The effect of diet-induced maternal obesity on offspring energy balance in a murine model and the therapeutic potential of a maternal dietary intervention with a fibre supplement

Maragkoudaki, Xanthi

Awarding institution:
King's College London

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The effect of diet-induced maternal obesity on offspring energy balance in a murine model and the therapeutic potential of a maternal dietary intervention with a fibre supplement.

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A thesis submitted to King’s College London for the degree of Doctor of Philosophy in the Women’s Health Research

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Abstract

Introduction: Obesity now affects nearly 1 in 3 adults in the UK. It is estimated that 20% of pregnant women are obese. Increasing evidence associate obesity in pregnancy with susceptibility to obesity and metabolic syndrome in the child. Here an established mouse model of maternal obesity was employed to investigate energy balance and glucose metabolism in the offspring. Polydextrose (PDX) has been shown to improve glucose metabolism and, therefore may be beneficial in obese pregnancy.

Hypothesis: It was hypothesised that (a) maternal obesity has adverse effects on offspring energy balance and glucose metabolism and that (b) these adverse effects will be prevented by supplementation of the maternal diet with PDX during pregnancy and lactation. Moreover, it was investigated whether PDX supplementation in obese pregnancy is protective against the adverse influences of an obesogenic dietary exposure in adulthood.

Methods: Female mice were fed a control or an obesogenic diet, 6-weeks before mating and throughout pregnancy and lactation. A cohort of the obese dams was assigned to supplementation with 5% PDX in pregnancy and lactation. Maternal profiles were assessed during pregnancy. At 3 and 6-months of age offspring energy intake (EI), energy expenditure (EE) and Respiratory Exchange Ratio (RER) were measured by indirect calorimetry and glucose-tolerance-tests were performed. At 3-months the animals were challenged for 3-weeks with an obesogenic diet before re-estimation of EI, EE, and RER. Microbiota composition, mitochondria copy number and UCP gene expression was assessed as potential underlying mechanisms.

Results: Maternal supplementation with PDX improved reproductive success, increased water intake and decreased markers of inflammation during gestation in the dams. At 3 months of age, offspring of obese dams (OffOb) metabolic
parameters did not differ from offspring of control dams (OffCon). At 6 months OffOb were heavier (P<0.01), had lower RER (P<0.05) and lower EE (P<0.001) compared to OffCon. OffOb had impaired glucose metabolism compared to OffCon (P<0.05). Maternal supplementation with PDX prevented these defects. Following 3-weeks obesogenic dietary challenge OffOb demonstrated hyperphagia, decreased EE (P<0.05) and subsequently greater weight gain compared to controls (P<0.05), which were prevented by maternal PDX supplementation. Maternal obesity resulted in decreased mitochondria copy number at 30 days of age and altered microbiota composition at 6 months of age, which may mediate the changes observed later in life. Maternal supplementation with PDX, prevented mitochondrial dysfunction, increased the number of beneficial microbiota and the expression of UCP1 and 3 genes.

**Conclusions:** Maternal obesity adversely influences offspring energy balance, which is prevented by maternal intervention with PDX. PDX may, therefore, provide a potential therapeutic intervention in preventing the transgenerational acceleration of obesity.
Acknowledgements

I would like to express my heartfelt gratitude to my supervisors Dr Paul Taylor and Professor Lucilla Poston. First of all, I sincerely thank them for offering me the opportunity to do this PhD. I am also thankful for the opportunities to supervise students and to present at scientific conferences. But most importantly I am grateful for all the time and effort they dedicated to guide me, teach me and support me during these four years.

I would, also, like to acknowledge and thank Biological Sciences Research Council (BBSRC), Tate and Lyle and Tommy’s charities for sponsoring my project and my attendance to research conferences.

To continue, many thanks go to all the members of my research group and specifically Dr Anne Maj Samuelsson, and Dr Timothy South for offering me their help and advice whenever I needed it. I am, also, sincerely grateful to Mr Joaquim Pombo for all his help with in vivo work, as without him taking any breaks or even getting sick would have been impossible.

Moreover, I would like to thank Dr Anna Czajka for her time and effort on the mitochondria copy numbers experiments; Dr Emilie Stolarczyk for her help and supervision in relation to the microbiota composition; and Dr Georgia Papacleovoulou for her help with the adipocytokines measurements.

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Declaration

Unless specifically stated in the text, all work described in this thesis is my own, completed under the supervision of Dr Paul Taylor and Professor Lucilla Poston.

The following work was carried out in collaboration with external parties:

- Mitochondrial copy numbers were measured under the supervision of Mrs Anna Czajka (MitoDNA lab, King’s College London, UK).
- Microbiota composition analysis was performed under the supervision of Emilie Stolarczyk (Nutrition and Diabetes, King’s College London, UK).

No part of this thesis has been previously accepted for, or is currently being submitted for another degree.

Xanthi Maragkoudaki

April 2014
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<tr>
<td>AgRP</td>
<td>Agouti-related peptide</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate Nucleus</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>BIA</td>
<td>Bioelectrical Impedance Analysis</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>BMR</td>
<td>Basal Metabolic Rate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid protein</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>DIT</td>
<td>Diet-induced Thermogenesis</td>
</tr>
<tr>
<td>DOHaD</td>
<td>Developmental Origins of Health and Disease</td>
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<tr>
<td>ECW</td>
<td>Extracellular Water</td>
</tr>
<tr>
<td>EE</td>
<td>Energy Expenditure</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
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<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
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<td>FISH</td>
<td>Fluorescence In Situ Hybridization</td>
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<tr>
<td>FFA</td>
<td>Free Fatty Acid</td>
</tr>
<tr>
<td>FFM</td>
<td>Free Fat Mass</td>
</tr>
<tr>
<td>FM</td>
<td>Fat Mass</td>
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<tr>
<td>G</td>
<td>Gram</td>
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<td>GDM</td>
<td>Gestational Diabetes Mellitus</td>
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<td>Glucose Tolerance Test</td>
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<td>KCAL</td>
<td>Kilogram Calorie</td>
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<td>Acronym</td>
<td>Definition</td>
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<td>KG</td>
<td>Kilogram</td>
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<tr>
<td>LGA</td>
<td>Large for Gestational Age</td>
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<td>MC4</td>
<td>Melanocortin receptor 4</td>
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<td>Minutes</td>
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<td>Mitochondrial DNA</td>
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<td>Mt/N</td>
<td>Mitochondrial/nuclear ratio</td>
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<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<td>Non Alcoholic Fatty Liver Disease</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>OD</td>
<td>Obesogenic Diet</td>
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<td>Offspring of Control Dam</td>
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<td>Offspring of Obese Dam given polydextrose</td>
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<td>Polydextrose</td>
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<td>POMC</td>
<td>Pro-opiomelanocortin</td>
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<td>PVN</td>
<td>Paraventricular nucleus</td>
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<tr>
<td>RER</td>
<td>Respiratory Exchange Ratio</td>
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<td>Respiratory Quotient</td>
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<td>Resting Metabolic Rate</td>
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<td>Ribonucleic acid</td>
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Chapter 1

Introduction
Chapter 1: Introduction

1.1 Obesity in pregnancy

1.1.1 The global obesity epidemic

Only two decades ago, obesity was not considered to be a public health problem as relatively few individuals were affected. However, during the last twenty years obesity has increased rapidly and now we are confronted with the obesity pandemic. The World Health Organization (FAO/WHO) (World Health Organization, 1997) first acknowledged obesity as a public health problem in 1997, since then obesity has become one of the main economic and health burdens of the 21st century (Organization, 2000; Ogunlewe et al., 2008).

Obesity is characterized by abnormal or excessive fat accumulation. It is defined, according to WHO, as a Body Mass Index (BMI) exceeding 30 kg/m\(^2\) and a person is considered to be overweight if their BMI is above 25 kg/m\(^2\). The risk for type 2 diabetes and cardiovascular diseases increases gradually when the BMI is above 20 kg/m\(^2\) (Flegal et al., 2007). Furthermore, increasing evidence associates obesity with some types of cancer (Maruthur et al., 2009; Whitlock et al., 2012) and non-alcoholic fatty liver disease (Pagadala & McCullough, 2012; Koliaki & Roden, 2013). By tackling obesity we therefore have the potential to reduce the risk of many other chronic diseases.

The prevalence of obesity varies by country (Figure 1.1) according to WHO’s latest report (World Health Organization, 2011). In the United States, over a third of the adult population is obese (Mitchell et al., 2011). Likewise, in the UK 25% of the adult population is considered obese, categorizing it as one of the most obese nations in the world (Sutton, 2011). Overall, in Europe more than 20% of all adults are obese, the rates being higher in Eastern, Southern and Middle Europe (Ogunlewe et al., ...
Latin America is also greatly affected; in Mexico for instance, 27% of men and 57% of women were estimated to be obese in 2010 (Rtveladze et al., 2013). Also, in developing countries the prevalence of obesity and physical inactivity are constantly rising (Ogunlewe et al., 2008).

![Prevalence of obesity map](image)

**Figure 1.1 The obesity epidemic, WHO 2011** (World Health Organization, 2013)

The 20th century's radical changes in lifestyle and food abundance have inevitably made a major contribution to the obesity problem. Increased food availability along with the sedentary lifestyle that characterizes, most particularly developed countries, but more recently also low to middle income countries, appear to be the perfect “obesogenic” stimulants. In the past, man had to be physically active in order to obtain his food by hunting or cultivating, in contrast to modern societies where food supply is abundant and access to food requires little or no physical activity (Prentice, 1997).
Arguably, some people are more susceptible to obesity than others. The exact reasons underlying an individual's susceptibility are not fully understood. However, twin, adoption and multigenerational studies suggest that about 45-75% of an individual's body composition variation is attributed to genetic factors (Farooqi & O'Rahilly, 2007). The inheritable component of obesity is certainly not attributable to a single gene, but rather is thought to be “polygenic” in origin, suggesting that the combined effects of a cluster of genes would determine 'heritable' obesity (Farooqi & O'Rahilly, 2007). As many as 113 candidate genes have been associated with increased bodyweight (Alfredo Martinez et al., 2007). One example is a peroxisome proliferator-activated receptor gamma (PPARG) polymorphism that leads to a Proline to Alanine (Pro12Ala) substitution at codon 12, resulting in a less active receptor towards both synthetic and natural ligands. A meta-analysis, including 19,136 subjects demonstrated a positive association between a Pro12Ala variant and higher BMI (Mariet al., 2008). Most widely recognized is the association between obesity risk and single nucleotide polymorphisms (SNPs) in intron 1 of the Fat mass obesity gene (FTO), which is located in chromosome region 16q12.2, (Frayling et al., 2007). Of these, the most common FTO variant rs9939609, is correlated with increased BMI and subsequently with increased risk of developing diabetes from 7 years upward, and has been identified in multiple ethnic groups (Scuteri et al., 2007).

Genetic factors are not the only contributors to the predisposition to obesity. Other putative reasons to the development of obesity which have been reviewed and identified include: infections, advanced maternal age, altered circadian rhythms, reduced variability in ambient temperatures, epigenetic changes, endocrine disruptors, pharmaceutical-induced weight gain and early life exposures (under the umbrella of the ‘developmental origins of health and disease’ hypothesis) (McAllister et al., 2009). The ‘developmental origins of obesity’ forms the main subject of this
thesis, specifically in relation to the influence of maternal obesity on the offspring’s susceptibility to obesity and related disorders.

1.1.2 Obesity in pregnancy

The increasing prevalence of obesity in women of childbearing age is a paramount problem. In the USA 29% of women aged 20 to 39 are clinically obese. Figure 1.2 shows the prevalence of obesity among adult women, highlighting the severity of the problem (World Health Organization, 2013).

It is difficult to ascertain the prevalence of maternal obesity in the UK because data for maternal weight is not routinely collected in antenatal care. However, according to the Health Survey for England 2008, 18.8% of women aged 25-34 were obese and 13.9% of those aged between 16-24 (Aresu et al., 2009). In the USA across 9 states, a 70% rise in the incidence of obesity in pregnancy during the space of a decade between 1993-2003 was recorded by the Pregnancy Risk Assessment Monitoring (PRAM) surveillance system (Kim et al., 2007b).

Figure 1.2 Obesity among adult women (World Health Organization, 2013)
1.1.3 Pregnancy complications in obese pregnant women

In addition to previously mentioned morbidities in the non pregnant population, about 39% of pregnancies in obese women are associated with adverse outcomes (Bautista-Castano et al., 2013). The most common complications include preeclampsia, gestational diabetes, and emergency caesarean sections due to complications arising during labour, as well as follow-up complications, such as infection following obstetric surgery (Waller & Dawson, 2005; Poston, 2012). Moreover, there are risks for the child as there is an increased risk of macrosomia, fetal congenital problems and stillbirth (Nelson et al., 2010; Ruager-Martin et al., 2010).

In addition, accumulating evidence suggests that maternal obesity may also play a significant role in the predisposition of the offspring to the development of chronic diseases in later life (Symonds et al., 2009).

1.2 Developmental origins of Health and Disease hypothesis (DoHAD)

Critical developmental windows and environmental influences during fetal and early postnatal life are considered to underpin the early life origins of health and disease in later life. These periods are characterized by plasticity because of rapid cell proliferation and organ development; therefore the developing child is particularly vulnerable to environmental exposures (Symonds et al., 2009).

1.2.1 Maternal nutritional deprivation and offspring risk of disease

The first epidemiological evidence showing an influence of early nutritional status on the risk of disease in later life was provided by retrospective studies in Norway published by Fordahl et al. Different geographical regions showed different
cardiovascular mortality rates that were not associated with lifestyle, but were positively associated with early infant mortality (Forsdahl, 1977, 1978). Subsequent studies, by Professor David Barker and colleagues, investigated the association of low birthweight with both neonatal and adult mortality rates in the UK between 1921-1925. The geographical pattern of mortality rates in adults from ischemic heart disease (IHD) very closely matched the pattern of neonatal mortality, with higher death rates occurring in the more deprived areas of the UK and Wales. The study was one of the first that was widely acknowledged in the field of developmental programming (Barker & Osmond, 1986), providing inspiration for scientists of the next generation. Subsequently a retrospective study, facilitated by meticulously collected birth records, demonstrated a strong association of low birthweight with sudden death from ischaemic heart disease among men born in Hertfordshire between 1911-1930 (Barker et al., 1989).

Moreover, evidence from the Dutch famine of 1944, known as the “Hunger Winter”, suggested that the effects of maternal nutrient restriction on the offspring’s development was dependent on the stage of the development at the time of the exposure (Lumey et al., 1993). Maternal exposure to famine was associated with glucose intolerance and obesity (Ravelli et al., 1998). Interestingly, the incidence of obesity in adulthood in the offspring was increased if the mother was exposed to famine in the first half of pregnancy (Ravelli et al., 1999) but not at other periods of gestation.

Hypertension, coronary heart disease, obesity and insulin resistance form the basis of the metabolic syndrome (WHO, World Health Organization, 2000). The association of these factors with maternal under-nutrition and subsequent low birthweight led to the development of the “thrifty phenotype hypothesis”, as proposed by Hales and Barker (Hales & Barker, 1992). This hypothesis suggests
that a fetus exposed to a nutritionally poor environment in utero, undergoes permanent changes in glucose-insulin metabolism, which would facilitate survival if nurtured in a similar environment of nutritional deprivation; subsequently this would provide it with an evolutionary advantage. However, when “catch-up growth” occurs as a consequence of a nutritionally rich postnatal environment then, the risk of developing obesity and other aspects of metabolic syndrome increases (Barker, 1998). This theory, born from retrospective data has since been tested, proven and improved upon.

Considering the increasing prevalence of maternal obesity and gestational diabetes, it was later suggested that overnutrition may also have an impact on fetal developmental programming, as a result of intrauterine hormonal imbalance (Gluckman & Hanson, 2004a, b).

1.2.2 Associations of maternal obesity with offspring phenotype - Epidemiological Studies

1.2.2.1 Fetal Overgrowth

The terms ‘high birthweight’, large for gestational age (LGA, >90th centile of population birthweight) or ‘macrosomia’ (>4Kg) all are routinely used to describe infants who are larger than normal at birth. Some high birthweight infants may be appropriately large because their size is genetically determined, and they may not be fat, but muscular (Ong, 2006). Increased weight at birth may also reflect maternal overnutrition, as the availability of nutrients to the fetus determines its growth rate, subsequent birthweight, body size and adiposity (Langer, 2000). Both maternal obesity and gestational diabetes have been independently associated with the risk of ‘large LGA” infants (Ehrenberg et al., 2004).
Pedersen first proposed in 1950s (Pedersen, 1954) that greater delivery of glucose due to gestational diabetes of the mother may result in fetal hyperglycaemia which evokes exaggerated insulin secretion leading to subsequent fetal overgrowth. Thirty years later, it was shown that fetal hyperinsulinaemia was also partially attributed to increased free fatty acids, ketones and amino acids crossing the placenta (Freinkel, 1980); suggesting that maternal obesity can also considered an important risk factor (Whitaker & Dietz, 1998).

1.2.2.2 Fetal Overgrowth and predisposition to obesity in later life

Fetal birthweight has been associated with the development of metabolic syndrome in childhood (Boney et al., 2005) and predisposition to obesity in later life (Curhan et al., 1996a; Curhan et al., 1996b). A U-shaped correlation curve between birthweight and the risk of developing type-2 diabetes (Harder et al., 2007) and a J-shaped between birthweight and adult BMI (Martorell et al., 2001) have been suggested. A recent meta-analysis (Zhao et al., 2012) of fifteen studies involving over 200,000 individuals concluded that high birthweights (>4kg) and not low birthweights were positively associated with an increased risk of obesity in adulthood. High birthweight infants are more likely to develop obesity in later life as shown in the cohorts in Iceland in 1988 and 1994 (Olafsdottir, Skuladottir et al. 2006).

1.2.2.2.1 Assessing Neonatal Body Composition

Whilst many studies have addressed the relationship between birthweight and adiposity it is now widely appreciated that birthweight is a very inexact measure of fetal adiposity, and that direct estimation of neonatal body composition is required in determining the early life origins of obesity (Ward et al., 2013). Contemporary studies are now measuring fat mass at birth using DEXA scans (Schmelzle & Fusch,
2002), air displacement plethysmography (the ‘pea pod’ technique) (Ellis et al., 2007) or by skin fold thickness (Hays & Patterson, 1987).

1.2.2.3 The effect of gestational diabetes mellitus (GDM) on the offspring predisposition to obesity

Studies among Pima Indians have shown that children born to diabetic mothers have an increased risk of developing diabetes and obesity (Pettitt et al., 1993). This association is unlikely to be confounded by genetics or socioeconomic status, as it has been demonstrated in a study of siblings; children born after the mother was diagnosed with gestational diabetes were at increased risk of developing diabetes compared to their siblings who were born prior to the diagnosis (Dabelea et al., 2000). Increased BMI, as well as increased fasting glucose and insulin have been found in the offspring of diabetic mothers, according to recent studies among European and North American populations (Burguet, 2010; Lawlor et al., 2011; Philipps et al., 2011). A large study in Sweden has also shown that an independent effect of gestational diabetes on offspring BMI. When the study data was adjusted for maternal BMI, children whose mothers were diabetic in pregnancy had higher BMI by 0.94 kg/m² greater (C.I.:0.32-1.52) compared to their siblings that were born before their mother was diagnosed with diabetes (Lawlor et al., 2011).

1.2.2.4 The effect of gestational weight gain on the offspring predisposition to obesity

Gestational weight gain (GWG) also plays an important role in excessive fetal growth. GWG among normal weight women varies greatly, being dependent on, ethnicity, maternal age and parity (Viswanathan et al., 2008). The effect of maternal obesity and GWG on the offspring’s risk of disease is often attributed to fast fetal overgrowth, subsequent high birthweight and fetal adiposity which causes
predisposition to increased fat mass throughout life (Lawlor et al., 2012). Several observational studies have recently shown an association of GWG with offspring predisposition to obesity; others have found no association (Whitaker, 2004; Koupil & Toivanen, 2008). The controversial results might be attributable to measurement errors and absence of attention to confounding factors in the earlier reports, as the evidence confirming an association of GWG with child obesity is increasing as the studies improve in design, and adjustment for confounding variables (Lawlor et al., 2012). Evidence from the Avon Longitudinal Study of Parents and Children demonstrated that GWG in early pregnancy was linearly associated with infant adiposity whilst more than 500 g per week GWG in mid pregnancy was linked with increased adiposity, adverse lipid and inflammatory profiles and blood pressure (Ness, 2004). According to the Southampton Women’s Survey, GWG from the beginning of pregnancy to the 34th week was positively associated with fat free mass at 4 years old and fat mass at 6 years (Crozier et al., 2010). In another large cohort study (sample n=11994), it was demonstrated that each 2.3 kg of GWG would be correlated with an expected increase of 0.6 kg in adolescence (Oken et al., 2008b). The relevant odds ratio (O.R.) after adjusting for all confounders was 1.08 (C.I. 1.05-1.13). In addition, another retrospective cohort study has shown a U-shaped association between GWG and offspring obesity at the ages of 18 and 35-36 (Oken et al., 2008b). Evidence from a sibling study suggested no effect of GWG on offspring BMI when the mother had normal weight before pregnancy but a 0.06 difference (95%, C.I. 0.01-0.12) between siblings for each additional kilogram at gestation when the mother was overweight or obese at the beginning of pregnancy (Stuebe et al., 2009). In a recent review, it has been concluded that GWG is positively associated with all the indicators of increased adiposity, such as skin fold thickness, waist circumference and fat mass (Reynolds et al., 2010).
1.2.2.5 The effect of maternal obesity on the offspring predisposition to obesity

Similar to the reports addressing the influence of GWG, it is difficult to establish a causative association of maternal obesity per se, with the increased risk of obesity in offspring. However, the quality of the studies have improved with time, and a recent metanalysis suggested an independent relationship between maternal obesity and offspring obesity (Yu et al., 2013). This included 45 studies, after screening 665 studies from 1970 to 2012, and concluded that obesity/overweight pre-pregnancy increases the risk of offspring obesity/overweight in later life (OR, 1.95; 95% CI, 1.77–2.13; and OR, 3.06; 95% CI, 2.68–3.49), respectively (Yu et al., 2013).

