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EFFECT OF MATERNAL DIET ON
OFFSPRING METABOLIC PROGRAMMING:
CHANGES INDUCED BY CARBOHYDRATE QUALITY

Theodora Sideratou
A thesis submitted in fulfilment of the requirements
for the degree of Doctor of Philosophy
Faculty of Science
The University of Sydney
2014
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On this page I get my chance to express the gratitude for those that have significantly influenced not only the completion of this PhD but also my life in so many ways.

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This PhD project represents a growth as a researcher and professional but also as a person that has been marked by every single person I crossed paths with.

“The direction in which a man’s education starts will determine his future in life”.

Plato (400BC)
Published abstracts and conference presentations

2014  Oral presentation and published abstract
IASO 12th International Congress on Obesity
Kuala Lumpur, Malaysia.
“FTO gene expression under low or high glycemic index (GI) diet in
pregnant mice and pups”.
Sideratou T; Bell-Anderson K; Brand-Miller J.

2013  Poster presentation and published abstract
IUNS 20th International Congress of Nutrition
Granada, Spain.
“FTO gene expression under low or high glycemic index (GI) diet in
pregnant mice and pups”.
Sideratou T; Bell-Anderson K; Brand-Miller J.

2011  Poster presentation
“Reproductive, Maternal & Child Health Research Theme Day”
Sydney, Australia.
“FTO gene expression under low or high glycemic index (GI) diet in
pregnant mice and pups”.
Sideratou T; Bell-Anderson K; Brand-Miller J.

2011  Poster presentation, NSNZ & NSA Joint Annual Scientific Meeting
Queenstown, New Zealand.
“FTO gene expression under low or high glycemic index (GI) diet in
pregnant mice and pups”.
Sideratou T; Bell-Anderson K; Brand-Miller J.

Note: Presenting author is underlined.
Abstract

**Background** Maternal diet and gestational diabetes have important implications for offspring health and disease. The fat mass and obesity associated gene (FTO) has the strongest relationship to increased body fat mass in humans, potentially via its effect on appetite and insulin sensitivity. Leptin plays a key role in regulating energy intake and expenditure, including appetite and hunger. Our hypothesis was that the expression of the Fto and leptin gene is influenced by the quality of carbohydrate in the maternal diet. Specifically, we postulated that the expression of Fto gene in the hypothalamus of the offspring of high glycaemic index (GI) diet-fed mothers would be higher than that of low GI diet-fed mothers, whereas the opposite would be observed for leptin gene expression.

**Aim** To compare glucose metabolism and mRNA expression of the Fto, leptin and other appetite-regulating genes in hypothalamic, adipose and/or other tissues of offspring of female mice fed high vs. low GI starch diets throughout pregnancy. Female C57BL/6 mice were randomly assigned to a low GI, high GI diet, or standard chow from 4 weeks of age and then mated with males who were fed the standard chow. In Study 1, male pups (n = 40) were weaned at the end of postnatal week 4 and divided into 2 subgroups, one following the chow diet and one their mother’s diet until 20 weeks of age (i.e. early life and life-long exposure respectively). At postnatal week 18, glucose tolerance and insulin sensitivity were determined. At week 20, the pups were sacrificed for tissue collection (hypothalamus, brown adipose tissue, liver, visceral and subcutaneous fat, red muscle and white muscle). Tissues were analysed for gene expression and histological findings. In
Study 2, female mice were sacrificed at gestation day 16-18 and placentas recovered. Gene expression was measured by quantitative real-time PCR.

**Results** Fto gene expression in the hypothalamus of offspring fed high GI starch from conception to postnatal week 20 was 2.5-fold higher than those fed low GI starch for the same time period (p = 0.01). Similarly, placental Fto gene expression was 3.8-fold higher in mothers fed the high GI starch diet vs. the low GI diet (p = 0.0003). By contrast, both early life and life-long exposure to a high GI diet were associated with lower Fto gene expression in white muscle (p = 0.0001). In the visceral adipose tissue, leptin gene expression was 3.3-fold higher in offspring exposed to the low GI diet from conception to week 20 and 4.4-fold higher in the offspring exposed to the low GI diet in early life. Plasma levels of the ‘hunger’ hormone ghrelin were significantly lower in offspring who received the low GI diet throughout life (202.0 ± 49.5 vs. 81.3 ± 21.6 pg/mL in high vs. low GI respectively, p = 0.02), while levels of the ‘satiety’ hormone leptin were higher (0.62 ± 0.18 vs. 3.88 ± 1.4 respectively, p = 0.001). The co-expression of hypothalamic appetite genes AGRP/NPY and POMC/CART was also differentially regulated by the nature of the dietary carbohydrates. In this study, differences in diet, gene expression and hormone levels were not accompanied by differences in phenotype, including body weight, glucose tolerance or insulin sensitivity. Similarly, despite interesting trends, the histological findings did not show a definitive effect of the maternal diet on offspring liver tissue morphology. Offspring who were weaned to chow at week 5 demonstrated similar trends to those fed high vs. low GI diets throughout life.
**Conclusion**  This study suggests that carbohydrate quality, specifically the GI and/or rate of starch digestion in maternal diets, can differentially regulate the expression of the Fto and leptin gene in offspring tissues even in the absence of phenotypic differences. Furthermore, the nature of the dietary carbohydrates can regulate the well recognised co-expression of AGRP/NPY and POMC/CART in the hypothalamus. The findings imply that epigenetic changes induced in early life underlie observed differences in fat mass and insulin resistance in previous studies of high vs. low GI feeding. It is therefore possible that changes in the nature of the carbohydrate in human diets may explain in part the current epidemic of obesity in children and adults. Dietary interventions during pregnancy that reduce the rate of digestion of carbohydrate and postprandial glycaemia deserve further research.
Statement of authorship

The work described in this thesis was performed between March 2010 and March 2014 in the School of Molecular Bioscience (Bldg G08) at the University of Sydney. Except where specifically noted, none of this material has been presented for any other degree and the work was originally created by myself and my academic supervisors, and executed by me.

Theodora Sideratou
Abbreviations

ACTH       Adrenocorticotropic hormone
AGRP       Agouti-related peptide
BAT        Brown adipose tissue
BMI        Body mass index
BW         Body weight
CART       Cocaine and amphetamine regulated transcript
CC         Chow diet-fed pups from chow diet mothers
Chow       Standard chow diet or group
ELISA      Enzyme linked immunosorbent assay
Frw        Forward primer
FTO        Fat mass and obesity associated gene (referring to human genetics)
Fto        Fat mass and obesity associated gene (referring to animal genetics)
G          grams
GI         Glycaemic Index
GLP1       Gucagon-like peptide 1
GTT        Glucose tolerance test
H & E       Haemotoxylin and eosin stain
HC         Chow-fed pups from high GI diet mothers
HGI        High GI diet-fed group of mothers
HH         High GI diet-fed pups from high GI diet mothers
Hypo       Hypothalamic tissue
iAUC       Incremental area under the curve
IL-6       Interleukin 6
IP         Intraperitoneal
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ITT</td>
<td>Insulin tolerance test</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilograms</td>
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<tr>
<td>L</td>
<td>Litres</td>
</tr>
<tr>
<td>LC</td>
<td>Chow diet-fed pups from low GI diet mothers</td>
</tr>
<tr>
<td>LGI</td>
<td>Low GI diet-fed group of mothers</td>
</tr>
<tr>
<td>LL</td>
<td>Low GI diet-fed pups from low GI diet mothers</td>
</tr>
<tr>
<td>MC3R</td>
<td>Melancortin 3 receptor</td>
</tr>
<tr>
<td>MIN</td>
<td>Minutes</td>
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<tr>
<td>MJ</td>
<td>Mega-joules</td>
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<tr>
<td>mL</td>
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<td>Milli-metres</td>
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<tr>
<td>n</td>
<td>number</td>
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<tr>
<td>NAFLP</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NS</td>
<td>Not significant</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>POMC</td>
<td>Pro-opriomelanocortin</td>
</tr>
<tr>
<td>PP</td>
<td>Pancreatic polypeptide</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide YY</td>
</tr>
<tr>
<td>Rev</td>
<td>Reverse primer</td>
</tr>
<tr>
<td>RM</td>
<td>Red muscle tissue</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SAT</td>
<td>Subcutaneous adipose tissue</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Abbreviation</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
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<tr>
<td>VAT</td>
<td>Visceral adipose tissue</td>
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<tr>
<td>Vs.</td>
<td>Versus</td>
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<td>WAT</td>
<td>White adipose tissue</td>
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<td>WM</td>
<td>White muscle tissue</td>
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<td>µL</td>
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<td>µmol</td>
<td>Micro-moles</td>
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Chapter 1  Review of the Literature
Chapter 1  Introduction

1.1 Obesity

The past two decades have been indelibly marked by an epidemic of obesity. The World Health Organisation (WHO) identifies obesity as the fifth leading cause of death and a major contributor to the burden of type 2 diabetes, ischaemic heart disease and certain cancers. The physiological, psychological and economic costs span all cultural and ethnic backgrounds (1). Financial costs begin to increase at a body mass index (BMI) \( \geq 25 \text{ kg/m}^2 \) and include both direct (medical and non-medical) and indirect expenses (2). Indeed, indirect costs such as decreases in workforce productivity and premature mortality are often higher than direct costs. In the USA in 2008, the medical costs alone were estimated to be USD147 billion (3).

In Australia, the most recent population-based data are derived from the Australian Diabetes, Obesity and Lifestyle (AusDiab) study (4-6) conducted in 1999-2000, with a 5-year follow-up study in 2005, and a 12-year follow-up in 2012. The prevalence of obesity in the adults was reported to be 27% in 2011-2012 and projected to reach 34% by 2025. In 2010, average annual direct and indirect costs to the Australian government were estimated to be AUD56 billion (4).

The burden of obesity is not limited to its economic impact. It also has a significant social impact because obese individuals have a lower quality of life and more functional limitations (2, 7, 8). Clinical depression and a range of other psychosocial disturbances are common, yet substantial weight loss brings about a marked improvement in psychological comorbidity (9). The impact on quality of life remains
even after adjusting for demographics, health habits, medical conditions (9). Sturm (10) summarised it in this way: “obesity has a greater negative impact on quality of life than 20 years of aging”.

Obesity and its metabolic sequelae are not a modern phenomenon. Over 2400 years ago in ancient Greece, Hippocrates stated that “sudden death is more common in those who are naturally fat than in the lean” (11). He also wrote “corpulence is not only a disease itself, but the harbinger of others”. Even today, most clinicians do not recognise obesity as a disease in itself. It was not until 1660 that the word “obesity” was first used although the condition was relatively uncommon (12). Three hundred years later, however, obesity became an epidemic afflicting most high-income countries, followed soon after by many middle- and low-income countries. In 2008, a total of 1.46 billion adults worldwide were classified as overweight (BMI >25 kg/m²) and 502 million as obese (BMI >30 kg/m²). At the same time, around 170 million children (aged <18 years) globally were classified as overweight or obese, double that of earlier estimates (13).

In 2012 WHO estimated that 2.8 million people worldwide died every year due to obesity and its complications (14). Clearly socio-political shifts have contributed to the rise. From wars and famine in the first half of the 20th century, many nations underwent a transformation in the second half that included a microbiologically safe, abundant, cheap and palatable food supply. Many researchers have identified the onset of global obesity with the availability of energy dense fast-foods coupled with the adoption of a sedentary lifestyle. However, others believe that the origins of obesity are far more complex (15).
In the last decade, research around obesity has focused on the interplay between individuals and their environmental influences (lifestyle and nutrition) as well as potential genetic determinants (16). Health professionals acknowledge that weight loss is a constant challenge and unlikely to be sustained over the long term. The paradigm has therefore shifted and prevention of weight gain has become the goal for health practitioners and researchers globally (17).

Most studies of long-term weight gain have evaluated current behaviours that could be targeted for change over time. The scientific world has proposed several strategies to prevent obesity and reduce the risk of development of metabolic syndrome and associated disorders (18). These include lifestyle aspects such as increased physical activity and a healthy diet. The recommended dietary pattern proposes that carbohydrates (mainly complex carbohydrates) should contribute to around 50% of the total daily calorie intake, healthy fats to around 30% of the total daily calorie intake and an increased consumption of fruits and vegetables (19). Clearly there is no one single solution to the problem of obesity. Simple advice such as balancing the calories consumed and those expended e.g. “eat less and exercise more” is rarely effective.

In 2011, Mozaffarian et al. conducted a prospective investigation of three separate cohorts involving ~120,000 non-obese individuals, free of chronic diseases, that were followed up over several years (17). As expected, several lifestyle choices were independently associated with long-term weight gain, including consumption of specific foods and beverages, physical activity, alcohol use, television watching and smoking. It also showed that on average weight gain in non-obese populations is
very gradual. Nonetheless, this accumulated weight over time had serious health implications including metabolic dysfunction, type 2 diabetes, cardiovascular disease and cancer.

The most important finding, however, was that the magnitude of weight gain varied for specific foods and beverages. Per serving they found that potatoes, refined grains and other processed foods were associated with the greatest weight gain per unit of time, while increased consumption of vegetables, nuts, fruits, and whole grains were associated with weight loss. The authors suggest that consumption of higher fibre foods slows digestion and increases satiety, resulting in a reduced intake of highly caloric processed foods and lower caloric intake in general. Interestingly, several dietary metrics that are currently emphasized (such as fat content, energy density, and added sugars), did not reliably identify the dietary factors that were found to be associated with long-term weight gain. For example, most of the foods that were positively associated with weight gain were starches or refined carbohydrates. This is not the first study suggesting that carbohydrate quality could be more critical than what we have thought to date.

In 1994, Brand-Miller and Colagiuri published the *Carnivore Connection* hypothesis (a revised edition was published in 2012) (20). This hypothesis argues that during human evolution we experienced over several dietary patterns; from diets with mostly plants in early hominids to those with a high animal:plant ratio and scarcity of dietary carbohydrate, and then more recently to high-carbohydrate, high-glycaemic index carbohydrates that characterise modern diets. These dietary changes are likely to have precipitated metabolic adaptations, particularly in relation to
glucose metabolism and insulin sensitivity. Carbohydrate quality changed markedly during the industrial revolution in the period 1760-1840 and again with developments in food processing (e.g. extrusion technology) (21). New high-speed steel roller mills produced finely ground cereal flours, removing the fibre and resulting in increased starch gelatinization during cooking. Prior to that, cereals were consumed whole or coarsely ground or flaked, with the endosperm and fibre intact (20). With this modern high carbohydrate dietary pattern, the digestion and absorption of the carbohydrates changed radically. Changes were less dramatic for meat, fruit and vegetables since we still consume these in much the same form.

Many researchers have noted that the recommended reduction of calories from fat has resulted in a compensatory increased consumption of refined, high GI carbohydrates (bread, rolls, pizza, white rice, ready-to-eat cold cereals) and added sugars (22, 23). Indeed, some argue persuasively that refined carbohydrates are a greater cause of metabolic disturbance than saturated fat and sedentary lifestyle (24). Nevertheless, a low-fat, high-carbohydrate diet still remains the most common dietary recommendation, although carbohydrate quality is now stressed and minimally processed foods such as whole grains, legumes, and vegetables are favoured.

The National Institutes of Health USA currently spends nearly $800 million a year on research designed to unravel and understand the metabolic, genetic and neurological foundations of obesity. Unfortunately, despite this expenditure, real progress is not being made and the prevalence of obesity continues to climb (13). New approaches are therefore needed. One approach is the science of epigenetics.
In deciding and designing the direction of my PhD research, I focused on the connections that interested me most. In the following paragraphs, my aim is to set the scene for the research decisions I was led to take based on my interpretation of the scientific evidence that was available to me. A schematic representation of the train of thought that initiated this research is indicated in Figure 1 below:

**Figure 1.** Schematic representation of the interplay between factors influencing the risk of obesity and their impact on later susceptibility to disease. (PA = physical activity, NCD = non communicable diseases, CVD = cardiovascular disease).
1.2 Genes and obesity

Every year, scientific conferences bring together the latest updates and possible treatments for obesity. In the strictly medical field, surgery and drugs are often the focus (13). But the data on the use of the existing drugs to treat obesity are not promising (25). Sibutramine and orlistat have serious side effects making their use as obesity solution agents problematic. The possibility to identify genetic targets that could offer a valuable solution and better understanding of how BMI is determined, has become a very promising and exciting field.

In 1962, geneticist James V. Neel, published the "Thrifty Genotype" hypothesis in his paper "Diabetes Mellitus: A 'Thrifty' Genotype Rendered Detrimental by 'Progress'?" (26). He proposed that there has been a natural adaptation selective process during our hunter-gatherer evolutionary existence with periods of feast and famine that have created natural selection of thrifty genes, i.e. genes that “save” and store energy. These adaptations now predispose people to chronic diseases of the modern civilization such as obesity and type 2 diabetes. According to Neel, the genes encode for proteins that are involved in maintaining energy balance and important metabolic functions such as fat oxidation. Based on this hypothesis, the thrifty gene variants, born as an evolutionary advantage, became a disadvantage in the modern obesogenic society.

A large number of studies, some of them using monozygotic and dizygotic twins to observe closely the interaction between gene and environment, have supported the hypothesis (27). Yet, the hypothesis still remains controversial and often criticized as
there are arguments that the time periods and the length of the famines were not enough to justify a natural selection (28).

In 2008, biologist John Speakman presented an alternative explanation, referred to as the “drifty hypothesis”, suggesting that adiposity could be the result of random mutations and genetic drift as a result of the interaction with the environment (28, 29). According to Speakman, periods of food insecurity have been common, occurring about once every decade. Nevertheless, since those periods were not marked with significant mortality, they become unimportant for genetic natural selection. Instead, Speakman suggests that what occurred was a series of random allelic mutations with frequencies that also randomly drift. He called them the ‘drifty genes’, and he argues that the “genetic lottery” can predispose to obesity and other modern metabolic disorders. As George Bray, a leading obesity researcher says, “the genetic background loads the gun, but the environment pulls the trigger” (30).

Genome-wide association studies and further investigations on the gene-environment interactions are increasingly promising (31). There are at least 37 known single nucleotide polymorphisms (SNPs) that are associated with obesity (31) and gene–environment and gene–behaviour interactions, including interactions in the intrauterine environment that require further study. The new field of epigenetics focuses on the study of heritable changes in gene expression that are not due to changes in DNA sequence, but to other chromosomal changes that occur as a result of environmental influences (32-34). Epigenetic mechanisms that modify the expression of genes and contribute to obesity must be investigated with valid methodologies that minimise the number of environmental variables (13).
A recent review (35) summarised advances in obesity genetics, describing promising research that may make personalized obesity therapy a near reality. Monogenic obesity resulting from a single gene or chromosomal region mutation is rare, yet this form of obesity has allowed us to investigate and better comprehend the neuroendocrine factors that are implicated in the development of obesity. An important single gene mutation that opened the door on this direction, was that observed in the leptin gene in the ob/ob mouse strain that led to the discovery of the leptin–melanocortin signalling pathway and its putative role in energy homeostasis and body weight regulation. Since then, several genes involved in this pathway have become targets of interest, stimulating research on protein products such as the prohormone pro-opiomelanocortin (POMC) and melanocortin 4 receptor (MC4R) (36).

Polygenic obesity, referred to as ‘common obesity’, is believed to be the result of the combined effect of genetic variants and SNPs in multiple genes (37). These variants and their interaction with environmental risk factors have become a promising field for research. Linkage analysis and genome wide association studies looking for association of genes with type 2 diabetes and obesity, have indicated and subsequently confirmed through replication, several genes that seem to be significantly associated with an increased body mass index (BMI) and adiposity (38).

The most compelling evidence came from a gene expressed mainly in the hypothalamus, located on chromosome 16q12.2 region, referred to as the fat mass and obesity-associated FTO gene (39-41). After its first discovery, it was found that FTO belongs to the superfamily of Fe(II) 2-oxoglutarate (2-OG)-dependent
dioxygenases and encodes a 2-oxoglutarate–dependent nucleic acid demethylase (42, 43). The amino acid sequence of the transcribed FTO protein showed high similarity with the enzyme AlkB which oxidatively demethylates DNA. The researchers of these discoveries discussed the possibility that the catalytic activity of FTO may regulate the transcription of genes involved in metabolism by nucleic acid demethylation or it may act as a nucleic acid repair enzyme. Evidence suggests that a breakdown of genomic repair processes leads to obesity and metabolic syndrome (44). Furthermore, in vitro, FTO demethylates m6A-containing RNA which implies that the FTO gene is involved in reversible chemical modification of RNA (45, 46).

Numerous studies since have shown an association with obesity traits (47, 48) of various SNPs in this gene, all presenting within a 47-kilobase block including parts of the first two introns and exon 2 of FTO (49). A table summarising the associations between the variants of the fto gene and obesity traits is shown in Appendix 1. But it was one particular variant that maintained its association with BMI and type 2 diabetes, even after data from several separate cohorts were adjusted for BMI (41). This was rs9939609, a cluster of 10 SNPs in the first intron of FTO. At the time, this variant had the strongest association between a gene and obesity risk (50). However, most recent developments have indicated that all of the identified FTO SNPs within a 47-kilobase (kb) region of high linkage disequilibrium in introns 1 and 2 of FTO (each of them with different frequencies in the various populations) belong to the same highly correlated cluster. All are associated with BMI and other obesity-related traits at similar significance levels (51). Individuals homozygous for risk alleles of the associated SNPs weigh approximately 3 kg more than individuals homozygous
for non-risk alleles, underscoring the significant phenotypic impact of these common variants (52).

FTO is not only statistically the strongest associated gene but also that with the largest effect size (53), even though its physiological role and the mechanism of its association to increased adiposity and weight need more investigation. The high expression of this gene in the hypothalamus, the regulatory centre of energy balance, led researchers to believe that FTO may influence adiposity through its impact on energy homeostasis (54).

The intronic position of these variants means they do not affect the amino acid sequence of the expressed protein. Rather, they affect the regulation or expression of the gene or that of a nearby gene or genes (55) and it can be tissue specific (or not). All these possibilities are now being explored in modern research.

In the early stages after the discovery of the FTO gene, a study showed that the expression of FTO in the hypothalamus was regulated by feeding and fasting (56), suggesting a role in the control of energy homeostasis. Indeed FTO was significantly up-regulated (+~40%) in the hypothalamus of rats after 48 hour food deprivation. Two similar studies conducted in mice showed the reverse outcome; Fto mRNA transcription levels in the brain of fasted mice were reduced. Nevertheless, these associations point towards a connection between this gene and obesity through mechanisms of feeding and fasting (42, 57). When considering these outcomes, the researchers looked at other genes associated with monogenic severe obesity, which appeared to be regulated by diet. For example, brain derived neurotrophic factor
(BDNF) expression has been shown to be altered by consumption of high fat diet (58, 59). This encouraged them to further explore the possibility that FTO was nutritionally regulated (55).

1.2.1 Human studies on the association of obesity and the FTO gene

In 2009, a cross-sectional analysis suggested that consuming a high-fat diet accentuated susceptibility to obesity by the FTO variant (60). However, in 2011 the same researchers examined the association between fat intake and mortality as a function of FTO genotype. Their results were inconclusive because although the FTO genotype was associated with both fat mass and lean mass, the level of fat intake modified only the association with fat mass (61). Of course, as cross-sectional studies, they do not investigate directly the effect of diet or more specifically of the type of dietary fat on FTO gene. Furthermore, it has been shown very difficult to obtain accurate data on fat intake in human studies.

Two large studies, the GOLDN and the BPRHS studies, have explored the association between fat and carbohydrate intake and FTO gene variation with BMI (62). Interestingly, participants with two copies of the FTO risk allele had a higher mean BMI compared to the other genotypes only when they had high saturated fat intake. There were no significant associations with total carbohydrate intake.

The Diabetes Prevention Program (DPP) study was a US randomised controlled trial in which 27 centres participated with over 3,500 high risk participants randomised to either a pharmaceutical intervention, intensive lifestyle modification (including a significant weight loss by eating less fat and fewer calories) or placebo, to prevent
the development of type 2 diabetes. The baseline and one year results were analysed to look for associations between the outcomes and the FTO gene variant rs9939609 (63). The study showed that at baseline the high risk A allele was associated with greater weight and BMI, but not adiposity or the change at one year in any anthropometric trait. Their data were adjusted for age, sex and ethnicity. They also examined the possible influence of the genotype on the effects of the various treatments at one year. It appeared that the risk A allele was associated with a greater increase in subcutaneous adipose tissue in the placebo group, but not the others. A limitation of this study, as in all human studies, is that the nutritional recommendations the individuals were given were generalised (lowering fat intake, in particular saturated fat) and lacked precision.

Similarly, in the Finnish Diabetes Prevention study (64), researchers investigated whether the FTO gene variant was associated with body weight and BMI and long-term weight changes. Approximately 500 participants with impaired glucose tolerance were randomized to control and lifestyle intervention groups. At baseline, the high risk homozygous FTO genotype female carriers had higher BMI than subjects with other genotypes and this was maintained throughout the four years of follow-up. This association was not observed in men. The FTO genotype did not appear to have any influence on weight reduction in either of the groups. Again here, the advice for the intervention group was to reduce saturated fat intake (use low-fat dairy and meat products and margarines or vegetable oils rich in monounsaturated fatty acids). It was also recommended to consume more than 50% of daily calories from carbohydrates. In 2012, after further data analysis in this study, an update was published indicating that at baseline and during the follow-up, the association
between FTO and BMI was more pronounced in those having a diet high in fat and low in carbohydrates and fibre. However, a limitation of the Finnish study was that the statistical power was low because it involved only ~500 participants. Nevertheless, the study was further confirmation of a link between the dietary factors, FTO genotypes and obesity.

