10 μM), mesenteric vessel responsiveness was also not different between groups (Figure 5.16 F).
Figure 5.16 ACh-induced relaxation in vessels incubated with CBX / TRAM-34 / Apamin / L-NAME / INDO

A) Mesenteric vessel responsiveness following cumulative additions of vasodilator acetylcholine (ACh) measured as % change from initial resting diameter after pre-constriction with PE (10 µM)
in the presence of CBX (100 µM). B) Mesenteric vessel responsiveness following cumulative additions of vasodilator ACh measured as % change from initial resting diameter after pre-constriction with PE (10 µM) in the presence of L-NAME (100 µM), INDO (10 µM) and CBX (100 µM). C) Mesenteric vessel responsiveness following cumulative additions of vasodilator ACh measured as % change from initial resting diameter after pre-constriction with PE (10 µM) in the presence of TRAM-34 (1 µM) and Apamim (30 µM). D) Mesenteric vessel responsiveness following cumulative additions of vasodilator ACh measured as % change from initial resting diameter after pre-constriction with PE (10 µM) in the presence of TRAM-34 (1 µM), Apamim (30 µM), L-NAME (100 µM) and INDO (10 µM). E) Mesenteric vessel responsiveness following cumulative additions of vasodilator ACh measured as % change from initial resting diameter after pre-constriction with PE (10 µM) in the presence of INDO (10 µM). F) Mesenteric vessel responsiveness following cumulative additions of vasodilator ACh measured as % change from initial resting diameter after pre-constriction with PE (10 µM) in the presence of L-NAME (100 µM) and INDO (10 µM). * P < 0.001 for UN-S versus all other groups. ** P < 0.001 for UN-GH versus UN-S. All data are means ± SEM, n = 10 per group.
5.3.4 Discussion on pre-weaning GH treatment ameliorates maternal UN programmed endothelial dysfunction

Programmed hypertension has been shown in a number of maternal undernutrition models (Section 1.3.2). In the present study, we have demonstrated that pre-weaning GH treatment normalised blood pressure and improved vascular responsiveness in adult offspring born to mothers who were undernourished during pregnancy. Previous studies have shown the beneficial effects of GH treatment reducing age-related hypertension in rats (489). Furthermore, Yang et al. showed that GH treatment may improve cardiac function by both increased myocardial contractility and decreased peripheral vascular resistance in the rat heart (490). In the present study, we observed an average ~18mmHg reduction of blood pressure in pre-weaning GH treated offspring to hypertensive UN-S offspring, which is in line with previously reported data. Furthermore, our study highlights that the modification of GH-IGF axis in early life have long term beneficial effects on the cardiovascular system.

Our observations indicate that the beneficial effects of early life GH treatment on vascular function are likely to be due to alterations in EDHFs and NO mediated vasodilatation. Impaired endothelium-dependent vasodilatation is a common pathological feature consistently observed in human patients and experimental animal models of hypertension (491-493). The endothelium produces a number of signalling molecules, including EDHFs, NO and prostaglandins, which regulate endothelial - vascular smooth muscle communication and lead to vasodilatation (494, 495) (Figure 5.17). Among the three main vasodilation mechanisms, the EDHFs pathway is the largest contributor to smooth muscle relaxation in resistance vessels. The EDHFs pathway involves the activation of Ca^{2+}-activated K^+ channels, myoendothelial gap junction mediated signal transfer and/or a few other molecular signalling that leads to vascular smooth muscle hyperpolarization (495). In the present study, the gap junction uncoupler CBX significantly inhibited mesenteric vessel relaxation of UN-S offspring which was completely reversed in pre-weaning GH treated offspring, indicating that GH may be beneficial to the vascular function via gap junction related EDHFs mechanisms. Interestingly, in the combined presence of the NO synthase inhibitor L-NAME, PGI2 blocker indomethacin, and the K^+ channels blockers apamin and TRAM-34, vessel relaxations were significantly reduced in UN-S animals compared to controls and completely reversed by GH treatment, which suggests a role of other EDHFs signalling mechanisms involved in vessel dilation that may not involve K^+ channels.
Overall, there are three main pathways that are responsible for vasodilation. Briefly, 1) NO pathway: sheer stress can force the opening of Ca$^{2+}$ channels and increase Ca$^{2+}$ in endothelial cells. Increased Ca$^{2+}$ concentration activates NO synthesis. NO diffuses to smooth muscle, binds to its receptor soluble guanylate cyclase which leads to hyperpolarization via cGMP pathway. 2) PGI pathway: PGI$_2$ is produced by cyclooxygenase from arachidonic acid. It signals adenylyl cyclase and increases production of cAMP, leading to smooth muscle relaxation. 3) EDHFs pathway: there are a number of mediators involved in this pathway. EDHFs can induce hyperpolarization in endothelial cells via K$^+$ channel activation. This hyperpolarization signal is then transferred through gap junctions to smooth muscle and cause relaxation. Additionally, some EDHFs can directly act on smooth muscle after releasing from the endothelium. NO: nitric oxide, EDHFs: endothelial-derived hyperpolarizing factors, PGI: prostaglandin I$_2$, cGMP: cyclic guanosine monophosphate, cAMP: cyclic adenosine monophosphate. Adopted from Vanhoutte et al. (496) and Giles et al. (495)
The current study also suggests that GH treatment during postnatal development may, in part, affect NO mediated pathways. When NO production was blocked by L-NAME and ODQ, vessel relaxation in response to ACh was significantly reduced in the UN-S offspring, and improved in UN-GH offspring. A number of studies have demonstrated that GH can increase NO production via directly inducing the activity of endothelial nitric oxide synthase and indirectly by increasing IGF-1 concentrations (497). GH deficiency is associated with endothelial dysfunction and decreased NO production (498), and administration of recombinant human GH has been shown to increase NO production and sequentially improve endothelial dysfunction in adult patients with GH deficiency (499). We have previously shown that circulating IGF-1 concentrations are reduced at birth in the offspring of undernourished mothers (102), which may potentially have adverse effects on endothelial development. Although not tested immediately following GH treatment in the current study, we speculate that offspring plasma IGF-1 concentrations may be rescued in UN-GH group given the observation on the immediate weight gain responses during the treatment period (Section 5.2).
5.4 Additional discussion

5.4.1 Limitations

Although 50% food restriction in rats results in an offspring phenotype that has a number of similarities to human fetal growth restriction, the clinical translation of this study needs caution, as the current model does not produce the most common pathological changes in human IUGR in Western society - maternal placental insufficiency. In the case of maternal placenta insufficiency, reduced fetal nutrient supply is often accompanied with fetal hypoxia and other primary maternal conditions such as preeclampsia. Further studies using different models will be needed to confirm the effect of GH treatment in a more complex pathological condition.