The following provides some examples of mother-child studies, which have addressed the relationship between maternal obesity and childhood obesity. In a cohort study, in USA, following 802 children, maternal obesity was the strongest predictor of offspring obesity in childhood, adolescence and young adulthood (Rooney et al., 2010). In an early study by Whitaker, 24.1% of children (total 8494) were obese by the age of 4 if their mothers were obese during the first trimester of pregnancy compared to only, 9.0 % of children born to normal weight mothers. Moreover, after controlling for confounding factors such as socioeconomic status, education, parity and others, the relative risk for children to develop obesity if their mother was obese was 2.0 (95% confidence interval [CI]: 1.7 - 2.3) (Whitaker, 2004). Evidence from 13,188 3-years old singleton children, included in the UK Millennium Cohort Study, showed that 23% of the children were overweight or obese and that increased weight in the children was independently associated with pre-pregnancy BMI above 25 (O.R. 1.28, 95% confidence interval [CI]: 1.14 to 1.45) (Hawkins et al., 2009). A recent prospective cohort study, in Alabama, has demonstrated increased risk ratio of children becoming obese at 5 years old if their
mother was overweight (RR: 2.30, 95% CI: 1.29–4.11) or obese before pregnancy (RR: 2.53, 95% CI: 1.49–4.31) (Janjua et al., 2012). In the Norwegian Mother and Child Cohort study (Stamnes Kopp et al., 2012), pre-pregnancy increased BMI was positively associated with increased child BMI at 3 years of age; each unit increase in maternal BMI was associated with an increase in offspring BMI of 0.034 BMI units. Interestingly, bariatric surgery in women has been associated with improved insulin sensitivity and lipid profile in the offspring compared to siblings who were born when the mother was still obese (Smith et al., 2009).

The Amsterdam Born Children and their Development, ABCD study (van Eijsden et al., 2011), is a very large population cohort study, which is investigating the effect of maternal lifestyle, nutrition, socioeconomic status and health, amongst other factors, on offspring outcomes later in life. Also, a new large cohort study, the LIFE study, is now in progress by University College London investigating the parental influences on child health and development (UCL2013). Finally, the Jerusalem study is a birth retrospective cohort of 1400 adults investigating how GWG and pre-pregnancy BMI influences cardiovascular health and adiposity in the offspring. It has already been shown that increased GWG and pre-pregnancy BMI increases cardiometabolic risk factors in the young offspring (Hochner et al., 2012).

In summary there is substantial evidence to suggest a strong influence of maternal obesity, gestational diabetes and gestation weight gain on offspring predisposition to obesity. The exact mechanisms of these associations need to be elucidated in order to better design effective intervention studies, and subsequently new policies, which aim to stop this intergenerational cycle of obesity.
1.3 Animal Models of maternal obesity and consequent relationship with offspring health and disease

Animal studies have been invaluable in proving causality in the research of developmental origins of health disease and in identifying mechanistic pathways for the intergenerational effects of obesity. Controlled nutritional interventions that would not be feasible in humans can be performed on animals sharing a similar or identical genetic background. Thus, confounding factors in human studies can be controlled whilst maternal interventions and their influences in the offspring can be investigated, especially in rodents, over a relatively short time span.

A number of different experimental protocols have been developed in order to investigate the influences of *in utero* exposures to the offspring. Figure 1.3 summarises the experimental protocols that will be further explained in this section.

![Diagram of Animal Models](Image)

**Figure 1.3 Summary of the protocols of developmental programming.**
1.3.1 Animal models of maternal undernutrition

In light of the evidence from early epidemiological studies, the first animal studies focused on the effect of maternal undernutrition on the offspring development. Models of maternal undernutrition were first developed by Snoeck to mimic the Dutch Hunger Winter (Snoeck et al., 1990), and Hoet and colleagues in Leuven, Belgium in the early 1990s (Hoet et al., 1992), and then widely investigated in various laboratories world wide. Calorific restriction or protein restriction during rodent pregnancy results in low birthweight of the offspring and subsequent postnatal catch-up growth (Langley & Jackson, 1994; Woodall et al., 1996) especially when some animal models are reared on a normal diet, or when catch up growth is exaggerated by reducing the litter size (Hales & Ozanne, 2003). Whilst the protocols differ in terms of dietary composition, and postnatal diet, these models have demonstrated a major influence of maternal nutrient restriction on the developing pancreas. This was first demonstrated by Hoet, and then Hales, as well as demonstrating pathways of insulin signalling (Dahri et al., 1995; Hoet & Hanson, 1999; Hales & Ozanne, 2003). The influence on the cardiovascular system is less clear, although some reports suggest development of hypertension and cardiovascular dysfunction (Langley-Evans et al., 1999; Torrens et al., 2003; Torrens et al., 2008). A common finding has also been the reduction of nephron number in the offspring kidney which could contribute to the development of hypertension (Abdelnabi et al., 2000).

Other studies, predominantly in rodents, have reduced uterine blood flow to limit nutrient availability for the developing fetus, this is achieved by uterine artery ligation. The offspring born under these condition express an abnormal phenotype, particularly impaired glucose homeostasis (Neitzke et al., 2011). Placental cotyledon reduction in sheep has also been used for the same purpose (Galan et
al., 2001), and others (Poore & Fowden, 2004) have investigated ‘within litter’ birthweight variation as a model to study the influence of low birthweight. Reduced intake of either micronutrients (e.g. iron) or macronutrients (e.g. protein) has also been associated with lower birthweight, and a predisposition to both hypertension and diabetes (Bertram & Hanson, 2001; Ozanne & Hales, 2002).

In addition, the animal models are used to examine the significance of the timing of critical developmental “windows” (Hoet & Hanson, 1999; McMillen & Robinson, 2005).

1.3.2 Animal models of maternal overnutrition: rodents

Studies that have fed high saturated fat diets to rodents (specifically mice and rats) during gestation and lactation have shown that the offspring had an increased risk of developing increased adiposity (Bayol et al., 2008; Samuelsson et al., 2008; Howie et al., 2009; Liang et al., 2009; Nivoit et al., 2009; Rajia et al., 2010), hypertension and endothelial dysfunction (Khan et al., 2003; Samuelsson et al., 2008; Liang et al., 2009; Samuelsson et al., 2010), hepatic steatosis (Elahi et al., 2009; Mouralidarane et al., 2013), fatty pancreas (Oben et al., 2010b), glucose intolerance (Taylor et al., 2005; Rajia et al., 2010), insulin resistance (Bayol et al., 2008; Samuelsson et al., 2008; Liang et al., 2009; Nivoit et al., 2009) and leptin resistance (Ozanne & Hales, 2002; Kirk et al., 2009; Poston, 2011). Some rodent studies have also made observations of increased adiposity and reduced growth of skeletal muscle (less muscle fibres and reduced cross-sectional muscle area (Bayol et al., 2005) due to a high fat maternal diet. Since rodents tend to reduce their food intake when they are fed a high calorific diet high in fat content, some of the studies above, including those from our laboratory have added sugar to the diet which appears to overcome this homeostatic control, and leads to a faster increase in fat mass (Gosselin et al.; Samuelsson et al., 2008).
Another common diet used is the “junk food” or cafeteria diet where the animal has ad libitum access to a western type, high-calorific/high sugar/high fat diet. Offspring of dams fed a “junk food” diet demonstrated increased adiposity, especially in females, reduced muscle force, hyperglycaemia, hyperinsulinaemia and hyperlipidaemia (Bayol et al., 2008; Bayol et al., 2009).

Others have induced maternal obesity by feeding a diet rich in sugars rather than fats. For example, a study in which rats were fed a diet isocalorific to the control, but higher in fructose during lactation only resulted in increased bodyweight, decreased hypothalamic sensitivity to exogenous leptin, hyperphagia in addition to reduced hypothalamic expression of several anorexigenic signals in the offspring at two months of age (Nivoit et al., 2009; Alzamendi et al., 2010; Samuelsson et al., 2013). Results from our group have shown that maternal diets high in sugar resulted in increased adiposity and impaired glucose homeostasis in the offspring, whilst the effects were more exaggerated in the females (Samuelsson et al., 2013).

In this thesis a murine model of maternal obesity, which has been developed and established in our group (Samuelsson et al., 2008; Oben et al., 2010a; Oben et al., 2010b) has been used. The "obesogenic" maternal diet was designed to mimic the content of a typical ‘Western’ type diet. The dams were fed this diet for six weeks before breeding and during gestation, as well as during lactation. These animals demonstrated increased appetite and gained weight. The offspring had increased appetite, increased body weight, plasma leptin, dyslipidaemia, adipocyte hypertrophy, insulin resistance and reduced pancreatic insulin content. There was also evidence for abnormal cardiovascular function including raised systolic blood pressure, increased heart rate and endothelial dysfunction compared to their control counterparts (Samuelsson et al., 2008). The same experimental design was later applied for a study on 30-day-old rats in order to examine metabolic and cardiovascular function prior to the development of adiposity and hyperleptinaemia.
(Samuelsson et al., 2010). The animals presented a similar phenotype to the mice in that they were hyperinsulinaemic, hyperphagic and hypertensive. Additionally, they demonstrated enhanced sympathetic tone compared to controls, as characterized by reduced heart rate variability and reduced baroreflex sensitivity. In another study our group has reported an influence of the maternal obesity on behaviour in the adult offspring (Fernandes et al., 2012) analogous to that reported in some mother-child studies (Rodriguez et al., 2008). Maternal obesity in these studies was associated with symptoms relevant to attention deficiency disorder.

1.3.2.1 Windows of susceptibility: gestation versus lactation

The period of lactation is also an important developmental window in animals and man, but it is practically difficult to investigate independent effects of lactation and gestation, thus in animal studies the method of cross-fostering is commonly used in which litters from dams fed obesogenic diets are fostered to control dams and vice-versa. In a cross-fostering study in rats (Khan et al., 2005), the effect of exposure to a maternal high fat diet either in utero or during the suckling period was associated with offspring elevated systolic blood pressure, increased adiposity and hyperinsulinaemia. However, it was also shown that the effects were more severe if the animals were exposed to the high fat diet during only the postnatal period, indicating a protective role of exposure in utero. Cross-fostering studies highlight the importance of the lactation period in rodents as brain development during that period is still in progress (Taylor & Poston, 2007). Animals whose mothers were fed a low protein diet were cross-fostered to control dams during lactation, they presented reduced longevity compared to the offspring whose mother had low protein diet during lactation (Ozanne & Hales, 2004). Nevertheless, the lactation period has been identified as critical for the growth of mice as overnutrition at the early postnatal period resulted in accelerated growth, while undernutrition resulted in restricted growth in the offspring (Kappeler et al., 2009).
Exposing the dams to overnutrition during lactation or gestation also highlights the importance of each critical “window” of development. High fat diet consumption during lactation resulted in increased risk of developing diet-induced obesity in male mice (Tsuduki et al., 2013). Recent evidence has shown that maternal high fat feeding during the lactation period leads to impaired neuronal projections, possibly via abnormal insulin signalling (Vogt et al., 2014). This evidence highlights the importance of the lactation period in rodents. However, interpretation regarding the human analogues requires attention as humans are born much more mature than rodents. Moreover, it is important to acknowledge that exposure to overnutrition during pregnancy may continue to have an effect on maternal physiology during lactation and subsequently to the offspring.

However, cross-fostering in rats has been shown to influence the metabolic and cardiovascular function of offspring when compared to controls suckled by their biological mothers. Cross-fostered mice demonstrated hyperphagia, increased body weight and increased female glucose tolerance. Males had increased abdominal adiposity, systolic blood pressure and also an enhanced endothelium-dependent relaxation. Female mice had increased renal noradrenaline. These findings highlight the importance of caution when interpreting the results of various developmental programming studies that use cross-fostering as a tool in the rat. Careful selection of the controls and improved techniques will be required in the future (Matthews et al., 2011).

1.3.3 Models of maternal overnutrition in species other than rodents

Other animal such as sheep and non-human primates have also been used in studies investigating the effects of maternal overnutrition, although due to cost and length of time for the offspring to reach maturity, they are used less often than rodents. These animals have the advantage of being precocial, that is that the
offspring are born relatively mature, compared to altricial species such as rats and mice, and therefore more relevant to human infants. Rattanatray et al. (2010) showed that when embryos from female ewes overfed prior to conception were transplanted to lean mothers, the adiposity of the offspring was correlated with the donor's weight. These findings indicate a periconceptional influence of over-nutrition independent of gestational over-nutrition (Rattanatray et al., 2010). Maternal diet-induced obesity in ewes results in neonatal adiposity, which may result from increased placental fatty acid and amino acid transport (Long et al., 2010). Ewes coming from obese dams showed hyperphagia and gained weight when they were exposed to *ad libitum* feeding at 19 months of age. Paradoxically they did not show a leptin peak during the first few days after birth like control animals but they did present a significant increase of cortisol levels at birth, suggesting different mechanisms compared to the control mice (Long et al., 2011). Maternal obesity in ewes was also associated with a maternal inflammatory response as reflected in increased expression of the toll-like receptor 4 (TLR4) (Zhu et al., 2010). Inflammatory mediators have also been implicated in 'programming' by Grayson et al. (2010), who showed that maternal over-nutrition for several years prior to gestation in the Japanese Macaque monkeys triggered increased proinflammatory cytokines in the fetal hypothalamus, suggesting an activation of local inflammatory response (Gosselin et al., 2004). Many of the monkeys fed the high fat diet did not develop obesity. However, the fetuses of dams that developed obesity due to the high fat diet and fetuses of those that were resistant to developing obesity both had increased levels of hepatic triglycerides. This highlights the importance of gestational over-nutrition, independent of pre-pregnancy bodyweight in this model (McCurdy et al., 2009). Also remarkably, when a sub-group of these dams were fed a control diet during gestation the hepatic triglycerides in the offspring improved. In another study by the same group, maternal over-nutrition resulted in fetal growth restriction, increased stillbirth and reduced fetoplacental blood flow (Frias et al.,
Also, a maternal high fat diet resulted in increased plasma insulin concentrations and endothelial dysfunction as suggested by thicker intima wall at 13 months of age (Fan et al., 2013). In addition, a long-term maternal high fat diet resulted in early behavioural problems in juvenile offspring, due to central serotonergic system dysfunction (Sullivan et al., 2010). Moreover, a maternal high fat diet also resulted in fetal growth restriction in baboons, which brings into question the relevance of these non-human condition where, large for gestational age deliveries are more common, although low birthweight may also occur. Other structural and functional changes have occurred in the baboon placenta due to maternal obesity including decreased placental syncyiotrophoblast amplification factor and increased placental and adipose tissue macrophage infiltration suggesting increased inflammation markers similar to humans (Farley et al., 2009). In this model it has also been found that fetal methionine and vitamin B12 were reduced indicating suppressed transplacental passage of these nutrients. Moreover, maternal obesity is associated with altered methylation status, thyroid axis and fetal hypothalamic expression of orexigenic peptides in the hypothalamus (Su et al., 2001). Finally, it was demonstrated that a maternal high fat/high sucrose diet induces fetal cardiac fibrosis and differential expression of cardiac miRNAs, implying a potential role in the altered heart development observed in previous studies (Maloyan et al., 2013).

These studies support the developmental origins of health and disease hypothesis and further highlight the influence of maternal overnutrition/obesity. Precocial species are very important in determining the specific mechanisms, which may have been identified in studies in the altricial species.
1.3.3.1 The additive effect of postnatal diet

Another approach has been used to investigate the influence of postnatal exposure to high fat and/or high sugar diet on offspring prenatally exposed to an obesogenic diet. A preference to ‘junk’ food was demonstrated in rats exposed to a ‘junk’ food diet both prenatally and postnatally which further contributed to the development of fatty liver disease (Bayol et al., 2007; Bayol et al., 2010). Rats exposed to a cafeteria diet in utero and postnatally demonstrated greater reduction in β-oxidation and increased hepatic lipogenesis (Bouanane et al., 2010). Evidence from a study in mice showed that long term exposure of dams to a high fat diet resulted in increased bodyweight, adiposity, blood pressure and serum cholesterol among the female offspring exposed to the high fat diet prenatally and/or postnatally compared to the offspring of control dams weaned onto a control diet (Elahi et al., 2009). Similarly, male rat offspring of dams fed a high saturated fat diet were hyperglycaemic only when they were exposed to the same diet post-weaning. However, there was an additive effect of maternal obesity and post-weaning diet on bodyweight that became greater with time (Page et al., 2009). In addition, exposure to a high fat/high fructose diet prenatally and postnatally potentiates the susceptibility to renal and metabolic disturbances later in life in the rat (Jackson et al., 2012). Moreover, male offspring of obese rat dams demonstrated impaired metabolic flexibility when they were fed a high fat diet, because they were not able to adapt the substrate utilization according to the substrate availability (Borengasser et al., 2011). Simar et al (Simar et al., 2012) have recently shown that maternal obesity and a high fat diet post-weaning had a significant additive effect on bodyweight. However, the interaction between the maternal obesity and the post-weaning high fat diet led to decreased skeletal muscle glucose transporter 4 and increased monocarboxylate transporter 1 protein. A greater effect of maternal obesity is observed in offspring fed a high fat or/or a high sugar diet post-weaning in the above studies. These observations reflect
that children who are being exposed to the same high calorific diet that their mother consumes develop early childhood obesity.

In humans, after a small interval in fat deposition in infancy, adiposity accelerates again around six years of age and it has been suggested that premature adiposity onset, presumably associated with dietary excess in early life, is associated with increased risk of obesity in adult life (Eriksson et al., 2002). Thus, early exposure to high fat diet in experimental animals could mask any effects of maternal obesity on offspring adiposity and metabolic profile in young animals. However, exposure to an obesogenic diet in adulthood would be expected to exacerbate the effect on the offspring phenotype due to maternal obesity. In a study by White et al (White et al., 2009) eight week old offspring of high or low fat fed rats dams were fed either a low or high fat diet for 12 weeks; the group that was exposed to the high fat diet prenatally and in adulthood had higher bodyweight and greater adiposity than offspring of the low fat fed dams fed the high fat diet. This evidence suggests that maternal obesity predisposes the offspring to developing obesity when they are exposed to obesogenic diet in adult life despite being lean in early life.

1.3.4 Summary

It is very important to acknowledge that animal studies have limitations. Some of these limitations may be interspecies differences; the use of inbred strains and the dietary exposures used for the studies may not match human experiences, and in themselves may induce biological and psychological stress. As mentioned above maturity at birth in altricial species such as rodents is not the same as in the human infant (Hartung, 2008). Because mice and rats are born relatively immature compared to humans, the early postnatal period could be equivalent to the last months of pregnancy in humans (Poston, 2010). The non-human primate provides a good model as their genetic code is closer to humans; however, due to ethical
considerations and high cost they are not the most commonly used models. Moreover, ewes are also not a cost or time effective model as they can only breed in the same months every year. Mainly for these reasons rodents are still the most commonly used model. Furthermore, the nutritional interventions differ greatly either in their consistency or in their ratios of the macronutrients, and for this reason it is difficult to compare the results and subsequent interpretation (Ozanne, 2001). If the different protocols used in different studies were reported in more detail it would facilitate standardisation of the methods used, and subsequently the evidence from these could be more conclusive. Despite these reservations, there is a remarkable degree of commonality reported amongst the phenotypes, suggesting that maternal obesity rather than individual dietary components may be causative.

1.4 Prenatal and early postnatal factors in obese pregnancy associated with developmental origins of obesity in the offspring

The maternal or early postnatal life exposures, which lead to persistent changes in offspring metabolism and thereby to obesity, are not yet well understood. However, several well described alterations in the metabolic and hormonal maternal profile due to obesity in pregnancy have been implicated. The maternal ‘exposures’ that may be affecting the fetus will now be discussed.

1.4.1 Inflammatory mediators

Adipose tissue is a metabolically active system, involved in the production of adipokines, autocrine, paracrine and endocrine signals (Kennedy et al., 1997; Fruhbeck et al., 2001; Catalano, 2003). The increased adipose tissue present in
obese pregnant women results in increased production of these signals, demonstrating an increase in serum concentration of inflammatory markers compared to lean women. More specifically obesity in pregnancy resulted in increased concentrations of interleukin-6 (IL-6) and sensitive C-reactive protein, which has been previously associated with increased risk of developing type 2 diabetes in the non-pregnant population (Ramsay et al., 2002).

Several studies have investigated whether these inflammatory mediators may play a causative role in development of offspring obesity. One report has shown that administration of IL-6 and tumour necrosis factor (TNF) to lean rats on days 8, 10 and 12 of gestation resulted in 30-40% increase in the adipose tissue mass of the offspring compared to their control counterparts (Dahlgren et al., 2001). A study in nonhuman primates has shown that prolonged maternal high fat feeding resulted in widespread activation of proinflammatory cytokines, and that this was implicated in the development of the offspring’s hypothalamus, and subsequently to early onset of weight gain (Grayson et al., 2010).

Based on the above, it could be hypothesised that circulating inflammatory markers from the mothers may be transported across the placenta and permanently influence fetal development, subsequently predisposing the offspring to obesity and associated disorders.