Several human studies have indicated that a possible mechanism of association between FTO and obesity is through an effect on energy intake and not energy expenditure (65-69). In one of the latest studies, Wardel and colleagues (66) assessed the data from a population based twin cohort of more than 3000 children in the United Kingdom that measured habitual appetitive behaviour. In agreement with previous studies, they confirmed that one copy of the A allele (in rs9939609) increased body weight by 1.5 kg in adults and children. They also showed a strong association with higher waist circumference. But a unique finding from this research group was that the carriers of two copies of the A risk allele had significantly lower satiety responsiveness compared to the other genotypes, even after controlling for BMI. The researchers suggested that FTO may have a direct effect on appetite and this could be the mechanism that in turn influences adiposity. This theory is also supported by a study in 2009, in which 16.5% more children with at least one risk A allele reported loss of control eating, compared to the low risk allele T children. In this study, children with the risk allele also consumed more calories from fat than those with the low risk allele (70). Thus, the above findings together with the fact that the FTO gene is mainly expressed in the hypothalamus suggest this gene plays a role in the control of satiety or appetite (71).
A new hypothesis was tested by Sovio and colleagues in 2011 (72), in relation to an age-dependent association between the FTO gene and BMI in children. They conducted a meta-analysis of eight cohort studies, looking at data derived from early infancy to 13 years of age. They also observed the timing of BMI adiposity rebound (AR), which is inversely related to the risk of later obesity and used as a marker of an advanced BMI-for-age. The FTO risk allele was associated with a higher BMI from the age of five years onwards, though the opposite was observed before the age of two and a half years. This suggested that the effect of this gene followed a shift in the age scale leading simultaneously to lower BMI during infancy and higher BMI in childhood, indicating an association with the timing of AR and its metabolic consequences (risk of increased adult BMI and type 2 diabetes). Similarly, a review of the data collected during the Fels longitudinal study indicated there was no association between the FTO gene and growth status or velocity until the age of three (73).

Meanwhile in Spain, a research group was analysing data derived from the placentas of women that took part in a birth cohort study (74). They were investigating an association between FTO gene expression in the placenta and fetal growth, weight gain and length. Placental FTO expression was positively associated with fetal weight and length but also with increased fetal-to-placental weight ratio. Nevertheless, there was no difference in the placental FTO expression levels among the babies with the various genotypes for the rs9939609 SNP (AA, AT, TT).

Finally, as part of a larger multicentre international study “HELENA” designed to investigate the interactions between environmental influences and cardiovascular
disease, an analysis was undertaken on the association of the FTO gene and serum leptin concentrations in 655 adolescent participants (75). The results indicated the high risk A allele was positively associated with higher serum leptin levels. The association remained after all data were adjusted for adiposity and other confounding factors such as physical activity. This suggested the A allele may be linked with leptin resistance thus indicating that the mechanism of action may lie in the control of energy balance.

In summary, human studies to date indicate that the association between obesity and the FTO gene is the result of the effect of the high risk A allele on appetite and energy intake, with a reduction in satiety and a positive association with increased dietary fat/saturated fat intake (53).

1.2.2 Animal studies on the association of obesity and the FTO gene

Animal models, and particularly mice models, are a valuable tool in the research of human disease due to their physiology, anatomy and genes being very similar. They also allow us to research and apply protocols in shorter periods of time and in tightly controlled environmental conditions than what is possible with humans. Moreover, sophisticated techniques allow us not only to observe wild types, but also to genetically engineer animal models that better serve the aims of our research projects (76).

In 2008, the expression of the Fto gene was investigated in pre-existing mouse models of obesity under different dietary manipulations (fed vs. fasted) (57). The data showed Fto gene expression was significantly higher in the hypothalamus
compared to other tissues such as adipose, pancreas and liver. In both lean and obese mice, Fto expression in the hypothalamus was lower in fasted compared to fed animals. Similar outcomes were published in another study (42), where laser-dissected hypothalamic arcuate nuclei of mice (freely feeding, fasted, and fasted + daily injections of leptin) were analysed for differences in Fto expression levels. Fto expression in the two fasted groups was 60% lower compared with the fed group. Although these results varied from those in fed vs. fasted rats (56), the scientific world proposed two likely causes for the discrepancy; firstly the difference between these two animals in their sensitivity to starvation and secondly the diverse methodological protocols that could impact on Fto gene expression (77). Despite the inconsistency, the results indicated that Fto mRNA expression could be regulated by nutrition.

In 2009, the first specifically produced animal model to study the Fto gene and its putative role in energy homeostasis was created by Fisher and colleagues (78). It was a knockout mice generated by replacing exons 2 and 3. An important outcome during the generation of this mutant animal model was that the knockout mice were produced according to the Mendelian ratio. So, the authors suggested that Fto is not essential for embryonic development (78). Their data showed that Fto knockout mice had significantly less adiposity and lean body mass indicative of postnatal growth retardation. Additionally, these mice had an elevated metabolic rate, a result that at that time contrasted with the outcomes of the published human studies (79). The findings of this study were strongly criticized on the grounds it was under-powered and had several issues in regards to the measurement and calculation of energy expenditure.
To obtain a better understanding of the functional role of the Fto protein product, Gao and colleagues (80) produced two mouse models: one with a full body deletion of the Fto gene and the second with a conditional deletion of the Fto gene only in the nervous system. They observed that the whole body deletion of the Fto gene was associated with postnatal growth retardation and confirmed the outcomes published by Fisher and colleagues (78). An important contribution of this study was that mice with the conditional deletion in the nervous system were phenotypically similar to the full body knockout mice thus presenting similar postnatal growth retardation. This was interpreted as a strong indication that Fto gene protein acts in the brain to regulate growth. A puzzling finding of this study compared to human studies at the time was that both knockout models did not show any association with either the chow or the high fat diet to which they were exposed.

Meanwhile, a study conducted in Göttingen minipigs (81) indicated that after 11 weeks of either chow or high cholesterol feeding, Fto gene expression in the brain cortex was higher in the cholesterol-fed minipigs compared to the controls. Another study published the same year, was conducted on sheep (82), aiming to shed light into the interaction between the in utero environment and the regulation of the FTO gene. Pregnant animals were either fed to meet 100% of their energy requirements or were nutrient restricted by 50%. The study showed that prenatal nutrient restriction did not alter Fto gene expression in the hypothalamus of the newborn. But when these lambs of nutrient restricted mothers were exposed for one year to an obesogenic environment, Fto gene expression in the hypothalamus was up-regulated.
Church et al. (83) engineered three new mice models that globally expressed one, two or three additional copies of the Fto gene. They conducted a study to compare the effects of these additional gene copies in relation to obesity in mice that were fed standard chow diet and high fat diet. They showed that there was a dose-dependent increase in weight and fat mass with the different Fto over-expression models both in the chow and the high fat-fed mice. Also, there was a positive increase in food intake relative to the level of Fto over-expression. This again was observed in both diet groups. Finally, they also reported that mice with three additional copies of the Fto gene fed a high fat diet were glucose intolerant. These outcomes opened new perspectives in the research around this gene, as they suggested that systematic overexpression of FTO was associated with obesity (i.e. higher food intake and possibly glucose intolerance) and therefore the search for a therapeutic mechanism that acts on this gene became a research target.

The same research group that had previously used mice (42), conducted a new study to investigate the effects of high fat feeding on Fto expression in rats (84). They demonstrated that expression was 2.5 fold higher in the arcuate nuclei of the high fat-fed vs. chow-fed rats. Interestingly, in this study the reduction in Fto expression in the arcuate nuclei of the chow-fed rats was associated with an increased food intake. This finding was consistent with their previous observation in the arcuate nuclei of mice (42) and suggest a possible effect of Fto on appetite.

Finally, a recent study on mice hypothalamic cell cultures (85) indicated that 12 hours of glucose deprivation down-regulated Fto mRNA expression by 50%, whereas just 6 hours of amino acid deprivation resulted in 60% reduction in Fto
transcript levels. This suggests that FTO expression is altered by the availability of both amino acids and glucose. Taken together, these findings suggest that both the quantity and quality of nutrients available to the body alter the regulation of the FTO gene in the appetite-specific areas of the brain. It is therefore conceivable that specific nutritional interventions may counteract the effects of the high risk genotype of the FTO gene.

1.3 The relationship between the Developmental Origins of Health and Disease hypothesis and nutritional regulation of gene expression

1.3.1 The Developmental Origins of Health and Disease (DOHAD)

There has been much debate in the last 20 years in regards to the developmental origins of adult disease hypothesis, originally formulated by Dr David Barker (86-88). It postulated that environmental factors, particularly maternal nutrition, act in early life to programme the risks for adverse health outcomes, such as cardiovascular disease, obesity and the metabolic syndrome in adult life (89, 90). The hypothesis initiated a new field of research, known today as the Developmental Origins of Health and Disease (DOHAD) (91). This branch of research investigates how environmental factors, particularly nutritional insults, during the phase of developmental plasticity in the womb, impact structures of organs in the fetus and interact with genotypic variation, leading to chronic disease in adult life (i.e. metabolic syndrome, type 2 diabetes, etc.).

Recent research provides compelling evidence in support of the DOHAD hypothesis (92). Developmental plasticity in utero gives rise to phenotypes better suited to that specific environment (88, 93) and maternal nutrition has the potential to impact fetal
growth and lead to disease in later life (92), particularly where the environment differs from the one in which the fetus grew. This assumption of “programming”, the ability to change structures, functions and responses in the new animal, is the central concept of this theory (94). These changes, not necessarily evident at birth (94), have been ascribed to epigenetic modifications of the gene that alter expression but convey no alteration of the DNA sequence (95). The epigenome is most vulnerable to environmental factors during the periconceptional and intrauterine period, thereby increasing the risk of adult disease more so than later exposure (96).

Nutrient imbalance in fetal life has been shown to be associated with higher risk of hypertension, type 2 diabetes and obesity in later life even when no changes in fetal growth rates are observed (97). The proposed epigenetic mechanisms elicited by nutritional factors include DNA methylation, histone modification and non-coding microRNAs (98, 99). Table 1.1 (below) summarises data from two reviews (98, 99) and highlights important research demonstrating the role of these mechanisms in physiological and clinical outcomes.
Table 1.1 Examples of the association of nutritional factors and metabolic effects through epigenetic mechanisms. Table adapted from Canani et al. (98) and Milagro et al. (99).

<table>
<thead>
<tr>
<th>Epigenetic mechanisms</th>
<th>Nutritional factors</th>
<th>Metabolic effects</th>
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<tbody>
<tr>
<td>DNA methylation</td>
<td>Vitamin B12</td>
<td>Insulin resistance, obesity</td>
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<tr>
<td></td>
<td>Folate</td>
<td>Adiposity, Insulin resistance</td>
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<td></td>
<td>Eicosapentaenoic acid</td>
<td>n-3 Polyunsaturated fatty acid metabolism</td>
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<tr>
<td></td>
<td>Docosahexaenoic acid</td>
<td>n-3 Polyunsaturated fatty acid metabolism</td>
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<td></td>
<td>Arachidonic acid</td>
<td>n-6 Polyunsaturated fatty acid metabolism</td>
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<td></td>
<td>Ascorbate</td>
<td>Antioxidant processes</td>
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<td></td>
<td>Genistein</td>
<td>Body weight</td>
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<td>Soy isoflavones</td>
<td>Body weight, insulin sensitivity</td>
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<td></td>
<td>Low protein diet</td>
<td>Impact on the amino acid response pathway and cholesterol metabolism</td>
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<td></td>
<td>High fat diet</td>
<td>Impact on leptin gene in adipose tissue and melanocortin receptor 4 in brain</td>
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<td></td>
<td>Hyper-caloric diets</td>
<td>Impact on the expression of genes involved in the control of appetite and energy metabolism</td>
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<td></td>
<td>High fat diet and dietary protein restriction</td>
<td>Obesity, metabolic syndrome and impaired glucose metabolism</td>
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<td></td>
<td>Calorie restriction</td>
<td>Increased adrenal mass and cortisol response to stress</td>
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<td>miRNA</td>
<td>Curcumin</td>
<td>Inflammation response, body weight</td>
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<tr>
<td></td>
<td>Soya, retinoic acid</td>
<td>Cancer prevention and therapy</td>
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<td>High cholesterol diet</td>
<td>Obesity</td>
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<td>Dietary methyl deficiency</td>
<td>Non-alcoholic steatohepatitis and liver cancer</td>
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<td>Histone modifications</td>
<td>Retinol</td>
<td>Antioxidant processes</td>
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<td></td>
<td>Tocopherols</td>
<td>Antioxidant processes</td>
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<tr>
<td></td>
<td>Resveratrol</td>
<td>Body weight, liver steatosis</td>
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<td></td>
<td>Sulforaphane</td>
<td>Adipocyte differentiation</td>
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<td></td>
<td>Dietary protein restriction</td>
<td>Type 2 diabetes</td>
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<td></td>
<td>High fat diet / calorie restriction</td>
<td>increased histone 4 acetylation in adipose tissue</td>
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<tr>
<td>Histone and DNA methylation</td>
<td>Epigallocatechin 3-gallate</td>
<td>Weight reduction, insulin sensitivity, liver steatosis</td>
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<td></td>
<td>Choline</td>
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<td>Betaine</td>
<td>Liver steatosis, insulin resistance</td>
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<td>Methionine</td>
<td>Insulin resistance, obesity</td>
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<tr>
<td></td>
<td>Serine, Glycine, Histidine</td>
<td>Amino acid metabolism</td>
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1.3.2 Possible epigenetic mechanisms

There is now a large body of evidence based on both human and animal research on the association of prenatal nutritional exposure and the risk of adult obesity and metabolic disorders (92, 97, 99, 100). Nonetheless, the support coming from human population studies has been limited (97) and does not prove a cause-effect relationship. The advantage of animal experiments is they allow observations of how specific modifications to the diet of the mother can produce permanent effects in the metabolism of the fetus (101). Indeed, the most recent studies in rodents have demonstrated that the central nervous system, part of which is the hypothalamic energy homeostasis circuit, develops in utero and continues to mature in the suckling period (102, 103). Different nutritional exposures and modifications during this critical developmental period have led to outcomes in support of the early origins hypothesis. Indeed, Coan et al. (104) were able to show that even subtle variations in dietary composition of the maternal diet lead to functional adaptations in placental phenotype that optimise nutrient transfer to the fetus and regulate fetal growth.

One of the nutritional factors known to have a significant epigenetic effect linked with obesity is glucose (99, 105). Maternal blood glucose crosses the placenta into the fetal circulation and can directly influence the epigenetic profile (106). Using ultrasound fetal measurements, Farah et al. showed that maternal glycaemia influenced birth weight, fetal adiposity and distribution of adipose tissue in women without diagnosed gestational diabetes mellitus (GDM) (107). The Hyperglycaemia and Adverse Pregnancy Outcomes (HAPO) study confirmed that maternal glucose levels below those diagnostic of diabetes were associated with increased birth weight and cord-blood serum C-peptide levels (a measure of fetal insulin secretion).
Bouchard et al. (106) demonstrated that higher glucose concentrations during the second trimester of pregnancy were associated with lower placental adiponectin DNA methylation. Lower methylation levels were also associated with higher insulin resistance during the last two trimesters of pregnancy. Because adiponectin is suspected to have insulin-sensitizing properties, these epigenetic adaptations have the potential to induce changes in glucose metabolism in the mother and offspring. Finally, Deierlein et al. found that higher maternal glucose concentrations were proportionally associated with higher BMI in offspring at 3 years of age (108). These findings together strengthen the evidence that maternal glucose plays an important role in intrauterine fetal programming.

GDM offers an almost perfect example for researchers to better investigate the connections between maternal glucose levels and epigenetic effects during gestation. Maternal diabetes in pregnancy is strongly associated with an increased rate of offspring childhood obesity, impaired glucose tolerance, and type 2 diabetes later in life (109). It was indeed these observations in GDM that prompted the first investigations on the impact of in utero conditions on the risk of developing chronic diseases and gave grounds to the early origins of adult disease theory (110). The most plausible mechanism is believed to be the increased glucose transfer to the fetus caused by the maternal hyperglycaemia which leads to an increased fetal insulin secretion. Fetal hyperinsulinaemia occurs during the critical time window of brain development when the hypothalamic regulation centre (and thus metabolism and energy regulation systems) are being laid down (111, 112). A study on the placentas of GDM-affected mothers found functional modifications at the leptin and
adiponectin genes that influenced the regulation of energy metabolism in the offspring, leading to obesity (110).

These effects of maternal hyperglycemia are further supported by recent animal work that demonstrates that metabolic memory (the phenomenon where diabetes complications persist and progress after glycaemic recovery is achieved) is heritable and its transmission correlates with hyperglycaemia-induced DNA hypomethylation and altered gene expression (113). This study used a zebrafish model in which the daughter tissue, which was not exposed to hyperglycaemia but was derived from tissue that was, showed the same associations.

Mühlhäusler and her group conducted several studies in sheep investigating the development of the hypothalamic appetite regulatory neural network in the fetus and the effects of intra-fetal glucose infusion during late gestation (114, 115). They found that components of the central neural network that regulate appetite and energy balance in the adult sheep are expressed already in the developing fetal brain and have the potential to operate immediately post-partum (114). When they conducted continuous intravenous infusion of glucose in the fetuses of sheep from 130-140 days of gestation (115), they found that it significantly increased the expression of POMC in the arcuate nucleus of the fetal sheep hypothalamus, despite the fact that glucose infusion did not increase circulating concentrations of leptin. Further studies in sheep have shown that maternal overnutrition increases hypothalamic expression of the appetite inhibitor proopiomelanocortin, PPARγ and LPL expression in the adipose tissue of the postnatal lamb, as well as leptin mRNA expression in perirenal adipose tissue in the fetal sheep in late gestation (116-119).
Finally, Mohmaad et al. (102) conducted a rodent study to investigate the possible epigenetic modifications associated with the Npy and Pomc genes induced by a high carbohydrate diet in the developmental period. In an earlier study, the same research group had shown that rearing of newborn rats on a high-carbohydrate milk formula resulted in hyperinsulinaemia, which persisted in the post-weaning period and led to adult-onset obesity. In the second study, the high carbohydrate milk formula administered to neonatal female rats resulted in altered epigenetic regulation of the genes involved in the hypothalamic energy homeostatic mechanism. This suggested that the changes could contribute to the development of obesity in the adult rat. Together, these findings stimulated my interest in investigating the high and low end of normal maternal glucose exposure and its programming effects during the in-utero and early post weaning period.

1.4 The glycaemic index of foods, its clinical applications and outcomes

Carbohydrates present in different foods have distinct physiological effects, including those on postprandial glycaemia and insulinaemia, which can influence the rate of digestion, appetite (hunger and satiety), fuel partitioning and metabolic rate. The type and quality of carbohydrate foods in human diets has been found to have a major impact on health outcomes (120). The glycaemic index (GI) is a food classification based on the postprandial blood glucose response relative to a reference food, gram for gram of carbohydrate (121). The glycaemic load (GL), the mathematical product of the GI and the amount of carbohydrate, encapsulates both the quality (i.e. GI) and quantity of carbohydrate, and is the single best predictor of postprandial glycaemia (122). In overweight and insulin resistant individuals
consuming high GI/GL diets, glycaemic spikes and insulin demand are excessively increased. Foods can be classified as low GI (GI <55), medium GI (GI 55-69) or high GI (≥70) (123). The given value of a carbohydrate containing food is calculated using the average incremental area under the curve (iAUC) of the blood glucose levels within a 2 hour period elicited by 50 g of available carbohydrate portion of that food, relative to the iAUC elicited by 50 g of pure glucose (124). The GI reflects the way the carbohydrates in a given food raise blood glucose levels and the rate it is cleared from the bloodstream within this 2 hour period. Slower digestion and absorption of carbohydrate elicited by a low GI food results in a more moderate increase in the blood sugar levels with a lower peak level when compared to a high GI food (123).

Many studies have investigated dietary strategies to limit postprandial hyperglycaemia, including high-protein diets, Mediterranean-style diets and low GI diets. Compared with conventional healthy diets, diets with a low GI carbohydrate composition have shown greater weight loss, metabolic advantages such as improved diabetes control, and have been associated with reduced risk of developing type 2 diabetes, improved satiety, increased utilization of stored fuels and improved weight management (20, 125).

Obesity, excess weight, diabetes and pregnancy influence metabolic and endocrine pathways and therefore production of hormones and growth factors in different ways (125, 126). A recent study investigated whether the GI can impact health in people with or without the metabolic syndrome (127). Using a cross-over feeding design with a low and a high GI diet in normal and overweight/obese (but otherwise healthy)
subjects, they measured biomarkers of inflammation and obesity, including leptin, adiponectin, C-reactive protein and IL-6. The low GI diet was associated with decreased serum C-reactive protein levels and increased adiponectin concentrations in participants with a high body adiposity. Thus, the low GI diet reduced inflammation and increased adiponectin (an insulin-sensitising factor) in obese people, suggesting that the quality of carbohydrate foods plays a significant role in the prevention of obesity-related metabolic dysfunctions. Similarly, a recent meta-analysis on the possible beneficial effects of long-term low GI diet, indicated that pro-inflammatory markers such as CRP were also positively influenced by the diet (128).

Evidence shows that pregnant women and those of reproductive age are often at risk of obesity or already obese, and therefore have an increased risk of adverse pregnancy outcomes (125, 129). However, the priority of a mother’s diet is to ensure sufficient supply of nutrients for the fetus to grow. If there is excessive weight gain in pregnancy, the complications include gestational diabetes, pre-eclampsia, post-partum weight retention leading to later obesity, large-for-gestational age (LGA) babies and others. Reproductive outcomes have also been correlated with the glycaemic load (GL) of the diet, with high GL diets associated with greater risk of poor outcomes (125, 130). In the Danish National Birth Cohort (125), birth weight increased by 36 g from the lowest to highest GL quintile, with 14% increased risk of large for gestational age infants (LGA) in the highest vs. lowest GL quintile. Among normal-weight and overweight women, higher rates of gestational weight gain were detected in the highest vs. lowest GL quintile. A review of the evidence on the GI of the periconceptional, gestational and lactating diet and the metabolic effects on the
offspring’s health concluded that a low GI maternal diet can alter maternal blood glucose production, insulinaemia and reduce adiposity as well as fetal and placental insulin and glucose regulation, fetal growth, birth weight and offspring adiposity (131).

Louie et al. published a systematic review article which focused on the evidence regarding the effect of dietary GI on maternal and fetal nutrition (132). Taking into account the increasing incidence of high birth weight and its association with increased risk for obesity in adulthood, the review highlighted the importance of strategies to lower maternal blood sugar levels thus avoiding fetal hyperinsulinaemia and oxidative stress, both linked to the development of obesity. The human studies indicated that a low GI diet can improve pregnancy outcomes in both healthy and GDM affected women. This conclusion was also supported in the more recent review (133). Indeed, a controversial point of the GI concept has been its cogency and power of application in people with various metabolic profiles. The reproducibility of GI values and their impact in healthy, hyperinsulinaemic or diabetic subjects was tested by Lan-Pidhainy et al. in 2011 (126). They demonstrated that the GI values of the defined carbohydrate foods are valid in all subjects regardless of their metabolic status.

Danielsen and colleagues recently published the results of an investigation on the association between the GI of the maternal diet during pregnancy and the metabolic profile in the adult offspring (134). The study was based on the data recovered from the Danish Fetal Origin cohort in 1988 and follow-up offspring data in 2008 and 2009. They were able to show that high dietary GI in pregnancy was significantly
associated with the metabolic syndrome. An increasing maternal GI diet led to young adult offspring presenting higher levels of cholesterol, HOMA-IR, plasma leptin and insulin. When the quantity of this type of carbohydrates was increased, there were associations with blood pressure and waist circumference as well in the young female adults.

The data derived from epidemiological research can therefore be seen to complement the findings in animal models. Importantly, animal studies have allowed a more controlled identification of the effect of the dietary GI on the metabolic outcomes and epigenetic effects that can contribute to later development of disease.

A detailed study conducted in rats indicated that the GI had an independent effect on body composition (135). The research was based on multiple experiments and showed that rats on a high GI diet, consuming the same amount of food ad libitum and gaining the same amount of weight for 7 weeks compared to the low GI group, developed hyperinsulinaemia. Also, the high GI-fed rats gained the same weight as the low GI group even though consuming less food from week 8 onwards which indicated they were metabolically compromised. The authors suggested it could be due to lower resting energy expenditure or activity due to decreased lean body mass. The study also included an experiment in the obesity-prone C57BL/6J mice. On a high GI diet, these mice had lower lean body mass and nearly twice the body fat compared to the low GI-fed mice with the same mean body weight.