Sex-specific effects are often observed in studies related to developmental programming (Section 1.3.5). Due to logistical constraints, adipose tissue and endothelium function were only examined in male offspring. Given the similar postnatal growth trajectories and adiposity in female offspring, it is possible that female offspring may have similar outcomes related to adipose tissue function. Nonetheless, further experiments on female offspring are necessary to make any such conclusions.

CBX has been shown to be the most potent blocker of gap junctions (500), however, it is known to have non-specific effects on varied ion channels and cellular processes, although conflicting reports exist (501, 502). While using CBX to block gap junctions in endothelium functional studies is still a widely accepted method in the field (488, 503), the interpretation of relevant results needs to take the non-specificity of CBX into account.

5.4.2 Future direction

Studies by others (Section 1.4.1) and unpublished data from our group suggested that maternal UN can lead to offspring liver steatosis. Given the liver weight change (Table 5.2) and altered systemic inflammation profile (Table 5.4) observed in this study, a key interest is to evaluate the features of NAFLD in these offspring in future work.
Chapter 6. Discussion

This thesis addressed two main challenges in the DOHaD paradigm. Firstly, to identify potential mechanisms involved in the developmental programming phenomenon, particularly in the setting of two different maternal nutritional adversities - obesity and undernutrition. Secondly, we sought to evaluate the effectiveness of nutritional and pharmaceutical intervention strategies during critical early life periods to reverse detrimental programming effects observed in offspring. Importantly, we have also examined sex-specific effects in offspring following different early life programming stimuli. In this final section, we summarise our findings in relation to these challenges and suggest future directions to build on our current knowledge and understanding.

6.1 Mechanisms and pathways

Developmental programming is a complex phenomenon, which often affects multiple systems in an exposed individual (Figure 1.2). Significant evidence has suggested a central role for chronic low-grade inflammation in the development of obesity and metabolic syndrome (165, 504). However, there is a relative paucity of data examining the role of inflammatory processes in the setting of developmental programming of metabolic dysfunction despite its known role in the etiology of obesity and type 2 diabetes (Section 1.3.4). Our studies now provide further evidence that priming of offspring inflammatory responses represent a potential mechanism underpinning developmental programming (Section 4.2, 5.3). The fetal liver is the most significant contributor to the early immune system before birth (505, 506). In our maternal obesity models (Section 4.2), programmed neonates were shown to have altered hepatic pro-inflammatory gene expression profiles, suggesting a potentially impaired immune system at birth. Interestingly, we observed maternal systemic pro-inflammatory profile induction in parallel with altered offspring hepatic inflammatory profiles (Section 4.2). It is possible to speculate that maternal obesity induced pro-inflammatory cytokines may act as a primary insult that set the baseline of offspring inflammatory responses, which may contribute to the later development of IR and other metabolic dysfunction (Section 1.3.4). Indeed, recent studies have shown that maternal immune activation using a bacterial immunostimulant can modify the immune response of offspring in adulthood (507, 508). In the maternal undernutrition model (Section 5.3), offspring born to undernourished mothers displayed a potent immunophenotype featuring enhanced systemic inflammation, increased adipose tissue pro-inflammatory cytokine secretion and macrophage infiltration. A follow-up study by our
group on bone marrow macrophages in the same cohort suggests a heightened sensitivity to immune stressors arising following maternal undernutrition (Appendix VIII). Additionally, it has been shown that adipose macrophage infiltration and increased pro-inflammatory cytokines can lead to systemic IR and endothelial dysfunction (165, 509, 510). Although not tested directly in our studies, we speculate that the primed macrophages may at least partially contribute to the IR and endothelial dysfunction observed in our maternal undernutrition study (Section 5.3). Taken together, our findings provide further evidence that inflammation may play an important role in the instigation of developmental programming and therefore therapeutic strategies aimed at addressing the inflammatory state may be a useful approach to ameliorate both the immediate consequences (e.g. maternal) and later metabolic sequelae in offspring following an adverse early life nutritional environment.

Further, in addition to inflammatory pathways, we have shown that leptin may also play a key mechanism in the initiation of developmental programming (Section 3.3, 4.3, 5.2). Leptin is a key adipokine that gates energy balance and reproductive fitness (Section 1.3.4, 3.4). Previous studies by our group and others suggested that leptin resistance may be a common mechanism in the developmental programming of obesity in the setting of both maternal obesity and undernutrition (Section 1.3.4). The observations made in the current thesis (Section 4.3, 5.2) agree with and add to the current understanding. Interestingly, the expression of the leptin regulated gene, IGFBP-2, was reduced as a result of maternal undernutrition (Section 5.2). Although, the physiological role of IGFBP-2 remains poorly understood, we speculate that there might be a potential link between leptin and GH-IGF axis which may potentially contribute to developmental programming observed following maternal undernutrition. Additionally, we observed a similar trend between neonatal plasma leptin concentration and altered timing of offspring first pubertal onset (Section 3.3, 4.3). While these observations are associative and therefore we can not make firm conclusions about the role of leptin on the timing of pubertal onset in the present study, our data does encourage further investigation on prepubertal leptin regulation in order to detail the specific pathways involved in developmental programming of reproductive maturity.

A third potential pathway related to mechanisms of developmental programming and supported by our studies involves the regulation of food intake and appetite control (Section 4.3). We observed a profound high energy food preference concomitant with hyperphagia in offspring born to MO diet induced obese dams (Section 4.3). Increased maternal glutamate concentrations and offspring leptin concentrations suggest potential modifications in the central regulation of food intake (Section 4.4). Interestingly, female MO offspring only
exhibited excessive weight gain when given the choice of high energy food, but not during the period of chow feeding alone, despite the fact that female offspring showed a long period of increased chow intake (Section 4.3). This observation highlights that programmed high energy food preference is a significant factor for the development of obesity in the developmental programming.

Another pathway known to be involved in developmental programming in the setting of maternal undernutrition is that of the GH-IGF-1 axis. Our work showed that maternal undernutrition leads to increased hepatic GHR expression in both male and female adult offspring (Section 5.2) which may critically affect the growth trajectory - as the responsiveness of liver to GH-induced IGF-1 production is dependent on the expression of GHR (448). Although not tested directly in our study, we speculate that glucocorticoids may play a role in this increased GHR expression. Prenatal stress conditions including maternal undernutrition and IUGR are known to be associated with increased materno-fetal glucocorticoid concentrations in both human and experimental animal models (511). Ex vivo studies in rat hepatocyte and human adipocyte have demonstrated that glucocorticoids can regulate the expression of GHR in a dose-dependent manner - low dose glucocorticoid exposure can induce GHR expression while high dose glucocorticoid exposure can suppress GHR expression (512, 513). Additionally, it has been shown in a sheep model that within a physiological range the plasma cortisol concentration in utero is positively correlated with the abundance of GHR in the fetal liver (514). We therefore speculate that altered materno-fetal glucocorticoid concentrations may permanently modify offspring GHR expression and thus lead to changes in offspring postnatal growth. When exposed to low dose glucocorticoid concentrations caused by mild stress, offspring may increase GHR which subsequently leads to rapid postnatal catch-up growth. On the other hand, exposure to high concentrations of glucocorticoids caused by severe stress, may cause offspring to down-regulate GHR which subsequently leads to permanent growth retardation. Although further investigations are essential to make such conclusions, the relationship between glucocorticoid and GHR may present a cross-talk pathway between hypothalamic–pituitary–adrenal axis and GH-IGF-1 axis, and potentially provide a fine-tune mechanism that regulates postnatal growth according to the early life stress stimuli. This thesis suggests and lends support to such a novel hypothesis.