1.4.2 Maternal glucose, fetal hyperinsulinaemia and hyperleptinaemia

Obesity is strongly associated with insulin resistance and hyperglycaemia (Catalano et al., 2003; Catalano et al., 2009; Catalano, 2010). Insulin sensitivity during late gestation is reduced further than 50% in obese women. In contrast with normal pregnancies, fasting glucose during early gestation in obese pregnancies is not decreased (Mills et al., 1998) and insulin sensitivity during late gestation is reduced
further than 60% (Catalano et al., 1991; Nelson et al., 2010). The Hyperglycaemia and Adverse Pregnancy Outcome (HAPO Study Cooperative Research Group) study (HAPO Study Cooperative Research Group, 2010) has reported that increased maternal glucose in non-diabetic women is strongly correlated with fetal fat mass. Glucose is readily transported across the placenta and stimulates the fetal pancreatic beta cells leading to hyperinsulinaemia in the fetus, and thereafter to fetal growth and macrosomia, which has been associated with obesity in later life (Bo et al., 2003; Roman et al., 2011). One suggested mechanism is increased fetal adipocyte insulin sensitivity during gestation, which predisposes to obesity (Hofman et al., 1997). Alternatively, as discussed below, fetal hyperinsulinaemia has been implicated in altered development of the central pathways regulating energy balance.

Another potential mediator of offspring obesity is maternal and/or fetal hyperleptinaemia. Leptin, which is secreted by the adipocytes, acts to decrease food intake and increase energy expenditure in adults (Friedman & Halaas, 1998; Bouret & Simerly, 2004). In rodent models there is evidence of a postnatal leptin surge, which has been implicated in the normal development of the hypothalamus (see below), and not at this stage in energy balance (Ahima et al., 1998). An exaggerated surge has been implicated in offspring leptin resistance and subsequently to susceptibility to obesity (Ahima et al., 1998; Ahren & Scheurink, 1998). Some have suggested that this leptin surge does not correlate with fetal body fat, as fetal adipocytes produce low levels of leptin in late gestation, this suggests that the source of leptin is from the mother rather than the fetus (Ahima et al., 1998; Velkoska & Morris, 2011). As leptin cannot cross the placenta during pregnancy, it is possible that a maternal contribution to this surge is derived from the milk (Horvath & Bruning, 2006; Samuelsson et al., 2008). Alternatively, our group and others have implicated glucose stimulated fetal adipocyte proliferation, since the exaggerated
The postnatal leptin surge was associated with increased fetal adipose Ob gene expression (Kirk et al., 2009). In turn (see below), this has also been implicated in altered development of the central pathways regulating energy balance. Interestingly in offspring of obese sheep, the regular neonatal peak of leptin at six to nine days postnatal seems to be dysregulated due to early increased cortisol levels, which results in an increased stimulus to fat cell leptin production occurring when capacity to secrete leptin is rapidly exhausted (Long et al., 2011).

### 1.4.3 Lipids

During pregnancy, basal fat oxidation increases by 50-80%, whilst significant hyperlipidaemia occurs (Okereke et al., 2004; Nelson et al., 2010). In obese pregnancies, hyperlipidaemia is aggravated, as total and very low-density lipoprotein (VLDL) triglycerides increase while plasma high-density lipoprotein (HDL) decreases and low-density lipoprotein (LDL) remains the same (Merzouk et al., 1998; Ramsay et al., 2002; Rajasingam et al., 2009; Nelson et al., 2010). Moreover, reduced insulin efficacy in lipolysis results in increased fatty acids in obese patients (Sivan et al., 1999). The increased free fatty acids and triglycerides can cross the placenta and subsequently increase the fetal blood concentrations (Han et al., 2005). There is a strong association in human pregnancies between maternal hyperlipidaemia with fetal growth and subsequently with childhood obesity (Catalano et al., 2009). According to the Pune Maternal nutritional study, which included 631 women, increased cholesterol and triglyceride concentrations at 28 weeks were associated with increased birthweights (Kulkarni et al., 2013). However, the types of fat are important, as there is evidence that polyunsaturated fats may be protective. Donahue et al (Donahue et al., 2011), have also shown that supplementation with omega 3 polyunsaturated fatty acids was correlated with lower childhood adiposity as measured by skin fold thickness. Similarly, supplementation with polyunsaturated
fatty acids in diabetic rats prevented macrosomia suggesting a role of maternal lipid profile on offspring development (Makni et al., 2011).

1.4.4 Maternal microbiota

The microbiome is a robust ecosystem consisting of many microbial communities (Collado et al., 2012). These commensal bacteria enable metabolism and the synthesis of fatty acids and vitamins via encoding enzymatic pathways (Turnbaugh et al., 2006). Diet plays the most predominant role in the structure of the microbiome and even short-term changes in substrate availability due to dietary changes can influence the bacteria populations (David et al., 2014). The development of human microbiome, though, is presumed to start at birth by exposure to the maternal microbiota. The mode of delivery and the infant’s diet will further develop the infant microbiome (Collado et al., 2012). Evidence, however, show that microbial colonization of the human body may begin earlier than at birth as bacteria have been found in umbilical cord, placenta, amniotic fluid and also in meconium (Jimenez et al., 2005; Jimenez et al., 2008; Satokari et al., 2009). Therefore, it can be assumed that prenatal maternal exposures including high fat diet or obesity per se may influence maternal microbiome and subsequently play an independent role in the development of offspring’s microbiota.

Overweight pregnant women had increased numbers of *Staphylococcus*, *Enterobacteriaceae*, and *Escherichia coli* and reduced numbers of *Bifidobacterium* and *Bacteroides* compared to normal weight pregnant women (Santacruz et al., 2010). Moreover, increased gestational weight gain has been associated with a decrease in *Bacteroides* (Collado et al., 2008). In relation to the above, infants born to women with excessive GWG during pregnancy showed lower levels of bifidobacteria than those from women with normal GWG (Palmer et al., 2007). In addition, large for gestational age infants (indicative of increased GWG) have been
shown to have significantly different and less diverse gut microbiota compared to appropriate for gestational age infants (Karlsson et al., 2011). These early differences may be critical for the offspring metabolism as according to a prospective study, which included 50 children, decreased number of Bifidobacterium at infancy was associated with increased bodyweight at the age of seven (Kalliomaki et al., 2008). Moreover, it has been recently shown in macaques that maternal high diet influenced the offspring gut microbiota at 1 year of age. The juveniles gut microbiota was compared to adults gut microbiota on either control or high fat diet. Even though the post-weaning diet was the most predictive factor structuring the juvenile gut microbiota; exposure to control post-weaning diet did not fully reverse the dysbiosis attributed to the exposure to maternal high fat diet (Ma et al., 2014).

There have been several attempts to improve maternal microbiota in order to influence infants’ microbiota and pregnancy outcomes. Administration of probiotics two-four weeks before delivery, as reviewed recently (Sanz, 2011), has been shown to be effective in preventing preterm delivery in women at risk (Othman et al., 2007), suggesting a beneficial effect on pregnancy outcomes. Moreover, administration of prebiotics combined with dietary counseling improved glucose metabolism during pregnancy compared to a placebo group and dietary intervention only (Laitinen et al., 2009). Additionally, administration of L.rhamnosus three weeks before and four weeks after delivery induced specific changes in the transfer and initial establishment of bifidobacteria in the infant (Collado et al., 2012).

The evidence summarized here, even though limited, suggest a role of maternal gut microbiota on infant’s microbiota, which is suggested to be associated with the risk of developing obesity in later life. Moreover, an intervention aiming to improve the maternal microbiota could potentially prevent the adverse effects of maternal obesity on the offspring. The current study will explore whether maternal obesity affects the
offspring microbiome and whether an intervention with fibre will influence this effect.

1.5 Pathways of obesity; the role of energy balance and basal metabolic rate

Having determined the potential maternal exposures in relation to overnutrition, which lead to offspring obesity, the pathways, which are likely to be disrupted and which will ultimately lead to obesity will now be considered. These necessarily include energy balance and in particular basal metabolic rate. After discussion of these, the evidence of these pathways being abnormal in offspring of obese mothers compared to offspring of control mothers will be reviewed.

1.5.1 Energy Balance

Energy represents the ability of a system to perform work. According to the first law of thermodynamics, energy cannot be destroyed or created but is transferred from one form to another. Animals including humans derive their energy from nutrients. In relation to energy metabolism, both excess and insufficient nutrient intake may be detrimental for human health as they can lead to severe diseases like diabetes or chronic nutrient imbalance, respectively (Lanham-New et al., 2011).

The energy balance equation is simple and if only one of the parameters alters a negative or a positive energy balance results which consequently leads to weight loss or gain, respectively (Figure 1.4).

\[
\text{Energy Intake} - \text{Energy Expenditure} = \text{Energy Balance}
\]

In other words, if the total energy contained in the body is not altered $\Delta \text{Energy stores} = 0$, then Energy intake equals the energy expenditure (Schoeller, 2009).
Figure 1.4 Depiction of energy balance equation as it applies to body weight regulation.

1.5.2 Energy intake

Energy intake for an individual is the amount of chemical energy that enters the body and can be released by metabolism (Kleiber, 1975a). The energy content of any food is measured when the food is completely oxidised to water, carbon dioxide and nitrogen, the total heat that is released is the gross energy of the food. The gross energy for each macronutrient varies, for example it is 17.7 kJ/g for the sucrose but 39.3 kJ/g for fat (Geissler, 2005). However, the gross energy does not represent the actual energy available to the body, since no potential oxidisable substrate can be considered as available before it is ready for cell oxidation. Thus, the metabolisable energy differs from the unabsorbed energy that is excreted as
faeces or the energy that may be excreted in urine due to incomplete metabolism (Kleiber, 1975b; Schoeller, 2009).

The probable chemical energy that is usually stored as fat but also sometimes as glycogen and protein represents energy storage. Observing the obesity rates retrospectively, the rise of obesity could be a consequence of only modest energy imbalance during a short period of time (Ferrannini, 1988). According to the National Health and Nutrition Examination Survey (NHANES) data the average weight of a 40-year-old male between the periods of 1976-1980 and 2002-2004 increased by 7.4 kg, or 0.5 kg/year, which can be translated to a daily imbalance of only 10 kcal/day (Ferrannini, 1988; Cunningham, 1991). However, interestingly the energy intake in the UK has remained the same according to the National diet and Nutrition Survey the energy intake has remained stable from 2008-2012 suggesting an even greater role of energy expenditure in the development of obesity (Whitton et al., 2011; Food Standards Agency & Public Health England, 2014).

### 1.5.3 Energy expenditure

The energy output is heat released by the body though basal metabolic rate (BMR), physical activity and adaptive thermogenesis due to various environmental stimuli (such as diet-induced thermic effect and thermogenesis by adaptation to the heat or cold) (Groff & Gropper, 2000; Geissler, 2005). BMR is the most important component of energy expenditure (figure 1.5) of a sedentary person, as it accounts for 60% of the total energy expenditure while physical activity accounts only for 30% and thermogenesis for the remaining 10% (Levine, 2005b) as seen in the equation below.
Energy Expenditure = (0.6 x Resting Energy Expenditure) + (0.3 x Physical Activity) + (0.1 x Thermogenesis)

Figure 1.5. Schematic depiction of the different parameters of the Energy Expenditure

During recent decades, it is not only the rise in energy dense foods that has contributed to the obesity increase, but also the decrease in physical activity, with most people live a sedentary lifestyle. According to the 2008 Health Survey for England 61% of men and 71% of women did not exercise at least five times a week for 30 minutes, as the Department of Health recommends (Aresu et al., 2009). As these rates were based on self-reported physical activity a study using accelerometers was also conducted and concluded that of the 1,998 men and 2,509 women involved in the study, only 6% and 4% respectively, met the government’s guidelines.
Diet-induced thermogenesis (DIT) or alternatively, the ‘thermic effect of food’ is the amount of energy consumed in the process of digesting, absorbing and metabolising nutrients (McArdle *et al.*, 2006). Different macronutrients require different amounts of energy to process and thus incur differing amounts of thermogenesis (Westerterp *et al.*, 1999; Westerterp, 2004). Nowadays adaptive thermogenesis does not play an important role as people spend most of their time in controlled temperature indoors. It is more likely therefore that there are greater differences in DIT than adaptive thermogenesis between people. Interestingly, DIT is observed to be impaired among obese individuals, and is suggested to play a causative factor in the development of obesity (Schutz *et al.*, 1984; Bogardus *et al.*, 1985; Golay *et al.*, 1989; Thomas *et al.*, 1992b).

To date there has been no thorough investigation into whether reduced DIT can be ‘programmed’ *in utero*. However ‘genetically obese’ ob/ob mouse models demonstrate impaired DIT before the onset of obesity (Trayhurn *et al.*, 1977). This single observation suggests that DIT could be an obesogenic risk factor, or at least associated with other obesogenic risk factors, rather than obesity being the cause of DIT impairment.

Any alterations in basal metabolic rate would be detrimental for the individual as basal metabolic rate is the main component of energy expenditure. The primary outcome of this study is the effect of maternal obesity on basal metabolic rate, which will be discussed in more detail below.

**1.5.4 Basal metabolic rate**

An individual’s metabolism in a basal state is measured by basal metabolic rate (BMR). BMR was described by Harris and Benedict (Harris & Benedict, 1918) as the heat production of an individual being at “complete muscular repose”, in
postabsorptive condition (10-12h after the last meal). In other words, it represents the metabolic activity of tissues and cells and only the vital physiological functions (SACN, 2009). BMR measurements are made 12 hours after the last meal, completely at rest and at thermoneutral temperature (about 23 °C), when these conditions are not met fully then the measurement is known as the Resting Metabolic Rate (RMR) (Black, 2000). When RMR is not measured at the standardised metabolic state, it may include additional metabolic activity such as the thermic effect of food, thus the heat produced may be higher than the energy that would be produced for BMR. BMR is associated with arousal, therefore, it is slightly higher than the sleeping metabolic rate (SACN, 2009).

The most common way to measure energy expenditure, and subsequently BMR, is based on the measurements by indirect calorimetry and direct calorimetry. Indirect calorimetry measures energy expenditure based on oxygen consumption (VO$_2$) and carbon dioxide production (VCO$_2$). Additionally, in systems of indirect calorimetry Respiratory Exchange Ratio (RER) is also measured. RER, that equals VO$_2$/VCO$_2$, is used to determine the nature of the oxidised substrates. RER is higher than 1 when there is 100% carbohydrate oxidation while when the RER is 0.7 fat is oxidised. In direct calorimetry heat production from the body, directly, is assessed in order to calculate energy expenditure (Lanham-New et al., 2011).

1.5.4.1 Determinants of BMR

1.5.4.1.1 Body size and composition

Basal and total energy expenditure are strongly correlated with body size, as both height and weight are determinants of energy expenditure (Durnin, 1996). Skeletal muscle plays an important role in the metabolic rate (Lanham-New et al., 2011); in contrast, the contribution of adipose tissue to BMR is small. Increasing body size of overweight and obese individuals increases both Fat Mass (FM) and Free Fat Mass
(FFM) (White & Seymour, 2005). It is suggested that higher percentages of body fat composition may have an effect on the mechanical efficiency of movement resulting in increased total energy expenditure (Prentice et al., 1996; White & Seymour, 2005). Therefore, overweight and obese people may have increased absolute BMR (Durnin, 1996; McAllister et al., 2009; Kaiyala & Schwartz, 2011) however, when body weight is taken into account BMR declines due to increased adiposity and decrease FFM (Prentice et al., 1996; Das et al., 2004). According to one study in humans (Bosy-Westphal et al., 2009), the effect of FM on BMR is relevant to the degree of adiposity. The effect is positively correlated until a fat mass of 40% but above that the importance of the effect is reduced.

1.5.4.1.2 Organs

By taking into account FFM most differences in BMR are attenuated, since FFM includes the heart, kidneys, liver and resting muscles that are all of high metabolic activity (Geissler, 2005). Even though the heart, spleen, kidneys, liver and brain (high metabolic rate organs) represent 5.5% of total body mass, almost 60% of total energy expenditure can be attributed to their function. It has been suggested that 5% of the BMR variability that remains unclear can be explained by high or low metabolic rate tissue activity (Javed et al., 2010). Moreover, variability in the mass of high metabolic rate organs, either due to differences between race groups or due to increasing age, has a strong effect on BMR (Javed et al., 2010). Body size, however, does not affect the metabolic activity of the organs among healthy individuals (Later et al., 2008). Therefore, variability in organs metabolic activity, that would subsequently influence BMR variability, may be determined by other factors, such as the sympathetic drive activity.
1.5.4.1.3 Age

BMR decreases with older age and this is mainly due the decreased FFM with ageing but some studies have showed only a small decline in BMR due to the reduction of FFM (Pannemans & Westerterp, 1995; Klausen et al., 1997; Piers et al., 1998; Bosy-Westphal et al., 2003; Johnstone et al., 2005). Apart from the FFM, other factors may have an effect with the decline of BMR with older age such as decline in sodium potassium ATPase activity, decreased muscle protein turnover and changes in mitochondrial protein permeability (Wilson & Morley, 2003). However, it is very difficult to identify the causal effects of ageing, as it could also be associated with age-related diseases affecting the metabolic rate of the organs (Bosy-Westphal et al., 2003).

1.5.4.1.4 Sex

Sex differences in BMR can mainly be attributed to differences in body size and composition. Women have less FFM and more FM than men, thus, lower BMR. By adjusting for body size and composition some studies have found that women still have lower BMR (Arciero et al., 1993; Poehlman et al., 1997), while, others have reported no difference (Klausen et al., 1997). However, according to evidence from the Helsinki Birth Cohort study (Sandboge et al., 2011) birthweight is correlated in a different way between men and women with RMR in adulthood. The correlation of birthweight with RMR is U-shaped among men while in women it is not significant when adjusted for FFM and FM. Thus, adult RMR is influenced by an early developmental influence that differs between men and women.

1.5.4.1.5 Hypothalamus

It was first discovered that the hypothalamus plays an important role in energy balance regulation as after lesion of discrete areas of the hypothalamus, obesity and nervosa anorexia occurred (Anand & Brobeck, 1951). The hypothalamus nuclei
produce specific neuropeptides that perform various tasks like the homeostasis of temperature, water, sleep, and reproduction (Luiten et al., 1987).

The release of thyroid hormone, which plays an important role on the regulation of energy expenditure and thermogenesis, by the thyroid gland, is induced by Thyrotropin-releasing hormone (TRH) via the release of Thyroid stimulating hormone (TSH) hormone from the pituitary. The terminals of TRH are in the hypothalamus in median eminence and the pituitary (Lechan & Jackson, 1982). Thus, the hypothalamic-pituitary-thyroid (HPT) axis is vital for energy homeostasis due to the regulation of thyroid hormone (Lechan & Jackson, 1982). The paraventricular nucleus (PVN) and other hypothalamic nuclei also stimulate neurons that are part of the sympathetic and parasympathetic autonomic nervous system (Luiten et al., 1987). The pathways of the autonomous nervous system can regulate energy expenditure by influencing the organ function as for example the heart rate (Ozanne, 2001; Remmers & Delemarre-van de Waal, 2011).

Moreover, the hypothalamus regulates the hormones that produce the signal of hunger, satiety and fat reserves of the body (Remmers & Delemarre-van de Waal, 2011), therefore they could influence positive energy balance and subsequently lead to adiposity and thus affect FFM and FM proportion.

Even though the hypothalamus does not affect BMR directly; by regulating the secretion of hormones, organ function and behaviour, BMR may be up or downregulated.

1.5.4.1.6 Endocrine state

The role of thyroxin on BMR is arguable (Astrup et al., 1992; Svendsen et al., 1993). Hyperthyroidism results in an increase whilst hypothyroidism results in decrease in BMR (Danforth & Burger, 1984). Despite the above, it remains unclear whether the
variation between individuals within the euthyroid zone is attributable for variations in BMR (Müller et al., 1989; Svendsen et al., 1993; al-Adsani et al., 1997; Rosenbaum et al., 2000; Johnstone et al., 2005).

Leptin has been shown to influence BMR (Nicklas et al., 1997; Toth et al., 1997; Jorgensen et al., 1998). However, BMR variations cannot be attributed only to leptin concentrations when adjusted for FM and FFM (Roberts et al., 1997; Martin et al., 1998; Bobbioni-Harsch et al., 1999; Johnstone et al., 2005). A problem in identifying any causal relationship would be the effect of FM on plasma leptin concentrations (Neuhauser-Berthold et al., 2000).

An increase in BMR has been noticed during the luteal phase of menstrual cycle that suggests an effect on energy expenditure by sex hormones (Solomon et al., 1982; Webb, 1986; Lovejoy, 1998; Day et al., 2005). Suppression of oestrogen with pharmacological agents has been shown to reduce BMR (Day et al., 2005).

1.5.4.1.7 Mitochondrial Dysfunction

Smith (1956) found that liver mitochondrial density is linked to body energy expenditure, implying that the controlling factor for the total oxygen consumption of the body could be the relative amount of mitochondria in any given tissue. In addition it has been previously shown that maximal oxygen consumption (\( \text{VO}_2 \max \)) rate and muscle performance is associated with mitochondrial function (Rasmussen et al., 2001). The above facts could be implying maternal influences, due to mitochondrial dysfunction, on the basal energy expenditure.

**1.5.4.2 Intra-individual variability of BMR**

It should also be noted that BMR might vary ±10% between individuals in the same age, sex, body size and FFM group. Intra-individual variability appears to be more common among women (Geissler, 2005), however the exact reasons for this
variability between individuals remain to be discovered. Johnstone et al (2005) have examined the factors influencing the variation in BMR, yet still have not been able to explain 26.6% of the variation (Figure 1.6). Therefore, developmental programming in the fetal period and genetic factors could account for the variation in the BMR that predisposes some individuals to have lower total energy expenditure.