Other studies have evaluated the effects of the GI on adiposity, glucose homeostasis and other metabolic parameters in the longer term. One study compared high and low GI diets matched for macro and micronutrients in mice up to the age of 40
weeks, an age equivalent to old age in humans (136). The high GI mice had lower fat oxidation, 40% greater adiposity and were more insulin resistant. Interestingly these effects were independent of energy intake since that was the same between the two diet groups. Similarly a study on 16 weeks and 44 weeks old mice (137) showed that the 16 weeks old high GI-fed mice had significantly higher body weight, increased fat mass and reduced insulin sensitivity. These outcomes were not as pronounced in the 44 weeks old high GI-fed mice indicating that the effect was greater in the younger years.

Lastly, Scribner’s group conducted another study to understand the short (6 weeks) and long term (20 weeks) effects of high vs. low GI diets on body composition, liver fat, glucose clearance and lipid metabolism in mice (136, 138). All high GI-fed animals showed a significant increase in body fat mass, greater liver fat and elevated lipogeneses. In addition, the mice exposed to the high GI diet for longer were more insulin resistant than the low GI-fed mice, and in the postprandial state had a slower switch to carbohydrate and fat oxidation.

1.5 Research aim and hypothesis

In view of earlier findings (as reviewed above), the overall goal of this research project was to investigate the effects of a high and a low GI maternal diet on offspring metabolic markers and expression of two key genes (Fto and leptin) in an animal model. The primary outcome was the relative difference in Fto expression in offspring hypothalamus, the site of highest Fto expression and regulation of energy balance. The secondary outcomes included Fto expression in maternal placenta, leptin expression in offspring adipose tissue, growth rate, glucose and insulin
tolerance, plasma leptin and ghrelin concentration, and other measures related to appetite control. Our final aim was to examine histological liver samples in the offspring of high and low GI-fed mothers to determine how early exposure to postprandial hyperglycaemia may contribute to liver lesions and/or morphological alterations. The study design allowed for observations of both early-life and life-long exposure in response to the two carbohydrate-modified diets.

Our hypotheses were:

1. The offspring of high GI-fed mothers will be more glucose intolerant and/or insulin resistant than those of low GI-fed mothers.
2. The concentration of circulating appetite/satiety hormones in offspring plasma will be differentially influenced by the exposure to the two treatment diets.
3. Fto gene expression in key tissues will be differentially regulated by the treatment diets. Specifically, Fto gene expression in the hypothalamus of pups of high GI-fed mothers will be higher than those of low GI-fed mothers, implying a direct epigenetic effect of maternal dietary GI. Fto gene expression in the placenta of the high GI-fed mothers will be higher than in low GI-fed mothers, providing a mechanism linking maternal with fetal expression.
4. Leptin gene expression in the adipose tissue will be differentially regulated by the treatment diets. Specifically, leptin gene expression in the adipose tissue of pups of high GI-fed mothers will be lower than those of low GI-fed mothers, implying a direct epigenetic effect of maternal dietary GI on leptin gene regulation.
5. High GI diet-fed offspring may present altered liver morphology due to greater glycogen and fat deposition resulting from postprandial hyperglycaemia.
6. Any adverse effects of early-life exposure to a high GI diet will be exacerbated by life-long exposure.
Chapter 2

Investigating the effect of early-life and life-long exposure to a high vs. low GI diet on the metabolic phenotype
Chapter 2  Study design and rationale

2.1 Introduction

In 1990, Barker proposed the initially controversial hypothesis that human metabolism is programmed in utero (87, 88, 139, 140), that common chronic illnesses such as cancer, cardiovascular disease and diabetes result not always from ‘bad’ genes and an unhealthy adult lifestyle, but from poor intrauterine and early postnatal health. Later described as the ‘developmental origins of health and disease’ (DOHAD), it is now one of the greatest arenas for experimental and clinical studies worldwide. In the space of a short timeframe, starting with the union of a sperm and an egg and finishing with birth, a single cell is transformed into a fetus. By birth the fetus has developed its own physiological attributes and the ability to live outside the womb. The developmental process is defined by the environment and the conditions in utero in which the embryo and fetus grow. Vulnerable stages of cell division and organ development are directly influenced by the conditions inside the womb.

This environment, governed by fetal nutrition via the placenta, programs the distribution of cell types, hormonal feedback and metabolic activity. The nutrition of the mother plays a critical role. As early as 2000, there was evidence that even minor modifications to the maternal diet, both before and during pregnancy, changed the metabolism of the offspring (88). A similar effect was replicated with the “pup-in-a-cup” model (141), thus becoming one of the most important examples of metabolic determination of the offspring by diet modification before and during pregnancy (142).
We now know that the mother’s diet shapes the metabolic programming of her fetus via specific modifications to genes, including histone modifications, gene hyper- or hypo-methylation or other pathways that are constantly being studied (95). Inappropriate fetal nutrition, orchestrated by the quantity and quality of nourishment obtained through the maternal–fetal interface, drive the future biological functions and health outcomes for the offspring (143). Langley-Evans described it thus: “Programming is the consequence of the innate capacity of developing tissues to adapt to the conditions that prevail during early life, which for almost all cell types in all organs is an ability that is present for only a short period before the time of birth” (97).

Animal experiments have enabled us to observe these effects of maternal nutritional manipulation and their influence on the phenotype and metabolic profile of the offspring, as well as the later signs of metabolic syndrome in adult life (144). Furthermore, they have allowed us to discover that differential nutritional exposures can “program” or alter the epigenome (145, 146). These experimental findings are consistent with associations observed in humans.

Fetal metabolism is largely based on the fetus’ principal energy substrate, glucose (105), which is transported across the placenta corresponding to the glucose concentration in the maternal circulation. The future offspring has an innate coping and adaptive capability to manage with changes in glucose supply, but these strategies may subsequently set the scene for metabolic disorders such as insulin resistance, obesity and diabetes mellitus (147). Gestational diabetes mellitus is one of the best examples of metabolic programming in utero. Intrauterine hyperglycemia,
leading to increased fetal plasma glucose and insulin concentrations, increases the rate of fetal growth, predisposing to high perinatal morbidity and mortality, as well as a long term predisposition to obesity, metabolic disorders and malignancy (117).

However, even maternal glucose levels that are below the diagnostic values of diabetes have been associated with increased birth weight and cord-blood serum C-peptide levels and hence fetal insulin levels (148). For this reason, diets which reduce postprandial glycaemia have the potential to improve pregnancy outcomes in normal women. Low glycaemic index (GI) diets are associated with reduced risk of type 2 diabetes and obesity-related disorders in prospective observational studies (128, 149, 150). Meta-analyses of randomized controlled trials show beneficial effects of low GI diets compared to high GI diets in overweight, obesity, type 2 diabetes (151-153), and gestational diabetes (132, 133).

Moses and Brand-Miller were the first to demonstrate that normal pregnant women following a lower GI diet had smaller infants with a lower ponderal index and reduced proportion of large-for-gestational age infants based on fetal centiles (130, 154, 155). Since then, several other RCTs in overweight women (130, 156-158) and women with a history of macrosomia (159) have shown improved obstetric outcomes on a low GI diet. Although birth weight is often used to gather information about the end point of fetal growth, it does not reflect the growth trajectory nor body composition (160) nor the metabolic health of the infant. However, observational studies also suggest that following a high GI diet in pregnancy adversely affects markers of the metabolic syndrome in the young adult offspring (134).
In designing the current study, we reasoned that an investigation of metabolic programming throughout pregnancy and early life required conditions and parameters that could be standardised and replicated. Therefore in this study, we chose a mouse model to examine the impact of two commercial isocaloric diets matched for macronutrients and differing only in their glycaemic index (GI) on the male offspring of pregnant mice. In addition to growth and weight gain, our aim was to examine metabolic and physiological responses including glucose and insulin tolerance, plasma leptin, ghrelin, adiponectin and other hormones that may be affected by the difference in GI of the two diets. This kind of diets, have previously been tested in mice and rats in diet intervention trials showing effects on glucose tolerance, food intake, weight and adiposity (135, 136, 161).

2.2 Materials and methods

2.2.1 Animals and diets

All animal work was approved by the Animal Ethics Committee of the University of Sydney (Protocol number L02/8-2010/2/5355). C57BL/6 mice were provided by the Animal Resources Center (Canning Vale, WA). Mice breeders were kept in indoor animal house facilities (temperature kept constant at 25° C) in the School of Molecular Bioscience Animal House of the University of Sydney (Figure 2.1a). In general, two to six same sex mice were housed together in standard cages containing clean paper pellets and paper rolls to shred. Mice had ad libitum access to their feed and water through a feeder tray within the lid of the cage. The light-dark cycle of the animal holding room was 12 h-12 h.
Figure 2.1a Animal house facilities and cages.

Photograph of the temperature-controlled cages. Food was placed in the insert of the cage and mice had ad libitum access. Water was always available through a bottle placed on top of the cage.

Two special diets and a standard chow, were obtained commercially from Specialty Feeds, Glen Forest, WA, Australia. The two special diets were of identical macronutrient composition and calculated energy density, with only the starch component being different (Table 2.1). As described by the company, the starch was mechanically manipulated in order to obtain a ‘gel crisp’ high amylose starch for the Low GI diet (70% amylose, 30% amylopectin) and a dextrinised high amylopectin starch for the High GI diet (90% amylopectin, 10% amylose). Both starches were extracted from conventionally-bred varieties of maize (corn, Zea mays). The diets were enhanced with small amounts of AIN93 vitamin premix that used wheat starch instead of sucrose or glucose as the carrier. The diets were not irradiated (as was normal practice) because an earlier adverse event suggested that the process may have reduced the availability of micronutrients during pregnancy. The adverse event is described in Appendix 3.
Figure 2.1b The High GI and Low GI feeds used in this study.

The High GI and Low GI feeds were matched for macro- and micro-nutrients and had similar appearance, texture and colour.
Table 2.1. A comparison of the main nutrients in the two special feeds and the chow diet. A more comprehensive table with the full nutritional information panel of each treatment diet can be found in Appendix 2.

<table>
<thead>
<tr>
<th></th>
<th>LOW GI</th>
<th>HIGH GI</th>
<th>CHOW</th>
</tr>
</thead>
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<td>19.40</td>
<td>19.60</td>
</tr>
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</tr>
<tr>
<td>AD fibre (g/100 g)</td>
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<td>4.70</td>
<td>Not specified</td>
</tr>
<tr>
<td>Digestible energy (MJ/kg)</td>
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<td>16.3</td>
<td>14.3</td>
</tr>
<tr>
<td>Total calculated digestible energy from lipids (g/100 g)</td>
<td>16.00</td>
<td>16.00</td>
<td>Not specified</td>
</tr>
<tr>
<td>Total calculated digestible energy from protein (g/100 g)</td>
<td>21.00</td>
<td>21.00</td>
<td>Not specified</td>
</tr>
</tbody>
</table>

2.2.2 Study design

A schematic representation of the design of the first study is shown below.
C57BL/6 female mice (n = 21) were delivered to the Animal House at 4 weeks of age and randomly assigned to one of the following diet groups: Low GI diet (LGI), High GI diet (HGI) and Standard Chow (CC) (7 female mice for each group). C57BL/6 male breeders were delivered at 3 weeks of age and consumed CHOW for the 4 weeks prior to mating. At this point, their diet was interrupted briefly (~ 15 h) when they were placed in female cages.

Mating was based on the “Breeding Strategies for Maintaining Colonies of Laboratory Mice”, Resource Manual by the Jackson Laboratory (http://jaxmice.jax.org/manual/#breeding). At birth only male pups were kept for this research. Female mice were made available to other researchers.

Male pups from the LGI and HGI groups were weaned at the beginning of week 5 and were further divided into two subgroups, one following their mother’s diet (LL and HH) and one following the CHOW diet (LC and HC). Male pups of the CHOW group were weaned at the same time and continued on CHOW (CC). This last group has been used as the group of reference and comparison for our analysis.

2.2.3 In vitro GI testing of diets

An in vitro starch digestion assay described by Englyst et al. (162) as ‘a method that predicts the glycaemic response’ was performed in triplicate. Rapidly available glucose (RAG) and slowly available glucose (SAG) were measured in order to describe the rate at which glucose from each diet became available for absorption.
Briefly, the *in vitro* digestion of starch from the high and low GI feeds was determined in duplicate samples. An enzyme solution was prepared by suspending 0.45 g of porcine pancreatic α-amylase (150 U/mg, Sigma A3176) in 16 mL of water with magnetic stirring for 10 min at 37 °C. The mixture was centrifuged (1,500 × g for 10 min) and 2 mL of amyloglucosidase (3,260 U/ml, Megazyme) was added to 10.8 mL of the above enzyme supernatant. The feeds were crushed into a fine powder using a laboratory mortar and pestle, and the corresponding amount containing 100 mg starch (dry weight) was dispersed in 4 mL of 0.1 M sodium acetate buffer (pH 5.2). After adding 1 mL of the freshly prepared enzyme solution, the mixture was incubated in a shaking water bath (37°C, 160 strokes/min). Aliquots (0.1 mL) were taken at the same time intervals as for GI testing and mixed with 1 mL of 95% ethanol. The glucose released was measured by the glucose oxidase-peroxidase reagent according to the supplier’s instructions (Megazyme International Ireland Ltd. Bray Co., Wicklow, Ireland).

2.2.4 Identification by ear-marking

At 4 weeks of age, male pups were anaesthetised using isoflurane (ISO) solution within a glass chamber. A surgical scissor was used to obtain one of six unique ear punches (three sites for the right and three sites for the left ear).

2.2.5 Food intake and weight monitoring

After weaning, the pup weights were recorded weekly using an electronic laboratory scale, until 20 weeks of age when they were sacrificed. Three 24 h food intake observations were conducted at weeks 9, 12 and 16. Mice were placed in custom made cages that contain an insert to allow catching spilled food. The experiment was
conducted according to cage grouping (n = 2-5 per group) to minimize stress of individual housing. Food intake measurements were recorded by weighing food pellets before and after 24 h and dividing by the number of mice in the cage. Any food spilled into the bottom of the cage was subtracted.

2.2.6 Glucose tolerance test

An intraperitoneal Glucose Tolerance Test (GTT) was conducted at week 17. Mice were fasted for 4-6 hours prior to testing (163, 164). Basal blood glucose levels were tested using an Accu-Check Performa glucometer (Roche Diagnostics Australia Pty Ltd) through a small incision on the tail. Mice were subsequently administered glucose (2 mg/g body weight of 50% w/v solution) via an intraperitoneal injection. Blood glucose levels were determined at 15, 30, 45, 60, 90 and 120 min after first injection and the incremental area under the curve (iAUC) was calculated in accordance with the method recommended by Wolever (165).

2.2.7 Insulin tolerance test

An intraperitoneal Insulin Tolerance Test (ITT) was conducted at week 18. Mice were fasted for 4-6 hours prior to testing. Basal blood glucose levels were tested using an Accu-Check Performa glucometer (Roche Diagnostics Australia Pty Ltd) through a small incision on the tail. Mice were subsequently administered insulin (Actrapid Novo Nordisk Pharmaceuticals Pty Ltd. 1U/kg body weight) via an intraperitoneal injection. Blood glucose levels were determined at 15, 30, 45, 60, 90 and 120 min after first injection. In the case of mice with very low readings at any point of the ITT, a glucose solution was injected and the animal removed from the
experiment. This was necessary in order to avoid coma or death due to hypoglycaemia.

The primary outcome was the rate of decline of glucose during the first 30 min after insulin injection. The rate was calculated from the slope of the line and interpreted as a measure of insulin sensitivity.

2.2.8 Mouse sacrifice, blood and tissue collection

At 20 weeks of age, mice from all groups were sacrificed by intraperitoneal injection with 100 µl of Lethabarb euthanasia solution. Mice were previously fasted for 4-6 hrs. Sacrifice took place in the surgery room of the Animal House away from other animals to avoid stress. After the injection, mice were observed until assured they were dead by absence of respiratory movement and corneal reflex.

With a cardiac puncture, blood was collected in sterile heparin coated tubes and separated by centrifugation (10000rpm, 1 min, 25°C, Eppendorf Centrifuge MiniSpin®). Plasma was transferred to a fresh tube, snap frozen in liquid nitrogen and stored at -80°C until further analysis.

The following tissues were collected: hypothalamus, brown adipose tissue (BAT), liver, visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT), red muscle (RM) and white muscle (WM). A sample from each liver tissue was fixed in 4% (w/v) phosphate-buffered paraformaldehyde for 48 hours at 4°C. Then tissues were transferred to phosphate-buffered saline solution and subsequently embedded in
paraffin and sectioned to allow for histological tests. Collected tissues were snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

2.2.9 Plasma analysis

Plasma samples collected at sacrifice were analysed by ELISA using a Multiplex Map Kit by Cardinal Bioresearch Pty Ltd (New Farm, Qld, Australia). Protease inhibitors were added during sample thawing. The following hormones were measured: ghrelin, leptin, adiponectin, insulin, Glucagon-like peptide 1 (GLP1), pancreatic polypeptide (PP), peptide YY (PYY), adrenocorticotropic hormone (ACTH), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNFα).

2.2.10 Statistical analysis

Data were statistically analysed using SPSS Statistics 19 (IBM) and Prism 6.0 (Graph Pad Software, USA). A one-way repeated measures analysis of variance (ANOVA) and two-tailed unpaired t-tests were performed to assess the difference between the studied variables amongst the groups. A normal distribution could be assumed for most variables but in the case of plasma hormone levels, a large within group variability was observed and significant deviation from normality was found, thus Kruskal–Wallis and Mann-Whitney U non parametric tests were used.

These comparisons were made in order to investigate the effect of varying exposure to the High GI, Low GI and CHOW diets, and more specifically to investigate the effects of:

a. Early-life exposure to the diet (comparison of HC and LC groups)

b. Extended (life-long) exposure to the diet (comparison of HH and LL groups)
Due to the large number of variables, a statistical $p$ value of $\leq 0.05$ was taken as marginal significance and $p \leq 0.01$ as statistically significant.

2.3 Results

2.3.1 Rate of digestion in vitro

A validated *in vitro* starch digestion assay was used as an indicator of the likely glycaemic response to each of the diets (162). The results (Figure 2.2) show that at the 20 min time point, the amount of rapidly available glucose (RAG) in the High GI feed was 55.6% higher than the Low GI feed ($p = 0.006$). Similarly, at 120 min of digestion, the amount of glucose released was 43.6% higher in the high GI feed ($p = 0.0006$). This trend continued for the whole testing period of starch digestion.

![Figure 2.2 Rate of starch digestion for the High GI feed (—) and the Low GI feed (—). Rate of digestion was determined by the Englyst procedure (162). Both feeds were tested in duplicate on three occasions, using 1 g of finely powdered pellet. RAG = $G_{20} - G_0$. The High GI feed had an excess of 93 mg of glucose per gram of feed available for absorption compared with the low GI feed at 20 min. SAG = $G_{120}$-](image-url)
The Low GI feed had 102 mg/g less glucose available for absorption and demonstrated an overall slower starch digestion over the 180 min period.

### 2.3.2 Food intake

In this study there were no differences in litter size (average n = 7) or conception rate. After weaning, male pups in each litter were subdivided into two diet groups. Overall five diet groups were created with chow diet being used as a control group. Figure 2.3 summarises the abbreviations used for the five diet groups and the number of pups in each group.

![Diagram of diet groups after weaning](image)

**Figure 2.3 Diet groups after weaning.**

CC: pups of the chow diet mothers that continued on chow after weaning  
HH: pups of the High GI diet mothers that continued the High GI diet after weaning  
HC: pups of the High GI diet mothers that were weaned to a chow diet  
LL: pups of the Low GI diet mothers that continued the Low GI diet after weaning  
LC: pups of the Low GI diet mothers that were weaned to a chow diet

Food intake was measured at week 9, 12 and 16 (Figure 2.4). The highest food intake was observed in week 9 for the CC and HC groups whereas the LL group showed the lowest intake, but there were no significant differences at any time point.
Similarly, at week 12 the HH group had the highest food intake (3.7 g/mouse/day) compared to all other groups, whereas the LL group had the lowest (3 g/mouse/day) but overall, the differences among the groups did not reach statistical significance (p = 0.4). In week 16 all groups showed very similar intake.
Figure 2.4 Average food intake. Values expressed as average g per kg of body weight. Chow n = 12, HC n = 12, HH n = 13, LC n = 12, LL n = 10. All data represent mean ± SEM. (NS = not significant).
2.3.3 Body weight

Body weights of the pups were measured at weekly intervals from 5 to 20 weeks of age. Figure 2.5 shows the weights at week 5 just before weaning. There was no significant difference in mean weight between CC and the treatment groups HGI and LGI. Body weight progression was similar in all groups. At weeks 16 and 19, LC showed a slightly higher (+7%) body weight than the HC group (p = 0.005) (Figure 2.6 A). All groups were comparable with the average body weight of ~31.4 g (±2.4) at week 19, similar to that described by the animal breeder (*Jax® Mice and Services*) (http://jaxmice.jax.org).

![Figure 2.5 Male C57BL/6 pups body weights at week 5.](image)

Pups of CC (n = 13), HGI (n = 28) and LGI mothers (n = 24). Data are expressed as mean ± SEM. Weights were recorded weekly to the nearest 0.1 g. (NS = not significant).

Subsequent comparisons between the groups were made in order to investigate the effect of varying exposure to the High GI, Low GI and CHOW diets, as shown in Figures 2.6 and 2.7:
A. Early-life exposure to the diet (comparison of HC and LC groups)

B. Extended (life-long) exposure to the diet (comparison of HH and LL groups)
Figure 2.6 Body weight progressions from week 5 to week 20. Data are expressed as mean ± SEM. A and B: no significant differences (NS) were observed at any time point. CC n = 13, HC n = 13, HH n = 15, LC n = 13, LL n = 11.

At week 20, immediately prior to sacrifice, LC were slightly heavier (+6.9%) than HC mice, (reaching the greatest average weight overall of 31 g) but the difference did not reach statistical significance.
Figure 2.7 Body weights at week 20 just prior to sacrifice. Data are expressed as mean ±SEM. No significant difference was observed. CC n = 13, HC n=13, HH n = 15, LC n = 13, LL n = 11. NS = not significant.

2.3.4 Glucose tolerance test

Intraperitoneal glucose tolerance tests (ipGTT) were conducted to investigate differences in glucose tolerance among the groups. After injection at 0 time, plasma glucose levels were determined at 0, 15, 30, 45, 60, 90 and 120 min. The incremental area under the curve (iAUC) was calculated and results are shown in Figures 2.8 and 2.9.

Overall, there were no significant differences in plasma glucose responses between the HC and LC groups, although the peak at 15 min was slightly higher in HC than LC (Figure 2.8A, p = 0.08). At 30 min, the HH group had marginally higher glucose levels than the LL group (p = 0.02), suggesting slower glucose clearance in HH, but
other time points were similar (Figure 2.8B). There were no significant differences in incremental area under the curve (iAUC) in HH vs. LL (Figure 2.9).
Figure 2.8 Plasma glucose responses to the intraperitoneal glucose tolerance test.

Blood glucose readings were performed after intraperitoneal glucose injection. Data represent mean ± SEM. A: no significant differences were observed. B: marginal significant difference at 30 min (p = 0.02). CC n = 8, HC n = 10, HH n = 10, LC n = 13, LL n = 10.
2.3.5 Insulin tolerance test

An intraperitoneal insulin tolerance test (ipITT) was performed to investigate insulin sensitivity. Insulin was injected and the rate of decrease in plasma glucose levels observed. The slope of the line between 0 and 30 min of the testing period can be used as a measure of insulin action.

Overall, there were no significant differences in insulin tolerance between HC vs. LC or HH vs. LL (Figure 2.10). Blood glucose at 30 min and at 45 - 120 min (p > 0.1) tended to be lower in the high GI groups (HC and HH) but this did not reach
statistical significance. CC had significantly lower plasma glucose levels at 0 min than LC and LL groups (p ≤ 0.01).

**A**

![Plasma glucose levels after intraperitoneal insulin injection. Data shown represent mean ± SEM. A: C vs. LC p = 0.004, C vs. HC p = 0.037. B: C vs. LL p = 0.001 and HH vs. LL at 45 – 90 min p > 0.01. CC n = 7, HC n = 7, HH n = 12, LC n = 7, LL n = 10.](image)

**Figure 2.10 Plasma glucose levels after intraperitoneal insulin injection.** Data shown represent mean ± SEM. A: C vs. LC p = 0.004, C vs. HC p = 0.037. B: C vs. LL p = 0.001 and HH vs. LL at 45 – 90 min p > 0.01. CC n = 7, HC n = 7, HH n = 12, LC n = 7, LL n = 10.
Figure 2.11 shows the average slope observed during the first 30 min after the intra-peritoneal insulin injection. No significant difference was observed among the groups.

![Slope](image)

**Figure 2.11 Average slope of plasma glucose levels in the first 30 min of ipITT.**

Data shown represent mean ± SEM. There were no significant differences between the groups. CC n = 7, HC n = 7, HH n = 12, LC n = 7, LL n = 10. NS = not significant.

### 2.3.6 Plasma hormones analysis

Plasma samples collected with a cardiac puncture at sacrifice were analysed using an enzyme-linked immunosorbent assay (ELISA) and the following hormones were measured: ghrelin, leptin, adiponectin, insulin, PYY, GLP-1, PP, ACTH, IL6 and TNFα. Only cardiac plasma samples that were equal or above 200 µl were used, and
therefore the number of samples per group vary. In our analysis, no outliers were found.