6.2 Intervention strategies targeting early life

Two different intervention strategies were applied to maternal obesity and maternal undernutrition setting respectively in this thesis. In the models using a maternal obesogenic
environment, we observed a number of beneficial effects of taurine in reversing programmed offspring metabolic and reproductive dysfunction in a sex-specific manner (Section 3.2, 4.2). One possible mechanism for the observed beneficial effects of taurine is taurine’s anti-inflammatory feature. As discussed in the last section, maternal immune activation may set the baseline of offspring inflammatory responses, which may influence offspring later health. In our studies, taurine supplementation completely normalised maternal systemic pro-inflammatory cytokines induced by a maternal obesogenic diet, and improved neonatal hepatic pro-inflammatory gene expression profiles (Section 3.2, 4.2). Taurine has been shown to normalise offspring pancreatic islet development in maternal low protein models (171, 252, 255, 257). Although we didn’t directly test pancreatic function per se, we are the first, to our knowledge, to report similar taurine protective effects on offspring long term glucose metabolism in the setting of maternal obesity (Section 4.3.6). Moreover, maternal taurine supplementation also impacted on offspring food intake, energy metabolism, and reproductive maturity (Section 3.3, 4.3). These effects might be related to taurine’s function as a central neurotransmitter or mediated via interactions with leptin as discussed in corresponding sections. However, further investigations, particularly on hypothalamic function, are needed to draw such conclusions.

Despite the beneficial effects on programmed offspring and some aspects of maternal health outcomes, we found that taurine supplementation lead to some adverse outcomes in control offspring and MO diet fed dams. Maternal taurine supplementation increased neonatal mortality in control pregnancies and exacerbated hepatic steatosis in high fat high fructose fed dams (Section 4.2). While these observations warrant further investigation, caution regarding the potential harm from taurine consumption during pregnancy should be considered.

In the maternal undernutrition model, pre-weaning GH hormone treatment completely normalised maternal UN-induced offspring hyperphagia, prevented rapid post-weaning growth and concomitant adiposity, adipose tissue dysfunction and endothelial dysfunction (Section 5.2, 5.3). Interestingly, we did not detect any obvious adverse effect of pre-weaning GH treatment in control animals on linear growth, glucose metabolism, adipose tissue or cardiovascular system. In clinical practice, GH has been suggested to treat short stature in children over 3-4 year old as per European and American guidelines (515, 516), and the safety of GH treatment is well-documented (517, 518). Given the remarkable beneficial effects of pre-weaning GH treatment observed in our studies and the well-documented clinical usage of GH in children, we propose that GH treatment in the early period of establishment of the
GH-IGF-1 axis may be a promising intervention strategy to prevent long term adverse developmental programming effects in growth restricted offspring.

*In summary*, the work described in this thesis, using rat models at both ends of the maternal nutritional spectrum (obesity and undernutrition), have further investigated the mechanisms of developmental programming and proved in principle that it is possible to use simple dietary or pharmaceutical intervention strategies during the early life period of developmental plasticity to combat the adverse long term programming effects. The current findings support the DOHaD hypothesis and broaden the understanding of the phenomenon particularly in relation to several inflammatory and metabolic pathways and potential efficacy of targeted intervention strategies. Further understanding of the mechanisms in relation to the phenomenon and interventions of developmental programming remains the main focus of future research in this area.
Appendix I

Review Article

Maternal Obesity and Developmental Programming of Metabolic Disorders in Offspring: Evidence from Animal Models

M. Li, D. M. Sloboda, and M. H. Vickers

Liggins Institute and the National Research Centre for Growth and Development, University of Auckland, Auckland 1142, New Zealand

Correspondence should be addressed to M. H. Vickers, m.vickers@auckland.ac.nz

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The incidence of obesity and overweight has reached epidemic proportions in the developed world as well as in those countries transitioning to first world economies, and this represents a major global health problem. Concern is rising over the rapid increases in childhood obesity and metabolic disease that will translate into later adult obesity. Although an obesogenic nutritional environment and increasingly sedentary lifestyle contribute to our risk of developing obesity, a growing body of evidence links early life nutritional adversity to the development of long-term metabolic disorders. In particular, the increasing prevalence of maternal obesity and excess maternal weight gain has been associated with a heightened risk of obesity development in offspring in addition to an increased risk of pregnancy-related complications. The mechanisms that link maternal obesity to obesity in offspring and the level of gene-environment interactions are not well understood, but the early life environment may represent a critical window for which intervention strategies could be developed to curb the current obesity epidemic. This paper will discuss the various animal models of maternal overnutrition and their importance in our understanding of the mechanisms underlying altered obesity risk in offspring.

1. Background

The current epidemic of obesity and related metabolic disorders has been seen as a symptom of affluence with the primary cause relating to the development of an obesogenic environment and ease of access to highly calorific foods and reduced energy expenditure in work and leisure activities [1].

The metabolic syndrome is characterised by the clustering of cardiovascular risk factors including diabetes, obesity, hyperlipidaemia, and hypertension and is likely the result of complex interactions between genes, dietary intake, physical activity, and the environment. Within the cluster of risk traits for the metabolic syndrome, insulin resistance and visceral obesity have been recognized as the most important causal factors [2]. A number of genes have been identified that are associated with obesity and metabolic syndrome in humans [1, 3], but the genetic component of this condition cannot account for the marked increases in the prevalence of obesity and metabolic syndrome in recent years. In this context, the developmental origins of health and disease (DOHaD) hypothesis has highlighted the link between the periconceptual, fetal, and early infant phases of life and subsequent development of adult obesity and the metabolic syndrome [4–6].

The mechanisms underpinning the developmental programming framework and the role of genetic versus environmental factors remain speculative. One general thesis is that in response to an adverse intrauterine environment the fetus adapts its physiological development to maximize its immediate chances for survival. These adaptations may include resetting metabolic homeostasis set points, endocrine systems, and downregulating of growth, commonly manifest in an altered birth phenotype. More recently the “predictive adaptive response” (PARs) hypothesis proposes that the degree of mismatch between the pre- and postnatal environments is a major determinant of subsequent disease risk [7, 8]. Thus, it is thought that whilst adaptive changes in fetal physiology may be beneficial for short-term survival in utero, they may be maladaptive in later life, contributing to adverse health outcomes when offspring are exposed to catch-up growth, diet-induced obesity, and other factors [8, 9].