![Figure 1.6 Partitioning of the variance in BMR, figure taken from (Johnstone et al., 2005)](image)

1.5.5 Evidence of early origins of energy imbalance due to exposure to maternal overnutrition

1.5.5.1 Energy intake

Hyperphagia in offspring is also significantly associated with maternal obesity. Both maternal undernutrition and protein restriction have been associated with increased food intake in the offspring (Vickers et al., 2000; Vickers et al., 2003; Bellinger et al., 2006). Offspring of obese dams were hyperphagic from four to six weeks compared to their control counterparts and this was associated with increased adiposity.
(Samuelsson et al., 2008). Also, offspring of dams fed with a junk food diet during pregnancy demonstrated increased appetite and preference for high fat foods (Bayol et al., 2007; Bayol et al., 2010). The hypothalamus plays an important role in the regulation of appetite. The arcuate nucleus (ARC), a group of neurons including neuropeptide Y (NPY), agouti-related peptide (AgRP) and pro-opiomelanocortin (POMC), situated above the median eminence, is thought to be central in energy homeostasis (Alfaradhi & Ozanne, 2011). In rodents its development is not completed until 20 days postpartum (Grove et al., 2005). Therefore, it is possible that the programming of appetite, at least in part, occurs in the early postnatal period (McMillen et al., 2005; Plagemann, 2005; Muhlhausler et al., 2006; Plagemann, 2006). Insulin and leptin have been involved in the development of the hypothalamus. POMC expression increases when the circulating leptin and insulin concentrations increase (Brown et al., 2006). Following, the observation of the neonatal leptin surge in neonatal rodents, Flier (Ahima et al., 1998) was the first to suggest that this may be playing a role other than control of food intake and weight, since there was no relationship between the leptin concentration and these parameters. Subsequently Bouret found that mice deficient in leptin (ob/ob) have incomplete ARC projections and dysfunctional leptin signaling (Bouret et al., 2004). Whilst the leptin-deficient mice become obese this is reversed with the administration of exogenous leptin (Campfield et al., 1995; Halaas et al., 1995; Pelleymouter et al., 1995). Our laboratory found that the leptin surge was exaggerated in the offspring of obese rats and that this was associated with abnormal development of the ARC projections to the PVN, which was attributed to development of leptin resistance (Ahren & Scheurink, 1998). Leptin treatment in neonatal rats aiming to mimic the exaggerated leptin surge in offspring coming from obese mothers also implied that leptin resistance may be programmed due to maternal obesity and increases the offspring susceptibility to obesity (Samuelsson et al., 2010). Thus our laboratory has proposed that the neonatal leptin surge is
suggested to mediate the hyperphagia observed in the offspring of obese dams (Kirk et al., 2009). Similarly, in non-human primates it has been shown that NPY and POMC are well expressed by 90% of gestation and intrauterine growth restriction increases NPY and decreases POMC, increasing the risk of developing increased appetite in later life (Li et al., 2013) This evidence confirms the mechanisms previously observed in altricial species and highlight the importance of the last period of gestation for the development of the hypothalamus.

Plagemann et al (1999), have investigated how overfeeding would affect the orexigenic and anorexogenic peptides in the hypothalamus. At first in offspring of dams with GDM (Plagemann et al., 1999a) and later in a model of overfeeding by litter size reduction (Plagemann et al., 1999b). The results in the two studies were similar suggesting that GDM leads to fetal overfeeding. The overfed rats had increased GAL-neurons in the ARC, which play a role in the regulation of food intake and body weight. By investigating further, decreased mRNA and immunopositivity in the GAL-neurons was associated with increased insulin in the hypothalamus. This evidence was against the known physiology suggesting hypothalamic resistance of the GAL-neurons to increased insulin levels. Insulin plays a critical role in neuronal differentiation and maturation (Nataf & Monier, 1992), thus increased levels peripherally and in the hypothalamus could be influencing the development of the ARC and subsequently to a hypothalamic resistance of the galaninergic system to elevated insulin. Therefore, early exposure to hyperinsulinaemia due to overfeeding could permanently predispose the offspring to hyperphagia and increase the risk of developing obesity.

Very recently Vogt et al, (Vogt et al., 2014) showed that the development of POMC and AgRP projections are impaired by maternal overnutrition during the critical period of lactation. In this study it was demonstrated that loss of insulin signalling in
POMC neurons neither improved POMC axonal innervation of the neuroendocrine compartment of the PVH, the DMH, or the LH nor has it prevented increased adiposity and insulin resistance. Thus, as maternal overnutrition during lactation results in increased levels of glucose and insulin, elevated leptin levels and free fatty acids in the milk possibly contribute to the impaired metabolic profile of the offspring exposed to maternal overnutrition during lactation.

**1.5.5.2 Energy Expenditure**

Offspring of dams exposed to a high protein diet had lower energy expenditure and higher body fat (Daenzer et al., 2002). These differences in energy expenditure, however, can be derived from differences in any of the components of energy expenditure. Our group has also shown that offspring of rats fed a high fat diet demonstrated reduced locomotor activity, measured by radiotelemetry, which was associated with increased adiposity (Khan et al., 2003). These studies suggest that lower physical activity could be mediating obesity, but the evidence to date is minimal.

There are no studies of which I am aware which have investigated diet-induced thermogenesis in offspring of obese dams. However, diet-induced thermogenesis has also been associated with obesity as offspring of female mice exposed to undernutrition during pregnancy did not present as profound an effect as controls. In a human study including 36 adolescents, it was shown that offspring of obese mothers had elevated concentrations of Thyroid Stimulating Hormone (TSH) and reduced VO₂, which could theoretically influence total energy expenditure (Wilms et al., 2010).
1.5.5.3 Evidence of developmental programming of basal metabolic rate

Basal metabolic rate is the largest component of energy expenditure but still largely unexplored. The following studies suggest a possible programming effect of maternal obesity on BMR.

In addition to the above an older cohort following 25 children of normal weight and obese parents for 12 years showed an association between increased parental weight and lower RMR/kg, especially for boys, which was closely related to BMR (Griffiths et al., 1990).

A study measuring RMR by Enhanced Metabolic Testing Chamber in 21 infants showed that infants born to overweight and obese biological mothers had a lower extrapolated 24 hours energy expenditure than those of lean mothers (P<0.05) (Rising & Lifshitz, 2008). Additionally, evidence showed that children of obese mothers slept more, a greater proportion of their energy intake consisted of carbohydrates and they tended to have a greater respiratory exchange ratio than their lean counterparts, showing that they were more prone to store energy derived from food as fat (Rising et al., 2003).

Recently, a study in mice has shown that intrauterine exposure to gestational diabetes results in lower energy expenditure and higher RER especially during the daytime hours. These results imply an impaired capability of offspring to from dams with gestational diabetes to switch from carbohydrate oxidation to lipid oxidation(Lau et al., 2011). This effect was observed before the development of the obese phenotype and is indicative of the potential programming role of the maternal phenotype to the BMR and the substrate oxidation of the offspring.
By elucidating the reasons for the variations in BMR, new strategies could be designed to adjust BMR and help prevent obesity. If lower BMR is programmed in utero, as we hypothesize, maternal interventions during pregnancy aiming at the improvement of maternal phenotype could subsequently benefit the offspring.

1.6 Other molecular mechanisms implicated in the predisposition to obesity in offspring of obese mothers

The molecular pathways, which are perturbed in the offspring as an effect of maternal obesity, are proposed to be regulators of energy balance. The development of obesity in the offspring could be attributed to dysregulation of the energy balance and subsequently adiposity and obesity.

1.6.1 Adipocyte metabolism

Offspring of rat dams fed a high palatable diet in pregnancy and lactation demonstrated adipocyte hypertrophy after weaning, however, it is not known if the effect was persistent (Bayol et al., 2005). In a cross-fostering study offspring of obese dams suckled by lean mice had approximately 40% higher PPAR-γ levels than controls on the same high fat diet (Shankar et al., 2008), suggesting increased adipogenesis due to maternal obesity. Similarly, offspring of obese mice at 12 weeks of age had increased leptin expression and decreased adiponectin expression in white adipose tissue (Fernandez-Twinn et al., 2012).

In addition, decreased capability to switch to fat oxidation could have an effect on adipocytes. Data from a twin study suggest that impaired UCP3 expression results in increased RER and thus reduced fat oxidation (Ukkola et al., 2001). The recent study of Lau et al. (Lau et al.), showed that offspring of diabetic mothers had reduced ability to switch to fat oxidation during the light hours, as usually occurs, which could lead to increased adiposity.
The above proteins and mechanisms could play a mediating role in increased adipogenesis and subsequently to the development of obesity. The growth of adipose tissue is not restricted, nor is the increase in fat cell number reversible (Faust et al., 1978; Corbett et al., 1986). Therefore, increased adipogenesis during early life or even in utero could have a detrimental effect on body composition in adult life.

1.6.2 Mitochondrial dysfunction

Mitochondrial diseases form a group of the commonest inherited disorders that associate with metabolism and can be associated with various symptoms and outcomes. About 10–15% of the mitochondrial diseases are attributed to mutations in mitochondrial DNA (mtDNA) (Mancuso et al., 2007; Debray et al., 2008). mtDNA is vulnerable to environmental influences and suboptimal environments can reduce quantity and increase random mutations within it (Wallace; Graziewicz et al., 2002; Mancuso et al., 2007; Debray et al., 2008). Although some changes due to environmental influences in embryonic mitochondria may be adaptive, others may be just developmentally disruptive (Knudsen & Green, 2004).

Insulin sensitivity was associated with mtDNA content in the offspring of type 2 diabetic patients but was inversely correlated with the components of metabolic syndrome such as blood pressure and waist/hip ratio (Song et al., 2001). Increased mtDNA content has been associated with the development of metabolic syndrome (Malik & Czajka, 2013). According to the data of a study in rats, both maternal exposure to malnutrition and high fat diet both resulted in an absence of increased ATP production and insulin release in response to glucose stimulation, which suggests an absence of islet sensitivity to glucose sensing (Song et al., 2001; Theys et al., 2011). Mitochondrial dysfunction has been previously correlated with insulin resistance in rats coming from obese dams (Taylor et al., 2005; Bruce et al.,
In a mouse study offspring of obese mice have been found to have a compromised complex II-III activity, but only in males. Nevertheless, no effect was observed in the mitochondrial ETC activity assays (Shelley et al., 2009).

Moreover, offspring of obese rats had higher RER suggesting impaired fatty acid oxidation, which is associated with mitochondrial function. The offspring of obese dams had reduced hepatic SIRT3 mRNA, mitochondrial protein content, ETC enzyme complexes I&II and fasting PGC1-α expression (Borengasser et al., 2011). Evidence from a study of mice demonstrated that offspring of high fat fed mice developed non-alcoholic fatty liver disease which was partly attributed to reduced hepatic ETC enzyme complex activity I,II,II and IV(Bruce et al., 2009). Finally, it has been shown that maximal oxygen consumption (VO₂ max) rate and muscle performance is associated with mitochondrial function (Rasmussen et al., 2001). This fact along with the evidence that obese adolescents from obese mothers had a decreased mitochondrial mass in contrast with those where only the fathers were obese imply a maternal influences in resting energy expenditure (Wilms et al., 2010).

### 1.6.2.1 Uncoupling Proteins

Uncoupling proteins are a family of proteins that have been identified in the inner mitochondrial membrane. They modulate the entrance of protons into the mitochondrial matrix without ATP oxidation (Ukkola et al., 2001), that leads to the heat generation of the adipose tissue (Schrauwen & Hesselink, 2002). Because of the above, we can draw the conclusion that UCPs participate in the energy metabolism (Ukkola et al., 2001). The most common proteins are the UCP 1, 2 and 3 that are expressed in different tissues and each has different role in energy metabolism.
UCP1 is expressed in brown adipose tissue and has been associated with adaptive thermogenesis (Cannon & Nedergaard; Ukkola et al., 2001). Evidence of the role of UCP1 in diet-induced thermogenesis derive by its downregulation during starvation due to reduced sympathetic nerve activity to brown adipose tissue (Lowell & Spiegelman, 2000). However, UCP1 knockout mice do not become obese. Therefore, UCP2 and UCP3 were suggested to also be involved in thermogenesis. It has been shown that carriers of common polymorphisms of UCP2 had increased respiratory exchange ratio (Ukkola et al., 2001), insulin resistance (Yu et al., 2005) and elevated TSH hormone and increased weight gain during a period of overfeeding (Ukkola et al., 2001). Moreover increased levels of UCP2 protected form diet-induced obesity in mice (Ricquier & Bouillaud, 2000). UCP3 is suggested to be a regulator of the effects of sucrose on energy balance (Levine et al., 2003) and involved in the oxidation of free fatty acids (Boily et al.) (Schrauwen & Hesselink, 2002). Finally, impaired UCP3 expression resulted in increased respiratory exchange ratio in a twin study (Ukkola et al., 2001), highlighting the role of UCP3 on the substrate oxidation. UCP3 progressing decreasing levels have been associated with the development of insulin resistance during high fat feeding (Senese et al., 2011). Moreover, UCP3 has been shown to protect from lipid induced mitochondria damage resulting from prolonged high fat feeding in mice (Nabben et al., 2011).

Evidence from studies in developmental programming indicate a role of maternal influences in offspring UCP gene expression. Maternal undernutrition during the last month of pregnancy results in UCP1 reduction in neonatal ewes (Budge et al., 2000). Consumption of 25% more of calories in ewes during gestation resulted in increased UCP1 expression in, increased thermogenesis and subsequently increased viability in the offspring (Budge et al., 2000). Moreover, the expression of UCP2 was
increased among rat male coming from dams fed a junk food diet and exposed to a postnatal junk food diet as well (Bayol et al., 2010).

1.6.3 The role of epigenetic modification of DNA in development of offspring obesity associated with maternal obesity

Alterations in the epigenome, including cytosine methylation of DNA, histone posttranslational modifications, and micro-RNA have been associated with metabolic diseases including obesity (Seki et al., 2012).

Epigenetic modifications secondary to overnutrition may result in metabolic imprinting or persistent alterations in genes involved in the regulation of energy homeostasis (Dabelea & Crume, 2011). Though epigenetic alterations are thought to underlie many of the aforementioned mechanisms (Gluckman et al., 2007; Gabory et al., 2011), relatively few studies have demonstrated evidence of epigenetic changes in the offspring as a result of maternal overnutrition (Seki et al., 2012). In addition, there is limited evidence in relation to the epigenetic characterization of humans at risk of T2D or obesity and most studies to date have focused on characterizing the methylation status of selected CpG sites in candidate genes (Drong et al., 2012).

However, obesity of both parents has been shown to affect DNA methylation status of newborn babies and at different loci suggesting a significant but different role of paternal and maternal obesity (Soubry et al., 2013). A study of 48 women showed that placental leptin gene DNA methylation levels were correlated with glucose levels in women with glucose intolerance (Bouchard et al., 2010). Similarly, maternal glycemia has been associated with DNA methylation changes at two adipokine (ADIPOQ) gene loci (C1, E2) in the placenta (Houde et al., 2013).
Similarly, animal models have been an invaluable tool in the study of epigenetic modifications secondary to maternal nutrition. Offspring of HF diet-fed rat dams have shown hypomethylation in the promoter regions of several genes thought to facilitate feeding behaviours (Vucetic et al., 2010). According to data from a rat model of maternal obesity FTO expression was elevated in both the hypothalamus and liver at weaning among the offspring of obese dams. Early overexpression of hypothalamic FTO correlated with increased adiposity and hyperphagia when exposed to high fat diet (Caruso et al., 2011).

In addition to changes in methylation patterns, histone modifications and changes in miRNA-mediated mechanisms have also been reported (Sinclair et al., 2007). A study in macaques found that maternal (HF diet-based) overnutrition impaired fetal chromatin structure by hyper-acetylation of several histones (Aagaard-Tillery et al., 2008). Moreover, maternal HF diet in mice not only resulted in upregulation of specific genes such as PPAR-alpha Igf2 and cpt-1a but also in altered levels of approximately 23 miRNAs by ~1.5-4.9-fold. It is suggested that these miRNAs may play an important role during early development and in offspring metabolism or if they have common targeted transcripts, to suppress protein synthesis (Zhang et al., 2009).

It is thought that certain epigenetic changes are potentially reversible due to the plasticity of the epigenome (Seki et al., 2012). This plasticity may persist throughout gestation, lactation and early life of animals, and so may provide opportunity for intervention against programmed epigenetic changes in utero; a concept already demonstrated in rats (Burdge et al., 2009) and mice (Dolinoy et al., 2006). For example, agouti mice were administrated either a normal diet or a methyl-supplemented diet that induces DNA hypermethylation during development. The
supplementation with methyl-donor rich foods before and during pregnancy resulted in a persistent hypermethylation at the AGRP locus and prevention of obesity in the offspring (Waterland et al., 2008). More recently, it has been shown that exercise intervention in the offspring of obese dams decreases the expression of FTO in the hypothalamus (Caruso et al., 2013). An intervention study has shown that siblings who were born following bariatric surgery of the mother had different methylation status and gene expression to their sibling. In particular, gene expression of glucose-metabolic and inflammation-related functions was altered leading to improved metabolic functions of these offspring (Guenard et al., 2013).

The evidence above, suggest that maternal obesity does have a negative effect on the offspring genome but it could be reversed by dietary and lifestyle interventions during gestation.

1.7 Maternal interventions aiming to improve obese pregnancy

1.7.1 Human Studies

As summarised previously, in a review of relevant studies in women and their children, observational studies have suggested independent associations between maternal obesity and offspring obesity; however observational studies cannot prove causality. The association of maternal obesity with the development of offspring obesity can be confounded by environmental and lifestyle factors relating to the mother or the child. Maternal education, lifestyle, smoking and socio-economic status have always been considered, among others, to be co-factors in the development of childhood obesity. The child’s physical activity and diet will also potentially play a major confounding role (Han et al., 2010). Intervention studies can provide a better insight into causality, as in the setting of a randomised controlled trial, the study bias can be minimised. To date, very few intervention studies on
obese or overweight women have followed the children to address the influence of the intervention on childhood obesity. Importantly, an intervention has first to be shown to be effective in changing factors in pregnancy which have been implicated in childhood obesity for example maternal fat mass, maternal diet, maternal glucose tolerance or neonatal fat mass or birthweight. Relevant interventions studies will now be briefly reviewed.

Most of the interventions have focused on reducing GWG by exercise, lifestyles and/or counselling interventions. It is generally considered, although not always the case, that GWG reflects maternal gain in fat mass. Interventions aiming at increased physical activity among the intervention groups have been used as reviewed by Streuling et al (Streuling et al., 2010; Streuling et al., 2011). The results, though, are controversial as evidence from some studies have shown a significant reduction in GWG in both obese (Weisman et al., 2011) and overweight women (Korpi-Hyovalti et al., 2011). However, others showed no difference either in obese (Guelinckx et al., 2010) or overweight (Luoto et al., 2011) women.

Other studies have placed the focus on diet, either on reducing fat intake (Polley et al., 2002; Luoto et al., 2011) and improving the quality of the dietary fats (Laitinen et al., 2009; Jackson et al., 2011) or decreasing the total glycaemic load (Rhodes et al., 2010) in women at risk but not necessarily obese. Dietary interventions have generally been targeted to achieving a reduction in GWG by improving nutritional habits during gestation. A study has shown that frequent communication and dietary advice to the intervention group resulted in lower GWG only among women with a normal pre-pregnancy weight (Phelan et al., 2011). However, a study using a multidisciplinary approach, did improve nutritional habits and reduced incidence of gestational diabetes among obese women in the intervention group (Quinlivan et al., 2011). It is noteworthy that in a meta-analysis by Thangaratinam (2012), it was found overall that diet was more likely to be changeable than physical activity.
Moreover, only dietary intervention showed a significant but small reduction in GWG and gestational hypertension among obese or overweight women. However, there was minimal evidence for any change in maternal or neonatal endpoints of clinical relevance.

In many of these studies the results are stratified for BMI but have not been undertaken solely in overweight or obese women. Among overweight and obese women only, very few intervention studies have been reported, which have been the subject of systematic reviews (Dodd et al., 2010). Only small effects of the interventions in relation to GWG have been observed, and no overall benefit on clinical outcomes, notably in terms of relevant childhood outcomes, in relation to any reduction in LGA or macrosomia have been seen.

Most of the intervention studies are not powered for clinical outcomes and design is generally poor and often it is not determined whether the intervention changes the behaviours for example diet and physical activity anticipated. Moreover most of the studies have attempted to reduce GWG by general dietary advice, without addressing the key issue in obesity and adverse pregnancy outcome, notably insulin resistance.

More recent studies have a different approach, targeting insulin resistance in obese pregnancies but not in GWG. The ROLO dietary intervention, from Dublin, targeted women who have previously delivered macrosomic infants. Despite showing no effect on macrosomia, the women included in the intervention did gain less weight and improved their glucose metabolism (Walsh et al., 2012). The LIMIT study is an intervention in obese and overweight women providing dietary and lifestyle advice to the intervention group (Dodd et al., 2011). Recently it was reported that even though the prevalence of large for gestation age (LGA) infants has not changed between the two groups (the primary outcome), macrosomia (a secondary outcome) was
significantly reduced (Dodd et al., 2014). The UPBEAT study (Poston et al., 2013), shortly to complete recruitment, from our group introduces an exercise intervention together with a low glycaemic index dietary intervention in pregnant women. The pilot study has reported changes in diet but not physical activity due to the intervention among obese women.

Other than the LIMIT study, and UPBEAT, which is yet to report, the studies above are mainly focusing on improving pregnancy outcomes at birth and are not investigating how offspring outcomes are affected. Very recently it was reported that neonatal infants from the intervention group in the ROLO study had reduced thigh circumference, indicative of reduced adiposity (Donnelly et al., 2014). The principle investigators of these three studies are now working together to standardise protocols for follow up of the children. 3 year-old follow up is currently underway in LIMIT and UPBEAT, and 5 year old in the ROLO study.