Figure 2.12 shows the average values of ghrelin detected in the plasma samples. Ghrelin levels in the CC group were almost 4-fold higher than LC pups (p = 0.04) but there were no significant differences between HC and LC. In contrast, ghrelin was 150% higher in HH compared to LL, although the difference was only marginally significant (p = 0.02). Overall, ghrelin levels were 2-fold higher (+126%) in groups exposed to high GI feeding (HC + HH) vs. low GI feeding (LC + LL, p = 0.02).

Leptin plasma levels were significantly higher (+525%) in the LL group compared to HH (p = 0.001, Figure 2.13). No significant statistical difference was observed among the other groups.

Adiponectin levels in the LC group were ~33% higher than HC, but this did not reach significance (p = 0.1, Figure 2.14). There was no significant difference between HH and LL. The CC group showed the highest adiponectin levels (CC vs. HC and HH, p = 0.008).

Plasma insulin levels are shown in Figure 2.15 below. CC had the highest mean concentration and HH the lowest, but none of the differences was significant.
Figure 2.12 Plasma ghrelin levels in 20 week old mice obtained by cardiac puncture. Data shown represent mean ± SEM. CC n = 6, HC n = 8, HH n = 8, LC n = 8, LL n = 8.

Figure 2.13 Plasma leptin levels in 20 week old mice obtained by cardiac puncture. Data shown represent mean ± SEM. CC n = 6, HC n = 8, HH n = 8, LC n = 8, LL n = 8.
Figure 2.14 Plasma adiponectin levels in 20 week old mice obtained by cardiac puncture. Data shown represent mean ± SEM. CC n = 6, HC n = 8, HH n = 8, LC n = 8, LL n = 8.

Figure 2.15 Plasma insulin levels in 20 week old mice obtained by cardiac puncture. Data shown represent mean ± SEM. No significant differences were observed among the groups. CC n = 6, HC n = 8, HH n = 8, LC n = 8, LL n = 8. NS = not significant.
Glucagon-like peptide 1 (GLP1), pancreatic polypeptide (PP) and peptide YY (PYY) were also analysed (Figure 2.16). GLP1 levels varied over a 2-fold range, but the difference between HH and LL did not reach significance (p = 0.07). Similar results were obtained for PP and PYY concentrations, where none of the differences among the groups reached statistical significance.

Finally, Figure 2.17 shows the results for adrenocorticotropic hormone (ACTH), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNFα) respectively. Although there was some variation among the groups, none of the differences reached statistical significance.
Figure 2.16 Plasma GLP-1, PP, PYY levels in 20 week old mice respectively.

Data shown represent mean ± SEM. No statistically significant (NS) differences were observed. For all three hormones: CC n = 5, HC n = 8, HH n = 8, LC n = 8, LL n = 8. NS = not significant.
Figure 2.17 Plasma ACTH, IL6, TNFα levels in 20 week old mice respectively.

Data shown represent mean ± SEM. No statistically significant (NS) differences were observed. For all three hormones: CC n = 6, HC n = 8, HH n = 8, LC n = 8, LL n = 8.
2.4 Discussion

In this chapter, contrary to our hypothesis, we were not able to show an association of dietary GI with food intake, body weight, glucose tolerance or insulin sensitivity. However, the present study did show that life-long exposure to a low GI diet impacts on plasma ghrelin (an orexigenic hormone) and leptin levels (an anorexigenic hormone). Ghrelin concentration was higher in mice exposed to the high GI diet, suggesting greater hunger overall, while leptin levels were lower, suggesting lower satiety. Together these hormonal differences suggest that a high GI diet increases the risk of overconsumption, although we could not detect differences in food intake or body weight up to 20 weeks of age. These associations were highly significant in pups continuously exposed to a high GI diet after weaning (HH), but the same trend was evident (but without reaching statistical significance) even in the early-life exposed pups (HC). These findings suggest that carbohydrate nutrition and/or glycaemic exposure has subtle effects during the gestational period that may not be revealed until later in adulthood. Circulating levels of the anorexigenic hormone leptin and orexigenic hormone ghrelin have been shown to play a crucial role in the development of overweight and obesity through hunger / appetite control and energy balance (166).

2.4.1 Rate of digestion in vitro

In humans and animal models the rate of starch digestion and absorption has been shown to be an excellent predictor of the glycaemic response to a starchy meal (167, 168). Englyst et al. (162) developed an in vitro methodology to measure the amount of rapidly available glucose (RAG) in a food sample, using digestive enzymes under standardized conditions. RAG is defined as the glucose released from starch within
the first 20 min of digestion. Slowly available glucose (SAG) is defined as the glucose released between 20 and 120 min. Resistant starch is the fraction that remained undigested after 120 min. Englyst’s group has shown that RAG is more highly correlated than SAG to the glycaemic response elicited by different foods. Since then, Englyst’s methodology has been widely used to predict GI values of unknown foods (169-171).

Amylose content, particularly the amylose/amylopectin ratio (172, 173), is known to have a direct impact on starch hydrolysis and digestibility. The two special diets provided by Specialty Feeds Pty Ltd were therefore formulated with same starch content, but differing in the amylose/amylopectin ratio. The in vitro testing allowed us to test and confirm that the two diets contained starches with different rates of digestion. At both 20 min and 120 min the high GI diet released a significantly higher amount of glucose compared to the low GI diet. Since RAG is the best predictor of the glycaemic response, at least in humans, we predicted that the High GI diet was likely to have produced a higher postprandial response than the Low GI diet in the mice participating in our experiment.

The 120 min in vitro results indicate that the Low GI diet contained a higher portion of starch that is fairly resistant to digestion, at least in humans. However, it is possible that mice digest raw starch to a much greater extent because they have evolved on native diets contained uncooked cereal grains. Nonetheless, it could be argued that fewer calories were available to the mice on this feed. If true, we might expect to have seen differences in rate of growth or food intake. However, we found similar food intake and no important differences in body weight between treatment
groups at 20 weeks of age. Thus, any metabolic differences between groups can be reasonably ascribed to lower postprandial glycaemia throughout the lifespan of these mice. We do not exclude though, the possibility that the Low GI feed could reduce overall calorie intake, alter body composition and be less obesogenic because it contains proportionately more resistant starch.

2.4.2 Food intake and metabolic profile

In the male pups, we conducted 24 h food intake measurements in triplicate at different ages to estimate average food intake in each diet group. Overall there were no significant differences in food intake at any time between the groups. This indicates that in the current study the GI of the maternal and offspring diet did not affect food intake for the first 16 weeks of life. We anticipated that the low GI-fed mice might consume less feed because the satiety potential of the low GI diet has been shown to be higher in some studies, but our data did not support this. It is also possible that the low GI diet contributed fewer kilojoules because some starch was resistant to digestion. However, the starting weights at weaning (week 5) did not differ significantly among the pups of the CHOW, High and Low GI-fed mothers and their progression remained similar for the rest of their development. In particular, looking at the extended exposure to the treatment diets (HH vs. LL), it is obvious that there was virtually no difference in body weight for the whole 20 week period.

These findings are in agreement with a previous study (136) investigating the long term effects of a high and low GI diet in mice. The body weight of the two treatment groups throughout the 38 weeks of observation did not differ significantly. Similarly energy intake calculated through the use of stools after a food intake observation
period, did not differ between the high and low GI-fed mice. In contrast, Isken et al. (174) showed that long-term exposure (up to 20 weeks) to a high vs. low GI diet leads to a moderate but significant increase in body weight in the high GI-fed C57BL/6 mice. However in the current study, a higher fat content in the high and low GI feeds may have produced the expected differences in phenotype. Previous studies have indicated that fat is the crucial stimulus for overconsumption, obesity and diabetes-related parameters in C57BL/6 wild-type mouse (175, 176).

A limitation of the current study is that we did not collect faeces or determine faecal energy in the mice during the 24h food intake experiment. Similarly, in the absence of data regarding oxygen consumption and carbon dioxide expiration, we were not able to determine net energy intake and expenditure. Our food intake measurements were made in triplicate at different ages but were based on group intake rather than individuals. We did this so as not to increase the stress levels of the mice, a factor known to alter appetite (177). However, even under these conditions, the mice may have altered their normal day to day eating pattern and some mice could have been affected more than others. This means that the average food intake results should be treated with caution. Maternal food intake and body weights would also have been informative, but we were wary of disturbing the mothers due to the serious adverse event that we faced previously (Appendix 3). Likewise, body composition measurements (total body fat, epidydimal fat) of the pups would have provided important insights but were not the focus of our study.

In our rodent model of early-life and life-long exposure, glucose clearance during the ipGTT was similar amongst pups exposed to the CHOW, High or Low GI diet. The
iAUC\textsubscript{glucose} were not significantly different, but there was some evidence that glucose levels were lower in the LL group. At the critical time of 30 min (the peak) plasma glucose concentration was significantly lower in the LL vs. HH group, suggesting faster glucose clearance in those exposed continuously to a low GI from conception to 17 weeks of age.

Similarly, during the ipITT we did not observe any significant difference in the rate of glucose decline (measured as the slope of the line between 0 time and 30 min) between the groups. But there was some evidence that the life-long low GI-fed pups (LL) were less sensitive to the insulin injected. Although this is usually interpreted as greater insulin resistance, it could be argued that the HH group has an inferior counter regulatory system that resulted in greater glucose fluctuation. Thus the LL group (and to some extent the LC group) may cope better with metabolic stress (in this case, a carbohydrate challenge), with avoidance of wide fluctuations in blood glucose levels.

Our findings in regard to GI and glucose homeostasis are in agreement with some studies but not others. Scribner et al. (136) reported no differences between glycaemic AUC following an oral GTT test in mice fed low and high GI diets, whereas Isken et al. (174) observed lower glucose AUC (i.e. greater glucose tolerance) in the low GI-fed mice but no difference in insulin AUC. Coate and Huggins (178) reported that low GI-fed mice, independently of the fat content of the diet, had lower glucose AUC than high GI-fed mice. In this case, the test conducted was a meal tolerance test instead of an intraperitoneal glucose and insulin challenge.
With 10-13 animals per diet group, our study was appropriately powered to detect biologically important differences in glucose tolerance and insulin sensitivity. However, the results and differences could have been influenced by the fact that for both the GTT and ITT, the amounts of glucose and insulin injected were calculated on the body weight of the pups and not their body lean mass (a more appropriate parameter for the study of metabolism). Although the experiments were conducted carefully, the Accu-check™ glucometer that was used in both experiments is not designed for animal use. It is not as accurate at blood glucose levels >20 mM, a concentration that is commonly exceeded in mice during a GTT. These weaknesses may have meant that true differences between the groups have gone undetected.

We did not find any significant differences in adiponectin or insulin plasma levels. There was a tendency for CC, LC and LL groups to display higher plasma adiponectin but these observations did not reach statistical significance. These outcomes are in agreement with a human study on the effect of low- and high- GI diets on chronic disease susceptibility biomarkers (127). A first analysis of their data indicated there were no statistical differences in the adiponectin levels by diet treatment, though a small difference (where low GI diet was associated with higher adiponectin concentration) was noted. After a stratified analysis by body fat mass, adiponectin increased significantly in the overweight/obese individuals that followed a low GI diet. Thus, their outcome resembles the higher adiponectin trend we observed in the chow and low GI fed healthy weight mice. It may be speculated that if there were overweight or obese mice in our study, adiponectin levels might have been significantly different between the low and high GI groups. In contrast, our fasting plasma insulin result contrasts that of a previous study in mice by van
Schortorst et al. (175) that showed significantly lower insulin levels in the low GI group.

Likewise, although there were some variations in the plasma levels of GLP-1, PP, PYY, ACTH, IL6 and TNFα between the groups, no significant differences were observed. A recent review on the effect of low vs. high GI diets on selected biomarkers (179) indicated inconsistent results. The human studies that were designed to maintain constant body weight showed that GLP-1, IL-6 and TNFα were not significantly affected by the GI values of the diet. However, in one study intended to induce weight loss, the low GI diet was associated with lower IL-6 and TNFα concentrations. The authors of the review acknowledged that formulating dietary recommendations between the GI and these biomarkers is premature due to the inconsistencies in the literature. Similarly, for PYY, ACTH and PP, to our knowledge there are no investigations conducted after meal testing (180, 181) making the comparison to our data difficult.

Finally, this study showed that life-long exposure to a high GI diet vs. low GI diet is associated with higher (+148.6%) ghrelin plasma levels and significantly lower (-525%) leptin levels. Even though we were not able to show a significant impact on ghrelin and leptin in the early-life exposed pups (HC and LC), they displayed a consistent and similar trend to that observed in the life-long exposed pups. Thus we argue that in both cases, early-life nutrition appears to play a role. To our knowledge these findings are novel. One study in mice reported higher leptin levels in the high GI-fed group (178). However, the diet composition was simultaneously altered for fat content and body fat may have been higher (and therefore leptin). In a human study, after 12 weeks of dietary intervention, a low GI diet significantly decreased...
leptin levels compared to a high GI diet (182). However, as this was a weight loss study, the changes in leptin were also correlated to reductions in fat mass. We believe our finding is reliable because plasma assays were conducted in duplicate using automated multi-level ELISA and in a large sample size for each diet group.

Since ghrelin is an important hormone for the stimulation of appetite, while leptin is the major satiety and appetite control hormone, our discovery has significant implications. Greater hunger before meals and less satiety between meals, even of a modest degree, are likely to spell more frequent and higher food intake over the longer term.

Huda et al. (183), in an investigation of the effects of ghrelin on appetite and energy expenditure in lean, obese and post-gastrectomy subjects, showed that the levels of ghrelin around traditional mealtimes play a significant role in the continuous ‘grazing’ pattern that is seen in obese subjects. Furthermore, in a very rare human condition that is congenital leptin deficiency, Farooqi et al. (184) showed that before and after 7 days of treatment with recombinant human leptin, treatment had a major effect on food intake. After leptin treatment, hunger ratings in the fasted state decreased, and satiety following a meal increased. Their experiment provided functional neuroanatomical insights into the mechanisms by which leptin, can interact with the recent ingestion of food to modulate spontaneous eating behaviour in humans.

In summary, contrary to our hypothesis, we were not able to show an association of dietary GI with food intake, body weight, glucose tolerance or insulin sensitivity.
However, our study demonstrated that extended exposure to a low GI diet increases circulating leptin levels and decreases ghrelin levels, an effect that is probably programmed already in utero. Together, the findings suggest that maternal and offspring diets that raise postprandial glycaemia, even within the normal range, have the capacity to program the metabolism of offspring in a manner that predisposes to overweight.
Chapter 3

Investigating the effect of early-life and life-long exposure to a high vs. low GI diet on FTO and leptin gene expression
Chapter 3  Study design and rationale

3.1 Introduction

The current epidemic of obesity has been often linked with the increased availability of energy-dense foods, sedentary lifestyles and genetic predisposition. This theory has not satisfied many in the scientific world because it does not explain the sudden increase in obesity observed in children and young adults (185). Increasingly convincing evidence supports the developmental origins of adult health and disease hypothesis, that is, that the origins of obesity and other chronic diseases lie in the early pre- and post-natal environment. Specifically, different nutritional exposures during the early developmental period, including fetal life and lactation, influence predisposition to the metabolic syndrome and obesity later in life (186).

A nutritional stimulus or insult during the critical period of organogenesis (fetal development) and immediate postnatal maturation (suckling) can have lasting programming effects. In pregnancies complicated by diabetes, hyperglycaemia in the early stages of pregnancy results in the up-regulation of the glucose transporters and leads to an accelerated fetal growth during late gestation (143). In turn, this leads to increased risk of child obesity and type 2 diabetes in young adulthood (110, 148, 187).

In rat studies, zinc deficient dams produce offspring that weigh less than controls at birth and up until six months of age, but have a higher body fat ratio. Maternal zinc restriction induced fasting hypoinsulinaemia in the offspring and impaired insulin response to a glucose challenge (188). Patel and colleagues have summarised studies...
showing the interaction between early life nutrition and metabolic programming (Table 3.1) (186). Thus nutritional stress or nutritional adequacy clearly influence metabolic imprinting. Effects can be either negative or positive outcomes in different stages of life, from conception to the post-weaning period and later in life.

The endocrine mechanisms that develop in suboptimal intrauterine conditions are thought to program metabolism throughout life. The main site where the defence response to stress is located is the hypothalamic-pituitary-adrenal axis (HPA). Exposure to physiological stresses during early stages of development are thought to create a pattern of stress response in the HPA that will follow the fetus throughout its neonatal and adult life (189).
Table 3.1 Summary of the metabolic programming effects induced by an altered experience in the immediate postnatal life. From Patel et al 2009 (186).

<table>
<thead>
<tr>
<th>A. Undernourishment (large-sized litter rat model)</th>
<th>B. Overnourishment (small-sized litter rat model)</th>
<th>C. The HC rat model (qualitative change in the nutrition)</th>
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<tbody>
<tr>
<td>• Reduced body weight gains for life</td>
<td>• Increased body weight gain beginning from the suckling period accompanied by hyperphagia.</td>
<td>a. Immediate effects (pre-weaning period)</td>
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<tr>
<td>• Reduced fasting plasma insulin levels</td>
<td>• Increased plasma levels of insulin and leptin for life</td>
<td>• Immediate increase in plasma insulin levels</td>
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<td>• Reduced insulin secretory response to a glucose stimulus</td>
<td>• Augmented insulin secretory response by isolated islets to a glucose stimulus</td>
<td>• Reduced plasma leptin levels</td>
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<td></td>
<td>• Altered responses by brain slices to various stimuli such as insulin, leptin and several neuropeptides</td>
<td>• Normal plasma glucose levels</td>
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<td>• Leftward shift in the response to a glucose stimulus</td>
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<td></td>
<td>• Increased expression of the preproinsulin and related transcription factor genes</td>
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<td>• Increased insulin biosynthesis</td>
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<td>• Altered pattern of several clusters of genes</td>
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<td></td>
<td></td>
<td>• Increased number of small-sized islets</td>
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<td></td>
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<td>• Increased number of islets per unit area</td>
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<td>• Modifications in the ANS regulation of insulin secretion</td>
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<td>• Increased mRNA levels of orexigenic neuropeptides</td>
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<td>• Decreased mRNA levels of anorexigenic neuropeptides</td>
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<td>• Increased protein content of neuropeptide Y</td>
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<td></td>
<td>• Increased hepatic lipogenic capacity</td>
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<td>b. Persistent effects (post-weaning period)</td>
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<td>• Persistence of hyperinsulinaemia</td>
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<td>• Hyperphagia resulting in adult-onset obesity</td>
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<td>• Abnormal response to a glucose tolerance test</td>
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<td>• Leftward shift to a glucose stimulus</td>
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<td>• Increased gene expression of preproinsulin</td>
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<td>• Alterations in several clusters of genes</td>
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<td>• Increase in insulin-producing mass</td>
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<td>• Modifications in the ANS regulation of insulin secretion</td>
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<td>• Increased mRNA levels of orexigenic neuropeptides</td>
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<td>• Decreased mRNA levels of anorexigenic neuropeptides</td>
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<td>• Increased neuropeptide Y protein content</td>
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<td>• Alteration in the protein levels of the proximal insulin signalling components</td>
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<td>• Increased lipogenic capacity of liver and adipose tissue</td>
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<td>• Increase in the size of the adipocytes</td>
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<td>c. Generational effects</td>
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<td>• Spontaneous transmission of the HC phenotype to the offspring</td>
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<td></td>
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<td>• Metabolic programming effects initiated during fetal development</td>
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The field of epigenetics has been defined as the study of “heritable changes in gene function that cannot be explained by changes in DNA sequence” (190). Without affecting the genotype the cell phenotype can be altered via structural adaptations or “marks” on the chromosomes that create and perpetuate altered activity states (191). Epigenetic changes can dictate the how, when, and where the sequence information will be used. This potential to determine functions and probabilities of disease has created specialised research fields focusing on possible cause-effect relationships. Nutrigenomics in particular focuses on how nutrients affect gene expression. There is now a large body of research exploring the influence of nutrition on methyl and acetyl groups that constitute the key epigenetic marks (190).

The intrauterine environment represents the most intense epigenetic activity period and thus, maternal diet is likely to have long-lasting effects on the development of non-communicable chronic diseases in adult life such as the metabolic syndrome, insulin resistance, type 2 diabetes, and obesity (98).

The international research project “Early Nutrition” (http://www.project-earlynutrition.eu) funded by the European Commission aims to find strategies for preventive intervention during early life (192). One of the branches of research in this project is focused on specific genes and single nucleotide polymorphisms (SNPs) that have been identified by genome-wide association studies and candidate genes association studies as being significantly associated with obesity. One of those genes is the fat mass and obesity associated (FTO) gene.
Genome wide association studies identify variants in the DNA, not genes themselves. FTO is a gene of nine exons on chromosome 16 in humans and chromosome 8 in mice. The original variant (SNP) associated with BMI and obesity in humans, was found in the first intron (rs9939609) (41). More single nucleotide polymorphisms were later discovered on the same intron (193), but rs9939609 has been the main variant of research interest (194). The risk allele frequency is close to 50% in Caucasians and Yorubans (an African ethnic group) and 14% in Asians, so it may affect the weight of almost half the world population.

However, it is now suggested that all of the identified SNPs of the same highly correlated cluster on introns 1 and 2 are associated with BMI and other obesity-related traits at similar significance levels (51). Although we know that FTO is an AlkB-like 2-oxoglutarate-dependent nucleic acid demethylase, the exact function of the FTO gene in relation to obesity remains elusive. It is not known whether there is cause-effect relationship or an interaction between nutrition and FTO gene expression (49, 77, 193-197).

Humans homozygous for the ‘obesity-risk’ A allele of FTO rs9939609 have a 1.7-fold increased risk for obesity compared with subjects homozygous for the low-risk T allele, and display overall increased ad libitum food intake, particularly fat consumption, and impaired satiety (198). Moreover, preschool AA children show obesity-prone eating behaviors, including increased food responsiveness and a tendency to eat in response to external cues, prior to the development of an association between FTO rs9939609 and BMI. Research to date suggests that the association between SNPs in FTO and BMI is predominantly driven by increased
energy intake.

The FTO gene is expressed in all fetal and adult tissues but at much higher levels in the hypothalamus and in particular in the arcuate nucleus (41, 57). It is also highly expressed in the human placenta (74) and associated with increased fetal weight and length. FTO demethylates N6-methyladenosine, a potential regulatory RNA modification, but the mechanisms by which FTO predisposes humans to obesity still remain unclear (199). These findings suggest that there is likely to be a prenatal effect on FTO expression.

One of the first animal studies on the function of FTO investigated its protein products and whether these were nutritionally regulated. Three groups of mice (freely feeding, fasted 48 h and fasted 48 h with daily injections of leptin) were compared. mRNA levels of FTO in the arcuate nucleus were reduced by 60% in fasted mice compared with controls and were not influenced by leptin supplementation (42). Further research showed that in rats exposed to a high fat diet for 10 weeks, FTO gene expression increased 2.5 fold in the arcuate nucleus of the hypothalamus (84).

Research around FTO gene is still challenging. The fact that its function is still unknown and that the obesity associated variants of this gene are located in an intronic area makes it difficult to point the exact mechanism by which this gene is implicated in obesity and other metabolic traits associated with non-communicable disease. There have been several hypotheses but none is definitive. Many studies suggests an association with hypothalamic control of energy intake, leptin and food intake, but others indicate a link to adipogenesis or energy expenditure (Figure 3.0)
(193). The potential physiological roles and mechanisms of the FTO gene are so vast that it is difficult to understand the cause-effect relationship of this gene with obesity and related traits. It is clear, however, that FTO expression can be regulated by nutrition and its expression may influence other processes in turn (194).

Figure 3.0 Putative physiological roles and potential cellular mechanisms of FTO. From: Larder et al. 2011 (193), Trends in Endocrinology & Metabolism.

More recently, Karra and colleagues (198) showed that adiposity-matched, normal-weight humans who were homozygous for the FTO obesity risk rs9939609 A allele had dysregulated circulating levels of the orexigenic hormone ghrelin and attenuated postprandial appetite reduction. Using functional magnetic resonance imaging in normal-weight AA and TT humans, they found that the FTO genotype modulates the neural responses to food images in homeostatic and brain reward regions. The AA and TT subjects exhibited divergent neural responsiveness to circulating ghrelin within brain regions that regulate appetite, reward processing, and incentive
motivation. In cell models, these researchers showed that FTO overexpression reduced ghrelin mRNA N6-methyladenosine methylation, concomitantly increasing ghrelin mRNA and peptide levels. Furthermore, blood cells from AA human subjects exhibited increased FTO mRNA, reduced ghrelin mRNA N6-methyladenosine methylation, and increased ghrelin mRNA abundance compared with TT subjects. Together, their findings suggest that FTO regulates ghrelin, a key mediator of ingestive behavior, and offer insight into how FTO obesity-risk alleles predispose to increased energy intake and obesity in humans.

In one of the earliest nutrigenomic studies, Kallio and colleagues (200) showed that two carbohydrate modified diets (i.e. different GI) differentially modulated gene expression in abdominal subcutaneous adipose tissue of men and women with the metabolic syndrome. The differences were evident even in the absence of changes in body weight. Genes involved in insulin signalling were down-regulated during the lower GI diet, and genes related to metabolic stress were up-regulated during the higher GI diet. This suggested that even a short exposure (12 weeks) could impact the risk of metabolic diseases via changes in gene expression. Unfortunately, the FTO gene was not studied in that trial.