Animal models have been extensively used to study the basic physiological principles underlying the DOHaD hypothesis and are essential to the search for the mechanistic
Appendix II

Developmental Programming of Nonalcoholic Fatty Liver Disease: The Effect of Early Life Nutrition on Susceptibility and Disease Severity in Later Life

Minglan Li, Clare M. Reynolds, Stephanie A. Segovia, Clint Gray, and Mark H. Vickers

Liggins Institute and Gravida: National Centre for Growth and Development, University of Auckland, Auckland 1142, New Zealand

Correspondence should be addressed to Mark H. Vickers; m.vickers@auckland.ac.nz

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Nonalcoholic fatty liver disease (NAFLD) is fast becoming the most common liver disease globally and parallels rising obesity rates. The developmental origins of health and disease hypothesis have linked alterations in the early life environment to an increased risk of metabolic disorders in later life. Altered early life nutrition, in addition to increasing risk for the development of obesity, type 2 diabetes, and cardiovascular disease in offspring, is now associated with an increased risk for the development of NAFLD. This review summarizes emerging research on the developmental programming of NAFLD by both maternal obesity and undernutrition with a particular focus on the possible mechanisms underlying the development of hepatic dysfunction and potential strategies for intervention.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a clinical term which refers to excess fat (>5% weight or volume) deposition in the liver in the absence of excessive alcohol intake. It is rapidly becoming one of the most prevalent liver diseases globally. Population studies utilising ultrasonography and magnetic resonance imaging (MRI) suggest that the prevalence of NAFLD is up to 30% in different countries studied to date including USA, Italy, China, and Japan [1]. Obesity is strongly associated with NAFLD. The incidence of NAFLD in severely obese populations is approximately 74%, and in developed nations 60% of NAFLD patients are obese [2–4]. With obesity rates increasing worldwide, particularly in developing societies undergoing nutritional transition, the prevalence of NAFLD is set to increase markedly in the near future [5, 6].

NAFLD represents a spectrum of pathological changes from isolated hepatic steatosis (fatty liver) without hepatocellular damage to nonalcoholic steatohepatitis (NASH, fatty liver with inflammation) which is the extreme form of the disease characterised by hepatocellular injury and inflammation with or without fibrosis [7]. The natural progression of NAFLD is not fully understood and long-term outcomes are dependent on pathological subtypes. The majority of isolated steatosis has a relatively benign outcome displaying slow progression over many years. However, 10–20% of cases progress to NASH, which is closely linked to hepatic cirrhosis and hepatocellular carcinoma (HCC), and carries a significantly increased mortality risk [8–13].

Although initially considered as a sequential progression from simple steatosis to accompanied inflammation, it is now widely accepted that the pathogenesis of simple steatosis and NASH is likely to progress via different mechanisms [13, 14]. The development of NASH consists of a number of events whereby steatosis and inflammation and cell damage may occur in parallel rather than in strict sequence [13]. The accumulation of fat in hepatocytes can be achieved via four main mechanisms: (1) increased free fatty acid and lipid uptake, (2) increased de novo lipogenesis, (3) decreased lipid oxidation, and (4) decreased hepatic very low density lipoprotein (VLDL)-triglyceride secretion, all of which have been reviewed in detail by Fabbri et al. [15]. On the other hand, in the case of NASH, as proposed by Tilg and Moschen, the evolution of steatosis and inflammation may enhance each other under a number of parallel processes [13]. These
Appendix III

Maternal taurine supplementation attenuates maternal fructose-induced metabolic and inflammatory dysregulation and partially reverses adverse metabolic programming in offspring

M. Li\textsuperscript{a}, C.M. Reynolds\textsuperscript{a}, D.M. Sloboda\textsuperscript{a,\textasteriskcentered b,} C. Gray\textsuperscript{a}, M.H. Vickers\textsuperscript{a,\textasteriskcentered *}

\textsuperscript{a}Liggins Institute and Gravida: National Centre for Growth and Development, University of Auckland, Auckland, New Zealand
\textsuperscript{b}Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada

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Abstract

Excessive fructose consumption is associated with insulin resistance (IR) and nonalcoholic fatty liver disease (NAFLD), and high fructose intake during pregnancy can lead to compromised fetal development in the rat. Evidence suggests that the amino acid taurine can ameliorate fructose-induced IR and NAFLD in nonpregnant animals. This study investigated the efficacy of taurine supplementation on maternal fructose-induced metabolic dysfunction and neonatal health. Time-mated Wistar rats were randomized to four groups during pregnancy and lactation: (a) control diet (CON), (b) CON supplemented with 1.5% taurine in drinking water (CT), (c) CON supplemented with fructose solution (F) and (d) F supplemented with taurine (FT). Maternal and neonatal weights, plasma cytokines and hepatic gene expression were analyzed. Maternal hyperinsulinemia, increased homeostasis model assessment of IR indices and elevated proinflammatory cytokines were observed in F group and normalized in FT group. Maternal fructose-induced hepatic steatosis accompanied with increased liver weight was ameliorated with taurine supplementation. Maternal hepatic sterol regulatory element-binding protein-1c and fatty acid synthase expression was significantly increased in the F group compared to the CON, CT and FT groups. Neonatal hepatic phosphoenolpyruvate carboxykinase expression was increased in male F neonates compared to the CON, CT and FT groups and was increased in female F and FT neonates compared to CON and CT. Interleukin-1β expression was decreased in male CT and FT neonates compared to other male groups. Hepatic tumour necrosis factor receptor-1 was lower in the male FT group than the F group. These results demonstrate that maternal taurine supplementation can partially reverse fructose-induced maternal metabolic dysfunction and may ameliorate adverse developmental programming effects in offspring in a sex-specific manner.

Keywords: Developmental programming; Taurine; Fructose; Inflammation; Fatty liver disease; Maternal nutrition; Animal models

1. Introduction

Over the last few decades, the use of fructose as a food additive to sweeten beverages and other processed foods has increased globally \cite{1} and correlates closely with the rise in obesity, metabolic syndrome and type 2 diabetes \cite{2}. Given the potential of excessive fructose consumption to induce adiposity and metabolic dysfunction, increased availability of products containing fructose has the potential to fuel the global obesity epidemic \cite{3–5}. Animal studies have shown that high-fructose diets can lead to a series of metabolic disorders including insulin resistance (IR), hypertension and nonalcoholic fatty liver disease (NAFLD) \cite{6,7}. Research from our group and others has shown that fructose consumption during pregnancy and lactation leads to hyperinsulinemia, hyperglycemia and hepatic steatosis in dams \cite{8–10}. However, there are few studies that examine the impact of intervention strategies to combat the detrimental effects of excessive fructose intake on the health and well being of pregnant mothers and their offspring.