1.7.2 Interventions on animal models of maternal obesity to prevent offspring adverse health outcomes

Animal models are not only effective in understanding the mechanisms underlying developmental programming but also provide a means of evaluating the efficiency or even the potential harms of some interventions before we introduce them to humans. Recently, some maternal interventions during developmental programming windows have proven beneficial for outcomes in the offspring of obese dams providing potential therapeutic pathways that could be tested in clinical trials. Some of these studies will be discussed below.
1.7.2.1 Dietary and physical activity interventions

A maternal dietary intervention (reduction in calorific intake) before mating in diet-induced obese rats resulted in offspring that had significantly improved serum leptin, insulin and triglyceride levels and reduced subcutaneous fat compared to the offspring of obese dams at weaning (Zambrano et al., 2010). In adulthood, the offspring showed decreased fat mass compared to the offspring of obese dams but increased fat mass compared to controls, so the intervention was not totally effective in reversing the phenotype. Similarly their blood glucose level was not significantly different from any of the two other groups.

The same group showed that an exercise intervention before and during pregnancy in the diet-induced obese rat model prevented the leptin and triglyceride increase that was observed in the offspring of obese dams at weaning. It is important to highlight that the dams in this intervention did not have different calorie intake or bodyweights suggesting that any changes observed in the offspring are due to differences in body composition (Nathanielsz et al., 2013; Vega et al., 2013).

Studies in ewes have shown that reduction of the maternal calorie intake during pregnancy in obese sheep (from 150% to 100% of habitual diet) prevented increases in fetal organ weight due to maternal obesity. At day 135 of gestation, the fetuses of obese dams had greater total kidney weight and total perirenal fat, left ventricular weights and thicknesses, right ventricular thicknesses, as well as reduced pancreatic weight, in comparison with the offspring of control and dams included in the intervention.

Together, these results from animal models not only indicate a role of maternal obesity on organ and tissue growth but also a therapeutic potential of interventions.
Moreover, it is important to conduct and compare studies in precocial and altricial species as findings may differ (Nathanielsz et al., 2013; Tuersunjiang et al., 2013).

1.7.2.2 Supplementation with antioxidants

Obesity in pregnancy has been associated with oxidative stress in the dams and the fetuses in rat studies (Lin et al., 2011) but also reduced oxidative defences have been reported in obese women (Sen et al., 2014). Therefore, supplementation with antioxidants is considered a potential therapeutic strategy.

A study in yellow agouti mice demonstrated that supplementation of genistein, a phytoestrogen with antioxidant properties (Han et al., 2009) to high fat fed dams during gestation resulted in offspring with reduced obesity risk compared to offspring from HF diet dams that did not receive the intervention (Dolinoy et al., 2006). This finding was thought to be due to the supplement causing changes in epigenetic marks, notably increased rates of methylation in the cellular DNA of the mice across multiple tissues.

Maternal supplementation with antioxidants (Vitamin A, C, E) in rats fed a ‘Western style’ diet has been shown to protect the offspring from glucose intolerance and increased adiposity (Sen & Simmons, 2010). Similarly, bitter melon supplement (known for its antioxidant properties) fed to high fat/high sugar diet-fed dams during gestation and lactation improved offspring lipid profiles, reduced markers of oxidative stress in their liver and increased markers of lipid oxidation like PPAR-γ (Ching et al., 2011).
1.7.2.3 Fibre supplementation

Despite limited studies there is some evidence showing a beneficial effect of maternal supplementation with fibre in obese pregnancy. These studies are going to be discussed in some detail in section 1.8, as one of the aims of my thesis, includes the investigation of how supplementation with fibre influences the offspring of obese dams.

1.8 Dietary fibre

In 2005, the WHO/FAO Codex Alimentarius Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) (FAO/WHO, 2005) defined dietary fibre as:

“Dietary fibre means carbohydrate polymers with a degree of polymerizations (DP) not lower than three, which are neither digested nor absorbed in the small intestine. A degree of polymerization not lower than three is intended to exclude mono- and disaccharides. It is not intended to reflect the average DP of a mixture.

Dietary fibre consists of one or more of:

- Edible carbohydrate polymers naturally occurring in the food as consumed
- Carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical means
- Synthetic carbohydrate polymers”

During the period in which obesity rates have increased, a significant change in diet has also taken place. While the total consumption of carbohydrates in the Westernized diet has increased, the consumption of fibre has fallen dramatically (Maskarinec et al., 2006; Ford & Frost, 2010). The recommended amount of fibre consumption in UK is 18 g of fibre per day according to the Department of Health (Whitton et al., 2011). In UK only 25% men and 29% of women consume the
recommended portion of fruits and vegetables per day and this proportion varies depending on different age groups (Aresu et al., 2009). However, the recommended fibre intakes may vary in different countries; to be more specific fibre recommendations published in the dietary reference intakes suggest that fibre recommendations are relevant to calorific intake thus the recommendation for men was 38 g/d in contrast to women's, 25 g/d (Slavin, 2005).

The benefit of fibre has been exemplified in a prospective cohort study of 74,091 women between 38-63 years old, in which an inverse association has been observed between weight gain and the consumption of foods rich in fibre (Liu et al., 2003). As the decreased fibre consumption could be linked with increased obesity prevalence, introducing fibre in foods could potentially be a strategy to ameliorate the current situation and improve weight management. The way that this effect of fibre may be achieved is likely to be attributed to several of its properties.

Increased fibre consumption has been associated with the replacement and reduction of energy dense meals and subsequently with reduced total energy and fat consumption (Dauchet et al., 2009). Moreover, long-term studies evaluating the effects of different dietary interventions suggest that increased fibre intake may benefit weight management by enhancing satiety (Rolls et al., 2004). Viscosity of some fibres is considered to be a potential mechanism mediating the appetite regulation linked to fibre consumption (Kristensen & Jensen, 2011). The role of fibre in satiety is, also, suggested to be mediated by its influence on gut hormones such as gastric inhibitory peptide (Tseng et al., 1996), glucagon-like peptide-1 (van Dijk & Thiele, 1999), and cholecystokinin (CCK). For example, a direct association of CCK has been reported between postprandial CCK and increased satiety (Holt et al., 2001; Burton-Freeman et al., 2002).
Foods rich in dietary fibre remain inindgested in the small intestine while entrapping/absorbing organic molecules such as glucose and subsequently influence their metabolism. Therefore, fibre health benefits are related to the low Glycaemic Index (GI) of fibre rich foods. Glycaemic index has been introduced in order to classify carbohydrates depending on their effect to post-meal glycaemia aiming at the glycaemic control of diabetic patients (Jenkins et al., 1981; Ford & Frost, 2010). Increasing evidence suggest that a low GI diet can increase insulin sensitivity along with High-Density Lipoprotein (HDL) and other positive metabolic consequences (Marsh & Brand-Miller, 2005). In young overweight men following an energy restricted low GI diet, mean resting energy expenditure (REE) was higher than in men consuming an energy restricted but high GI diet, despite the same weight loss in one week (Agus et al., 2000). A short term-study in 37 children found that consumption of a low GI breakfast was associated with increased satiety and a decreased energy intake at lunch compared to consumption of high GI breakfast (Warren et al., 2003). These properties of fibre are potentially relevant to obesity in pregnancy, which is associated with heightened insulin resistance. Moreover, plant-derived dietary fibre is rich in phytoconstituents, which have antioxidant properties (Sangeethapriya & Siddhuraju, 2014).

Moreover, when fibre is absorbed in the large intestine, it is fermented and provides carbohydrates to gut microbiota and produces short chain fatty acids (SCFAs) aiding colon health. This property of fibre is attributed to its potential prebiotic properties. “Prebiotics are non digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of intestinal bacteria intestinal bacteria associated with the host health” (Gibson & Roberfroid, 1995; Kristensen & Jensen, 2011). However, only the dietary carbohydrates that promote the growth of bacteria associated with host wellbeing, resist hydrolysis and are fermented by
intestinal microbiota are prebiotics as it has been reviewed (Kristensen & Jensen, 2011).

The influence of fibre supplementation has been tested in rodents. Rats that were fed a diet high in fibre from weaning demonstrated increased secretion of satiety hormones, upregulation of uncoupling protein-3 levels in brown adipose tissue (suggestive of increased thermogenic capacity) and improved expression levels of genes known to regulate glucose and lipid metabolism (Maurer et al., 2009). Moreover, rats that were fed a high fibre diet after weaning were less susceptible to weight gain and hyperphagia compared to rats that have been weaned to high protein diet (Maurer et al., 2010).

Despite the accumulating evidence and the increasing public awareness of fibre’s beneficial effects the busy, modern lifestyle makes it hard for people to plan their meals. As a result, the consumption of natural sources of fibre such as vegetables and fruits is usually neglected. Therefore, ‘functional foods’ which are rich in fibre may provide an alternative solution for the increase in the daily intake. For example, the addition of soluble fibre in ready-made meals such as ice cream or even dissolved in water could be a potential intervention.

1.8.1 Maternal supplementation with fibre

Despite the unequivocal beneficial effect of dietary fibre consumption on overall health there is limited evidence regarding its effect on pregnancy, mainly from animal studies. Maternal dietary supplementation with fibre could potentially attenuate the negative effects of maternal obesity on the offspring.

Fibre supplementation has been used as an intervention in rats. One study administered dietary fibre, from oats, to high fat fed diet dams until day 19.5 of gestation. The supplementation resulted in increased litter size and improved
reproductive success among the intervention group (Lin et al., 2011). Furthermore, fetal development and growth was improved by enhancing maternal, placental and fetal antioxidant defence capacities in the rats. The same group also replicated these findings more recently, showing that the same effects can be produced independently of maternal energy intake (Lin et al., 2012).

A similar intervention to the one presented in this study was a published study of a maternal intervention with prebiotics and fibre (inulin). The dams were given either the control diet or were supplemented with prebiotics and fibre during gestation. The offspring of the supplemented dams had increased muscle mass. Even though this intervention was not in obese dams it does show a potential beneficial role of fibre supplementation on offspring body composition (Desbuards et al., 2012).

The results from these maternal intervention studies in obese pregnant animals provide important evidence to inform on-going strategies in man, including a potential means for elucidating mechanisms, which would help in the identification of potential novel therapeutic agents, and subsequently the design of new clinical interventions. Interestingly, though, the studies mentioned above have also altered the overall diet composition, which could confound the interpretation of results. The aim of this study was to determine whether any beneficial effect of a dietary fibre supplementation could be achieved without altering the maternal diet composition. Such an intervention could be more realistic in obese mothers as they would not have to alter their dietary habits but just to include fibre as part of their daily dietary regime. As mentioned above several of the successful intervention studies employed dietary fibre; in this thesis I have also explored the potential for a dietary fibre intervention during pregnancy.
1.8.2 Polydextrose, a dietary fibre

In this thesis I have evaluated the influence of maternal dietary supplementation with polydextrose on offspring phenotype. The properties of this dietary fibre will now be discussed.

By definition polydextrose is now widely accepted as a dietary fibre. “Polydextrose is a randomly bonded polymer of glucose with some sorbitol endgroups” (Charalampopoulos et al., 2009). It was developed by Pfizer as a low calorie bulking agent that could be used with intense sweeteners in the food industry. It has a caloric value of 1 kcal/g, a mild sweet taste, a low glycaemic index and prebiotic properties. It is widely used in the food industry, mainly in confectionery, baking goods, fruit spreads, pasta and noodles, and pharmaceuticals (Charalampopoulos et al., 2009).

Introduction of polydextrose (PDX) in foods has been shown to be beneficial to various health outcomes in several studies. The addition of PDX and the replacement of sugar with lactitol in chocolate were associated with only a minor effect on participants’ plasma insulin and blood glucose, in contrast with the effect of control chocolate ingestion which increased both these parameters (Shimomura et al., 2005). Additionally, the triglyceride concentration was more elevated in the non-sugar chocolate group than in the control group. The same researchers followed this observation with a study in rats which showed that non-sugar fat emulsion containing lactitol and polydextrose had a lower effect on serum glucose and almost no effect on triglyceride levels compared with control fat emulsion containing sucrose (similar to chocolate) (Shimomura et al., 2005). In a similar study a chocolate milk containing 3 different concentrations of PDX was given to participants to assess the influence on subsequent food intake, and a dose dependent effect was demonstrated, the highest meal consumption being correlated with the lowest
consumption of PDX (Astbury et al., 2013). Moreover, in a study from China, 28 days supplementation of normal subjects with 4-12 g of PDX prevented increased glucose absorption by the small intestine and promoted its fermentation in the lower gut to produce short chain fatty acids (SCFAs) (Jie et al., 2000).

Polydextrose’s beneficial effects also relate to its action as a prebiotic. In addition to the above, fibre and in particular soluble fibre has a prebiotic action. Prebiotics were originally defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health" (Gibson & Roberfroid, 1995).

The effect of PDX on large intestine pH is similar to that of soluble dietary fibre, leading to alkalinisation, which in turn may affect the microbiome (Yoshioka et al., 1994). Indeed, supplementation with PDX, has been shown to simultaneously promote the proliferation of favourable intestinal bacteria and to reduce the acidity of the bowel (Jie et al., 2000). When PDX blended with sweetener in chocolate milk was given to men not only did it lower the energetic value of the chocolate drink, but it also had a beneficial effect on the gut microbiota and maintained abdominal discomfort at a low level (Beards et al., 2010). Furthermore, the addition of PDX to food has been shown to increase the abundance of bacteria (Peuranen et al., 2004) and that the colonic microbiota do not change with prolonged administration. Tolerance to high intake was also excellent (Achour et al., 1994).

The evidence for the potential health benefits of polydextrose together with the knowledge that it does not alter the caloric intake of the food consumed make it a good candidate for use as a potential intervention in obese pregnant women.
1.9 Aims and Hypotheses

As thoroughly discussed in the introduction, review of the literature reveals the necessity to further explore the influences of maternal obesity on offspring energy balance. In addition, attention is now drawn to the design of interventions aiming to the improvement of maternal physiological profiles during pregnancy and gestation. Therefore, the overarching goal of this thesis was to determine the role of maternal obesity on the disruption of offspring energy balance and to examine whether maternal supplementation with a soluble fibre in obese pregnancy, polydextrose can prevent or alleviate this disruption.

1.9.1 Hypotheses

The hypotheses tested during this PhD were:

1. Maternal obesity adversely influences offspring health through permanent alteration in energy balance. The enrichment of the maternal diet of obese mice with polydextrose, during critical periods of gestation and lactation will prevent these adverse effects and thereby improve offspring metabolic dysfunction.

2. Maternal obesity exacerbates the effects of obesogenic dietary challenge in adult offspring; while maternal polydextrose supplementation in obese pregnancy will confer protection against the consequences of the dietary challenge.

3. Supplementation of obese dams with the soluble fibre, polydextrose, during the critical periods of gestation and lactation will improve the maternal metabolic profile and subsequently benefit the offspring.
4. Maternal obesity results in altered mechanistic pathways involved in the regulation of energy balance. Supplementation of the obese dams with a soluble fibre will reverse the changes in the mechanistic pathways induced by maternal obesity.

1.9.2 Aims

Specific aims were devised in order to test the above hypotheses:

1. To investigate the influences of maternal obesity and the potential beneficial effect of maternal supplementation with polydextrose in offspring:
   i. Energy expenditure
   ii. Energy intake
   iii. Body composition
   iv. RER and Glucose metabolism.

2. To determine whether the dietary challenge in adult offspring has greater adverse outcomes in offspring of obese dams compared to offspring of control and of dams supplemented with polydextrose, in relation to:
   i. Susceptibility to bodyweight gain
   ii. Increased energy intake
   iii. Changes in energy expenditure
   iv. Changes in RER and glucose metabolism

3. To determine the effect of maternal supplementation with polydextrose in obese pregnancy, on:
   i. Reproductive success
   ii. Glucose metabolism
   iii. Water intake
iv. Energy Intake

v. Gestational bodyweight

vi. Adipokine profiles

4. To investigate the potential pathways affected by maternal obesity and by the supplementation with soluble fibre in obese pregnancy, with particular focus on:

i. Mitochondrial copy number.

ii. Uncoupling Protein (UCP) gene expression.

iii. Changes in microbiota
Chapter 2

Materials and Methods
Chapter 2 Materials and Methods

2.1 Methods applied for *in vivo* experiments

2.1.1 Animals

2.1.1.1 Dietary Composition and macronutrient intake

The murine model of maternal diet-induced obesity, which has previously been established in our group, was employed throughout in this study. The palatable obesogenic diet used mimics the dietary consistency of a Western-type diet and has been proven to be effective in inducing weight gain among female mice (Samuelsson *et al.*, 2008). Female C57BL/6J mice were fed either a standard chow diet (7% simple sugars, 3% fat, 50% polysaccharide, 15% protein (w/w) RM1, Special Dietary Services, UK, energy 3.5kcal/g, n=20) or a semi-synthetic energy-rich and highly palatable obesogenic diet (10% simple sugars, 20% animal lard 28% polysaccharide, 23% protein (w/w), Special Dietary Services, UK, energy 4.5kcal/g, n=30). The pelleted obesogenic diet was supplemented by ad libitum access to sweetened condensed milk (approximately 55% simple sugar, 8% fat, 8% protein, w/w, Nestle, SZ) with added micronutrient mineral mix (AIN93G, Special Dietary Services, UK) (Table 2.1, 2.2). Macronutrient and calorific intake were calculated from measured daily intake of pellets and milk (approximately 16% fat, 33% simple sugars, 15% protein, energy 4.0kcal/g) (Samuelsson et al, 2008).
<table>
<thead>
<tr>
<th>Diet</th>
<th>Control (RM1)</th>
<th>Obesogenic Diet (Pellets)</th>
<th>Obesogenic Diet (Sweet Condensed milk)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein (%)</strong></td>
<td>14.38</td>
<td>23</td>
<td>7.8</td>
</tr>
<tr>
<td><strong>Total Carbohydrate (%)</strong></td>
<td>61.73</td>
<td>38.83</td>
<td>55.3</td>
</tr>
<tr>
<td><strong>Polysaccharides (%)</strong></td>
<td>57.68</td>
<td>28.34</td>
<td>-</td>
</tr>
<tr>
<td><strong>Simple Sugars (%)</strong></td>
<td>4.05</td>
<td>10.49</td>
<td>55.3</td>
</tr>
<tr>
<td><strong>Fat (%)</strong></td>
<td>2.78</td>
<td>22.21</td>
<td>8.1</td>
</tr>
<tr>
<td><strong>Soya oil (%)</strong></td>
<td>-</td>
<td>4.32</td>
<td>-</td>
</tr>
<tr>
<td><strong>Saturated fat (%)</strong></td>
<td>-</td>
<td>17.89</td>
<td>5.6</td>
</tr>
<tr>
<td><strong>Corn oil (%)</strong></td>
<td>2.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Crude Fibre (%)</strong></td>
<td>4.65</td>
<td>6.17</td>
<td>Traces</td>
</tr>
<tr>
<td><strong>Energy (kcal/g)</strong></td>
<td>3.5</td>
<td>4.5</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Table 2.1 Composition of the experimental diets presented as percentages by weight (g/100g). The obesogenic diet consisted of both pellets and sweetened condensed milk. Due to varying moisture and nitrogen free extract content, columns will not sum to 100%.
2.1.1.2 Supplementation with Polydextrose (PDX)

Because polydextrose is a water-soluble fibre drinking water could be supplemented with PDX without altering the diet composition. Thus, it was concluded that any differences observed due to diet could be solely attributed to PDX. A part of the dams were supplemented with 5% (w/v) solution of PDX (Tate and Lyle, France) in the drinking water weekly. This concentration has previously been proven efficient in reducing insulin resistance in rats (Witaicenis et al., 2010) without toxicity. However, since the intervention in this study differed because the animals were supplemented in their drinking water; it was, therefore, investigated whether the water consumption in female adult mice (consuming either the obesogenic or the control diet) could differ. It was shown that the animals, which fed the obesogenic diet, consumed less water compared to their control counterparts (Animals on control diet 6.76±0.95 ml, n=6 versus obesogenic diet 3.83±0.62 ml, P<0.05; figure 2.1). This difference can be attributed to the addition of the sweet condensed milk, which provided the animals with an additional source of liquid. Thus, it was investigated whether increased concentration of PDX could be used with no safety. However it was shown that concentrations above 5 % induced diarrhoea as shown previously, (Witaicenis et al., 2010). This effect prompted us to keep the dosage to 5 % as previously published.
2.1.2 Animal husbandry

All studies were approved by Local Ethics Committee, and conducted under UK Home Office License. Female C57BL/6J mice, proven breeders (one previous litter) and approximately 100 days old (Charles River Laboratories, UK) were purchased and housed under controlled conditions (25°C, 12-hour light/dark cycle). The animals were housed in twos and were allowed to acclimatise for a week before being randomly assigned to either the control or obesogenic diet (see above) for 6 weeks, and then females were mated. The males were proven breeders (C57BL/6J) at 8-10 weeks of age. Following successful mating, the animals were maintained on the assigned diet throughout pregnancy and suckling. The animals were examined daily to confirm the presence of a copulation plug. The presence of a copulation plug was considered day zero of pregnancy. In the event < 10% gestational weight gain by gestational day 10 (a), animals were deemed 'not pregnant' and re-mated. The animals, which failed to become pregnant after a second mating, were killed by a schedule 1 method. 48 hours after birth, the litters were weighed and reduced to 3 males and 3 females from each dam, where appropriate. Small litters < 4 were not included in the study. The offspring were weaned to different post-weaning diets depending on the protocol. Specific n numbers are presented in each protocol.
2.1.3 Indirect Calorimetry

2.1.3.1 Principles of indirect Calorimetry

Heat loss occurring directly from a subject can be measured via direct calorimetry (Levine, 2005a). Typical methods of direct calorimetry are isothermal direct calorimeters or heat sink direct calorimetry (Kaiyala & Ramsay, 2011). Indirect calorimetry, however, estimates the heat production from gaseous exchange of the subject; more specifically, the ratio of oxygen (O₂) consumption and carbon dioxide (CO₂) production (Ferrannini, 1988; Arch et al., 2006).