Nonetheless, Kallio’s findings led us to hypothesise that two iso-energetic and nutritionally identical diets differing only in their GI (high vs. low) would evoke differential FTO gene expression at its main expression sites (the hypothalamus and the placenta). We hypothesised that there would be higher FTO expression and lower leptin expression in the high GI diet groups. Our hypothesis is underpinned by
previous evidence that FTO gene is involved in the processes of appetite and satiety, and with leptin expression.

**3.2 Materials and methods**

**3.2.1 Study design**

The study design has been described in detail in Chapter 2. Briefly, all animal work was approved by the Animal Ethics Committee of the University of Sydney (Protocol number L02/8-2010/2/5355). Animals were provided by the Animal Resources Centre (Canning Vale, WA). Mice breeders were kept in indoor animal house facilities (temperature kept constant at 25°C), where two to six same sex mice were housed together in standard cages containing clean paper pellets and paper rolls to shred. Mice had *ad libitum* access to their feed and water through a feeder tray within the lid of the cage. The light-dark cycle of the animal holding room was 12 h-12 h.

Feeds were obtained commercially from Specialty feeds, Glen Forest, WA, Australia. The purchased special diets were of the same energy density and all macronutrients were matched. The physical and chemical structure of the starch fraction was manipulated by the manufacturer in order to obtain a ‘gel crisp’, high amylose starch for the low GI diet (LGI: 70% amylose, 30% amyllopectin) and a dextrinised, high amyllopectin starch for the high GI diet (HGI: 90% amyllopectin, 10% amylose). Both diets were enhanced with AIN93 vitamin premix that used wheat instead of sucrose or glucose.
A schematic representation of the design of the two studies involved in this chapter is shown below.

1st study

C57BL female pregnant mice → Chow diet (CC) → male offspring → Chow

C57BL female pregnant mice → High GI diet (HGI) → male offspring → HGI

C57BL female pregnant mice → Low GI diet (LGI) → male offspring → LGI

2nd study

C57BL female pregnant mice → High GI diet (HGI) → Day 18-19 of gestation → Placenta recovery → PH

C57BL female pregnant mice → Low GI diet (LGI) → Placenta recovery → PL

As described in the previous chapter, 4 week old female C57BL/6 mice were randomly assigned to one of the following diet groups: low GI diet (LGI), high GI diet (HGI) and standard chow (CC). Male breeders (received at 3 weeks of age) were all fed a standard chow diet throughout. Their chow regime was interrupted for
breeding purposes only. Mice were maintained on their assigned diet for four weeks prior to mating.

Mating was based on the “Breeding Strategies for Maintaining Colonies of Laboratory Mice” Resource Manual by the Jackson Laboratory (http://jaxmice.jax.org/manual/#breeding). At birth only male pups were used for this research and female offspring were made available to other researchers. Male pups from the LGI and HGI groups were weaned at the end of postnatal week 4 and were further divided into two subgroups, one following their mother’s diet (LL and HH respectively) and one following the CHOW diet (LC and HC respectively). Male pups of the CHOW group were weaned at the same time and continued on CHOW (CC). This last group has been used as the group of reference and comparison for our analysis.

3.2.2 Ear-marking, food intake and weight monitoring

At 4 weeks of age, male pups were anaesthetised using isoflurane (ISO) solution within a glass chamber. A surgical scissor was used to obtain one of six unique ear punches (three sites for the right and three sites for the left ear). After weaning, the pups’ weight was recorded weekly until the age of five months when they would be sacrificed. Three 24 h food intake observations were conducted at weeks 9, 12 and 16. Mice were placed in custom made cages that contain an insert to allow catching spilled food.
3.2.3 Mouse sacrifice, blood and tissue collection

Study 1:
As described in chapter two, mice from all three groups were sacrificed at 20 weeks of age, by intraperitoneal injection with 100 µl of Lethabarb euthanasia solution. After the injection, mice were observed until death was confirmed by absence of respiratory movement and corneal reflex. The following tissues were collected: hypothalamus, brown adipose tissue (BAT), liver, visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT), red muscle (RM), and white muscle (WM). Collected tissues were snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

For the collection of the hypothalamus, the brains were free-hand rapidly but carefully removed from the skull and placed in the bottom of a glass Petri dish filled with 0.1 M sodium phosphate-buffered saline solution. Removal of the hypothalamus was conducted by dissecting away the optic chiasm from the anterior portion of the hypothalamus and the mammillary nuclei from the posterior of the hypothalamus. The entire hypothalamus was removed including the arcuate, ventromedial, dorsomedial, and paraventricular nuclei and was quickly separated and immediately snap-frozen in liquid nitrogen.

Study 2:
Female pregnant mice from the HGI and LGI groups were sacrificed at day 18-19 of gestation, close to the end of pregnancy. They were anaesthetised using a lethal dose of isoflurane (ISO) solution within a glass chamber. (An interperitoneal injection of Lethabarb was not appropriate for the recovery of the placenta (201),(202).) The
belly of the mouse was sprayed with ethanol and by a small incision, the skin was removed in order to access and open the peritoneum. Successively, the uterine horns were picked up at each distal end and placed in a petri dish containing phosphate buffered saline (PBS) solution. Figure 3.1a shows a photograph taken during a placenta recovery process.

![Figure 3.1a C57BL/6 pregnant mice and embryos.](image)

This photo was taken during a sacrifice for the recovery of the placentas. Each mouse had very similar appearance, with multiple embryos in their own amniotic sac and an individual umbilical artery connecting to the uterine artery.

Each embryo was treated separately in a separate petri dish containing PBS. The amniotic sac was opened and the embryo separated from the placenta by a slicing incision on the umbilical cord and pulling the cord and the attached embryo away from the placenta. The placenta was then snap-frozen in liquid nitrogen and stored at -80°C until further analysis.
3.2.4 RNA extraction

Total RNA extraction was conducted using RNeasy Lipid Tissue Mini Kit by Qiagen for tissues weighing ≥30 mg as per manufacturer’s instructions. For smaller tissues such as hypothalamus and placenta, RNA extraction was conducted using RNeasy Mini Kit by Qiagen. Briefly, for the first study the following tissues were used for purification of total RNA: hypothalamus, brown adipose tissue (BAT), liver, visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT), red muscle (RM) and white muscle (WM).

Tissue samples were disrupted and homogenised using QIAzol™ lysis reagent. For tissues that were larger and more fibrous, the Polytron™ handheld homogeniser was used for 10-15 seconds in order to achieve a homogeneous mixture. The resulting samples were allowed to stand at room temperature for 5 min after which 200 uL chloroform was added. The tissues were then vortexed for 30 sec and left at room temperature for 3 min. Tissues were then centrifuged (12,000 g, 15 min, 4 °C) using a microcentrifuge (Heraeus Biofuge Fresco™, Thermo Fisher Scientific). The supernatant aqueous phase containing RNA was transferred to a new tube where a 1:1 ratio of 70% ethanol solution was added. This was then transferred into the RNeasy column using extraction tubes provided in the Qiagen kit. After centrifugation and discard of the flow-through, the column was placed in a new Eppendorf rube where RNase-free (DEPC) water was added up to obtain a volume of 100 uL of solution.
3.2.5 Determination of RNA purity and yield

The RNA purity and yield were controlled using a NanoDrop (ND-1000) spectrophotometer (NanoDropTechnologies Inc, USA) and gel electrophoresis. The UV spectrophotometer was blanked with DEPC water and undiluted RNA samples (1.5 μL) were analysed. The determination of the yield and the purity of the RNA from protein and salt contamination was based on the A260 and A260:A280 and A260:A230 ratios.

A denaturing formaldehyde gel electrophoresis was used to determine the integrity of the purified RNA samples, using 18S and 28S ribosomal RNA as standard. Approximately 2 μg of the RNA sample was mixed with MOPS (20 mM MOPS, 5 mM NaOAc, 1 mM EDTA, pH 7.0), 20% formaldehyde, 50% (v/v) formamide, and 0.0125 μg ethidium bromide, obtaining a final volume of 10 μL. Samples were heated to 65 °C for 5 min and quickly chilled on ice.

Samples were mixed with 10 x loading buffer (25% (v/v) glycerol, 0.25% (w/v) xylem cyanol FF, 10 mM EDTA, pH 8.0), then were loaded onto a 1.5% (w/v) agarose gel (containing 1x MOPS, 2.2 M formaldehyde). Electrophoresis was carried out at 80 V applied for ~1 h. The agarose gel was then photographed under UV trans-illumination. The samples that showed clear bands with a 2:1 intensity of 28S:18S ribosomal RNA were determined good quality samples and used in the next step of the cDNA synthesis.
3.2.6 Synthesis of cDNA and primer design

Total RNA was reverse transcribed from 200 ng of total RNA in a final reaction volume of 20 μL, using Superscript® VILO™ cDNA Synthesis Kit (Life Technologies, city) according to manufacturer’s instructions. The mRNA sequences of the two target genes (Fto and leptin) were found at http://www.ncbi.nlm.nih.gov/ and the primers were designed using the ‘Primer3’ (v. 0.4.0) web tool, which is available at http://frodo.wi.mit.edu/primer3/. The search criteria used for choosing the correct primers were: primer length optimal at 20 bp, ~50% GC content, close or same Tm, amplicon size between 80-120 bp. The primers were not designed using intron spanning but they were designed to bridge the exon-intron boundaries within the gene of interest to exclude possible contamination by genomic DNA.

Once the pairs of the primers were chosen, the oligonucleotides were synthesised by Sigma Genosys (Sigma-Aldrich). The primers were experimentally validated with two quality control assays. First, a melt curve analysis, confirmed by agarose gel electrophoresis, verified that a single gene-specific product was produced. Each sample was run in triplicate. In addition we checked the amplification efficiency through a calibration curve. The slope of the Ct values versus the initial amounts of input material on a semi-log10 plot (of the best fit line) was calculated and we obtained an amplification efficiency > 90 %.
The sequences of the oligonucleotides were as follows:

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Length</th>
<th>Tm°C</th>
<th>GC%</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>Mus_FTO_fwd</td>
<td>20</td>
<td>64.0</td>
<td>60%</td>
<td>GTGAGGACGAGTCCAGCTTC</td>
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<td>18</td>
<td>66.4</td>
<td>61%</td>
<td>AGAGGAGCGAGCGACCAA</td>
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</table>

3.2.7 Quantitative Real-time PCR (qRT-PCR)

In PCR the cDNA targeted sequence is amplified using the specific primer pairs. We used the Fast SYBR® Green Master Mix (Applied Biosystems) containing Syber Green fluorescent dye which binds only double stranded DNA with an intensity proportional to the amount of double stranded product formed. The fluorescence after each amplification cycle was measured and the number of cycles required to achieve a pre-set level of double stranded DNA (threshold) determined. The more abundant the sequence in the initial cDNA preparation, the fewer cycles are required to achieve the threshold.

We used 96-well plates (Applied Biosystems) with a total reaction volume of 20 μL. According to the manufacturer’s instructions, we added 10 μL of Fast SYBR® Green Master Mix, 1.5 μL of forward and reverse primers and 7 μL of cDNA sample. Each sample was run in duplicate and in 1:1 and 1:8 dilutions. For the 18S ribosomal RNA primers which act as an endogenous control, additional 1:64 dilutions were conducted. We chose 18S rRNA as an internal control in relative RT-PCR rather than ACTB or GAPDH because it shows less variance in expression across a variety
of tissues and of treatment conditions than β-actin and GAPDH. Indeed in FTO gene analysis specifically, 18S is the dominant internal control (82, 103, 203, 204).

The amplification was performed in an ABI 7500 instrument (Applied Biosystems) with two-step thermocycler program. The first 5 min step was a 50 °C pre-incubation to remove contaminating amplified product from previous PCR assays. The second step consisted of 40 cycles comprising 3 seconds at 95 °C then 30 seconds at 60 °C. A melt curve was performed and all the Ct values were normalised to the appropriate 18S rRNA Ct value which was used as the reference sequence. Levels of 18S did not differ between groups. The gene expression was estimated by using the Ct (2ΔΔct) method (205).

3.2.8 Data and statistical analysis

Gene expression is presented as fold change compared to the control group, i.e. the chow-fed offspring of chow-fed mothers. Prism 6.0 (Graph Pad Software, USA) and SPSS Statistics 19 (IBM) was used for statistical analysis. One-way repeated measures analysis of variance (ANOVA) followed by two-tailed unpaired t-tests with the Bonferroni adjustment were performed to assess the difference between the groups for the expression levels of the two target genes. Due to the large number of variables we investigated, a statistical p value of ≤ 0.05 was taken as marginal significance and < 0.01 as significantly different.
### 3.3 Results

Study 1: The expression of the Fto gene varied considerably among the different tissues and also between treatment groups. Expression was 8-fold higher in the hypothalamus than in the next highest tissue, the red muscle (Figure 3.1b). Hypothalamic Fto gene expression was 2.5-fold higher in the HH group than LL (Figure 3.2, $p = 0.0001$) whereas HC and LC were not different (Figure 3.2, $p = 0.1$). The CC group was marginally different to two of the treatment groups: HC ($p = 0.03$) and HH ($p = 0.01$).

---

**Figure 3.1b Fto gene expression across tissues.** Data represent mean values ± sem.

The values were calculated for the control group (CC) and expression of Fto is relative to 18S.
Figure 3.2 Fto gene expression in the hypothalamus of 20-week male C57BL/6 mice. Values represent mean expression ± sem. CC n = 7, HC n= 6, LC n = 10, HH n = 5, LL n = 6.

In the brown adipose tissue (BAT), there were no significant differences among the diet groups (Figure 3.3).
Figure 3.3 Fto gene expression in the brown adipose tissue of 20-week male C57BL/6 mice. Values represent mean expression ±sem. No significant differences (NS) were observed. CC n = 7, HC n = 7, LC n = 8, HH n = 6, LL n = 7.

Similarly, in the liver tissue, there were no significant differences between HC and LC (p = 0.7) or HH and LL (p = 0.8). Fto gene expression in the CC group was marginally lower than in HC and LC (p = 0.01) (Figure 3.4).
Figure 3.4 Fto gene expression in the liver of 20-week male C57BL/6 mice. Values represent mean expression ± sem. CC n = 7, HC n= 7, LC n = 8, HH n = 6, LL n = 7. There were no differences between diets (HC vs. LC, or HH vs. LL) but CC lower than in HC and LC (p = 0.01)

In sub-cutaneous adipose tissue, there were no significant differences among any of the diet groups (Figure 3.5). In the visceral adipose tissue, however, there was significantly lower Fto gene expression in both HH and LL groups compared to the CC group (p<0.001, Figure 3.5).
Figure 3.5 Fto gene expression in the adipose tissue of 20-week male C57BL/6 mice. Values represent mean expression ± sem. No significant (NS) differences were found in the visceral adipose tissue. VAT: CC n = 7 , HC n = 7, LC n = 6, HH n = 6, LL n = 6. SAT: CC n = 7, HC n = 7, LC n = 7, HH n = 5, LL n = 7.
In the white muscle, Fto mRNA expression was nearly 3-fold greater in the LC group compared to HC (p = 0.0001) and 1.5-fold higher in LL group vs. HH (p = 0.0001) (Figure 3.6). The CC group was significantly higher only compared to HC (p = 0.001). By contrast, in red muscle, the HC group was 2-fold higher than LC (p = 0.0001) (Figure 3.6), while CC was higher than the remaining groups (p = 0.001).
**Figure 3.6 Fto gene expression in the muscle.** Values represent mean expression ± sem. WM: CC n = 5, HC n= 5, LC n = 4, HH n = 5, LL n = 5. RM: CC n = 5 , HC n= 7, LC n = 6, HH n = 7, LL n = 4 .
Leptin mRNA expression (Figure 3.7 and 3.8) was measured only in adipose tissue, since constitutive leptin expression has mainly been demonstrated in this tissue. In visceral adipose tissue leptin mRNA expression of the LC group was 4.4-fold higher than HC (p = 0.0001) and LL was 3.3-fold higher than HH (p = 0.0001). In subcutaneous adipose tissue, there were no significant differences between the groups. Even though LL showed a ~3.5–fold higher leptin expression than HH, this difference was only marginal because of the small number of animals (Figure 3.7, p = 0.02). The difference between LC and HC was not significant.

**Figure 3.7 Leptin gene expression in the visceral adipose tissue.** Values represent mean expression ± sem. VAT: CC n = 5, HC n= 6, LC n = 6, HH n = 6, LL n = 6.
Figure 3.8 Leptin gene expression in the subcutaneous adipose tissue. Values represent mean expression ± sem. CC n = 6, HC n = 7, LC n = 7, HH n = 4, LL n = 4.

Study 2: Placentas were recovered from the high GI (PH) and low GI (PL) mothers at 18-19 days gestation. Fto expression was 3.8-fold higher in the high GI-fed mothers compared to the low GI mothers (Figure 3.9, p = 0.0001).
Figure 3.9 Fto gene expression in the placenta. Values represent mean expression ± sem. PH, placentas recovered from high GI-fed mothers, n = 15. PL, placentas recovered from low GI-fed mothers, n = 18.
3.4 Discussion

To our knowledge, this is the first study to examine the direct effects of maternal high and low GI feeding on Fto and leptin gene expression in offspring. The findings show that Fto gene expression in key tissues is significantly regulated by both early-life and life-long exposure to different sources of dietary carbohydrate. The most important outcome was the 2.5-fold higher expression of Fto in the hypothalamus of pups exposed to a high GI diet vs. low GI diet from conception to 20 weeks of age (HH vs. LL). In the placentas of high-fed mothers, Fto expression was 4-fold higher compared with those fed a high GI diet. In contrast, there were no differences in expression of Fto in offspring liver or the adipose tissues, although surprising differences occurred in red and white muscle. There were also important differences in leptin gene expression, with 3 to 5-fold higher levels in the low GI fed pups compared to high GI fed offspring. Together these findings indicate an important role of carbohydrate nutrition and/or postprandial glycaemia in the in utero environment on the pattern of gene expression later in life.

The fat mass and obesity (FTO) gene has one of the strongest links with BMI in the human population. One in six people have the “risk” alteration and weigh 3 kg more than those with the unaltered gene, but it is not understood how this gene influences BMI and obesity. We set out to understand whether maternal diet affected offspring FTO expression using a mouse model. Although previous rodent studies demonstrate a role for FTO in energy homeostasis and body composition, the relationship between brain FTO mRNA levels and food intake remains controversial. Specifically, it is still unclear whether up-regulation of FTO expression is causal for obesity. However, in mice, Church et al. (83) showed that enhanced expression of Fto leads
to increased food intake and obesity in a dose-dependent manner. Therefore, in the present study, a 2.5-fold greater hypothalamic Fto expression in the pups exposed to a high GI diet vs. low GI diet, suggests that a high GI diet leads to greater risk for obesity and associated disorders. However, despite these differences in Fto gene expression, in the previous chapter we show no differences in food intake between the groups. We speculate that this is due to the low fat content of the diets in the current study, which probably causes low palatability for both the low and high GI feeds.

Although there are no similar studies to ours comparing the effects of maternal nutrition on Fto expression in the offspring, our results can be compared with findings in older animals fed different diets. Cheung at al. (85) also showed that Fto gene expression was down-regulated in mouse hypothalamic cells and embryonic fibroblasts deprived of amino acids. Tung et al.(84) reported that in rats exposed to a high fat diet, Fto gene expression in the arcuate nucleus of the hypothalamic area was up-regulated (2.5-fold) compared to the chow-fed control. In contrast, Gutierrez-Aguilar et al. (206) reported that Fto gene expression in the hypothalamus was down-regulated in rats fed a high fat diet compared to those fed chow. These inconsistent findings may be related to the length of exposure to the dietary intervention.

The findings of the present study do not support the hypothesis that the glycaemic impact of the diet influences Fto gene expression in tissues such as adipose and liver, tissues that are specialised for fat synthesis and storage. Indeed, there were no important differences between diet groups in relation to brown adipose tissue or sub-
In contrast, there were unexpected differences in red vs. white muscle tissue. In white muscle, Fto expression was significantly higher in both low GI groups (LC and LL) compared to their respective high GI groups (HC and HH). By contrast, in red muscle, expression was higher in the pups of mothers fed a high GI diet until weaning (HC vs. LC) but not between HH vs. LL. These inconsistent findings may relate to the different functions of white vs. red muscle. Red muscle (type 1 or slow twitch), is dense with capillaries and rich in mitochondria and myoglobin, giving the muscle tissue its characteristic colour. It carries more oxygen and can sustain aerobic activity using fats or carbohydrates as fuel for longer (207).

On the other hand, white muscle (type IIb, fast twitch) muscle is anaerobic and glycolytic, and is least dense in mitochondria and myoglobin. In small animals such as rodents this is the major fast muscle type, explaining the pale color of their flesh. It is possible therefore that carbohydrate nutrition during gestation influences the expression of genes regulating the fuel mix during prolonged strenuous exercise. In humans, low GI meals have been shown to extend endurance performance (208, 209), although this may be more related to the acute effects of glycaemia than gene expression over the longer term.

Interestingly, in line with our findings in muscle, McMurray et al (76) found that Fto appeared to have a critical role in the control of lean mass, independent of its effect on food intake. Removing FTO everywhere from conception had a dramatic effect on body composition and resulted in stunted growth and some lethality. Removing
FTO everywhere but only in adult animals resulted in better viability and normal growth but, surprisingly, reduced lean mass and increased fat mass with a change in the type of metabolic fuel being used. When they removed FTO from the hypothalamus of adult animals, these animals showed only a mild reduction in food intake and weight gain. Clearly, further research is needed to elucidate the meaning of changes in Fto expression in muscle vs. adipose tissues.

In the present study, we observed 4-fold higher expression of the Fto gene in the placentas of the high GI vs. low GI mothers. Placental programming by dietary manipulation is not yet widely researched and understood. Adaptations in placental phenotype have been observed in response to severely restricted dietary manipulations, which lead to a reduction of the nutrient transfer capacity of the placenta (104). These researchers showed that in mice, small alterations in the protein and carbohydrate content of the gestational diet led to significant differences in the expression of placental Igf2 on gestational day 16. Expression of the glucose transporter gene Slc2a1 and placental transfer of glucose were also differently affected by dietary interventions. Studies conducted in diabetic pregnancies have also shown that maternal gestational hyperglycemia may be involved in the pathogenesis of IR, impaired glucose tolerance, type 2 diabetes mellitus, the Metabolic Syndrome and subsequent cardiovascular diseases in adult offspring. A low GI maternal diet rather than a low carbohydrate diet, has been associated with measurable benefits to the offspring such as fetal and placental insulin and glucose regulation, fetal growth, birth weight and offspring adiposity (131, 133, 210). The differences in Fto gene expression in the placenta observed in the present study
suggest that postprandial glycaemia, rather than the dietary carbohydrate content, is the mechanism that regulates placental gene expression.

Our study also showed significantly higher expression of the leptin gene in visceral adipose tissue of offspring of low GI fed mothers (LC vs. HC and LL vs. HH). This can be interpreted in two ways i) as indicative of higher leptin production and therefore higher plasma leptin concentration or ii) indicative of greater body fat in the low GI fed pups. In humans and animal models, leptin concentration increases with higher body fat. Leptin gene expression in white adipose tissue has also been shown to be elevated in rats fed a high fat diet compared to a standard diet (211), indicating nutritional regulation of this gene. Nonetheless, in Chapter 2, we demonstrated that plasma leptin levels were higher in offspring of low GI mothers who were phenotypically the same as those of high GI mothers (similar body weight, insulin sensitivity and glucose tolerance). We also observed lower levels of the orexigenic hormone ghrelin in the low GI fed animals. Although an argument could be made regarding the possibility of leptin resistance in the low GI fed animals, as well as the fact that we cannot rule out differences in body fat composition, the weight of evidence suggests that high leptin gene expression in the low GI fed offspring results in higher leptin protein production and therefore greater satiety, and over the longer term, a reduced risk of excessive food intake and obesity.

The finding of higher expression of Fto and lower expression of leptin in the high GI offspring, in concert with the higher levels of ghrelin and lower levels of leptin in plasma (as described in Chapter 2) lead us to the conclusion that maternal diets with a high GI (or more specifically faster rate of carbohydrate digestion and absorption)
are likely to predispose to higher energy intake and greater risk of obesity in adult offspring. The fact that there were no phenotypic differences in the animals at the time of study strengthens the assumption that these are primary traits rather than secondary to higher body weight or body fat.

The strengths of our study include the study design which allowed us to observe the early in-utero impact of the maternal nutrition on the expression of Fto and leptin genes in the prenatal tissue, the placenta, and in adult tissues of the offspring. This is a valuable tool which brings power to the early programming theory. Furthermore, the fact that the treatment diets were identical apart from the source of the carbohydrate allows us to separate the effect of quantity of carbohydrate and other macronutrients from the quality of carbohydrate. The in vitro studies described in Chapter 2 confirmed that the rate at which glucose from each diet becomes available for absorption varied according to their GI. This reinforces the potential that low GI carbohydrate foods and diets may have on epigenetic programming and prevention of obesity and other non-communicable diseases.