Growing evidence from both human and animal studies suggests that the development of metabolic disorders during pregnancy is strongly associated with adverse effects on the long-term health of offspring \cite{11,12}. As proposed by the developmental origins of health and disease (DOHaD) paradigm, altered maternal nutrition during critical periods of developmental plasticity can alter offspring development, which in turn can lead to an increased risk of obesity and metabolic dysfunction in later life \cite{13}. We have previously shown that moderate fructose consumption during pregnancy can lead to changes in placental growth, hyperglycemia and hyperleptinemia in female offspring \cite{8}. Of importance, the fructose load in our experimental model is designed to provide the mother only 20% of total calories, thus highlighting that, even at relatively low concentrations, fructose can induce significant metabolic abnormalities in offspring. Further work by Rodriguez et al. \cite{14} demonstrated that maternal fructose intake could alter fetal leptin signaling. It is also important to delineate between the different forms of fructose. Most studies to date have utilized high-fructose corn syrup (HFCS) which is a commonly used sweetener in food and beverages. However, the terms HFCS and fructose are often, and incorrectly, used interchangeably. While pure crystalline fructose, as used in our previous work \cite{8}...
Appendix IV

Effects of Taurine Supplementation on Hepatic Markers of Inflammation and Lipid Metabolism in Mothers and Offspring in the Setting of Maternal Obesity

Minglan Li¹, Clare M. Reynolds¹, Deborah M. Sloboda¹,², Clint Gray¹, Mark H. Vickers¹*

¹Liggins Institute and Gravida: National Centre for Growth and Development, University of Auckland, Auckland, New Zealand, ²Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada

Abstract
Maternal obesity is associated with obesity and metabolic disorders in offspring. However, intervention strategies to reverse or ameliorate the effects of maternal obesity on offspring health are limited. Following maternal undernutrition, taurine supplementation can improve outcomes in offspring, possibly via effects on glucose homeostasis and insulin secretion. The effects of taurine in mediating inflammatory processes as a protective mechanism has not been investigated. Further, the efficacy of taurine supplementation in the setting of maternal obesity is not known. Using a model of maternal obesity, we examined the effects of maternal taurine supplementation on outcomes related to inflammation and lipid metabolism in mothers and neonates. Time-mated Wistar rats were randomised to either: 1) control : control diet during pregnancy and lactation (CON); 2) CON supplemented with 1.5% taurine in drinking water (CT); 3) maternal obesogenic diet (high fat, high fructose) during pregnancy and lactation (MO); or 4) MO supplemented with taurine (MOT). Maternal and neonatal weights, plasma cytokines and hepatic gene expression were analysed. A MO diet resulted in maternal hyperinsulinemia and hyperleptinemia and increased plasma glucose, glutamate and TNF-α concentrations. Taurine normalised maternal plasma TNF-α and glutamate concentrations in MOT animals. Both MO and MOT mothers displayed evidence of fatty liver accompanied by alterations in key markers of hepatic lipid metabolism. MO neonates displayed a pro-inflammatory hepatic profile which was partially rescued in MOT offspring. Conversely, a pro-inflammatory phenotype was observed in MOT mothers suggesting a possible maternal trade-off to protect the neonate. Despite protective effects of taurine in MOT offspring, neonatal mortality was increased in CT neonates, indicating possible adverse effects of taurine in the setting of normal pregnancy. These data suggest that maternal taurine supplementation may ameliorate the adverse effects observed in offspring following a maternal obesogenic diet but these effects are dependent upon prior maternal nutritional background.

Introduction
Obesity and overweight during pregnancy has become a major emerging issue for maternal and neonatal health over the past decade [1,2]. Periconceptional and gestational obesity are associated with insulin resistance (IR) and low-grade inflammation which increases the incidence of gestational diabetes, preeclampsia, miscarriage, and neonatal mortality and the long-term risk of developing metabolic syndrome [3–5]. A recent clinical study highlighted the relationship between intrahepatic fat and IR in women with previous gestational diabetes (GDM) [6], indicating mild hepatic steatosis in postpartum women may contribute to IR-related metabolic dysfunction.

In addition to metabolic disorders and adverse pregnancy outcomes, maternal obesity has been shown to impact the long term health of the offspring [7]. The developmental origins of health and disease (DOHaD) paradigm proposes that insults such as poor maternal nutrition during critical windows of development, can lead to an increased propensity in offspring to develop obesity and related metabolic and cardiovascular disorders in later life [8]. Both human studies [9,10] and animal models [11,12] clearly show a link between maternal obesity and heightened risk of metabolic disorders in offspring, yet relatively little is known about the mechanisms involved. Therefore, broad lifestyle recommendations remain the most common preventative strategies [7].

A number of studies have reported the effectiveness of taurine (2-aminoethanesulfonic acid) in treating IR [13–15]. Taurine is a sulphonic amino acid derived from methionine and cysteine metabolism and is found ubiquitously in all mammalian tissues. The synthesis and metabolism of taurine has known species-specific differences although taurine can be synthesised in vivo in both the human and rodent [16]. Taurine is involved in bile acid synthesis, osmoregulation, modulation of neurotransmitters, glucose homeostasis and insulin secretion [17,18]. Reports suggest that taurine supplementation can enhance insulin sensitivity.


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* E-mail: m.vickers@auckland.ac.nz
Appendix V

Preweaning GH Treatment Normalizes Body Growth Trajectory and Reverses Metabolic Dysregulation in Adult Offspring After Maternal Undernutrition

Minglan Li, Clare M. Reynolds, Clint Gray, and Mark H. Vickers

Liggins Institute and Gravida: National Centre for Growth and Development, University of Auckland, Auckland 1142, New Zealand

Maternal undernutrition (UN) results in growth disorders and metabolic dysfunction in offspring. Although dysregulation of the GH-IGF axis in offspring is a known consequence of maternal UN, little is known about the efficacy of GH treatment during the period of developmental plasticity on later growth and metabolic outcomes. The present study investigated the effect of preweaning GH treatment on growth, glucose metabolism, and the GH-IGF axis in adult male and female offspring after maternal UN. Female Sprague Dawley rats were fed either a chow diet ad libitum (control [CON]) or 50% of ad libitum (UN) throughout pregnancy. From postnatal day 3, CON and UN pups received either saline (CON-S and UN-S) or GH (2.5 μg/g-d CON-GH and UN-GH) daily throughout lactation. At weaning, male and female offspring were randomly selected from each litter and fed a standard chow diet for the remainder of the study. Preweaning GH treatment normalized maternal UN-induced alterations in postweaning growth trajectory and concomitant adiposity in offspring. Plasma leptin concentrations were increased in UN-S offspring and normalized in the UN-GH group. Hepatic GH receptor expression was significantly elevated in UN-S offspring and normalized with GH treatment. Hepatic IGF binding protein-2 gene expression and plasma IGF-1 to IGF binding protein-3 ratio was reduced in UN-S offspring and elevated with GH treatment. GH treatment during a critical developmental window prevented maternal UN-induced changes in postnatal growth patterns and related adiposity, suggesting that manipulation of the GH-IGF-1 axis in early development may represent a promising avenue to prevent adverse developmental programming effects in adulthood. (Endocrinology 156: 3228–3238, 2015)