Indirect calorimetry has a long history as people from the earliest times were linking respiration with body heat. The first attempt to build a calorimeter was by the English physician John Mayow (1643–1679) who was the first to discover that air was a mixture of gases (Figure 2.2). The calorimeter used in this instance consisted of an inverted bell jar over a water seal. The animal was placed in the jar and when breathing, the consumption of the air resulted in reduced pressure in the bell and water intruding. Despite establishing the air consumption, as the CO₂ was produced dissolved in the water, Mayow was unaware of the gas produced by the animal (Frankenfield, 2010).
Antoine Lavoisier, known for being the ‘Father of Modern Chemistry’, was the first to support that oxygen respired by the animal reacted in its lungs with carbon from the blood producing CO$_2$, which subsequently liberated heat directly from the oxygen (Lavoisier, 1780). Lavoisier suggested heat was a substance, which he called caloric, from which he derived the term calorimeter (Lutz, 2002; Kang, 2008; Frankenfield, 2010). Moreover, he demonstrated that the rate of oxygen consumption was dependent on a number of variables including environmental temperature and organism size. These variables suggested that experiments investigating metabolism needed to be performed under strict conditions (Henry, 2005). Lavoisier was the first to design an indirect calorimeter, using the same principles employed today and developing the first open circuit indirect calorimeter providing the animals with fresh air (Figure 2.3). Since then, the indirect calorimetry has been developed greatly and is used in numerous studies investigating energy expenditure.
Figure 2.3 Indirect Calorimeter by Antoine Lavoisier. On the left, a triple chamber construction is depicted. The upper right corner image shows a closed-circuit indirect calorimeter, which Lavoisier converted to open circuit direct calorimeter by including tubes that provided the mice with fresh air. The carbon dioxide produced was collected into bottles of alkali (Frankenfield, 2010).

The Labmaster, as shown in Figure 2.4, is an advanced system of an open circuit indirect calorimeter, which allows accurate measurement of $O_2$, $CO_2$ and substrate utilisation by Respiratory Exchange Ratio (RER). Animals are placed individually into metabolic cages and are provided with atmospheric air. The $O_2$ consumption ($VO_2$) and the $CO_2$ production ($VCO_2$) in each metabolic cage are measured, with correction for bodyweight. Heat production and RER are calculated by the predictive below by the software (as provided by the manufacturer).

$$RER = \frac{VCO_2}{VO_2}$$

$Heat production = CV \times VO_2$, where $CV = 3.815 + 1.232 \times RER$

These two metabolic parameters are the most important as they are indicators of resting metabolic rate and of the utilised substrate respectively.
Routine calibration against calibration gases of 20.90 Vol-% O₂ (accuracy ±0.1% relative), 0.05 Vol-% CO₂ (accuracy ± 1.0 % relative), 20.00 Vol-% O₂ (accuracy ± 0.1 % relative) and 0.95 Vol-% CO₂ (accuracy ±1.0 % relative). Weights for the feeding sensors were used at the beginning of each experimental period (time-points) and subsequently every 10 days thereafter.

2.1.3.2 TSE Labmaster

2.1.3.2.1 Measurement of energy expenditure

Energy expenditure (EE), respiratory exchange ratio (RER) and food intake were measured in the Labmaster (TSE, Hamburg) at 30 days, 3 and 6 months. At 30 days of age, 6 age-matched animals were weighed, and then placed in the metabolic cages (CaloCages). The animals remained in the cages for 48 hours. The first 24 hours was a period of acclimatisation; only the data from the last 24 hours were used in the analysis. Data points were collected in the Labmaster approximately every 20 minutes and hourly averages were calculated. The hourly averages were used to calculate daytime and night-time averages of the groups. The same procedure was repeated at 3 and 6 months of age.

Figure 2.4 The Labmaster. A. The processing unit where data are collected B. Calocages
2.1.3.2.2 Measurement of energy intake

The Labmaster was equipped with sensitive feeding and drinking sensors (Figure 2.5) that permitted accumulative food intake measurements. In the obesogenic diet group, the animals were provided with a pot filled with sweet condensed milk, which was weighed before the beginning and at the end of the experiment in order to calculate the consumption rate. The calorific intake was later calculated as the total quantity consumed multiplied by the calories contained in each gram of diet.

![Figure 2.5 The CaloCage and the food and drinking sensors. A. Sensor B. Drinking Bottle C. Feeding basket](image)

2.1.4 Body composition analysis by bio-impedance

Skeletal muscle plays an important role in the metabolic rate (Lanham-New et al., 2011). In contrast, the contribution of adipose tissue to basal metabolic rates (BMR) is small. When body weight is taken into account in obese animals, BMR declines due to increased adiposity and decreased fat-free mass (FFM) (Prentice et al., 1996; Das et al., 2004; Jin et al., 2009). Therefore, in obese animals it is important to have an accurate estimate of FFM and fat mass (FM) to accurately assess EE.

Bio-electrical impedance analysis measures the impedance of the body to the flow
of an electrical current. The electrical impedance (or conductivity) of a tissue depends upon its fluid and electrolyte content. Total body impedance is related to the amount of water in the body since body water is located primarily in free fat mass (Jin et al., 2009); total body water (TBW) varies between individuals according to the relative proportions of FFM and FM.

Total body impedance varies similarly but inversely according to the generally accepted model described by previously (Cornish et al., 1993).

\[ V = \frac{p \ L^2}{Z} \]

Where \( V \) = volume of total body weight (ml)
\( p \) = tissue resistivity (ohm/cm)
\( L \) = conductor length (cm)
\( Z \) = impedance (ohms)

Thus, assuming constant water content of FFM, and constant resistivity, TBW (or FFM) can be predicted from the measurement of whole body impedance (Z).

### 2.1.4.1 Body composition measurements by bio-impedance analysis

Measurements were made using the BIA model-Impedimed Imp SFB7. All animals were weighed and anesthetised with isoflurane (3–5% isoflurane/O\(_2\) mixture) for the duration of the BIA measurements. Anesthetised animals were placed flat on the abdomen on a non-conductive surface and the limbs extended perpendicular to the longitudinal axis. Four 25-gauge 1” needles (NN*2525R; Terumo Medical Corp, Elkton, MD, USA), bent at a 90° angle 5 mm from the tip, were inserted subcutaneously along the dorsal midline of the animal at the anterior edge of the
eye orbit (source 1), anterior edge of the pinna (detector 1), the sacral-caudal junction (detector 2) and fur line at the base of the tail (source 2) (Figure 2.6).

Figure 2.6 Placement of the electrodes on mice (Patel et al.). The Impedimed Imp SFB7 is shown on the right (A). Photo kindly provided by Dr Leigh Ward.

Electrodes and needles were attached according to the manufacturer’s guidelines. The length between the 2 detector needle electrodes were measured along the dorsal midline with a standard measuring tape and were incorporated into the BIS device calculations. Body proportion = 1.0, body density = 1.05 g/cm³, hydration constant = 0.732 and resistivity coefficients of pe = 325, pi = 752 for males and pe = 289, pi = 669 for females were used for all calculations. Whole body resistance and reactance readings were acquired using a single spectrum acquisition from 4 khz to 1000 khz. Three consecutive measurements were performed for a single positioning of the 4 needle electrodes in order to increase accuracy (Cornish et al., 1999). Needle electrodes were subsequently repositioned 2 additional times for a total of 9 measurements (3 positions, 3 measurements each). Acquired data was downloaded and processed using the provided bio-impedance software (ImpediVet Vet BIS1 v. 1.0.0.4).
2.1.4.2 Analysis and prediction of free fat mass

Since adult mice were approximately 1/10 the weight of adult rats, and, presuming similar body proportionality between the species, resistance coefficients were in the range of 5 to 15% of those defined for rats as it has previously been shown that resistant coefficient of 10% in rats can be successfully used (Chapman et al., 2010). Total body water (TBW) and ECW were predicted from the MFBIA measurement by using equations for rats (Thomas et al., 1992a):

\[ TBW = 309.9 \times \frac{L^2}{Z_c} + 30 \]
\[ ECW = 108.3 \times \frac{L^2}{R_0} + 13.8 \]

Where \( L \) is the inter-electrode distance, \( Z_c \), is the impedance at the characteristic frequency and \( R_0 \) is the resistance at zero frequency.

Predicted FFM was calculated from MFBIA-derived estimate of TBW as \( TBW/0.732 \). Subsequently, percentage of fat mass was calculated.

2.1.5 Glucose tolerance test

In order to determine whole body glucose tolerance, an intra-peritoneal glucose tolerance test (IPGTT) was performed using AlphaTRAK® Glucose meter (Abbott Animal health), which has been specially designed for and validated in mice and rats.

2.1.5.1 Measurement of glucose tolerance

Following an overnight fast, a cream containing lidocaine (2.5%) and prilocaine (2.5%) (EMLA cream 5%, AstraZeneca, UK) was applied as a topical anaesthetic to the tail. Once the analgesia had taken effect, fasting tail venous blood glucose was measured. Animals were then injected via the intraperitoneal (i.p.) route of administration with a glucose load (1 gram/kg) of glucose solution (10% glucose). Measurements of the blood glucose were taken at 15, 30, 60 and 120 minutes after the first injection using the glucose meter. Measurements were collected at each point while the animals were conscious and semi-restrained.
2.1.5.2 Data analysis

Data were expressed as mean ± SEM. The area under the curve for the selected groups were calculated and compared with the appropriate statistical test using Graphpad Prism 5 (GraphPad Software Inc., San Diego, California, USA). Statistical significance was assumed at the P< 0.05 level.

2.1.6 Organ Collection

At 30 days, 90 days and 14 months of age, animals were killed by rising concentration of CO₂ or cervical dislocation, in accordance with Schedule 1 of UK Home Office guidelines. The organs were removed and immediately snap frozen in liquid nitrogen for deoxyribonucleic acid (Bodnar et al.) extraction (Bodnar et al., 2003) and the fat pads (perineal, gonadal, inguinal and subcutaneous) were weighed.
2.2 Methods applied for *in vitro* experiments

2.2.1 RNA extraction from skeletal muscle and brown adipose tissue

2.2.1.1 RNA Extraction

Ullrich et al. in 1977 first extracted RNA using guanidinium isothiocyanate (Ullrich *et al.*, 1977), an innovative but very laborious method. Later, it was replaced by a single-step acidified phenol-chloroform homogenisation/precipitation method (Chomczynski & Sacchi, 1987). This method was modified for this study to extract total RNA from brown adipose tissue. RNA was dissolved by guanidine thicyanate and phenol, contained in Tri Reagent (Sigma-Aldrich). The addition of chloroform allowed the separation of the homogenate into three phases, from which the aqueous phase containing RNA was further precipitated.

In order to avoid RNA degradation and contamination, gloves were worn at all times and both gloves and workspaces were disinfected with TriGene disinfectant. Moreover, all samples were maintained on ice during the procedure and aliquots of RNA containing solutions were prepared at the time of extraction to avoid repeated freezing and thawing, which is known to lead to RNA degradation (Wilson & Walker, 2001).

2.2.1.2 RNA extraction procedure

Brown adipose tissue samples (30 mg weight) were placed in 1 ml Tri Reagent (Sigma-Aldrich, T 9424). The sample was then homogenised using a TissueLyser (Qiagen, UK) for 2 minutes at 25 Hz, using two 5 mm steel beads (Qiagen, UK) until the tissue had completely dispersed. Once homogenised, the samples were left to stand at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes. 200 µl of chloroform was added to all samples which, were then vortexed and incubated at room temperature for 15 minutes. Following
incubation, the samples were centrifuged in a microcentrifuge at 13000 rpm for 10 minutes at 4 °C. After centrifuging, the supernatant was carefully collected, in order to not disturb the interface, and transferred to sterile 2 ml eppendorf tubes. To precipitate the RNA, 500 µl of isopropanol (Sigma-Aldrich) was and the samples were centrifuged at 13,000 rpm for 10 minutes at 4 °C. The supernatant was removed carefully to avoid disturbing the RNA pellet. The RNA pellet was washed in 300 µl of ice cold 75 % ethanol and centrifuged at 13000 rpm for 5 minutes at 4 °C. The ethanol supernatant was disposed and the RNA pellets allowed to air-dry before adding 10 µl of distilled sterile water. A 2 µl aliquot was removed for quantification of RNA concentration and the remainder stored at -80 °C in 10 µl aliquots for further analysis later.

Once extracted, the purity of RNA was calculated by spectrophotometric absorbance at 260 nm using a NanoDrop® spectrophotometer (NanoDrop® ND-1000 Spectrophotometer, Nanodrop Technologies, Labtech, UK) relative to RNA free water alone. This reflects nucleic acid concentration so that one unit of optical density reflects 40 µg RNA/ml sample analysed (Wilson & Walker, 2001). In addition, the absorbance of each sample at 280 nm was measured. Subsequently, the 260/280 nm ratio was calculated; this ratio indicated any contamination from DNA/protein. When the ratio was ≥1.8 the sample was considered not to be contaminated (Wilkinson, 1995; Wilson & Walker, 2001; Wilson & Walker, 2010) and I proceeded to the synthesis of cDNA.

2.2.2 cDNA Synthesis

cDNA synthesis was performed using the Quantitect RT kit (Quantigen, UK). Firstly, the genomic DNA (gDNA) elimination reaction was set up on ice by adding 1 µl template RNA, 10 µl of water and 2 µl of gDNA wipeout buffer in 0.2 ml sterile eppendorf tubes. The samples were centrifuged for 30 seconds at 11800 rpm before
heating the mixture to 45°C for two minutes before standing on ice again. In each tube, 6 µl of mastermix including 1 µl of primer mix, 4 µl RT Quantiscript RT Buffer and 1 µl Quantiscript® Reverse Transcriptase were added and mixed by gentle pipetting. The samples were again centrifuged for 30 seconds at 11800 rpm. The samples were incubated for 5 minutes at 25°C to enhance binding of the random hexamers to the template RNA, then heated to 50°C for 60 minutes to allow cDNA synthesis and finally to 70°C for 15 minutes to inactivate the reaction. RNase/DNase-free water was added to give a final volume of 80µl. Each sample was diluted to 100 ng/ml using RNase/DNase free water (Qiagen, UK). All cDNA samples were stored at -80°C until required.

2.2.3 Polymerase Chain Reaction (PCR)

During PCR, a selected DNA sequence was amplified based on two primers (single-stranded DNAs), which were complementary to the opposite strands of the DNA sequence. The basic process of PCR was carried out over specific cycles of denaturation, hybridisation and synthesis resulting in the synthesis of several hundred million copies of the selected DNA fragment.

2.2.3.1 Oligonucleotide primers

Most primers used for qPCR were designed using Universal Probe Library Assay Design Centre, Roche Applied Science (https://www.roche-applied-science.com). Table 2.3 shows the forward and reverse sequences for all primers used for qPCR gene expression experiments.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Accession Number</th>
<th>Sequences</th>
</tr>
</thead>
</table>
| Mouse b2M     | NM_009735        | **Forward primer:** 5'-TTCAGTATGTTCGGCTTCCC-3'  
| (beta-2 microglobulin) |                  | **Reverse primer:** 5'-TGGTGCTTGTCTCAGTGACC-3'                                         |
| Mouse b- actin | NM_007393        | **Forward primer:** 5'-ATGGAGGGGAATACAGCC-3'                                                                                             |
|               |                  | **Reverse primer:** 5'-TTCTTTGCAGCTCTTTGGT-3’                                                                                          |
| Mouse GAPDH   | NM_001001303     | **Forward primer:** 5'-TTGATGGCAACAAATCCAC-3'                                                                                           |
| (glyceraldehyde-3-phosphate dehydrogenase) |                  | **Reverse primer:** 5'-CGTCCCCGTAGACAAAATGGT-3’                                                                                       |
| Mouse UCP1    | NM_009463.3      | **Forward primer:** 5'-GGCCTCTAGACACTCTTAA-3'                                                                                           |
| (Uncoupling protein-1) |            | **Reverse primer:** 3'-TAAGCCCAGCTGAGATTTGT-5’                                                                                          |
| Mouse UCP2    | NM_011671.4      | **Forward primer:** 5'-GGCCTCTACGACTCTGA-3'                                                                                             |
| (Uncoupling protein-2) |            | **Reverse primer:** 3'-GGGCACCTGTGGTGCTAC-5’                                                                                           |
| Mouse UCP3    | NM_009464.3      | **Forward primer:** 5'-GGATGCCTACAGAACCTG-3’                                                                                             |
| (Uncoupling protein-3) |            | **Reverse primer:** 3'-TTGTGAGTTGGGCAAGT-5’                                                                                             |

Table 2.2 Oligonucleotide primers used for the analysis of the selected gene expression. Forward and reverse sequences are included.
2.2.3.2 Reverse-transcriptase polymerase chain reaction (RT-PCR) protocol

Reverse transcription polymerase chain reaction (RT-PCR) allows the generation of complementary DNAs (cDNAs) using a reverse transcriptase enzyme and the amplification of a specific region of a gene of interest (Bustin, 2000).

Each RT-PCR reaction was made to total volume of 25 µl and consisted of: 3.5 µl cDNA, template 1 µl of each primer (forward and reverse), 12.5 µl HotStarTaq master mix (Qiagen, UK) and 7 µl RNase/DNase free water (Qiagen, UK). All RT-PCR reactions were conducted in a Px2 Thermal Cycler (Thermo Electron Corporation, UK). The PCR cycling conditions were as follows:

1. 95°C for 15 minutes
2. 3 step cycling: denaturation 94°C for 1 minute
3. Annealing at 60°C for 1 minute
4. Extension at 72°C for 2 minutes for a total of 40 cycles
5. Final extension at 72°C for 10 minutes.
6. RT-PCR products were stored at -20°C until required for making qPCR standards

2.2.3.3 DNA extraction from gel protocol

A 2% (w/v) agarose gel was made by dissolving high resolution agarose (Sigma-Aldrich, UK) in 0.5 % tris-borate EDTA/H2O (TBE) (diluted from 10 x TBE stock; Sigma-Aldrich, UK) and heated in a microwave on full power until all the agarose had fully dissolved. SYBR safe DNA dye (1 µl/10ml; Invitrogen, UK) was then added to the mixture. The mixture was left to set into a level horizontal gel tank cassette where a comb was inserted, allowing the appropriate-sized wells to form. The gel, after it set, was fully submerged in TBE.
In each well, 12 µl of sample consisting of 2 µl of loading buffer (6x DNA loading dye; Fermentas, UK) and 10 µl of PCR product were loaded. A 50 bp ladder (5 µl; Invitrogen, UK) was also run, to allow for size estimation of products. The gel was run at a constant voltage (120 V) for 1 hour or until the samples had migrated to an appropriate length as indicated by the loading dye (figure 2.7). Imaging of the gel was performed using the Gel Logic 2200 Pro Imaging System and Carestream Molecular Imaging software (Carestream Health, USA). The size of the bands matching the expected PCR product for each gene were removed from the gel using a sterile scalpel on a UV transilluminator (UVP, UK) and were then weighed. DNA was extracted using the QIAquick DNA extraction kit (Qiagen, UK). The extracted DNA was eluted in 20 ml buffer EB (Elution Buffer, Qiagen, UK) and subsequently stored at -20˚C until required.

![Figure 2.7](image_url)

**Figure 2.7** qPCR amplification product shown on 2 % agarose gel. The bands were later excised in order to extract DNA.

### 2.2.4 Calculation of gene copy number

In order to show how gene copy number was calculated the amplicon data for GAPDH will be used an example:

The average molecular weight (MW) of a dNTP (A, T, C, G) is 330 Da
• The conventional PCR product is double stranded (ds) cDNA, so the average dNTP MW is multiplied by a factor of two = 660 Da. This calculation assumes that there is an equal number of each dNTP in the amplified cDNA product.

• The molecular weight of GAPDH cDNA product is equal to the average dNTP molecular weight in cDNA multiplied by the total number of bp in the GAPDH amplicon: 660 x 150 = 99000 Da.

• Avogadro’s constant (6.02x10^{23}) is the number of molecules in one mole of a given substance, which is equal to the MW in grams:
  - 99000 g of GAPDH amplicon = 6.02 x 10^{23} GAPDH copies
  - 1 g of GAPDH amplicon = 6.08 x 10^{18} GAPDH copies
  - 1 ng of GAPDH amplicon = 6.08 x 10^{9} GAPDH copies

• The number of GAPDH copies in 1 µl can be calculated using the concentration of GAPDH cDNA obtained from the Nanodrop:
  - The concentration of GAPDH cDNA is 14.2 ng/µl
  - 6.08 x10^{9} copies in 1 ng x 14.2 ng/µl = 0.9 x10^{11} copies /µl

A serial dilution of the amplicon 1 x 10^{10} - 10^{1} were made as standards and stored at -80 °C until required for qPCR (real time quantitative PCR).

2.2.5 Real time quantitative PCR (qPCR)

Real time PCR is a technique used to measure a fluorescence signal emitted by DNA binding agents like SYBR green (Bustin, 2004). The signal increases, as more DNA molecules are available to bind to the fluorescent dye. Detection of signal from a sample begins when it is detected above background signal (Bustin, 2004). The
signal is measured at cycle threshold (CT) (Figure 2.8). Using a standard curve, which is constructed by running standards ($10^8$ to $10^1$ copies) of known copy numbers. The cycle number that the signal crosses the CT is used to calculate the absolute number of copies in the sample (Higuchi et al., 1993).