The weakness of our study should be noted. The biggest limitation is the use of a mouse model to test our hypotheses. It cannot be assumed that human offspring will display similar up-regulation of FTO in response to differences in the GI of maternal diet. Mice have evolved along a different evolutionary pathway with different native diet to humans. As an alternative, non-human primates could be employed although this would be expensive and difficult to undertake with large numbers of animals. Likewise, as humans, we consume real foods and mixed meals, and rarely do we consume a diet that has been manipulated in GI alone as was the case in the present...
study. The feeds we used were not purified and so their metabolic impact could be directly attributed to their starch content.

In summary, the findings of this research have important implications for the intrauterine programming of obesity. The perspective that a specific dietary choice, such as high or low GI carbohydrates, can regulate the expression pattern of regulatory and nutrient supply genes in key areas such as the hypothalamus, placenta, muscle and adipose tissues, suggest that changes in carbohydrate nutrition have contributed to our current obesity epidemic. Our findings also imply that a low GI diet is promising treatment approach that may substantially reduce the adult risk of developing diseases programmed in utero.
Chapter 4

Further findings;

Changes in expression of appetite genes in hypothalamus
Chapter 4 Changes in expression of appetite genes in hypothalamus

4.1 Introduction

In Chapter 3, we showed that leptin gene expression in visceral adipose tissue was upregulated by both early-life and life-long exposure to a low GI diet. Additionally, plasma leptin levels were 6-fold higher in the low GI pups after extended exposure to the diet, whereas plasma ghrelin levels were more than twice as high in the high GI pups. These outcomes led us to investigate other genes involved in the regulation of energy balance and appetite. This part of the research was carried on in collaboration with Seung Gwon Seo, a biochemistry honours student. The data presented below were therefore incorporated into his honours thesis (212), but the findings have been newly interpreted in the light of other results.

Both leptin and ghrelin have well recognised roles in the regulation of food intake, energy balance and body weight (213, 214). Leptin is an important adipose tissue-derived hormone released into the circulatory system as a feedback on adiposity level (215, 216). Leptin signalling, recognised by the hypothalamic leptin receptors, signals satiation and influences the expression and activity of other hypothalamic orexigenic and anorexigenic neuropeptides (Figure 4.1). In contrast, ghrelin is a gut-derived hunger-inducing hormone. Gastric emptiness is the main drive for ghrelin expression and associated with increased food intake (217). Interestingly, the functional role of ghrelin on food intake is also mediated through the hypothalamus (218), where it stimulates the expression of orexigenic neuropeptides and inhibits anorexigenic neurons in order to increase appetite.
Within the hypothalamus, situated in the arcuate nucleus, there are two neuronal populations responsible for the integration and processing of information related to energy homeostasis, followed by translation into signals influencing appetite and energy expenditure. The neurones containing neuropeptide Y (NPY) and agouti-related protein (AGRP) are stimulated by ghrelin and inhibited by leptin, with the net effect of increasing appetite. In contrast, the neurones containing proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) are stimulated by leptin and reduce appetite (219). The two neuronal populations act together to finely tune feeding behaviour and energy homeostasis (166, 213, 220, 221).
Figure 4.1. Interaction of peripheral signal hormones and central appetite regulation. Figure adapted from Klok et al. (166). Leptin, secreted from adipocytes, inhibits appetite by promoting co-expression of POMC and CART. Gut-originated ghrelin increases appetite by promoting co-expression of AGRP and NPY. (AGRP: Agouti-related protein, CART is produced by the CARTPT gene: cocaine-and-amphetamine-regulated transcript prepropeptide, NPY: Neuropeptide Y and POMC: pro-opiomelanocortin).

The central neurological orexigenic (appetite-stimulating) response is executed by AGRP and NPY in the arcuate nucleus (213). AGRP is an endogenous antagonist in the melanocortin system that controls feeding behaviour and energy homeostasis. Mice with AGRP mutations have shown increased obesity, insulin resistance, somatic growth and predisposition to tumorigenesis (222). Hypothalamic NPY is co-expressed with AGRP and its effect on food intake was first discovered after rats were injected with NPY directly into the hypothalamus (223). This elicited a strong
feeding response; a single injection of NPY rapidly increased appetite and food ingestion in a dose-dependent manner. Later, King et al. (224) corroborated the orexigenic properties of NPY, by blocking specific NPY receptors with synthetic antagonists. The direct injection of antagonists significantly reduced frequency and amount of feeding.

POMC also plays an important role in the melanocortin system and regulation of energy balance and body weight homeostasis (225). Several studies have shown decreased hypothalamic POMC mRNA expression in fasted rodents at the same time as orexigenic gene expression is increased (226). POMC mutation in humans has been associated with a hyperphagic phenotype (215) and its derivative α-Melanocyte-Stimulating Hormone (α-MSH) is involved in the appetite regulatory pathway (227).

Cocaine and amphetamine-regulated transcript (CART) is an anorectic neurotransmitter encoded by the CARTPT gene (CART prepropeptide). It is involved in food intake regulation by mediating short-term satiety and interacting with leptin (228). Although the mechanism is not yet understood, it appears that CART acts at hindbrain and hypothalamic sites to inhibit feeding.

Finally, we were interested in leptin receptor (LEPR), a single transmembrane-domain-receptor protein that in humans is encoded by the LEPR gene (229). LEPR functions as a receptor for the adipocyte-specific hormone leptin in the hypothalamus. Variations in the leptin receptor have been associated with obesity. In
the db/db mouse model of obesity and diabetes, for example, leptin receptor activity is deficient because the mice are homozygous for a point mutation in the LEPR (230).

The aim of these further investigations was to determine whether there were differences in the expression of hypothalamic appetite-regulating genes (AGRP, NPY, POMC, CART and LEPR) as a result of early-life and life-long exposure to a high vs. low GI diet.

Our hypotheses were:

1. High GI diet offspring will show higher hypothalamic expression of orexigenic AGRP and NPY
2. High GI diet offspring will show lower hypothalamic expression of anorexigenic POMC and CART
3. High GI diet offspring will show lower hypothalamic expression of leptin receptor gene, LEPR
4. Any adverse effects of early-life exposure to a high GI diet will be exacerbated by life-long exposure

4.2 Materials and methods

4.2.1 RNA extraction and primer design

As described in detail in Chapter 3, total RNA extraction from the whole hypothalamus was conducted using RNeasy Mini Kit by Qiagen as per manufacturer’s instructions. The RNA purity and yield were controlled using a NanoDrop (ND-1000) spectrophotometer (NanoDropTechnologies Inc, USA) and gel electrophoresis. For the cDNA synthesis, the sets of primers were designed using
the ‘Primer3’ (v 0.4) web tool, which is available at http://frodo.wi.mit.edu/primer3/. The search criteria were: primer length between 19-23 nucleotides long, amplicon optimal size 100 bp, melting temperature (Tm) between 58-62°C with Tm difference between primers not greater than 2°C, GC content of primers between 50-55% and no 3’ complementarity. The primers were not designed using intron spanning but they were designed to bridge the exon-intron boundaries within the gene of interest to exclude possible contamination by genomic DNA. Once the pairs of the primers were chosen, the oligonucleotides were synthesised by Sigma Genosys (Sigma-Aldrich). They were experimentally validated with two quality control assays. First, a melt curve analysis, confirmed by agarose gel electrophoresis, verified that a single gene-specific product was produced. Each sample was run in triplicate. In addition we checked the amplification efficiency through a calibration curve. The slope of the Ct values versus the initial amounts of input material on a semi-log10 plot (of the best fit line) was calculated and we obtained an amplification efficiency > 90 %.

Primers for the target genes, AGRP, NPY, POMC, CART and LEPR were selected as follows:

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<th>Oligo name</th>
<th>Sequence (5’- 3’)</th>
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<tr>
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</tr>
<tr>
<td>Mus_Lepr_rev</td>
<td>AGG GTC TGG TGT GGT CAA AAG</td>
</tr>
</tbody>
</table>
4.2.2 Quantitative Real-time PCR (qRT-PCR)

As described in Chapter 3, in PCR the cDNA targeted sequence is amplified using the specific primer pairs. We used the Fast SYBR® Green Master Mix (Applied Biosystems) containing Syber Green fluorescent dye which binds only double stranded DNA with an intensity proportional to the amount of double stranded product formed. The fluorescence after each amplification cycle was measured and the number of cycles required to achieve a pre-set level of double stranded DNA (threshold) determined. The more abundant the sequence in the initial cDNA preparation, the fewer cycles required to achieve the threshold. All the Ct values were normalised to the appropriate 18S rRNA Ct value which was used as the reference sequence. Levels of 18S did not differ between groups. Ct value which was used as the reference sequence. Gene expression was estimated using the Ct (2ΔΔct) method (205).

4.2.3 Data and statistical analysis

Gene expression is presented as fold-change compared to the control group, i.e. the chow-fed offspring of chow-fed mothers. Prism 6.0 (Graph Pad Software, USA) and SPSS Statistics 19 (IBM) was used for statistical analysis. One-way repeated measures analysis of variance (ANOVA) followed by two-tailed unpaired t-tests with the Bonferroni adjustment were performed to assess the difference between the groups for the expression levels of the target genes. Due to the number of variables we investigated in this part of the project, a statistical p value of ≤ 0.05 was taken as marginal significance and < 0.01 as significantly different.
4.3 Results

Overall, the high GI diet offspring expressed significantly more orexigenic genes than the low GI diet mice (LC and LL) and the chow group (Figure 4.2). AGRP mRNA expression level was approximately 2.5-fold higher in HC compared to the LC group (p = 0.0001). In the offspring exposed to a high GI diet for the entire 20 weeks (HH), AGRP expression was 1.9-fold higher compared to the LL group, but was not statistically significant (p = 0.2).

Hypothalamic NPY mRNA expression level also varied markedly between diet groups. As shown in the Figure 4.3, NPY mRNA expression was 3.1-fold higher in HH vs. LL. However, this difference was only marginally significant (p = 0.04). The difference between HC vs. LC did not reach statistical significance, and both were similar to CC (p = 0.1).

POMC gene expression (reflecting appetite inhibition) was significantly higher in HC vs. LC (p = 0.004), but there were no differences between HH, LL and CC (Figure 4.4). Similarly, CART mRNA gene expression in pups exposed to a high GI diet during early life (HC) had significantly higher expression than those exposed to low GI feeding (LC) (p = 0.003). In contrast, CART mRNA expression level among HH, LL, and CC groups was similar (Figure 4.5).
Figure 4.2 AGRP gene expression in the hypothalamus of 20-week male C57BL/6 mice. Values represent mean expression ± sem. CC n = 11, HC n = 7, LC n = 10, HH n = 7, LL n = 7.

Figure 4.3 NPY gene expression in the hypothalamus of 20-week male C57BL/6 mice. Values represent mean expression ± sem. CC n = 11, HC n = 7, LC n = 11, HH n = 9, LL n = 7.
Figure 4.4 POMC gene expression in the hypothalamus of 20-week male C57BL/6 mice. Values represent mean expression ± sem. CC n = 11, HC n = 6, LC n = 11, HH n = 9, LL n = 7.

Figure 4.5 CART gene expression in the hypothalamus of 20-week male C57BL/6 mice. Values represent mean expression ± sem. CC n = 6, HC n = 7, LC n = 11, HH n = 9, LL n = 7.
Figure 4.6 LEPR gene expression in the hypothalamus of 20-week male C57BL/6 mice. Values represent mean expression ± sem. CC n = 11, HC n= 7, LC n = 11, HH n = 9, LL n = 7.

LEPR mRNA expression was up-regulated in both HH and HC groups compared to CC. The difference between HC and LC was statistically significant (p = 0.001) but the difference between HH and LL was not (Figure 4.6)

4.4 Discussion

Our findings indicate that the offspring of high vs. low GI mothers display important differences in expression of key hypothalamic appetite-regulating genes. Pups exposed to a high GI diet throughout fetal and early post-natal life had 2.5-fold higher expression of the orexigenic gene AGRP (HC vs. LC, p = 0.0001) while those exposed life-long had 3-fold higher expression of the orexigenic NPY gene (HH vs. LL, p = 0.04). Paradoxically, however, expression of the two anorexigenic genes (POMC and CART) was also significantly higher in the HC group compared to LC
Similarly, LEPR gene expression was 2-fold higher in HC vs. LC (p = 0.001). Taken together, the findings imply that the source of carbohydrate has important epigenetic or programming effects on the developing hypothalamus, but the net result on appetite and energy balance is difficult to predict.

The finding of up-regulation of NPY and AGRP in the high GI offspring suggests they are more likely to be hyperphagic and show impaired energy intake regulation. However, as shown in Chapter 2, food intake, weight gain and body weight up to 20 weeks of age were similar between the treatment groups. The lack of difference in food intake may not be surprising in light of the significant up-regulated expression of the two anorexigenic genes, POMC and CART in high GI offspring (HC vs. LC). Early studies unrelated to dietary GI have reported that hyperglycaemia with hyperinsulinaemia is associated with enhanced satiety (231). Hence, increased glucose concentration along with a neurological gluco-sensing mechanism has the potential to up-regulate anorexigenic hormones such as POMC and CART, and therefore signal satiation. Thus in the context of the present findings, the simultaneous up-regulation of both anorexigenic and orexigenic genes may be a normal feedback, homeostatic response to ensure energy balance in a young growing animal.

Nonetheless, high GI-fed offspring may be predisposed to over-eating and persistent positive energy balance over the longer term. This is suggested by the fact that the absolute difference in orexigenic gene expression between high vs. low GI groups is larger (2.5 to 3-fold difference) than that of the anorexigenic genes (less than 2-fold). Additionally, the observed up-regulation of the hypothalamic leptin receptor gene
(LEPR) in early life high GI-fed offspring (HC vs. LC, p = 0.001) could imply reduced satiation through deficient leptin action in the hypothalamus. This interpretation is consistent with much lower concentrations of leptin in plasma (Chapter 2) and leptin gene expression (Chapter 3) in high GI-fed offspring, but contrary to our original hypothesis.

Previous animal studies have documented differences in the effect of the dietary GI on the phenotypic outcomes such as body weight and food intake. In rats, it was shown that the GI had an independent effect on body composition (135). Pair-feeding experiments showed that the high GI rats gained the same weight as the low GI group even though they consumed less food from week 8 onwards. The authors suggested it could be due to lower resting energy expenditure or activity due to decreased lean body mass. The study also included an experiment in the obesity-prone C57BL/6J mice. On a high GI diet, these mice had lower lean body mass and nearly twice the body fat compared to the low GI-fed mice with the same mean body weight. However, in this study the lower circulating leptin levels could suggest that fat mass might actually be lower in mice exposed to a high GI diet from conception to adulthood and thus more studies in this area are needed.

Scribner et al. investigated the long-term effects of dietary GI on adiposity and energy metabolism in mice. The diets were matched for macro and micronutrients up to the age of 40 weeks (136). The high GI mice had lower fat oxidation, 40% greater adiposity and were more insulin resistant. Interestingly, these effects were independent of energy intake since that was the same between the two diet groups. Similarly, a study in 16 weeks old mice (137) showed that the high GI-fed group had
significantly higher body weight and increased fat mass. Lastly, Scribner’s group conducted a later experiment to investigate the short (6 weeks) and long term (20 weeks) effects of high versus low GI diets on body composition, liver fat, glucose clearance and lipid metabolism in mice (136, 138). All high GI-fed animals showed a significant increase in body fat mass and greater liver fat.

Human studies have also reported that a low GI meal or diet is associated with enhanced satiety and inhibition of appetite (232-234). Many of these investigations were designed as single meal tests (234, 235) and satiety was often assessed subjectively (236). Despite these shortcomings, a recent meta-analysis of GI and energy intake indicated that children consuming a high GI diet have consistently increased food intake, irrespective of BMI (237). It is therefore plausible to suggest that the animals in the present study may be obesity-resistant, or alternatively, too young at 20 weeks of age to display differences in food intake and phenotype. It is possible that longer exposure to the diets would have produced results similar to those found in literature (234).

We also hypothesised that prolonged vs. early exposure to a high GI diet may be associated with exaggerated impact on appetite regulating gene expression. This appeared to be true for the NPY gene, and to less extent, the LEPR gene. But in the case of AGRP mRNA, the highest level of expression was found in HC rather than HH, suggesting pre-natal exposure to the high GI diet is the major determinant. In the case of POMC and CART expression, longer exposure may have even reversed some of the effects of early exposure, but experiments with larger numbers of animals are required to test this hypothesis. On the whole, it is evident that appetite
regulating gene expression is dependent on timing of exposure rather than length of exposure. It is also probable that a stronger metabolic challenge, such as access to a high-fat “western” diet may be necessary to provoke measurable outcomes and unmask the phenotypic characteristics of the animals in this timeframe.

The strengths of this study include the fact that it was adequately powered with a relatively large number of animals in each group (up to 11 in many cases). The effect on gene expression is also remarkably consistent, the high-GI fed offspring being the group with highest expression. Our conclusions are strengthened by the fact that there are no phenotypic differences at this stage, suggesting that differences in gene expression are primary rather than secondary to differences in body composition, insulin resistance or impaired glucose tolerance. However, a possible drawback could be considered the use of whole hypothalamus instead of just the arcuate nucleus (ARC) of the hypothalamus, where NPY, AgRP and the other neuropeptides are more highly expressed and differ from the rest of the hypothalamic regions.

This study provides insight into how a high GI diet increases the risk of obesity and other metabolic disorders. Previous studies have demonstrated that epigenetic modifications are responsible for altering the expression of key appetite regulating genes, including those studied in this chapter: NPY (238), POMC (239) and leptin (240). The field of epigenetics has become a subject of special interest in recent years because it provides a mechanism for explaining how perinatal nutrition becomes a determinant of later health outcomes (99). An epigenetic approach to obesity and associated metabolic disorders indicates that nutrition in early life is just
as important as an inherited predisposition in determining differences in BMI among individuals (241).

To our best knowledge, this is the first study to measure hypothalamic appetite-regulating gene expression in offspring of high vs. low GI mothers. We show that the well recognised co-expression of AGRP/NPY and POMC/CART is regulated by the nature of the dietary carbohydrates. Our novel findings provide molecular insights into how differences in carbohydrate nutrition, including rate of digestion and/or maternal post-prandial hyperglycaemia provoke differences in the regulation of orexigenic and anorexigenic genes in the hypothalamus.
Chapter 5

Investigating the effect of early-life and life-long exposure to a high vs. low GI diet on liver histology
Chapter 5  Study design and rationale

5.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is a group of chronic liver disorders, whose clinical manifestation can range from simple fatty deposits (hepatic steatosis) to much more marked fat accumulation in the hepatocytes combined with tissue degeneration, and lobular inflammation called non-alcoholic steatohepatitis (NASH). In this case the fat accumulation triggers an inflammatory response that leads to liver steatohepatitis and fibrosis (242).

NASH is associated with obesity, diabetes, insulin resistance (IR), and hypertriglyceridemia. In children, statistics show that NASH may be present in the prepubertal age group, particularly in males who are overweight or obese, even though clinical signs are rarely shown at such early age (243, 244). Liver biopsy is considered the gold standard in establishing the diagnosis, while slow, consistent weight loss has been shown to be the most effective treatment for NAFLD. A low glycaemic index diet has been shown to be effective in lowering BMI and this dietary treatment is commonly recommended for NAFLD (244).

Hyperinsulinaemia and hepatic insulin resistance are considered to be part of the metabolic profile of a patient diagnosed with NAFLD, but the mechanisms and pathways involved are still uncertain (245). Indeed, NAFLD is acknowledged as the hepatic manifestation of insulin resistance and the metabolic syndrome (246), with oxidative stress identified as the most plausible mechanism (247). Patients with NAFLD demonstrate decreased levels of antioxidants (245), a fact that enforces the
theory behind oxidative stress. Genetic influences are also recognised as having an important but still unclear role in development.

Exposure in-utero and later in life to several dietary factors and especially to macronutrients such as carbohydrates can directly influence the development and progression of NAFLD (248, 249). Indeed, an increased consumption of simple carbohydrates is considered one of the dietary risk factors for NAFLD (249, 250). High GI foods have been associated with increased hepatic fat in both rodents and humans, and simple carbohydrates, such as fructose, have been shown to lead to increased fat deposition in the liver by stimulating hepatic de-novo lipogenesis and decreasing lipid oxidation (250).

Little is known about the relationship between the FTO gene, insulin resistance and the clinical signs of NAFLD. FTO is not only expressed in the hypothalamus but also in almost every tissue related to metabolic disease, including the liver. In the past six years, there have been several animal studies that demonstrate a positive association between the FTO gene and lipid accumulation in the liver (251). Likewise, a recent human study showed that FTO overexpression increased the rate of lipogenesis and oxidative stress in myotubes (252). Although oxidative stress is thought to play a major role in the NAFLD lipid accumulation (253), the function of FTO remains elusive.

Guo et al. (251) explored changes in FTO expression in the liver of rats with NAFLD induced by high fat feeding. FTO gene expression was up-regulated along with increased oxidative stress. When they conducted in vitro overexpression experiments in human hepatocyte cells, they found that overexpression of the FTO
gene increased oxidative stress levels and was associated with greater lipid accumulation, which led to the conclusion that FTO activated the oxidative stress process and disrupted lipid metabolism.

The system for grading or staging fibrosis and steatosis of the liver was first developed in 1999 by Brunt et al. (254). Grading was based on a review of liver biopsies of adult patients with NASH and the terms mild, moderate, and severe were used to indicate the severity of the observed histological lesions. In 2005, an updated and validated scoring system was established to evaluate the whole spectrum of NAFLD that had improved features of inter-rater reproducibility and was used for both adults and children with any degree of NAFLD (246).

The full range of clinical manifestations of NAFLD observed in humans has not been totally replicated in animals. However, animal models are used in verifying hypotheses on the pathogenesis of NASH and in performing interventional studies (255). The same scoring system can be used for these purposes.

The aims of this part of the study were to observe and study any possible clinical signs of abnormal liver histology in mice fed chow versus the early life and life-long fed low GI and high GI diet groups. In the case of significant histological findings, we considered whether these could be correlated with Fto gene expression levels in the liver tissues of the different diet groups. Because higher postprandial glycaemia/insulinaemia encourages greater glycogen synthesis, we also endeavoured to quantify differences in glycogen deposition in the different treatment groups.
5.2 Materials and methods

5.2.1 Tissue collection

As described in Chapter 2, liver tissues were collected at 20 weeks of age from all five groups: CC, HC, HH, LC, LL. Liver tissue samples were fixed in 4% (w/v) phosphate-buffered paraformaldehyde for 48 hours at 4°C and then transferred to phosphate-buffered saline solution. Subsequently tissues were embedded in paraffin and sectioned to allow for histological assessment.

5.2.2 Tissue staining and analysis

The stains and histological specimens described below were analysed and interpreted under the guidance of Clinical Professor James Kench, at the Central Clinical School of Pathology, Royal Prince Alfred Hospital, University of Sydney, NSW (Australia). The interpretation of the histological results was based on the NASH scoring system as by Brunt (254) and Kleiner et al. (246).

The histological stains used in this study included Haematoxylin and Eosin (H&E), Milligan's Trichrome Method for connective tissue and Periodic Acid Schiff's (PAS) method for carbohydrates (or basement membrane). Haematoxylin and Eosin (H&E) is the most widely used staining procedure, involving a two-step process that uses hematoxylin solutions for nuclear staining and eosin solutions for cytoplasmic staining.

Liver sections were deparaffinised in two changes of xylene (10 min each) and hydrated through graded alcohols to water. Subsequently, tissues were stained in Harris's haematoxylin for 3 min and washed with water. Excess stains were removed
by 10 rapid dips in acid alcohol, followed by blue staining in Scott's Tap Water solution for 30 seconds. In this step the nuclei stain blue and cytoplasm remains unstained as confirmed under microscope.

For the second step, tissues were placed in 70% ethanol for 30 seconds and counterstained in Eosin (two changes 40 seconds each). Graded alcohols were used to dehydrate the samples by immersing the entire rack. Samples were then cleared in two changes of Xylene (2 min each) and mounted with DPX. In this process, cytoplasm, collagen, keratin and erythrocytes are stained pink to red. H&E staining allows an overview of the structure of the tissue which enables classification of the structures examined as normal, inflamed or pathological.

Milligan's trichrome method for connective tissue is a differential stain that colours collagen blue against a red background of hepatocytes and other structures. It stains the collagen present in the portal tracts and vessel walls, but also highlights the presence and distribution of reactive fibrosis as a result of liver injury, such as steatohepatitis.

Sections were stained in Weigert's haematoxylin for 5 min and passed into water and blue in Scott's solution. Sections were placed in the following order in these solutions: mordant solution for 7 min, 0.1% acid fuchsin solution for 8 min, 1% phosphomolybdic acid solution for 3 min, 2% orange G solution for 10 min, 1% acetic acid solution for 2 min, 1% fast green solution for 10 min and 1% acetic acid solution for 3 min. Subsequently sections were dehydrated using 95% alcohol. After
these stains, nuclei and muscle appear magenta, collagen appears green and red blood cells appear orange to orange-red.