Altering the early life maternal nutritional environment are associated with long term adverse health outcomes in offspring (1–3). It has been well established in both human and experimental studies that maternal undernutrition (UN) leads to an increased risk of obesity and metabolic and cardiovascular dysfunction in offspring in later life (1, 2, 4). Although the underlying mechanisms are not fully defined, developmental plasticity and fetal adaptations to an environment of poor nutrition lead to an altered developmental trajectory and accompanying changes in organogenesis and metabolic homeostasis (5). The predictive adaptive response theory proposes that in utero UN adjusts fetal metabolic set-points to match the predicted environment to ensure survival, however, if the subsequent postnatal environment is nutritionally enriched, these early adjustments become incongruous and can initiate rapid postnatal growth, contributing to metabolic dysfunction in later life (6).

GH and IGF-1 are key regulators of somatic growth. Dysregulation in the GH-IGF-1 axis plays an important role in developmental programming of metabolic dysfunction and related growth disorders arising as a consequence of early life UN (7). Offspring from UN mothers in experimental animal models and human infants with intra-
Appendix VI

Preweaning Growth Hormone Treatment Ameliorates Adipose Tissue Insulin Resistance and Inflammation in Adult Male Offspring Following Maternal Undernutrition

C. M. Reynolds, M. Li, C. Gray, and M. H. Vickers

Liggins Institute and Gravida, National Research Centre for Growth and Development, University of Auckland, Auckland 1023, New Zealand

It is well established that early-life nutritional alterations lead to increased risk of obesity and metabolic disorders in adult life. Although it is clear that obesity gives rise to chronic low-grade inflammation, there is little evidence regarding the role of inflammation in the adipose tissue of undernourished (UN) offspring. GH reduces fat mass and has anti-inflammatory properties. The present study examined the effect of maternal UN on adipose inflammation in adult offspring and whether GH treatment during a critical period of developmental plasticity could ameliorate metabolic dysfunction associated with a poor start to life. Sprague Dawley rats were assigned to chow (C) or UN (50% ad libitum; UN) diet throughout gestation. Male C and UN pups received saline (control saline [CS]/UN) or GH (2.5 μg/g/d; control growth hormone [CGH]/undernourished growth hormone [UNGH]) from days 3–21. Postweaning males were further randomized and fed either chow or high-fat diet until day 160. An ex vivo glucose uptake assay demonstrated adipose tissue from UN offspring displayed attenuated insulin-stimulated glucose uptake compared with CS, CGH, and UNGH. This was associated with increased insulin receptor, glucose transporter 4, and insulin receptor substrate 1 gene expression. Furthermore, UN demonstrated enhanced TNFα and IL-1β secretion from adipose explants and stromal vascular fraction cultures accompanied by increased adipose tissue gene expression of several key proinflammatory genes and markers of macrophage infiltration. Overall, UN offspring displayed a more potent immunophenotype, which correlated with decreased insulin sensitivity. Preweaning GH treatment negates these detrimental effects, indicating the potential for reversing metabolic dysfunction in UN adult offspring. (Endocrinology 154: 2676–2686, 2013)
Appendix VII

Introduction

Maternal undernutrition, a major risk factor for low birth weight has been shown to increase the risk of developing cardiovascular disease during adult-life in humans [1] and animal models [2,3]. The concept of undernutrition during gestation or early life having adverse effects on the offspring’s health as an adult, suggests that disease or metabolic disorders can be ‘programmed’ in utero by a nutritional insult during critical periods of early development [1]. The fetal programming hypothesis suggests that a nutritional insult during development will adapt to the immediate environment causing permanent alterations in tissue architecture, cell number and function, rendering the offspring metabolically disadvantaged at times of dietary fluctuations as an adult [1,4,5].

Elevated resting blood pressure and increased risk of cardiovascular disease caused by prenatal undernutrition has been characterised by endothelial dysfunction [3,6], tissue remodelling [7], reduced angiogenesis [8] and enhanced vascular superoxide production in adult offspring [9,10]. Hypertensive offspring of rats fed a diet of reduced total calorific intake (30–50%) have impaired vasodilator responses to sodium nitroprusside in small mesenteric resistance vessels [6] and endothelium-dependent responses in aortic rings [11]. Similarly, offspring from maternally undernourished dams have also shown blunted response to ACh in mesenteric arteries indicative of a decreased endothelium-dependent vasodilation [12]. Furthermore, nitric oxide (NO) is one of the major bio-active vasodilator molecules and the constitutive production of nitric oxide within the vascular endothelium is important for determining basal arteriolar tone. Maternal undernutrition has also been reported to inhibit nitric oxide synthase activity [13]. However, the development of altered vascular function and endothelial dysfunction in adult offspring is still poorly understood. Therefore, dysfunction of the vascular endothelium could either contribute to the onset of hypertension, or develop as a consequence, thus it is unknown whether the reported changes such as endothelial dysfunction are a cause or result of hypertension.

The deleterious effects of maternal undernutrition on an offspring’s tissue development and subsequent programmed...
Appendix VIII

Pre-Weaning Growth Hormone Treatment Ameliorates Bone Marrow Macrophage Inflammation in Adult Male Rat Offspring following Maternal Undernutrition

Clare M. Reynolds, Minglan Li, Clint Gray, Mark H. Vickers*

Liggins Institute and Gravida, National Research Centre for Growth and Development, University of Auckland, Auckland, New Zealand

Abstract

Maternal undernutrition (UN) is associated with the development of obesity and metabolic complications in adult offspring. While the role of inflammation in obesity and related comorbidities has been well established, there is little evidence regarding the effects of maternal UN-induced programming on immune function in male adult offspring. This study examines the effects growth hormone (GH), which is known to induce anti-inflammatory effects, on maternal UN-induced bone marrow macrophage (BMM) function in adult male offspring. Sprague-Dawley rats were assigned to chow (C) or UN (50% ad libitum; UN) diet throughout gestation. Male C and UN pups received saline (CS/UNS) or GH (2.5 μg/g/d; CGH/ UNGH) from day 3–21. Bone marrow hematopoietic cells were differentiated to a macrophage phenotype in the presence of M-CSF (50 ng/ml). Differentiated bone marrow macrophages (BMM) were stimulated with LPS (100 ng/ml) for 6 h. UNS-derived BMM had significantly increased secretion and expression of IL-1β and IL-6 following LPS stimulation. This was accompanied by increased expression of IL-1R1, IL-6R and TLR4. Pre-weaning GH treatment reversed this pro-inflammatory phenotype. Furthermore UNGH displayed increased expression of markers of alternative (M2) macrophage activation, mannose receptor and PPARγ. This study demonstrates that fetal UN exposure primes hematopoietic immune cells to a more potent pro-inflammatory phenotype with heightened cytokine secretion and receptor expression. Furthermore these pre-disposed to pro-inflammatory M1 macrophage phenotype which has wide-reaching and important effects in terms of obesity and metabolic disease.