**Figure 2.8 Example of real-time PCR result.** Raw fluorescence is shown for the unknown samples (multiple colours) and the standards (green) and non-template control (black). The cycle threshold (CT) is the point where the PCR machine first detects fluorescence above background noise, shown as red line.
(Figure 2.9). The specificity of the reaction is determined by the primers similar to conventional PCR. However, the product obtained after qPCR is confirmed by a melting curve, which represents a peak specific for a specific gene (Ririe et al., 1997; Al-Robaiy et al., 2001; Lekanne Deprez et al., 2002). Figure 2.10 illustrates the specificity of a melting curve for one single gene product. (Bustin, 2000, 2004).

Figure 2.9 Typical standard curve from real-time PCR, generated from fluorescence data, indicating the appropriate R2, and efficiency (E) values based on the standards from $10^8$ to $10^1$. 
Housekeeping genes were used to normalise the copy number and account for variations (error) in amount of cDNA loaded into each reaction (Bustin, 2000). Housekeeping genes should be highly expressed and not altered by the disease in question (Kelley et al., 1993), such as GAPDH.

### 2.2.5.1 Real time quantitative PCR (qPCR) protocol

Real Time qPCR was performed for all genes using the primers summarised in table 2.2 using SYBR Green chemistry (QuantiFAST SYBR green; Qiagen, UK) on a RotorGene 6000 (Corbett Research, Australia) machine.

In each sterile rotor well tube, 2 µl of cDNA/DNA with 8 µl mastermix containing 1µl primers (0.5 forward and 0.5 reverse), 5 µl SYBR Green and 2 µl RNAase/DNAase free water was added. Previously prepared standards from $10^8$ to $10^1$ copies and two non-template controls (NTC), in order to assess potential contamination, were also added to the rotor well. All samples were run in duplicates in order to minimise
intra-assay variability. Once the well rotor was prepared, it was heat-sealed and placed in the PCR machine (Technie Quantica®).

The steps of the qPCR were the following:

1. Pre-PCR cycle for 5 minutes at 95°C
2. 42 cycles of 95°C for 10 seconds denaturation step
3. 60°C for 30 seconds combined annealing/extension step.

Melt curve analysis was performed to confirm the presence of one single product. CT values and a standard curve were generated using the RotorGene software. All unknown sample quantification values should be within the dynamic range of the standard curve. Cut off values for efficiency and $R^2$ were 80% and 0.99 respectively. All gene qPCR products were sequence-verified and run on a gel to confirm product band size.

Quantification data for the genes of interest were expressed relative to the geometric mean of 2 housekeeping genes and assessed using GeNorm software.

### 2.2.6 Mitochondrial DNA Copy Number in Mouse skeletal muscle

Recently, mitochondrial DNA content has been used as an index of mitochondrial number. Because mitochondrial DNA differs from the nuclear genomic DNA, the ratio of mitochondrial versus nuclear genome, Mt/N (known as mitochondrial copy number) can be indicative of mitochondrial DNA content (Malik et al., 2011). The amount of mitochondrial DNA in a cell is indicative of mitochondrial activity, as the transcription of mitochondrial genes is proportional to their copy number (Hock & Kralli, 2009). Thus, alterations mitochondrial DNA content can be used as a marker of mitochondrial dysfunction (Malik & Czajka, 2013). This approach involves
isolation of genomic DNA from cells or tissues samples, and the use of qPCR to quantify a mitochondrial and a nuclear gene (Malik & Czajka, 2013).

2.2.6.1 DNA extraction from skeletal muscle

Total genomic DNA was extracted from ~ 25 mg of skeletal muscle tissue. Tissue was cut into small pieces using a sterile scalpel (Swann Morton, UK) and added to 160 µl of PBS (Sigma-Aldrich, UK) in a 2 ml RNase-free tube (Eppendorf, Germany) and briefly mixed by vortex. The sample was then homogenised using a TissueLyser (Qiagen, UK) for two minutes at 25 Hz using two 5 mm steal beads until the tissue had been visibly broken down. DNA was extracted from the lysate using the DNaesy blood and tissue kit (Qiagen, UK) as per manufacturer's recommendations for tissues samples. Having completed the DNaesy blood and tissue kit protocol, the extracted DNA was eluted in 100 ml of RNase/DNase free water. Samples were quantified to give a DNA concentration in ng/ml and were checked for DNA integrity, using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Labtech, UK). The absorbance of each sample at 260 nm and 280 nm in comparison to RNase/DNase free water was measured, allowing the calculation of a ratio of optical density of samples at 260:280 nm. The sample was considered satisfactory when the ratio varied between 1.8-2.0. Each DNA sample was diluted to 50 ng/ml in a total volume of 100 ml using RNase/DNase free water before being placed in a bath sonicator (Pulsatron 55; Kerry Ultrasonics Ltd., UK) for ten minutes in order to shear DNA and minimise dilution bias. Samples were then stored at -20°C until required for quantitative real time PCR (qPCR).
2.2.6.2 Mitochondrial DNA content in mouse skeletal muscle protocol

Genomic DNA was extracted from skeletal muscle from the offspring at 30 days of age. Real Time qPCR was performed using the primers listed in Table 2.3. The experiments were performed under the supervision of Mrs Anna Czajka (member of Dr Afshan Malik’s group, Diabetes & Nutritional Sciences Division, King’s College London, UK), using unique regions in the mouse mitochondrial sequence that are not duplicated in the nuclear genome. These sequences have not yet been published (Table 2.4). Data were expressed as Mt/N ratio: the qPCR derived copy number for the mouse mitochondrial genome relative to the mouse beta-2 microglobulin copy number.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Accession number</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse mitochondrion complete genome</td>
<td>NC_005089.1</td>
<td>Forward primer: 5’-CTAGAAACCCCGAAACCAAA -3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse primer: 5’-CCAGCTATCACCAAGCTCGT-3’</td>
</tr>
<tr>
<td>mouse b2M (beta-2 microglobulin)</td>
<td>NC_000068.8</td>
<td>Forward primer: 5’-CTAGAAACCCCGAAACCAAA -3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse primer: 5’-CCAGCTATCACCAAGCTCGT-3’</td>
</tr>
</tbody>
</table>

Table 2.3 Oligonucleotide primer sequences used to determine mitochondrial copy number

2.2.7 Biochemical analysis

Maternal bloods were collected and were allowed to clot for 2 hours at room temperature before centrifuging for 5 minutes at 13000 rpm. The sera were collected and aliquoted to tubes and stored at ≤ -80 °C until required. Adipocytokines were assessed in pooled serum samples from 5 animals per group with a Proteome Profiler Mouse Adipokine Array kit (R&D Systems) as per manufacturer’s guidelines.
The Proteome Profiler Mouse Adipokine Array allows the simultaneous measurement of relative expression levels of 38 mouse adipokines. Capture and control antibodies were spotted in duplicate on nitrocellulose membranes. Serum samples were diluted, mixed with a biotinylated detection antibody, and incubated overnight with the Proteome Profiler Mouse Adipokine Array. The membrane was then washed in order to remove unbound material. Streptavidin-HRP and chemiluminescent detection reagents were applied allowing the production of a signal at each capture spot corresponding to the amount of protein bound. The density of each protein was measured with Image J.

2.2.8 Microbiota Composition Analysis

2.2.8.1 Fluorescent in situ hybridisation of bacterial cells (FISH)
Investigation of gastrointestinal microflora by conventional bacterial culture is no longer the preferred method due to lack of sensitivity in assessment, as a significant proportion of the gut bacteria are resistant to culture (Berg, 1996), if not non-viable (Amann et al., 1995). Fluorescent in situ hybridisation is considered a more accurate method to obtain quantitative data on gut microflora (Harmsen et al., 2000b; Kalliomaki et al., 2001). FISH of bacteria cells is performed by using 16S rRNA-based oligonucleotide probes to count all the bacteria found in faecal samples (Amann et al., 1995; Langendijk et al., 1995). A 10-fold difference has been shown in the number of bacteria found by FISH analysis compared to classical bacterial cultivation (Harmsen et al., 2000a). Thus, FISH is now considered an improved and much more sensitive method in the investigation of host-microbe interaction (Kalliomaki et al., 2001).
2.2.8.2 Faecal sample collection

At weaning, 3 months and 6 months of age, faecal samples from the animals were collected in the morning in 2 ml Eppendorf tubes. The samples were collected directly from the animal into the tube to avoid contamination. Where possible, the faecal samples were collected directly from the gut after sacrificing the animals. Following collection, samples were snap frozen and kept at -80°C until required for analysis.

2.2.8.3 FISH FLOW protocol

The analysis of these samples was performed under the supervision of Dr. Emilie Stolarczyk, Division Diabetes and Nutritional Sciences, King’s College.

The protocol was divided in three distinct steps:

1. Fixation of the samples

The samples were weighed, and 500 µl phosphate buffered saline (PBS) was added for every 0.5 mg. In order to homogenise the samples, 3-4 glass beads were added prior to the addition of the samples. The samples were then placed in the tissuelyser for 5 minutes. The samples were prepared in aliquots of 200 µl each in 1.5 ml eppendorf tubes where 600 µl of 4% PFA (paraformaldehyde solution) was added. The samples were then fixed overnight at 4°C.

2. Permeabilisation

The fixed bacterial solution was spun at 6000 rpm for 1 minute in a centrifuge. The debris was removed and the fixed bacterial solution was placed in a new sterile 1.5 ml eppendorf with 200 µl PBS. The final solution was mixed well and centrifuged for 3 minutes. The supernatant was removed carefully and 1 ml Tris-EDTA was added to each sample. Afterwards, the samples were vortexed and centrifuged at 13000 rpm for 3 minutes. The supernatant was discarded as above and 1 ml Tris-EDTA with lysozyme (20mg lysozyme in 20 ml Tris-EDTA) was added and mixed well. The
solutions were then incubated for 10 minutes at room temperature before centrifuging them at 13000 rpm for 3 minutes and discarding the supernatant as above. Finally, 1 ml PBS was added to each sample before vortexing and centrifugation at 8000 rpm for 3 minutes. The supernatant was discarded again.

3. Hybridisation

For every sample, 1 ml of hybridisation buffer was added. Samples were then vortexed and centrifuged at 13000 rpm for 3 minutes before removing the supernatant. Another 240 µl hybridisation buffer was added to each sample. From each sample, 40 µl were added to the desired wells in the plate. The following procedures were all conducted under low lighting. In each well, 10 µl of the relevant probe mix (table 2.5) was added and the samples were covered in foil and incubated overnight at 37°C. 150 µl of hybridisation buffer was then added to each well and the mixture was homogenised with gentle pipetting. The plate was centrifuged at 4000 rpm for 10 minutes, before discarding the supernatant. To the remaining samples, 200 µl wash solution was added. Next, the plate was incubated at 39°C for 20 minutes and centrifuged at 4000 rpm for 10 minutes. Finally, 200 µl of PBS was added to each well and the samples were transferred into Fluorescence-activated cell sorting (FACS) tubes in order to be analysed with the cytometer.
## 2.2.8.4 Analysis with flow cytometry

The analysis was performed with flow cytometry as described by Rigottier-Gois et al (Rigottier-Gois et al., 2003). Cells were pelleted and resuspended in PBS for data acquisition by flow cytometry (HTS Fortessa, Becton Dickinson, USA).

A total of 20 000 events EUB 338-FITC positives (bacterial cells) were stored in list mode files. Subsequent analyses were conducted using FlowJo software (Tree Star, USA). Cell enumeration was performed by combining, in one hybridisation tube, one group Cy5-probe with the EUB 338-FITC probe. An FL1 histogram (Figure2.11, green fluorescence) was used to evaluate the total number of bacteria hybridising with the EUB 338-FITC probe. A gate was designed in this histogram representing

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence</th>
<th>Target</th>
<th>Label 5’</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB 338</td>
<td>pB-00159 GCTGCCTCCCCGTAGGAGT</td>
<td>Domain Bacteria</td>
<td>FITC</td>
</tr>
<tr>
<td>NON 338</td>
<td>pB-00243 ACATCCTACGGGAGGC</td>
<td>Negative probe</td>
<td>Cy5</td>
</tr>
<tr>
<td>Bac 303</td>
<td>pB-00031 CCAATGTGGGGAACCTT</td>
<td>Bacteroides</td>
<td>Cy5</td>
</tr>
<tr>
<td>Erec 482</td>
<td>pB-00963 GCTTCTTAGTCARGTACCG</td>
<td>Clostridium coccoides–Eubacterium rectale</td>
<td>Cy5</td>
</tr>
<tr>
<td>Lab 158</td>
<td>pB-03928 GGTATTAGCAYCTGTTTCCA</td>
<td>Lactobacillus-Streptococcus group</td>
<td>Cy5</td>
</tr>
<tr>
<td>Bif 164</td>
<td>pB-00037 CATCCGGCATACCACCC</td>
<td>Bifidobacterium</td>
<td>Cy5</td>
</tr>
</tbody>
</table>

Table 2.4 Panel of group- and species-specific 16S rRNA-targeted oligonucleotide probes
the total number of bacterial cells in the sample and was used to build an FL4 histogram (Figure 2.11, red fluorescence) to directly estimate the proportion of cells targeted by the group Cy5-probe in the sample. The proportion of cells was corrected by eliminating background fluorescence, which was measured using the negative control NON 338-Cy5 probe. Results were expressed as cells hybridising with the group-Cy5 probe as a proportion of the total bacteria hybridising with the EUB 338-FITC bacteria domain probe or normalised to the basal conditions as indicated.

..Figure 2.11 Representative FACS plot. EUB+ and gated cells. FL1 histogram, green fluorescence is the total number of bacteria hybridising with the EUB 338-FITC probe. FL4 histogram, red fluorescence, shows the proportion of cells targeted by the group Cy5-probe in the sample.

2.3 Statistical Analysis

Data are expressed as means ± SEM. The statistical analysis was performed with GraphPad Prism 5, (GraphPad Software Inc. San Diego, California, USA). The data were analysed with two-way ANOVA followed by Bonferroni post-hoc test in order to examine the effect of maternal diet, the intervention and the interaction between them on offspring phenotype. When comparing more than two groups, one-way ANOVA followed by Bonferroni post hoc test was used. When comparing the means
between two groups, student’s t-tests was used. Statistical significance was considered when the P value is ≤0.05.

Based on power calculations, in order to identify a 10 % difference in energy expenditure (representing the average inter-individual variability) with 95% confidence, 12 animals in each experimental group were required. The calculations were based on the standard deviations found in the pilot study (section 2.4). As the main outcome of this study was energy expenditure it is possible that this sample size was inadequate for the study of other parameters such as the RER where greater variation was observed.

2.4 Pilot Study

In order to optimise breeding protocols and Labmaster methods a pilot study was conducted in an ongoing cohort of animals employing the standard murine model of maternal diet-induced obesity.

2.4.1 Introduction

As discussed in detail in chapter 1, maternal overnutrition and/or obesity has a detrimental effect on offspring phenotype resulting in an heightened risk of developing increased adiposity (Bayol et al., 2008; Samuelsson et al., 2008; Howie et al., 2009; Liang et al., 2009; Nivoit et al., 2009; Rajia et al., 2010), hypertension and cardiac hypertrophy and contractile dysfunction (Khan et al., 2003; Samuelsson et al., 2008; Liang et al., 2009; Samuelsson et al., 2010), endothelial function, hepatic steatosis (Elahi et al., 2009) fatty pancreas (Oben et al., 2010b) glucose intolerance (Taylor et al., 2005; Bayol et al., 2008; Rajia et al., 2010); and insulin (Samuelsson et al., 2008; Bayol et al., 2009; Nivoit et al., 2009) and leptin resistance (Ozanne & Hales, 2002; Kirk et al., 2009; Poston, 2011). These effects are also exacerbated when the offspring are exposed to high fat/high sugar diet postnatally (Bayol et al., 2008).
However, the effect of maternal obesity on offspring energy expenditure is largely unexplored. There is limited evidence, as previously mentioned, showing that offspring of obese parents have reduced resting metabolic rate (Griffiths et al., 1990); whilst children of obese mothers sleep more (Rising et al., 2003). Infants born to overweight mothers also had lower 24 hours RMR (Rising & Lifshitz, 2008). In mice, it has been shown that intrauterine exposure to gestational diabetes results in lower energy expenditure and higher RER especially during the daytime hours (Lau et al., 2011).

To my knowledge, there is no study that has looked into the effect of diet-induced maternal obesity in the mouse in offspring energy expenditure and particularly in BMR. Moreover, the potential effect of postnatal offspring exposure to an obesogenic diet has not been investigated. Thus, in this study we aimed to investigate the effect of maternal obesity and the potential effect of a high fat high sugar post-weaning diet primarily on offspring energy balance and additionally on offspring substrate oxidation.

2.4.2 Hypothesis

I hypothesized that decreased obese offspring’s BMR may be attributed to maternal obesity. Exposure to the obesogenic diet post-weaning will exasperate the effect in the offspring metabolism.

Aims

• To measure the energy expenditure of adult offspring of control and obese dams weaned to either control or high fat/high sugar diet
• To measure the RER of the four adult offspring experimental groups
• To determine whether there were differences between the offspring calorific intake
• To assess if the bodyweights between the offspring experimental groups differed.

2.4.3 Methods

2.4.3.1 Breeding protocol
As described above, C57BL/6J female, proven breeders (one previous litter) at approximately 100 days old (Charles River Laboratories, UK), were maintained under controlled conditions (25°C, 12-hour light/dark cycle). After one week of acclimatization, they were allocated to either the obesogenic diet or the control diet /RM1 (control diet) for 6 weeks before mating and during gestation and lactation. The females were mated with C57BL/6J non-obese males, approximately 100 days old (Charles River Laboratories, UK). Body weights and food intake were measured weekly.

The offspring born from either obese or control dams were weaned to either the obesogenic diet or standard chow, thus we had four groups of animals. The final groups were:

• Offspring of control dams weaned onto standard chow (OffCon/Con)
• Offspring of control dams weaned onto obesogenic diet (OffCon/Ob)
• Offspring of obese dams weaned onto standard chow (OffOb/Con)
• Offspring of obese dams weaned onto obesogenic diet (OffOb/Ob)

2.4.3.2 The experimental design
At 5 months (± 10 days) of age, the offspring were weighed and placed in the metabolic cages (Labmaster, TSE) for 48 hours. Measurements were taken on average every 20 minutes. Animal bedding from the home cage was used to reduce the stress and to aid acclimatization. A total of 24 hours measurements were taken, following the acclimatization period and matching the dark/light cycle, from 7am to
7pm and 7 pm to 7 am respectively. RER and energy expenditure were measured every 20 minutes. In the analysis, averages for every hour of the 24-hour period for each experimental group were calculated and subsequently averages for day and night time.

### 2.4.4 Statistical Analysis

Data are expressed as mean ±SEM. Two-way ANOVA followed by Bonferroni post hoc test was used in order to examine the effect of maternal, postnatal diet and the interaction between them on the offspring metabolic parameters measured. Statistical significance was considered when P≤0.05.
2.4.5 Results

2.4.5.1 Maternal Data

2.4.5.1.1 Rates of successful pregnancies

<table>
<thead>
<tr>
<th></th>
<th>Obese Dams Reproductive</th>
<th>Control Dams Reproductive Rates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Successful</strong></td>
<td>37.5 %</td>
<td>77.7 %</td>
</tr>
<tr>
<td><strong>Cannibalization</strong></td>
<td>25 %</td>
<td>16.6 %</td>
</tr>
<tr>
<td><strong>Unsuccessful</strong></td>
<td>37.5 %</td>
<td>5.5 %</td>
</tr>
</tbody>
</table>

Table 2.5 Successful pregnancy rates among obese and control dams (obese dams: n=24, control dams: n=18), Successful was considered a pregnancy of a litter of more than 4 pups while unsuccessful was an animal's inability to conceive and/or deliver.

According to the data summarized in table 2.6 the obese dams had 40.2% reduced successful pregnancies compared to control dams. These differences were not significant but prompted us to include a bigger number of obese dams in the full study.
2.4.5.1.2 Maternal pre-mating bodyweight

**Figure 2.12 Maternal pre-conception body weights.** (Control diet n=6 BW= 23.5 vs. Obesogenic Diet n=6 BW= 30.4g, n=6) Data expressed as mean ±SEM, * represents P<0.05 ***represents P<0.001.

Preconception maternal body weights were recorded on a weekly basis to ascertain suitability for entry into the breeding programme. The dams on the obesogenic diet weighed on average 30.4 g before mating while the control ones 23.5g. Thus, the obese animals were 29.3 % heavier at the time of mating (n=6, P<0.001, Figure 2.12).
2.4.5.2 Offspring outcomes at 5 months of age

2.4.5.2.1 Offspring Bodyweights

Maternal obesity did not significantly affect the offspring body weight. However, male offspring (Figure 2.13) weaned onto the obesogenic diet demonstrated significantly increased body weight compared to those weaned onto control diet (Con/Con: 31.7±0.72 n=8 and OffOb/Con: 30.6 ± 0.89 n=5 versus OffCon/Ob: 46.5±1.5g n=8, OffOb/Ob: 47.4±1.7g n=6, P<0.001) due to the postnatal diet which accounted for 85% of the variance.

In the female offspring groups (Figure 2.13B), the effect of the maternal diet alone was not significant. The effect of postnatal diet was highly significant accounting for 88% of the total variance (P<0.001). Increased body weights were observed in the OffCon/Ob (42.1g ±1.4, n=6) and OffOb/Ob (47.5±1.7g, n=6) groups compared to the ones weaned onto control diet (OffCon/Con: 26.6±1.1 g n=6, OffOb/Con: 24.3 ± 0.4, n=5). Finally, the interaction of the maternal diet and the postnatal diet significantly accounted for 4 % of (P<0.05) of the variance in female body weights.