Periodic Acid Schiff's (PAS) method for carbohydrates was used to identify glycogen deposition. This is the most widely used technique in carbohydrate histochemistry and is positive for structures containing neutral sugars. Sections were deparaffinised and hydrated to distilled water and placed in 1% periodic acid solution for 15 min in order to oxidise glycol groups to aldehydes. Schiff's working solution was used (1:3 dilution for glycogen, fungi and carbohydrate) to create a stable product with the aldehydes. Sections were stained with haematoxylin for 3 min followed by 10 rapid dips in acid alcohol. Samples were placed in Scott’s Bluing Solution for 30 sec and dehydrated from 95% alcohol. Glycogen, mucopolysaccharide, fungi and basement membrane should stain pink, nuclei blue and other tissues remain colourless.

5.2.3 Statistical analysis

The data were analysed using Prism 6.0 (Graph Pad Software, USA) and IBM SPSS Statistics 19. P values were determined by Fisher’s exact test for categorical variables and Wilcoxon rank sum test for continuous variables. Odds ratios (ORs) and 95% confidence intervals were determined from multiple ordinal logistic regression analysis.

5.3 Results

Results rated by the NASH scoring system (246) are shown in Table 5.1. Successful stains were obtained from all treatment groups with the following liver sample
numbers: CC n = 6, HH n = 9, HC n = 5, LC n = 7 and LL n = 4. Average total liver weights were 1.2 g and did not vary significantly among the groups (p = 0.4).

**Glycogen deposition**

Glycogen was semi-quantitatively assessed either as 0 (no staining on PAS sections), 1 (focal or diffuse, pale (low intensity) PAS staining), 2 (diffuse moderate staining or focal strong intensity PAS staining) or 3 (diffuse strong staining). Figure 5.1 shows an example of liver sample for each score. No significant differences were observed between the groups (p = 0.481). However, there was a notable difference in the frequency of score 3 with CC and HC, indicative of strong glycogen deposition (33%). In group LL half of the samples showed focal low intensity stains indicating minimum glycogen deposition, whereas only 25% showed diffuse moderate stains, the maximum score given to this group. None of the LL samples indicated strong glycogen deposition. In contrast, 40% of the HH samples displayed diffuse moderate stains and another 40% scored the highest grade 3 (diffuse strong staining), indicating a more severe glycogen deposition in this group. Because of the small number of samples within each group, there was no overall statistical significance in glycogen deposition among the treatment groups.
**Figure 5.1 Various grades of glycogen deposition.** On the left, original magnification is 40x. On the right, the same sample is shown with original magnification 200x. (A) shows staining grade = 0, (B) shows staining grade = 1, (C) shows staining grade = 2 and (D) shows staining grade = 3.
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<th>Score</th>
<th>CC</th>
<th>HC</th>
<th>LC (%</th>
<th>HH</th>
<th>LL</th>
<th>HC/LC</th>
<th>HH/LL</th>
<th>p value</th>
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<td>0</td>
<td>1 (20.0%)</td>
<td>0</td>
<td></td>
<td>0.700</td>
<td>1.00</td>
</tr>
<tr>
<td>Microvesicular steatosis</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none or &lt;10% cells with few tiny vacuoles</td>
<td>0</td>
<td>2 (33.3%)</td>
<td>7 (77.8%)</td>
<td>4 (57.1%)</td>
<td>4 (80.0%)</td>
<td>2 (50.0%)</td>
<td>0.342</td>
<td>0.683</td>
<td></td>
</tr>
<tr>
<td>few tiny vacuoles in 10-33% cells</td>
<td>1</td>
<td>2 (33.3%)</td>
<td>2 (22.2%)</td>
<td>1 (14.3%)</td>
<td>1 (20.0%)</td>
<td>1 (25.0%)</td>
<td>0.342</td>
<td>0.683</td>
<td></td>
</tr>
<tr>
<td>few tiny vacuoles in &gt;34% cells</td>
<td>2</td>
<td>2 (33.3%)</td>
<td>0</td>
<td>2 (28.6%)</td>
<td>0</td>
<td>1 (25.0%)</td>
<td>0.342</td>
<td>0.683</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1 Baseline histologic characteristics by diet group. Data show the number of samples observed for each score/code (and % of total number of samples). C = 6, HC n = 9, LC n = 7, HH n = 5, LL n = 4. P values were calculated using the Fisher’s exact test.
**Lobular necrosis**

There was marginally significant evidence of increased necrosis in the liver lobules of group HC vs. LC (11.1% versus 0% had marked focal necrosis respectively, \( p = 0.045 \)), but no difference between HH vs. LL (\( p = 0.810 \)). Nonetheless, there was a trend for the group LL to show none to scanty focal necrosis, whereas 40% of the HH group showed signs of moderate lobular necrosis. CC samples were evenly distributed between scanty focal to moderate necrosis.

**Hepatocyte ballooning**

While hepatocyte ballooning is difficult to reproducibly recognise and grade when mild as in our case, a score of 0 (none), 1 (a few ballooned cells) or 2 (many cells/prominent ballooning) was subjectively assigned. Overall, there were no significant differences between any of the groups.

**Lobular inflammation**

Lobular (also known as intra-acinar) inflammation was quantified as 0 (none or \(<1\) focus/field), 1 (\(<2\) foci/field), 2 (2-4 foci/field) or 3 (>4 foci/field). The lobular inflammatory infiltrate observed in some cases, consisted almost entirely of lymphocytes, with a few neutrophils observed occasionally. However, no findings were statistically significant.

**Portal tract inflammation**

Portal tract inflammation was assessed as none, mild, moderate or heavy/severe. The portal inflammatory infiltrate consisted of lymphocytes. Again no significant
histologic findings were observed ($p > 0.700$), although interestingly only groups HC
and HH showed signs of moderate portal inflammation.

*Fibrosis and steatosis*
There was no evidence of significant fibrosis (defined according to Brunt 1999 and
Kleiner 2005 (246, 254)), and no architectural distortion. Likewise, macrovesicular
steatosis, defined as cytoplasmic vacuole or vacuoles as large as or larger than the
nucleus, was not detected in any of the samples. However, microvesicular steatosis
was evident. Although it is difficult to grade microvesicular steatosis using light
microscopy, the following grading system was applied: 0 (none or $<10\%$ cells with a
few tiny vacuoles), 1(a few tiny vacuoles/cell in 10-33% cells), 2 (a few tiny
vacuoles in $>34\%$ cells) and 3 (numerous tiny vacuoles/cell in $>34\%$ cells). Figure
5.2 illustrates an example for each grade. Some samples from the CC, LC and LL
groups scored 2, indicating few tiny vacuoles/cell in $>34\%$ cells. None of the
differences reached significance compared to the other groups.
Figure 5.2 Scoring of microvesicular steatosis. On the left, original magnification is 40x. On the right, the same sample with original magnification 200x. (A) shows score = 0, (B) shows score = 1 and (C) shows score = 2. Score 3 was not observed in any of the histological samples.

5.4 Discussion

In the present study, there were no significant differences in the liver histological findings between the high GI and the low GI pups at 20 weeks of age. Thus there is no support for our hypothesis that a high GI diet consumed in pregnancy or beyond
increases the risk of liver pathology in the offspring. However, some findings suggest there is a need for further research with a greater number of animals and at a later time in the life.

Non-alcoholic fatty liver disease (NAFLD) is an acquired metabolic stress-related liver disease with histological manifestations ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (249). Carbohydrates in the diet could play a key role in the development and progression of NAFLD. In particular, a high consumption of sucrose, fructose and/or high GI carbohydrates (a source of rapidly digested and absorbed glucose) has been identified as a risk factor, whereas dietary fibre and low GI foods have been recognised as protective factors against NAFLD (249).

In animal studies, high fat, western-style maternal diets have been associated with epigenetic changes during early development and liver changes similar to NAFLD (256). Prenatal caffeine exposure has also been shown to increase the risk of NAFLD through effects on intrauterine glucose and alterations in lipid metabolism (248). These changes were associated with increased foetal hepatic lipogenesis and reduced lipid output in utero, effects that reappeared in later life and aggravated the risk for NAFLD. At the present time, however, there is no direct evidence of epigenetic effects of carbohydrates in the maternal diet.

Here we investigated the role that the GI of the maternal and offspring diet might play in the development of NAFLD. Although there were some interesting trends, none of the differences reached statistical significance. This was the case in relation
to glycogen deposition as well as signs of inflammation, ballooning, necrosis or steatosis. Pups exposed to the treatment diets either in utero or also later in life did not show significantly altered liver histological findings that could indicate a major or lesser predisposition to NASH and NAFLD. For this reason, no correlations could be made with the earlier finding of differences in gene expression.

The outcomes of this study contrast with findings of a recent study in a multi-ethnic cohort of children, all with hepatic steatosis. In this instance, children whose diets were higher in foods containing fructose (soft drinks, fruit juice) or high GI and GL, had higher risk of NAFLD (257). Interestingly, all children were overweight or obese in contrast to our animal model, where the pups in all five diet groups were of normal weight. Thus the physical effects of carbohydrate quality on the liver may not be evident unless body fat composition is also compromised. It is possible that a longer exposure (more than 20 weeks) to the treatment would have produced differences in weight and histological findings in the liver.

In a previous study (138) 129S2/SvPas mice that had been assigned to either low GI diet or high GI diet for 25 weeks had the same total body weight, but the high GI group had significantly greater body fat mass and altered hepatic fat storage. It is possible that these mice were more ‘carbohydrate and fat sensitive’ than the obesity-prone C57BL/6 mice used in our study. It is also possible that the additional 5 weeks of exposure to the diet might have been a factor that influenced the timing of appearance and accumulation of hepatic fat deposition.
Some findings should be noted. Our study suggests there is a tendency for higher prevalence of diffuse strong staining (score 3, indicative of greater glycogen deposition) in CC, HC and HH groups (more than a third of the samples were affected) compared to LC and LL. In contrast, 75% of LL had no or only pale focal staining, while ~60% of LC had diffused moderate staining. Overall, these results imply that an exposure in utero or beyond to either chow or a high GI diet increases glycogen deposition in the liver. This is itself is not pathological but provides additional evidence that the high GI diet did indeed increase postprandial glycaemia and/or insulinaemia. Similarly, evidence of moderate lobular necrosis, was noted in the HH group (40% were affected) while LL pups had either no or light signs. This could indicate that continuing life-long exposure to a high GI diet increases the risk of pathological changes in the liver.

These findings are consistent with those of an observational study in humans that investigated the relationship of liver steatosis to dietary GI (258). The prevalence of high grade liver steatosis increased significantly across the ranges of dietary GI (from low to medium to high). The high grade steatosis in the subjects of the high GI diet was twice as frequent as in the low or medium GI diet subjects, even though there were no differences in BMI between the subjects.

The limitations of our study should be noted. In particular for this part of the study we could obtain only a small number of liver samples, particularly for the HH and LL diet groups. This was due to the small population of pups in each diet group (~10 pups/groups) and also to difficulties with the collection of the samples. It would be
desirable to repeat the study with a greater number of animals so that the power of
the study is adequate for the purpose of liver histology.

In summary, despite interesting trends, the histological findings of this study do not
show a definitive effect of the GI of the maternal and offspring’s diet on liver tissue
morphology. Further studies on a greater number of liver tissues in both older and
young animals are warranted.
Chapter 6

General discussion and conclusions
Chapter 6  General discussion and conclusions

This research project investigated the effect of two carbohydrate-modified diets during the critical period of in-utero development. Our hypothesis was that a maternal diet containing quickly digested vs. slowly digested starch would have adverse outcomes on metabolic markers and expression of key genes that increased the risk of offspring obesity. In male offspring of high vs. low GI C57BL/6 female mice, we studied effects on growth rate, food intake, glucose and insulin tolerance, and other markers of metabolism. Simultaneously, we obtained data on the expression of the Fto gene in the hypothalamus, adipose and muscle tissues, liver and placenta. We also determined leptin gene expression in visceral adipose tissue and other genes involved in regulation of appetite and energy homeostasis (AGRP, NPY, POMC, CART and LEPR) in hypothalamic tissue. Finally we examined liver histology for signs of differences arising from different maternal diets. The study design allowed for observations of both early-life and life-long exposure in response to the two carbohydrate-modified diets.

To our knowledge, this is the first study to examine the direct effects of maternal and life-long exposure to high vs. low GI feeding on Fto and leptin gene expression in offspring. Table 6.1 summarises the most important associations we were able to detect in relation to an exposure to a high vs. low GI diet. Here we highlight and interpret the findings in the context of the current scientific literature.
**Table 6.1 Significant associations of early-life and life-long exposure to a low GI diet.** For the effect of early life exposure, offspring of low GI-fed mothers were switched over to a chow diet after weaning at 4 weeks of age (LC group). The life-long exposure effect was obtained in offspring that continued a low GI diet until 20 weeks of age (LL group). The comparison group in each case is the high GI-fed group (HC and HH respectively).

<table>
<thead>
<tr>
<th>Nutritional exposure</th>
<th>Associations</th>
</tr>
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<tbody>
<tr>
<td><strong>Early-life exposure to a low GI</strong></td>
<td>• ↓ FTO in placenta&lt;br&gt;• ↑ FTO in white muscle&lt;br&gt;• ↓ FTO in red muscle&lt;br&gt;• ↑ Leptin in VAT&lt;br&gt;• ↓ AGRP in hypothalamus&lt;br&gt;• ↓ POMC in hypothalamus&lt;br&gt;• ↓ CART in hypothalamus&lt;br&gt;• ↓ LEPR in hypothalamus</td>
</tr>
<tr>
<td><strong>Extended exposure to a low GI</strong></td>
<td>• ↓ FTO gene expression in hypothalamus&lt;br&gt;• ↑ FTO expression in white muscle&lt;br&gt;• ↓ FTO expression in red muscle&lt;br&gt;• ↑ Leptin expression in VAT&lt;br&gt;• ↑ Leptin expression in SAT&lt;br&gt;• ↓ NPY expression in hypothalamus&lt;br&gt;• ↓ plasma ghrelin&lt;br&gt;• ↑ plasma leptin</td>
</tr>
</tbody>
</table>
6.1 Outcomes on phenotype and metabolic parameters

Our first hypothesis was that glucose tolerance and insulin sensitivity in the offspring of low GI-fed mothers would be higher than those of high-GI-fed mothers. In the current study, however, these parameters were not affected by either early-life nor life-long exposure to the diets. Therefore, our findings do not support our initial assumption. Similarly, we were not able to show a difference in food intake, growth or body weight between the groups. High and low GI diets have produced differences in phenotype in some human studies, with high GI diets being associated with a higher degree of fatness and higher body weight (128, 131, 259-261). Similarly, many (but not all) studies in animal models have produced differences in body fat, glucose tolerance and/or insulin resistance (175, 178, 262, 263). Pawlak et al., for example, using partially pancreatectomised pair-fed rats, reported no differences in growth rate or body weight, but significant differences in body composition, glucose tolerance and insulin sensitivity (135). Mice on the high-GI diet, however, had almost twice the body fat of those on the low GI diet after 9 weeks (135, 136). The use of pancreatectomised animals was the key because beta-cell mass is reduced, increasing the risk of impaired glucose tolerance and its metabolic sequelae.

Nutritional regimes (especially in rodents) on the association of the GI with body fatness often address other macronutrient issues at the same time, such as high vs. low fat, or high vs. low protein (175, 262, 263). We propose that using a higher fat content in our high and low GI feeds may have produced the expected differences in phenotype. Previous studies have indicated that fat is the crucial stimulus for
overconsumption, obesity and diabetes-related parameters in C57BL/6 wild-type mouse (175, 176).

In the high and low GI diets used here, the percentage of energy from fat was 7%, whereas for the chow diet it was 4.6%. In studies where the focus of the treatment diets is the fat content, any percentage below 11% is considered a low fat diet and above 50% is a high fat diet (175). Therefore, we can confidently say that the diets we used in the present study were low fat diets and this could have compromised the ability to show phenotypical differences between the groups.

In addition, the in vitro testing allowed us to test and confirm that the two commercial high and low GI diets contained starches with different rates of digestion. Since rapidly available glucose in the best predictor of glycaemic response in humans, it was likely that the high GI feed would have produced a higher postprandial glucose response. However, this testing does not necessarily translate to the glycaemic impact in animals after consumption, and it is possible that the absolute GI difference between the two feeds was not big enough to produce different effects on phenotype.

We also anticipated the concentration of circulating hormones involved in the regulation of appetite and satiety would have been differentially influenced by exposure to the two treatment diets. We showed that life-long exposure to a low GI diet was associated with a 6-fold greater fasting plasma leptin and 2-fold lower ghrelin (LL vs. HH). Even though we were not able to show a significant impact on ghrelin and leptin levels in the early-life exposed pups (LC vs. HC), the data showed
a consistent and similar trend to that observed in the life-long exposed pups (LL vs. HH). Thus we argue that in both cases, early-life nutrition appears to play an important predisposing role. The high glycaemic exposure during the gestational period has therefore produced subtle effects that appear to be exaggerated after extended exposure.

The concurrent use of a chow diet as a reference, allows us to conclude the two carbohydrate-modified diets produced responses that fell within the typical range expected of C57BL/6 mice. Chow diets, however, are not standardised and may vary in glycaemic impact because ingredients vary from time to time, as well as from manufacturer to manufacturer. The lack of information on the GI of the chow diet precludes further assumptions on the extent to which the diet after weaning affects phenotype.

We found no significant differences in the other hormones measured in fasting plasma samples (adiponectin, insulin, PYY, GLP-1, PP, ACTH, IL6 and TNFα). As discussed previously, we may have seen differences if they were accompanied by an overweight phenotype. Therefore, in view of this limitation, future research in this arena should consider the use of animal feeds from raw materials of a known GI value, and pre-tested in trained meal-fed animals. This would provide a clearer picture on the effect of maternal and later life exposure to a high vs. low GI diet. In addition, we recommend the use of feeds with a higher fat content in order to aim for phenotypical differences. Data should then be collected both before and after the phenotype appears. The recent landmark study by Solon-Biet and colleagues (264) demonstrates the importance of interactions of fat, protein and carbohydrate energy
for long term health, longevity and body composition in the C57BL/6 mouse. Their findings should therefore guide the decision on the composition of the two treatment diets. Ideally, the macronutrient (fat, protein and carbohydrate) composition of the feed should also be fine-tuned so as to produce an animal with some degree of excess adiposity.

6.2 Effects of gene expression

Our next hypothesis was that gene expression (Fto, leptin, AGRP, NPY, POMC, CART and LEPR) in key tissues would have been differentially regulated by the two treatment diets. This hypothesis was confirmed, demonstrating a likely epigenetic regulatory effect of the glycaemic impact of the diet on these genes.

In particular, the results obtained on Fto gene were consistent and robust. Maternal exposure to a high GI diet from conception onwards was associated with ~4-fold higher Fto gene expression in the placental tissues recovered at late gestation. This suggests that postprandial maternal glycaemia within the normal range has a profound influence on metabolism of the placenta, including the expression of the Fto gene. Furthermore, life-long exposure to a high GI diet was associated with 2.5-fold higher hypothalamic Fto mRNA expression. Although this trend was not evident in the early-life exposed pups, the findings in placenta as well as pups exposed for longer suggest early programming sets the scene for the future pattern of hypothalamic Fto gene expression. Since enhanced expression of Fto is associated with increased risk of obesity in rodents (83), the lower expression levels in such important tissues as the hypothalamus and the placenta suggest that a low GI diet can reduce the risk for obesity and associated disorders.
Likewise, the results we obtained on leptin gene expression were highly significant. Leptin mRNA levels in the visceral adipose tissue were >3-fold higher in the low GI-fed groups (both early-life and life-long exposure) than in the high GI-fed offspring. This, together with the results on fasting plasma leptin levels (6-fold higher in life-long exposed pups) and in parallel with the fact that these mice did not display phenotypical differences, suggests that the leptin differences are primary outcomes and not secondary to increased body weight, insulin resistance or glucose intolerance.

Although leptin is a major regulator of food intake and energy homeostasis, it is not the only one. Leptin exerts inhibitory feedback on anabolic hypothalamic peptides such as NPY and positive feedback on catabolic peptides such as POMC, the precursor of the melanocortin-3/-4 receptor agonist α-melanocyte-stimulating hormone (265). When leptin central signalling is disrupted by genetic modifications, NPY expression increases and POMC expression decreases, putting the organism into an anabolic state, and increasing the risk of developing obesity (265). So it could be predicted from an examination of the hypothalamic NPY and POMC expression, that in the low GI mice (LC and LL) mRNA NPY levels would be lower and POMC higher, reducing the risk of obesity. The opposite effect would be expected for the high GI groups (HC and HH) since their leptin gene expression in the VAT was significantly lower.

Indeed, there was a clear trend for NPY gene expression in the hypothalamus. There was ~3-fold lower NPY mRNA expression level in the LL group, and we observed a similar trend in the LC group. This implies a reduced risk of obesity. However, the results on POMC expression were puzzling. POMC mRNA expression in LC was
significantly lower than HC, suggesting lower levels of POMC in the circulation and increased risk of obesity. No differences were observed between the long-life exposed groups (HH and LL).

Overall, however, there are much greater absolute differences in the expression of orexigenic genes, AGRP and NPY in HH vs. LL, with no significant differences in the anorexigenic genes, POMC and CART. This implies greater anabolic effects and greater risk of obesity as a result of life-long exposure to a high GI diet vs. low GI diet. The findings confirm that the source of carbohydrate has important epigenetic and/or programming effects on the developing hypothalamus, but the net result on appetite and energy balance is difficult to predict.

Taken together, our novel findings provide molecular insights into how differences in carbohydrate nutrition, particularly the rate of digestion and/or maternal post-prandial hyperglycaemia, provoke differences in the regulation of the expression of obesity associated genes as well as regulation of orexigenic and anorexigenic genes in the brain’s appetite centre, the hypothalamus. Thus, epigenetic changes are a potential mechanism that underlines the observed changes in gene expression in the present study and need to be further investigated in future studies.

Other genes should also receive attention in the context of maternal dietary carbohydrate. Godfrey and colleagues showed that higher methylation of the retinoid X receptor-α (RXRA) on chromosome 9, was associated with fat mass and % fat mass in two cohorts of British children (266). Interestingly, the degree of methylation was also linked to lower maternal carbohydrate intake in early
pregnancy and higher neonatal adiposity in this population. Thus epigenetic studies on the degree of methylation or histone modification on genes such as RXRA will provide a more in depth understanding of the how the quality of carbohydrate regulates influences the risk of obesity.

Recent research has also identified a novel gene located in the broad neighbourhood of FTO called Iroquois homeobox protein 3 gene (IRX3) (52). IRX3 encodes a transcription factor involved in regulating the expression of other genes (including FTO) which is highly expressed in the brain, and consistent with a role in regulating energy metabolism and eating behaviour. Although IRX3 is located far from FTO gene, the researchers showed that the obesity-associated regions in FTO were physically in contact with the promoter of IRX3. Mice lacking the IRX3 gene weighed 25–30% less than mice with a functional IRX3 gene and did not gain weight on a high-fat diet and were resistant to metabolic disorders such as diabetes. In view of these findings, it would be informative to determine IRX3 expression in future research.

6.3 Histological findings

In the present study, despite interesting trends, the histological findings did not show a definitive effect of the GI of the maternal and offspring’s diet on liver tissue morphology. Thus our hypothesis that a high GI diet consumed in pregnancy or beyond increases the risk of liver pathology in the offspring, is not supported.

However there was a tendency for higher prevalence of diffuse strong glycogen deposition in CC, HC and HH groups (more than a third of the samples were affected)
compared to LC and LL. Overall, these results imply that an exposure in utero or beyond to either chow or a high GI diet increases glycogen deposition in the liver. This in itself is not pathological but provides additional evidence that the high GI diet did indeed increase postprandial glycaemia and/or insulinemia.

For the purpose of liver histology the study needed a greater number of samples. A future study on the effects of maternal high vs. low GI diet on liver histology should include a greater number of samples to increase the statistical power of the outcomes.

6.4 Summary of the effect of prenatal exposure to the low GI diet

The findings of the present study are novel and provide a unique first approach of the effects of low GI diet during the prenatal period and lactating period. We show that FTO gene expression was 4-fold lower in the placentas of the low GI-fed mothers. Though placental programming by dietary manipulation is not yet widely researched and understood, animals studies on adaptations in placental phenotype have been observed in response to severely restricted dietary manipulations, and showed a reduction of the nutrient transfer capacity of the placenta (104). And in diabetic pregnancies, studies show that maternal gestational hyperglycemia may be involved in the pathogenesis of IR, impaired glucose tolerance, type 2 diabetes mellitus, the Metabolic Syndrome and subsequent cardiovascular diseases in adult offspring. A low GI maternal diet rather than a low carbohydrate diet, has been associated with measurable benefits to the offspring such as fetal and placental insulin and glucose regulation, fetal growth, birth weight and offspring adiposity (131, 133, 210).
Therefore, the differences in Fto gene expression in the placenta observed in the present study suggest that postprandial glycaemia, rather than the dietary carbohydrate content, is the mechanism that regulates placental gene expression.

We also show that Fto gene expression was higher in the white muscle and lower in the red muscle, indicating the possibility that carbohydrate quality during gestation can differentially influence the expression of genes in the muscle, in a way dependant to the nature and function of the particular muscle type. Furthermore we show that leptin gene expression in the visceral adipose tissue was 4.4-fold higher in the pups exposed to a low GI diet early in life. We assume that this high leptin gene expression in the low GI fed offspring results in higher leptin protein production and therefore greater satiety, and over the longer term, has the potential to reduce the risk of excessive food intake and obesity.