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* E-mail: m.vickers@auckland.ac.nz

Introduction

There is considerable evidence that exposure to adverse fetal and early life nutritional and environmental stressors result in increased risk of adult disease [1]. The predominant focus of the “developmental origins of health and disease” (DOHaD) hypothesis has largely centred around perturbations in metabolic homeostasis [2,3]. However, recent studies have provided insight into alteration of immune function in response to gestational nutritional imbalances [4]. Indeed maternal nutrition is associated with placental inflammation and aberrant immune activity which may alter nutritional set points established throughout gestation and the neonatal period enhancing the risk not only for obesity-induced metabolic dysfunction but also chronic inflammatory conditions later in life [5]. Furthermore there is significant evidence that maternal undernutrition (UN) has detrimental effects on the development of both primary and secondary lymphoid organs [6].

The innate immune system is the first line of defense against invading organisms. However, dysfunctional activation of these innate immune cells contributes to the pathogenesis of both metabolic and chronic inflammatory disorders [7]. Macrophages represent a critical part of this system. They originate in the bone marrow as monocytes and once differentiated are distributed throughout most body tissues. In addition to their role in inflammation, macrophages are key mediators of metabolic function and tissue remodeling. Given their multifunctional nature, macrophages display significant phenotypic plasticity. Classically activated (M1) macrophages typically produce high levels of pro-inflammatory cytokines (interleukin (IL)-12, IL-1β, Tumor necrosis factor (TNF)α and IL-6) while alternatively activated (M2) macrophages are characterized by anti-inflammatory IL-10 and IL-4 [8]. Macrophages originate early in embryonic development and as such may be vulnerable to maternal programming [9]. Despite this, the role of developmental programming on long-term immune function has not been comprehensively investigated. Furthermore viable therapeutic treatments to address immunological disparities in relation to maternal UN-induced programming remain unexplored.

Several studies have reported beneficial effects of growth hormone (GH) on parameters of metabolic function in the offspring of UN mothers [10,11]. Indeed, recent evidence from this group has determined that pre-weaning GH treatment ameliorates hypertension in these animals [12]. Traditionally GH has been implicated in the regulation of key components of lipid and glucose homeostasis however growing evidence has established GH as a contributor in the development of immune...
Appendix IX

Early-life growth hormone treatment to offspring of undernourished mothers alters metabolic parameters in primary adipocytes in adulthood

C. M. Reynolds¹,², M. Li¹,², C. Gray¹,², and M. H. Vickers¹,²

¹Liggins Institute and ²Gravida, National Research Centre for Growth and Development, University of Auckland, Auckland, New Zealand

Abstract
Maternal undernutrition (UN) is associated with the development of obesity and metabolic complications in adult offspring. This study investigated the impact of preweaning growth hormone (GH) treatment on adipocyte functionality in adult male offspring. Sprague-Dawley rats were assigned either standard (C) or undernourished (UN) diet (50% ad libitum) throughout gestation. Postnatal day 3–21, male C/UN pups received either saline (CS, UNS) or GH (2.5 μg/g/d; CGH, UNGH) by subcutaneous injection. Primary adipocytes were isolated following the collagenase digestion of adipose tissue. Primary adipocytes from UN offspring had significantly increased the secretion of pro-inflammatory cytokines accompanied by increased cytokine/cytokine receptor expression. This correlated with increased TLR4/NF-κB signaling. While increased inflammatory potential was not observed in adipocytes derived from UNGH offspring, there was a clear alteration in the expression of genes relating to carbohydrate and metabolite transport. Overall, preweaning GH treatment alters detrimental patterns of development, which predispose UN offspring to obesity and insulin resistance.

Introduction
Obesity is quickly becoming the most damaging condition facing global health and is instrumental in the pathology of insulin resistance, type 2 diabetes and cardiovascular disease (Ahima, 2006). While lifestyle factors such as calorie-rich diets and lack of exercise are key to the development of obesity, the role of the maternal and intrauterine environment has now been shown to influence long-term health outcomes in the offspring (Gluckman et al., 2008; Hanson & Gluckman, 2011). Epidemiological data along with animal models have repeatedly demonstrated that maternal undernutrition (UN) during pregnancy “programs” an obese phenotype in offspring (Hanson & Gluckman, 2011; Vickers, 2011). The “developmental origins of health and disease” (DOHaD) hypothesis suggests that this early-life nutrient deprivation alters nutritional set points, thereby allowing the offspring to readily adapt to an adverse postuterine environment. However, this renders offspring less capable of responding to postnatal nutrient abundance, resulting in an obese phenotype (Barker, 2007; McMillen & Robinson, 2005). Given the current epidemic of global obesity, there is growing interest in determining mechanisms relating to the origins of obesity and associated comorbidities and developing preventative strategies at an early age.

Growth hormone (GH) is well recognized as a regulator of somatic growth and development (Moller & Jorgensen, 2009) and plays a major role in glucose and lipid homeostasis (Nam & Lobie, 2000). As a regulator of lipolysis, GH and its receptor (GHR) is expressed on both adipocytes and other cell types of adipose tissue. However, treatment with exogenous GH remains controversial; despite evidence of reductions in fat mass (Nam et al., 2001), side effects from increased lipolytic activity such as elevated blood lipid profiles are associated with cardiometabolic complications and have also been associated with diabetogenic effects (Yuen et al., 2013). However, evidence of reduced adipose functionality in patients with GH deficiency suggests an important role for GH in the development of adipose tissue (Carroll et al., 2004). We have shown in the rat model that adult GH treatment can reduce hypertension and obesity in offspring induced as a consequence of maternal UN (Vickers et al., 2002). Recent work by our group has now also demonstrated that early-life exposure to GH can ameliorate the detrimental effects of maternal UN-induced obesity, insulin resistance and cardiovascular disease (Gray et al., 2013; Reynolds et al., 2013a,b). This implies that the restoration of GH at an early developmental stage “resets” adipose tissue development, thereby altering the trajectory toward obesity.
Appendix X