Finally, our findings indicate that the offspring of low GI mothers display important differences in expression of key hypothalamic appetite-regulating genes such as AGRP, POMC, CART and LEPR. This shows that the well recognised co-expression of AGRP/NPY and POMC/CART is regulated by the nature of the dietary carbohydrates and therefore, differences in carbohydrate nutrition, including rate of digestion and/or maternal post-prandial hyperglycaemia can provoke differences in the regulation of orexigenic and anorexigenic genes in the hypothalamus. We propose that the source of carbohydrate has important epigenetic or programming effects on the developing hypothalamus, but the net result on appetite and energy balance is difficult to predict.
6.5 Strengths and weaknesses of this study

The strengths of our study include the use of an animal model which allowed us to observe the early in-utero impact of the maternal diet on the expression of Fto and leptin genes in prenatal tissue (the placenta) and in adult tissues of the offspring. This is a valuable tool which brings power to the early programming theory. Furthermore, the fact that the treatment diets were identical apart from the source of the carbohydrate allows us to separate the effect of quantity of carbohydrate and other macronutrients from the quality of carbohydrate. The in vitro starch digestion we conducted on the commercial feeds confirmed that the rate at which glucose from each diet becomes available for absorption varied according to their GI. This reinforces the results obtained as it demonstrates it is the quality of carbohydrate and not the amount that has an epigenetic impact.

The weaknesses of our study should be noted. The biggest limitation is the use of a mouse model to test our hypotheses. It cannot be assumed that human offspring will display similar up-regulation of FTO in response to differences in the GI of maternal diet. Mice have evolved along a different evolutionary pathway with different native diet to humans. As an alternative, non-human primates could be employed although this would be expensive and difficult to undertake with large numbers of animals. Likewise, as humans, we consume real foods and mixed meals, and rarely do we consume a diet that has been manipulated in carbohydrate alone as was the case in the present study. Also, body composition measurements (total body fat, epididymal fat) and tissue/organ weights of the pups would have provided important insights and better understanding of some of the outcomes in the present study. However, we placed the highest priority on extracting and snap-freezing all the tissues as fast as
possible to increase the reliability of the gene expression studies. Finally, we need to consider that an exposure to an obesogenic diet post weaning, as we can considered the High GI, is required to bring out programmed phenotypes characterised by obesity related indicators.

6.6 Significance of this study

Obesity is a complex metabolic disease that is characterised by the interaction of obesity susceptibility gene variants with diet composition and other environmental factors. The investigation of gene–diet interactions is becoming increasingly important in the attempt to understand the etiology and pathophysiology of nutrition-related disorders. The knowledge that some macronutrients can interact with obesity-associated genetic variants presents the opportunity for more effective preventative intervention and provides the basis for personalised nutrition therapy that will more effectively address the current epidemic of obesity.

The definition of functional foods is not universal but the most common one is: “foods or dietary components that may provide a health benefit beyond providing basic nutrition” (19). Nutrition and food science are making rapid advances. Only 25 years ago, the brain was believed to be insulin-insensitive, but today it is widely accepted that insulin is a central regulator of neural function and hippocampal metabolism, and a key player in multiple neuropsychological conditions (267). Today, our carbohydrate foods are more likely to be highly processed (or even ultra-processed), more finely milled, “instantised”, and separated from natural components such as fibre that act as a barrier to slow enzymic digestion. The quality of carbohydrate in the human diet has therefore undergone a rapid change that dictates
marked increases in postprandial glycaemia and insulinaemia. Hence a low GI diet, by improving postprandial glucose homeostasis and insulin action throughout life, represents an inexpensive, safe and realistic alternative in the context of behaviourally sustainable nutrition. During pregnancy it has the potential to epigenetically program the offspring to lower the risk of obesity and metabolic disorders. The choice of low GI carbohydrates therefore has not only a therapeutic basis, but one that is without the inherent risks associated with lower carbohydrate, higher protein and higher fat diets (268).
References


adulthood may have greater impact than reducing obesity prevalence in childhood. Obes Rev 2013;14(7):523-531.


84. Tung YCL, Ayuso E, Shan XY, Bosch F, O'Rahilly S, Coll AP, Yeo GSH. Hypothalamic-Specific Manipulation of Fto, the Ortholog of the Human Obesity Gene FTO, Affects Food Intake in Rats. Plos One 2010;5(1).


102. Mahmood S, Smiraglia DJ, Srinivasan M, Patel MS. Epigenetic changes in hypothalamic appetite regulatory genes may underlie the developmental


151. Thomas DE, Elliott EJ, Baur L. Low glycaemic index or low glycaemic load diets for overweight and obesity. Cochrane Database of Systematic Reviews 2007(3).


212. Seo SG. A maternal high glycaemic index diet alters central appetite gene expression in offspring at weaning. Thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Medical Science (Honours) School of Molecular Bioscience, University of Sydney, October 2013.


## Appendix 1

Associations between variants of the FTO gene and obesity traits
(BMI, waist circumference/ waist–hip ratio, fat percentage/ fat mass)

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<tr>
<th>FTO variant</th>
<th>Associated trait</th>
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<tr>
<td>rs1121980</td>
<td>BMI</td>
</tr>
<tr>
<td>rs1558902</td>
<td>BMI, WAIST</td>
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<td>rs9939609</td>
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<tr>
<td>rs8050136</td>
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<td>rs12149832</td>
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<td>rs1421085</td>
<td>BMI, Fat %, WAIST</td>
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</table>

## Appendix 2

**Modified AIN93G Rodent Diet all CHO as Dextrinised Starch**

SF10-081

A semi-pure diet formulation for laboratory rats and mice based on AIN-93G.
- All CHO has been replaced with dextrinised starch.
- This diet is designed to have a high glycaemic index.

<table>
<thead>
<tr>
<th>Calculated Nutritional Parameters</th>
<th>Ingredients</th>
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</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Casein (Acid)</td>
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<tr>
<td>Total Fat</td>
<td>Dextrinised Starch</td>
</tr>
<tr>
<td>Crude Fibre</td>
<td>Canola Oil</td>
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<td>DL Methionine</td>
</tr>
<tr>
<td>% Total calculated digestible energy from lipids</td>
<td>Calcium Carbonate</td>
</tr>
<tr>
<td>% Total calculated digestible energy from protein</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td></td>
<td>AIN93 Trace Minerals</td>
</tr>
<tr>
<td></td>
<td>Potassium Citrate</td>
</tr>
<tr>
<td></td>
<td>Potassium Dihydrogen Phosphate</td>
</tr>
<tr>
<td></td>
<td>Potassium Sulphate</td>
</tr>
<tr>
<td></td>
<td>Choline Chloride (75%)</td>
</tr>
<tr>
<td></td>
<td>AIN93 Vitamins</td>
</tr>
</tbody>
</table>

**Diet Form and Features**
- Semi pure diet, 12 mm diameter pellets.
- Pack size 5 Kg, vacuum packed in oxygen impermeable plastic bags, under nitrogen. Bags are packed into cardboard cartons to protect them during transit. Smaller pack quantity on request.
- Diet suitable for irradiation but not suitable for autoclave.
- Lead time 2 weeks for non-irradiation or 4 weeks for irradiation.
<table>
<thead>
<tr>
<th>Calculated Amino Acids</th>
<th>Calculated Total Vitamins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valine</td>
<td>Vitamin A (Retinol)</td>
</tr>
<tr>
<td>Leucine</td>
<td>4 000 IU/Kg</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Vitamin D (Cholecalciferol)</td>
</tr>
<tr>
<td>Threonine</td>
<td>1 000 IU/Kg</td>
</tr>
<tr>
<td>Methionine</td>
<td>Vitamin E (a Tocopherol acetate)</td>
</tr>
<tr>
<td>Cystine</td>
<td>78 mg/Kg</td>
</tr>
<tr>
<td>Lysine</td>
<td>Vitamin K (Menadione)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1 mg/Kg</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Vitamin C (Ascorbic acid)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>None added</td>
</tr>
<tr>
<td></td>
<td>Vitamin B1 (Thiamine)</td>
</tr>
<tr>
<td></td>
<td>6.1 mg/Kg</td>
</tr>
<tr>
<td></td>
<td>Vitamin B2 (Riboflavin)</td>
</tr>
<tr>
<td></td>
<td>6.3 mg/Kg</td>
</tr>
<tr>
<td></td>
<td>Niacin (Nicotinic acid)</td>
</tr>
<tr>
<td></td>
<td>30 mg/Kg</td>
</tr>
<tr>
<td></td>
<td>Vitamin B6 (Pryridoxine)</td>
</tr>
<tr>
<td></td>
<td>7 mg/Kg</td>
</tr>
<tr>
<td></td>
<td>Pantothenic Acid</td>
</tr>
<tr>
<td></td>
<td>16.5 mg/Kg</td>
</tr>
<tr>
<td></td>
<td>Biotin</td>
</tr>
<tr>
<td></td>
<td>200 µg/Kg</td>
</tr>
<tr>
<td></td>
<td>Folic Acid</td>
</tr>
<tr>
<td></td>
<td>2 mg/Kg</td>
</tr>
<tr>
<td></td>
<td>Inositol</td>
</tr>
<tr>
<td></td>
<td>None added</td>
</tr>
<tr>
<td></td>
<td>Vitamin B12 (Cyancobalamin)</td>
</tr>
<tr>
<td></td>
<td>103 µg/Kg</td>
</tr>
<tr>
<td></td>
<td>Choline</td>
</tr>
<tr>
<td></td>
<td>1 470 mg/Kg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Calculated Total Minerals</th>
<th>Calculated Fatty Acid Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>Myristic Acid 14:0</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>Palmitic Acid 16:0</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.32%</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.10%</td>
</tr>
<tr>
<td>Chloride</td>
<td>0.12%</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.40%</td>
</tr>
<tr>
<td>Sulphur</td>
<td>0.23%</td>
</tr>
<tr>
<td>Iron</td>
<td>75 mg/Kg</td>
</tr>
<tr>
<td>Copper</td>
<td>7.3 mg/Kg</td>
</tr>
<tr>
<td>Iodine</td>
<td>0.2 mg/Kg</td>
</tr>
<tr>
<td>Manganese</td>
<td>18 mg/Kg</td>
</tr>
<tr>
<td>Cobalt</td>
<td>No data</td>
</tr>
<tr>
<td>Zinc</td>
<td>53 mg/Kg</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>0.15 mg/Kg</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.3 mg/Kg</td>
</tr>
<tr>
<td>Cadmium</td>
<td>No data</td>
</tr>
<tr>
<td>Chromium</td>
<td>1.0 mg/Kg</td>
</tr>
<tr>
<td>Fluoride</td>
<td>1.0 mg/Kg</td>
</tr>
<tr>
<td>Lithium</td>
<td>0.1 mg/Kg</td>
</tr>
<tr>
<td>Boron</td>
<td>2.1 mg/Kg</td>
</tr>
<tr>
<td>Nickel</td>
<td>0.5 mg/Kg</td>
</tr>
<tr>
<td>Vanadium</td>
<td>0.1 mg/Kg</td>
</tr>
</tbody>
</table>

Calculated data uses information from typical raw material composition. It could be expected that individual batches of diet will vary from this figure. Diet post treatment by irradiation or auto clave could change these parameters. We are happy to provide full calculated nutritional information for all of our products, however we would like to emphasise that these diets have been specifically designed for manufacture by Specialty Feeds.
## Modified AIN93G Rodent Diet all CHO as Gel Crisp Starch

**SF10-084**

A semi-pure diet formulation for laboratory rats and mice based on AIN-93G.
- All CHO has been replaced with Gel Crisp starch.
- This diet is designed to have a low glycaemic index.

### Calculated Nutritional Parameters

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>19.40%</td>
</tr>
<tr>
<td>Total Fat</td>
<td>7.00%</td>
</tr>
<tr>
<td>Crude Fibre</td>
<td>4.70%</td>
</tr>
<tr>
<td>AD Fibre</td>
<td>4.70%</td>
</tr>
<tr>
<td>Digestible Energy</td>
<td>16.3 MJ/Kg</td>
</tr>
<tr>
<td>% Total calculated digestible energy from lipids</td>
<td>16.00%</td>
</tr>
<tr>
<td>% Total calculated digestible energy from protein</td>
<td>21.00%</td>
</tr>
</tbody>
</table>

### Ingredients

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (Acid)</td>
<td>200 g/Kg</td>
</tr>
<tr>
<td>Gel Crisp Starch</td>
<td>636 g/Kg</td>
</tr>
<tr>
<td>Canola Oil</td>
<td>70 g/Kg</td>
</tr>
<tr>
<td>Cellulose</td>
<td>60 g/Kg</td>
</tr>
<tr>
<td>DL Methionine</td>
<td>3.0 g/Kg</td>
</tr>
<tr>
<td>Calcium Carbonate</td>
<td>13.1 g/Kg</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>2.6 g/Kg</td>
</tr>
<tr>
<td>AIN93 Trace Minerals</td>
<td>1.4 g/Kg</td>
</tr>
<tr>
<td>Potassium Citrate</td>
<td>2.5 g/Kg</td>
</tr>
<tr>
<td>Potassium Dihydrogen Phosphate</td>
<td>6.9 g/Kg</td>
</tr>
<tr>
<td>Potassium Sulphate</td>
<td>1.6 g/Kg</td>
</tr>
<tr>
<td>Choline Chloride (75%)</td>
<td>2.5 g/Kg</td>
</tr>
<tr>
<td>AIN93 Vitamins</td>
<td>10 g/Kg</td>
</tr>
</tbody>
</table>

### Diet Form and Features

- Semi pure diet: 12 mm diameter pellets.
- Pack size 5 Kg, vacuum packed in oxygen impermeable plastic bags, under nitrogen. Bags are packed into cardboard cartons to protect them during transit. Smaller pack quantity on request.
- Diet suitable for irradiation but not suitable for autoclave.
- Lead time 2 weeks for non-irradiation or 4 weeks for irradiation.
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<th>Calculated Amino Acids</th>
<th>Calculated Total Vitamins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valine</td>
<td>Vitamin A (Retinol)</td>
</tr>
<tr>
<td>1.30%</td>
<td>4 000 IU/Kg</td>
</tr>
<tr>
<td>Leucine</td>
<td>Vitamin D (Cholecalciferol)</td>
</tr>
<tr>
<td>1.80%</td>
<td>1 000 IU/Kg</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Vitamin E (a Tocopherol acetate)</td>
</tr>
<tr>
<td>0.90%</td>
<td>78 mg/Kg</td>
</tr>
<tr>
<td>Threonine</td>
<td>Vitamin K (Menadione)</td>
</tr>
<tr>
<td>0.80%</td>
<td>1 mg/Kg</td>
</tr>
<tr>
<td>Methionine</td>
<td>Vitamin C (Ascorbic acid)</td>
</tr>
<tr>
<td>0.80%</td>
<td>None added</td>
</tr>
<tr>
<td>Cystine</td>
<td>Vitamin B1 (Thiamine)</td>
</tr>
<tr>
<td>0.06%</td>
<td>6.1 mg/Kg</td>
</tr>
<tr>
<td>Lysine</td>
<td>Vitamin B2 (Riboflavin)</td>
</tr>
<tr>
<td>1.50%</td>
<td>6.3 mg/Kg</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Niacin (Nicotinic acid)</td>
</tr>
<tr>
<td>1.00%</td>
<td>30 mg/Kg</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Vitamin B6 (Pyrindoxine)</td>
</tr>
<tr>
<td>1.00%</td>
<td>7 mg/Kg</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Pantothenic Acid</td>
</tr>
<tr>
<td>0.30%</td>
<td>16.5 mg/Kg</td>
</tr>
<tr>
<td></td>
<td>Biotin</td>
</tr>
<tr>
<td></td>
<td>200 ug/Kg</td>
</tr>
<tr>
<td></td>
<td>Folic Acid</td>
</tr>
<tr>
<td></td>
<td>2 mg/Kg</td>
</tr>
<tr>
<td></td>
<td>Inositol</td>
</tr>
<tr>
<td></td>
<td>None added</td>
</tr>
<tr>
<td></td>
<td>Vitamin B12 (Cyanocobalamin)</td>
</tr>
<tr>
<td></td>
<td>103 ug/Kg</td>
</tr>
<tr>
<td></td>
<td>Choline</td>
</tr>
<tr>
<td></td>
<td>1 470 mg/Kg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Calculated Total Minerals</th>
<th>Calculated Fatty Acid Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>Myristic Acid 14:0</td>
</tr>
<tr>
<td>0.47%</td>
<td>Trace</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>Palmitic Acid 16:0</td>
</tr>
<tr>
<td>0.32%</td>
<td>0.30%</td>
</tr>
<tr>
<td>Magnesium</td>
<td>Stearic Acid 18:0</td>
</tr>
<tr>
<td>0.10%</td>
<td>0.10%</td>
</tr>
<tr>
<td>Sodium</td>
<td>Palmitoleic Acid 16:1</td>
</tr>
<tr>
<td>0.12%</td>
<td>No data</td>
</tr>
<tr>
<td>Chloride</td>
<td>Oleic Acid 18:1</td>
</tr>
<tr>
<td>0.16%</td>
<td>3.90%</td>
</tr>
<tr>
<td>Potassium</td>
<td>Gadolec Acid 20:1</td>
</tr>
<tr>
<td>0.40%</td>
<td>0.10%</td>
</tr>
<tr>
<td>Sulphur</td>
<td>Linoleic Acid 18:2 n6</td>
</tr>
<tr>
<td>0.23%</td>
<td>1.50%</td>
</tr>
<tr>
<td>Iron</td>
<td>a Linolenic Acid 18:3 n3</td>
</tr>
<tr>
<td>75 mg/Kg</td>
<td>0.98%</td>
</tr>
<tr>
<td>Copper</td>
<td>Arachadonic Acid 20:4 n6</td>
</tr>
<tr>
<td>7.3 mg/Kg</td>
<td>No data</td>
</tr>
<tr>
<td>Iodine</td>
<td>EPA 20:5 n3</td>
</tr>
<tr>
<td>0.2 mg/Kg</td>
<td>No data</td>
</tr>
<tr>
<td>Manganese</td>
<td>DHA 22:6 n3</td>
</tr>
<tr>
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</tr>
<tr>
<td>Cobalt</td>
<td>Total n3</td>
</tr>
<tr>
<td>No data</td>
<td>0.98%</td>
</tr>
<tr>
<td>Zinc</td>
<td>Total n6</td>
</tr>
<tr>
<td>53 mg/Kg</td>
<td>1.51%</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>Total Mono Unsaturated Fats</td>
</tr>
<tr>
<td>0.15 mg/Kg</td>
<td>3.98%</td>
</tr>
<tr>
<td>Selenium</td>
<td>Total Polyunsaturated Fats</td>
</tr>
<tr>
<td>0.3 mg/Kg</td>
<td>2.50%</td>
</tr>
<tr>
<td>Cadmium</td>
<td>Total Saturated Fats</td>
</tr>
<tr>
<td>No data</td>
<td>0.50%</td>
</tr>
<tr>
<td>Chromium</td>
<td></td>
</tr>
<tr>
<td>1.0 mg/Kg</td>
<td></td>
</tr>
<tr>
<td>Fluoride</td>
<td></td>
</tr>
<tr>
<td>1.0 mg/Kg</td>
<td></td>
</tr>
<tr>
<td>Lithium</td>
<td></td>
</tr>
<tr>
<td>0.1 mg/Kg</td>
<td></td>
</tr>
<tr>
<td>Boron</td>
<td></td>
</tr>
<tr>
<td>2.1 mg/Kg</td>
<td></td>
</tr>
<tr>
<td>Nickel</td>
<td></td>
</tr>
<tr>
<td>0.5 mg/Kg</td>
<td></td>
</tr>
<tr>
<td>Vanadium</td>
<td></td>
</tr>
<tr>
<td>0.1 mg/Kg</td>
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</tr>
</tbody>
</table>

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Appendix 3

Summary of adverse and unexpected event

On 25th February 2011 we noticed 4 dead pregnant female mice. Mice seemed fine for the majority of the time before the event but the last 2-3 days started losing weight and unexpectedly died. The same happened with other female pregnant or lactating mice in the following weeks. All mice (females and males) were monitored daily and weights were taken regularly. No other symptoms were observed except a rapid weight loss.

From the first event, the veterinary doctor was contacted and a post mortem examination was conducted. Together with the dead lactating females, all pups were culled due to starvation. From the 16 female adult mice, 7 were found dead. All male mice (n=8) survived. The mice were initially divided into 2 groups following either a low glycaemic or a high glycaemic irradiated diet. Trio breeding was used according to the approved protocol. On 8th March, the protocol was terminated and after consultation with the vet, all mice changed to a chow diet with addition of a vitamin supplement and a hydration gel, to monitor developments. From that day onwards, mice were looking better and gaining weight. Nevertheless, mice that survived were still excluded from the protocol and given to other researchers for different projects.

The outcomes of the post mortem examination and the veterinary doctor’s report were as follows: “Post mortem examinations were conducted on lactating females exhibiting weight loss and pups from affected mothers. Histological examination of a wide range of organs revealed occasional mild to moderate lesions in a small
proportion of tissues in the mothers but these were not consistent; there were no significant abnormalities in the pups. Serological tests for 14 infectious agents were negative and haematological assessment of one mother was unremarkable.

Despite comprehensive investigation, there were no findings that pointed to a specific cause for this problem. There are, however, several features of the case which suggest an association with the specially formulated diets. These include the recovery of the animals following reinstatement of standard diet and the fact that no similar problems were observed in other animals on standard diet elsewhere in the facility during the time of the illness.

After discussion with the manufacturer of the specialty diets, it was established that both diets contained the same raw materials as diets supplied to other customers around the same time, none of whom had reported problems. It is also worth noting that most of the ingredients in these diets are human food grade so should be of high quality.

The one suspect factor here appears to be the use of irradiation to decontaminate the feed. Irradiation is used widely for this purpose and since it is known that certain vitamins can be partially broken down during the irradiation process, many standard rodent diets are fortified with key nutrients to prevent deficiency. In the present case, however, the diets were based on a special dietary formula (AIN-93G) developed for nutritional studies. While this formula is designed to meet the increased nutritional demands of pregnancy and lactation, it is not fortified to make up for possible losses during treatments such as irradiation. The fact that only lactating females developed
any detectable illness suggests that any nutrient losses due to the irradiation would only be marginal since the males (which of course were not facing the same nutritional demands as the lactating females) remained healthy.

It is also at least theoretically possible that the irradiation treatment had induced the formation of toxic oxidation products in the feed. If this had been the case, however, one would have expected both sexes to be affected; it would also be expected that a similar effect would occur when standard diet is irradiated but the widespread and successful use of irradiated standard diets not support this. It is also believed that toxic oxidation products are more likely to be a problem in diets containing much higher levels of fat that is the case with the diets in this case.

In conclusion, I believe there is strong circumstantial evidence that this outbreak was the result of a nutritional deficiency induced by nutrient loss during irradiation of the feed. Unfortunately, confirmation by nutrient assay is not feasible due to technical complexities and lack of sufficient quantities of specimen for analysis.”

In view of the above findings, in collaboration with the vet and after consulting researchers in other universities doing similar work, we retained that the low and high GI diets were damaged during the irradiation and therefore many nutrients important for pregnancy and lactation e.g. folate, vitamin A, were lost or significantly decreased. This could have led to insufficient nutrient intake from the female pregnant and lactating mice. Our next action was to restart the protocol with new mice using a new batch of the low GI and high GI diets that was not irradiated. The diets were ordered from Australia (Specialty Feeds) and thus the risk of bacterial
infection was minimized. While it was important to consider the contamination risks posed by non-irradiated feed in animal facilities, these risks were greatly reduced with the sort of semi-pure custom diets used in the present study since they were based on human food grade ingredients. Furthermore, non-irradiated special diets were used in other animal facilities for some years without evidence of infection being introduced. The new trial started in October 2011.
Appendix 4

List of chemicals

• Amyloglucosidase (Megazyme International Ireland Ltd. Bray Co., Ireland)
• Chloroform (Sigma-Aldrich)
• Eosin
• Ethanol (200 proof) (Sigma-Aldrich)
• Fast SYBR® Green Master Mix (Applied Biosystems)
• Formaldehyde (37% (w/v)) (Sigma-Aldrich)
• Glucose oxidase-peroxidase (Megazyme International Ireland Ltd. Bray Co., Ireland)
• Haematoxylin
• Insulin (Actrapid Novo Nordisk Pharmaceuticals Pty Ltd)
• Isopropanol (Sigma-Aldrich)
• Multiplex Map Kit by Cardinal Bioresearch Pty Ltd (New Farm, Qld, Australia)
• Oligonucleotides (Sigma Genosys, Sigma-Aldrich)
• Porcine pancreatic α-amylase (Sigma-Aldrich)
• QIAzol™ lysis reagent (Qiagen)
• RNeasy Lipid Tissue Mini Kit (Qiagen)
• RNeasy Mini Kit (Qiagen)
• Superscript® VILO™ cDNA Synthesis Kit (Life Technologies, Australia)
• Xylene