Gray C, Li M, Reynolds CM, Vickers MH. Let-7 miRNA profiles are associated with the reversal of left ventricular hypertrophy and hypertension in adult male offspring from mothers undernourished during pregnancy following pre-weaning growth hormone treatment. *Endocrinology*. 2014; 155(12), 4808-4817.
Let-7 miRNA Profiles Are Associated With the Reversal of Left Ventricular Hypertrophy and Hypertension in Adult Male Offspring From Mothers Undernourished During Pregnancy After Preweaning Growth Hormone Treatment

Clint Gray, Minglan Li, Rachna Patel, Clare M. Reynolds, and Mark H. Vickers
Liggins Institute and Gravida, National Centre for Growth and Development, University of Auckland, Auckland, 1023, New Zealand

Maternal undernutrition (UN) is known to cause cardiac hypertrophy, elevated blood pressure, and endothelial dysfunction in adult offspring. Maternal UN may also lead to disturbances in GH regulation in offspring. Because GH plays a key role in cardiac development, we used a model of maternal UN to examine the effects of neonatal GH treatment on cardiac hypertrophy, cardiac micro RNA (miRNA) profiles, and associated gene regulation in adult offspring. Female Sprague-Dawley rats were fed either a standard control diet (CON) or 50% of CON intake throughout pregnancy (UN). From neonatal day 3 until weaning (d 21), CON and UN pups received either saline (S) (CON-S, UN-S) or GH (2.5 μg/g/d) (CON-GH, UN-GH). Heart structure was determined by hematoxylin and eosin staining, and miRNA was isolated from cardiac tissue and miRNA expression analyzed using Cardiovascular miRNA gene Arrays (SABiosciences Ltd). Maternal UN caused marked increases in cardiac hypertrophy and left ventricular cardiomyocyte area, which were reversed by preweaning GH treatment. Systolic blood pressure was increased in UN-S groups and normalized in UN-GH groups (CON-S 121±2 mmHg, CON-GH 115±3 mmHg, UN-S 146±3 mmHg, and UN-GH 127±2 mmHg). GH treatment during early development facilitated a reversal of pathological changes in offspring hearts caused by UN during pregnancy. Specific cardiac miRNA profiles were exhibited in response to maternal UN, accompanied by up-regulation of the lethal-7 (LET-7) miRNA family in GH-treated offspring. miRNA target analysis revealed a number of genes associated with inflammation and cardiovascular development, which may be involved in the altered cardiac function of these offspring. Up-regulation of the LET-7 family of miRNAs observed in GH groups may mediate the reversal of cardiac hypertrophy observed in adult offspring males of UN mothers. (Endocrinology 155: 4808–4817, 2014)

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he life of the developing embryo is reliant upon the seamless, precise, and complex transitions that occur during fetal heart development and function, and any deviation from these precisely controlled mechanisms will result in dire and often fatal consequences to the developing fetus (1). Correlations between birth weight and coronary heart disease incidence were reported nearly 25 years ago from the observations of Barker and Osmond (2) and Barker et al (3). Subsequently, epidemiological studies and experimental animal models have shown that adult predisposition to cardiovascular disease (CVD) arise from events occurring during fetal and early postnatal development (4, 5). Further research has shown that maternal undernutrition (UN) throughout pregnancy results in adult offspring altered cardio-metabolic profile, cardiac remodeling, and hypertension, which are all thought to be

Abbreviations: ABAT-1, 4-aminobutrate aminotransferase; CON, control; Ct, cycle threshold; CVD, cardiovascular disease; GABA, y-amino butyric acid; LV, left ventricular; MI, myocardial infarction; miRNA, micro RNA; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; RV, right ventricular; S, saline; SBP, systolic blood pressure; SNORD, Small nucleolar RNA; UN, undernutrition; VEGF, vascular endothelial growth factor.
Early Life Exposure to Fructose and Offspring Phenotype: Implications for Long Term Metabolic Homeostasis

Deborah M. Sloboda,1 Minglan Li,2 Rachna Patel,2 Zoe E. Clayton,2 Cassandra Yap,2 and Mark H. Vickers2

1 The Department of Biochemistry and Biomedical Sciences, McMaster University, 1280 Main Street West, HSC 4H30A, Hamilton, ON, Canada L8S 4K1
2 The Liggins Institute and Gravida: National Centre for Growth and Development, University of Auckland, Auckland 1142, New Zealand

Correspondence should be addressed to Deborah M. Sloboda; sloboda@mcmaster.ca

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The consumption of artificially sweetened processed foods, particularly high in fructose or high fructose corn syrup, has increased significantly in the past few decades. As such, interest into the long term outcomes of consuming high levels of fructose has increased significantly, particularly when the exposure is early in life. Epidemiological and experimental evidence has linked fructose consumption to the metabolic syndrome and associated comorbidities—implicating fructose as a potential factor in the obesity epidemic. Yet, despite the widespread consumption of fructose-containing foods and beverages and the rising incidence of maternal obesity, little attention has been paid to the possible adverse effects of maternal fructose consumption on the developing fetus and long term effects on offspring. In this paper we review studies investigating the effects of fructose intake on metabolic outcomes in both mother and offspring using human and experimental studies.

1. Dietary Trends in Today’s Society

The prevalence of obesity and diabetes has increased radically on a global scale over the past two decades to a point where these conditions are now considered to be “epidemic.” There are an estimated 312 million obese adults worldwide and at least 155 million children who are either overweight or obese [1]. This problem is not limited to developed countries; rates of obesity have tripled in developing countries as they adopt a western lifestyle characterised by less physical activity and over indulgence in high-calorie processed foods [1, 2]. The increase in obesity has been accompanied by an increase in the incidence of type 2 diabetes, particularly in areas that have undergone fairly rapid urbanisation [3, 4]. Advances in food processing technologies have significantly contributed to an influx of inexpensive, energy dense diets [5] that are readily available worldwide.

Countries tend to undergo nutritional transitions from traditional grain-based diets to high-fat high-sugar diets as affluence increases and populations become more urban [6]; countries with the highest incomes have the highest fat and sugar intakes [5]. Throughout the world, the consumption of fat and sugar is on the rise; their availability per capita has increased by more than 20% since 1977 and the consumption of nonsucrose caloric sweeteners has also seen an upsurge since 1962 [5, 7, 8]. Increasing attention is being paid to the large increase in sweetened beverage consumption worldwide which has been primarily driven by increased sweetened beverage intake which makes up a substantial proportion of the total increased caloric load in many developed countries [9]. Shifts in the use of fructose and high fructose corn-syrup from sucrose in sweetened beverages as well as in processed foods have been suggested to play a role in the obesity trend [10–13], although in some populations sugar intake may be on the decline [14]. Although evidence exists suggesting that excessive fructose intake could be one determinant in the aetiology of the metabolic syndrome long term impaired hepatic pathophysiology (Nonalcoholic Fatty Liver Disease, NAFLD)}
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234


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