![Figure 2.13](image)

Figure 2.13  (A) Male Offspring body weights, (B) Female Offspring body weights weaned on either control or obesogenic diet. White bars represent offspring of control mother and black bars offspring of obese mothers. Data expressed as mean ±SEM. *** represents p<0.001. Two-way ANOVA observed an interaction represented as $$, P<0.01, n=5-8
2.4.5.2.2 Offspring Calorific Intake

Offspring calorific intake was not significantly associated with maternal obesity. Males, (Figure 2.14A), weaned onto the obesogenic diet had a significantly increased calorific intake compared to controls (Con/Con: 22.3±1.36 kcal n=8, OffOb/Con: 17.1± 0.9 kcal n=5 versus OffCon/Ob: 36.7± 4.1 kcal n=8 and OffOb/Ob: 39.8 ± 3.4 n=6, (P<0.001) due to the obesogenic diet, which accounted for 61% of total variance and was therefore the main source of variation in the data.

In females, (Figure 2.14B), maternal diet did not affect calorific intake but the effect of the postnatal diet resulted in an increase in the calorific intake accounting for 25% of total variance (Con/Con 20 ± 2.7 kcal n=6, OffOb/Con: 18.6 ± 1.2 kcal n=5 versus OffCon/Ob: 33.9 ± 7.2 kcal n=5, OffOb/Ob: 26.4 ± 3.7 kcal n=6, P<0.01).

Figure 2.14 Offspring calorific intake (A) Male Offspring (B) Female Offspring calorific intake weaned on either control or obesogenic diet. White bars represent offspring of control mother and black bars offspring of obese mothers. Data expressed as mean ± SEM, *** represents p<0.001, * represents P<0.05, n=5-8
2.4.5.2.3 Offspring Respiratory Exchange Ratio

There was no significant difference between the RER measurements among the male offspring, either during daytime (Figure 2.15A) or during night-time (Figure 2.15B).

Similarly, among the females, maternal obesity alone did not have an effect on RER (Figure 2.15 C, D). Obesogenic postnatal diet, however, accounted for 24% of total variance and resulted in a decrease in RER (P<0.05) during daytime. The mean RER for the daytime (Figure 2.16 C) was 0.94± 0.03 (n=6) for the Con/Con; the 0.87 ± 0.02 (n=5) for the OffOb/Con; 0.83 ± 0.02 (n=6) for the OffCon/Ob; and 0.82 ± 0.03 (n=6) for the OffOb/Ob.

Figure 2.15 Offspring RER (A) Male offspring daytime RER, (B) male offspring night-time RER, (C) female offspring daytime RER, (D) female offspring night-time RER weaned on either control or obesogenic diet. White bars represent offspring of control mother and black bars offspring of obese mothers. Data expressed as mean ± SEM, * represents P<0.05, n=5-8
2.4.5.2.4 Offspring Energy Expenditure

There was no significant difference between the male offspring (Figure 2.16 A,B). In the females (Figure 2.16 C,D) the effect of maternal obesity on EE was not found to be significant. The groups exposed to the postnatal obesogenic diet (OffCon/Ob, OffOb/Ob) had significantly lower EE during daytime (P<0.01) and night-time (P<0.001) compared with Con/Con and OffOb/Con offspring, as the postnatal obesogenic diet accounted for 41% and 45% of the variation in EE during daytime and night-time respectively. [For day: (Con/Con: 15 ±0.8 kcal/h/kg n=6, OffOb/Con: 13.4 ± 0.6 kcal/h/kg n=5 versus OffCon/Ob: 12.4 ± 0.4 kcal/h/kg n=6 and OffOb/Ob: 12.37 ± 0.5 kcal/h/kg n=6, P<0.01) and for night: (Con/Con: 16.7 ±0.9 kcal/h/kg n=6, OffOb/Con: 15.02 ± 0.7 kcal/h/kg n=5 versus OffCon/Ob: 13.4 ± 0.3 kcal/h/kg n=6 and OffOb/Ob: 13.5 ± 0.5 kcal/h/kg n=6, P=0.01).

Figure 2.16 Offspring energy expenditure (EE) (A) Male offspring daytime (B) male offspring night-time (C) female offspring daytime (D) female offspring night-time EE weaned on either control or obesogenic diet. White bars represent offspring of control mothers and black bars for offspring of obese mothers. Data expressed as mean ± SEM, *** represents P<0.001** represents P<0.01, n=5-8.
2.4.6 Discussion

This pilot study was conducted in order to optimise the Labmaster apparatus and compare our results with the literature values from previously published studies using indirect calorimetry in mice.

2.4.6.1 Maternal Data

Preconditioning on the obesogenic diet proved successful in increasing weight gain by approximately 30% in 6 weeks, therefore the same obesogenic diet with the addition of fortified sweetened condense milk was used in all subsequent protocols. The differences between the control and the obese dams in relation to reproductive success were not significantly different. Nevertheless, the poor reproductive success among the obese dams prompted us to increase the number of obese females that were mated. The animals were moved into different rooms during the study, which could have induced stress in the pregnant animals. Therefore, pregnant animals in future studies remained in the same room during all the study and handling of the pregnant animals was limited to avoid inducing stress.

2.4.6.2 Offspring Data

Different outcomes were observed between male and female groups; therefore, data could not be combined to increase power male and female offspring data are presented separately throughout this thesis.

2.4.6.2.1 Body weight

At 5 months of age, maternal diet alone did not affect the offspring body weight but the effect of postnatal diet was evident in both male and female mice. Whilst there was no apparent interaction of maternal obesity with postnatal diet among males, exposure to both maternal and postnatal obesogenic diet resulted in increased body weight among the females. The interaction of postnatal and prenatal diet has been shown before as summarized in paragraph 1.3.3.1. Exposure to high fat diet both
prenatally and/or postnatally results in increased body weight, increased adiposity, raised blood pressure and serum cholesterol among the female offspring compared to their control counterparts (Elahi et al., 2009) Possibly the effect of prolonged exposure to the obesogenic postnatal diet had masked any underlying effect of maternal obesity. It has been shown before (Page et al., 2009), that the effect of maternal obesity and post-weaning diet became greater with time in male offspring. Female offspring, on the other hand, demonstrated increased bodyweight when they were exposed to maternal obesity and the obesogenic diet post-weaning. The differences between males and females could be attributed to differences in body composition that were not apparent without fat mass measurements. Therefore, measurement of fat mass in a non-invasive way in order to be able to follow up the offspring was considered necessary.

2.4.6.3 Calorific Intake

Calorific intake, contrary to previous studies from our group (Samuelsson et al., 2008), was not increased in the offspring of obese mothers weaned onto control diet. Both males and females showed increased calorific intake if they were exposed to the obesogenic postnatal diet. Maternal obesity, though, did not affect the appetite of the offspring. Measuring calorific intake is difficult due to the different methods used in different studies and the differences in diets. In this protocol the calorific intake was measured in the Labmaster during 24 h while in our previous study, it was measured weekly. In order to be consistent and more accurate in all the studies following energy intake was measured in the Labmaster.
2.4.6.4 Energy Expenditure

Heat production of the animals in the Labmaster (TSE) indicates the EE, which particularly reflects BMR during the daytime when the animals are at rest (Geissler, 2005). Maternal diet alone did not have a significant effect on offspring EE. In males the postnatal diet did not have an effect either. The EE values among males, during the consumption of the control diet, were approximately 12 kcal/h/kg for daytime and 14 kcal/h/kg during the active night-time phase. In the females the consumption of the obesogenic diet reduced the energy expenditure from approximately 15 kcal/h/kg (on standard chow) to 12 kcal/h/kg during the daytime and from 16.5 kcal/h/kg to 13 kcal/h/kg during the nighttime. These values are in accordance with those in the literature (Pfluger et al., 2008; Van Klinken et al., 2012).

The females significantly decreased their energy expenditure on to the post-weaning obesogenic diet. Even when raised under identical condition laboratory animals may differ in body size, which could introduce significant bias to the study (Even & Nadkarni, 2012). The values of EE were corrected for bodyweight to avoid bias introduced due to the mechanical effort of carrying the excess weight. However, this difference could be attributed to increased adiposity in this group, as fat mass (FM) has small contribution to BMR. Thus, measurements of adiposity in future studies would be necessary in order to accurately control for any potential bias.

Neither the interaction between maternal obesity and postweaning obesogenic diet or maternal obesity alone significantly influenced EE. In relation to the animals on the control diet, it is possible that they demonstrate differences later in life, as the BMR further decreases with age (Kane et al., 2008). Similarly, it would be interesting to examine whether there are differences apparent in early life due to maternal obesity that may disappear with age. Regarding the animals on the postnatal
obesogenic diet, the effect of the diet for 5 months could be so overwhelming that any changes in metabolism due to maternal obesity are masked.

2.4.6.5 Offspring Respiratory Exchange Ratio

Finally, I investigated how maternal obesity and postnatal obesogenic diet may affect RER, which is an indicator of the substrate oxidation. Maternal obesity did not have a significant effect on offspring RER, therefore I compared the OffCon/Con and OffCon/Ob RER with previously published data. The interpretation of the RER is very difficult, thus, scientific papers often lack coherency (Simonson & DeFronzo, 1990; Arch et al., 2006). When RER approaches 1, then glucose is oxidised, while a value close to 0.7 indicates fat oxidation. During the daytime when mice rest, they usually switch to fat oxidation (Lau et al., 2011). In our experiments the effect of the obesogenic postnatal diet significantly decreased the RER. Similarly, a previous study has shown a reduction of RER due to short term and long term exposure to obesogenic diet compared to consumption of standard chow (Longo et al., 2010). However, the baseline values in that study were lower compared to ours ranging to 0.76 to 0.86. However, the animals used were 8 months old while the ones in our were 5 months old, suggesting that different values may be attributed to age. In other studies the values of RER were similar to the current study (Boily et al., 2008; Church et al., 2009). The higher values we observed in the offspring weaned onto the obesogenic diet may be explained by the higher sugar content in our obesogenic diet.

Despite a trend of lower RER due to maternal obesity among female offspring on standard chow, no significant difference was identified. However, the reduced RER among the animals in the obesogenic diet is usually interpreted as increased fat oxidation. However, the values did not approach 0.7 but remained higher than 0.83
in all groups. A possible explanation of this effect could be the fact that when hyperinsulinaemia occurs, glucose oxidation reaches a plateau and glucose is thought to be metabolized in a non-oxidative way and be stored as glycogen (Thiebaud et al., 1982; Thiebaud et al., 1983; Simonson & DeFronzo, 1990). Animals coming from obese mothers have been shown previously to be hyperinsulinaemic (Bayol et al., 2008; Samuelsson et al., 2008; Bayol et al., 2009; Samuelsson et al., 2010). Assessment of insulin resistance in future studies would confirm these suggestions.

<table>
<thead>
<tr>
<th>Substance</th>
<th>$O_2$ Consumed During Oxidation, l/g</th>
<th>$CO_2$ Produced During Oxidation, l/g</th>
<th>RQ</th>
<th>Heat Produced/ Gram Oxidized, kcal</th>
<th>Heat Produced/ Liter, kcal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen</td>
<td>0.829</td>
<td>0.829</td>
<td>1.00</td>
<td>4.18</td>
<td>5.05</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.786</td>
<td>0.786</td>
<td>1.00</td>
<td>3.96</td>
<td>5.04</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.746</td>
<td>0.746</td>
<td>1.00</td>
<td>3.74</td>
<td>5.01</td>
</tr>
<tr>
<td>Lipid</td>
<td>2.019</td>
<td>1.427</td>
<td>0.70</td>
<td>9.46</td>
<td>4.69</td>
</tr>
<tr>
<td>Protein</td>
<td>0.966</td>
<td>0.774</td>
<td>0.80</td>
<td>4.32</td>
<td>4.48</td>
</tr>
</tbody>
</table>

Comparison of $O_2$ consumption, $CO_2$ production, respiratory quotient (RQ), and heat production during the oxidation of various substrates in vivo.

Table 2.6 RER and heat production relevant to substrate oxidation (Simonson & DeFronzo, 1990)

2.4.7 Future Suggestions arising from the pilot study

During this pilot study several observations were made that aimed to the improvement of future studies. First of all, I have had comparable values to those in the literature confirming the credibility of our methods. However, increased sample size is strongly required in order to conclusively delineate the associations between maternal obesity and energy balance and understand the possible different mechanisms in males and females. In addition, differences between male and female offspring were demonstrated, similar to humans, (Lazzer et al., 2010), highlighting the importance data assessment according to sex.
I have suggested that lower RER could be indicative of insulin resistance. To confirm that the RER was indicative of insulin resistance glucose tolerance tests should be carried out at the same time as measurements of RER.

The animals on the obesogenic diet were exposed to the diet very early in life resulting in a great weight gain, which is reflecting great adiposity, in offspring of control dams and obese dams. Therefore the effect of postnatal diet could be masking the effect of maternal obesity on EE. In humans being overweight in childhood is an independent risk factor for developing obesity in adulthood (Ebbeling et al., 2002). However, it is undeniable that some adults are more susceptible to weight gain when they are exposed to a Western diet than others. Therefore, exposing the animals on the obesogenic diet in adulthood for a short period could help us investigate whether maternal obesity together with the postnatal diet influence EE, independently from the development of great adiposity.

Finally, changes in body composition could influence the results. Therefore, it is necessary in the future to use analysis for covariance (ANCOVA) in order to correct for lean mass, if a change in body composition in the offspring is identified (Arch et al., 2006).

This study provided us with a good insight regarding the effects of maternal obesity on offspring energy balance and helped identify limitations of the study, aiding our understanding of the requirements for future studies, to identify more conclusive associations.
Chapter 3


3.1 Introduction

As recently reviewed (Yu et al., 2013) the literature from human mother-child cohort studies suggests an independent relationship between maternal obesity and the risk of offspring obesity, following adjustment for confounders such as maternal smoking, maternal gestational weight gain, childhood breast feeding and diet. Whilst independent relationships between maternal and childhood obesity and related metabolic dysfunction have been observed in human studies, causality is difficult to prove. As described in Chapter 1 (Introduction) animal models across several species, have nonetheless provided strong support for ‘developmental programming’ of cardio-metabolic dysfunction in the offspring secondary to maternal obesity.

A series of studies from our laboratory have demonstrated that the offspring of obese mice at 6 months of age had increased food intake, increased body weight and adipocyte hypertrophy (Samuelsson et al., 2008; Oben et al., 2010a; Oben et al., 2010b), all indicative of disturbed energy balance. However, as detailed in Chapter 1, very little work has been undertaken in either our laboratory or others to determine the various component elements of the energy balance ‘equation’ in the offspring of obese rodents, although there has been some suggestion of altered energy expenditure (Borengasser et al., 2011; Lau et al., 2011). In the study described in this chapter, experiments are presented which have address the different elements of the energy balance ‘equation’ in offspring of obese dams with particular emphasis on energy expenditure.
Potential candidate developmental programming vectors have been identified in the dysmetabolic milieu of obese pregnancy. Hence, fetal and neonatal exposure to glucose, insulin, leptin, lipids and inflammatory mediators have been implicated in fetal macrosomia, increased adiposity and permanent alteration in energy balance regulation, resulting in childhood obesity and the transgenerational acceleration of obesity (Poston, 2012, O'Reilly and Reynolds, 2013, Drake and Reynolds, 2010). Diet-induced obesity in rodent dams, as in obese human pregnancy, is associated with maternal hyperinsulinaemia, hyperlipidaemia and glucose intolerance in pregnancy and/or lactation (Taylor et al., 2003, Srinivasan et al., 2006, Samuelsson et al., 2008, Nivoit et al., 2009, Holemans et al., 2004, Chen et al., 2008).

In human and animal studies, attention has turned to methods for prevention of developmental programming by maternal obesity through dietary and/or pharmacological interventions in the mother to improve maternal glucose homeostasis and metabolic sequelae of obesity and thereby reduce the adverse effects on metabolic function in the offspring. The experiments described in this chapter therefore have also addressed the potential intervention of maternal dietary supplementation to improve metabolism and pathways of energy balance in the offspring. During the period that obesity rates have increased, the consumption of dietary fibre has also been reduced, suggesting an association between reduced fibre consumption and the development of obesity (Maskarinec et al., 2006; Ford & Frost, 2010). Polydextrose is a soluble dietary fibre with a calorific value of 1 kcal/g, a neutral taste, low glycaemic impact and prebiotic properties. The introduction of PDX to the diet in previous studies in adult humans and animals has been proven to be beneficial as it can increase satiety; lower insulin levels and decrease cholesterol and triglycerides concentrations (Shimomura et al., 2005). Therefore, PDX supplementation in obese women (and mice) offers a potential means to improve
metabolic profiling during pregnancy and to positively impact on the health of the offspring.

3.2 Hypothesis

Maternal obesity adversely influences offspring health through permanent alteration in energy balance. The enrichment of the maternal diet of obese mice with PDX, during critical periods of gestation and lactation will prevent these adverse effects and thereby improve offspring metabolic dysfunction.

3.2.1 Aims

- To investigate whether maternal obesity in mice influences body composition and energy balance (energy expenditure and calorific intake) in the offspring.
- To determine whether polydextrose supplementation in pregnant obese mice reverses any changes in body composition and energy balance (energy expenditure and calorific intake) observed in the offspring of obese dams.
- To determine whether any observed changes in pathways of energy balance in the offspring of obese dams are associated with altered glucose homeostasis.
- To determine whether maternal supplementation with polydextrose reverses any alterations in glucose homeostasis observed in offspring of obese dams.
3.3 Methodology

3.3.1 Experimental design

A summary of the protocol is shown in Figure 3.1.

Female C57BL/6J mice were fed either a standard chow diet (RM1) (n=18) or an obesogenic diet (n=46) for 6 weeks before mating and during gestation and lactation, as described in Chapter 2.1.1. Following successful mating, a sub-group of obesogenic diet-fed dams were assigned to the same diet supplemented with 5% PDX (n=12) during gestation and lactation or to continued maintenance on the obesogenic diet (n= 34) leading to three groups (control (Con), obese (Ob) and obese +5%PDX ObP), (Figure 3.1).

ObP were supplemented with PDX in the drinking water (5% w/v; Tate and Lyle). This concentration has previous proven efficacy in reducing insulin resistance in adult rats (Witaicenis et al., 2010) without toxicity and without altering the calorific intake.

All pups were weighed at 48 hours after delivery, and each litter with more than 6 pups was then reduced to 3 males and 3 females. Litters with less than four pups were not used. The offspring groups were:

- Offspring of Control dams (OffCon, n=15)
- Offspring of Obese dams (OffOb, n=15)
- Offspring of Obese dams supplemented with PDX (OffObP, n=7)

After weaning, all offspring were assigned to the standard chow diet. At 30 days of age the energy expenditure (EE), RER and calorific intake of the offspring (OffCon, OffOb and OffObP) were measured using the Labmaster apparatus. The measurements were repeated at 3 and 6 months of age (Figure 3.1).
At 6 months of age, part of the animals was sacrificed; organs were collected and the fat pads (retroperitoneal, gonadal, mesenteric and subcutaneous) were weighed.

Figure 3.1 Schematic representation of the experimental design. Female dams were fed either an obesogenic or a control diet. Following successful mating a part of the obese dams were given a supplementation with PDX. The offspring of the dams were weaned to control diet and were followed up to 6 months.

3.3.2 TSE Labmaster

EE, RER and food intake in the offspring, were measured in the Labmaster at 30 days, 3 and 6 month time points. At each time point, 6 age-matched animals were weighed, and then placed in the metabolic cages (CaloCages) for a period of 48 hours. The first 24 hours was a period of acclimatisation; only data from the last 24 hours were used in analysis. Data points were recorded in the Labmaster approximately every 20 minutes and hourly means calculated.
3.3.3 Bioimpedance Analysis

The free fat mass of the offspring was measured by bioimpedance (Bio Impedimed) at 3 months of age. The body composition was assessed before measurements in the LabMaster apparatus for indirect calorimetry. This was designed to enable the calculated values of FFM and FM to be used in the calculation of BMR if necessary. Details of the bioimpedance analysis are described in Chapter 2.1.5.

3.3.4 Glucose Tolerance test

A glucose tolerance test was performed on offspring at 30 days, 3 months and 6 months of age (n=6) as described in Chapter 2.1.6. Following the test, the animals were placed in the Labmaster CaloCages.

3.3.5 Body composition by fat pad weight

At 6 months of age, animals were sacrificed by rising concentrations of CO₂. Fat pads were removed and weighed. White adipose tissue and brown adipose tissue samples were collected and snap frozen for RNA analysis.

3.3.6 Statistical Analysis

Data are expressed as mean ± SEM. Student’s t-test was used for comparison between two groups and one-way ANOVA analysis followed by Bonferroni post-hoc tests for comparison between multiple groups. Statistical significance was considered when the P value is <0.05.

3.4 Results

The litter size and the pup size of these animals are presented in detail in chapter 5. In summary, maternal obesity resulted in smaller litter size (P<0.05) but there were no differences in birthweight.
3.4.1 Offspring 30 days old

There were no significant differences in bodyweights or any parameters of energy balance at 30 days of age, between the male (Figure 3.2) or female (Figure 3.3) offspring of the OffCon, OffOb or OffObP groups.

**Figure 3.2 Male offspring at 30 days of age.** Bodyweights (A), Daily Calorific Intake (B), mean respiratory exchange ratio (RER) during daytime (C), mean RER during night-time (D) and mean daytime energy expenditure (EE) (E) and mean night time energy expenditure (EE) (F), in male offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) at 30 days of age (n=7-15)
**Figure 3.3. Female offspring at 30 days of age.** Bodyweights (A), Daily Calorific Intake (B), mean respiratory exchange ratio (RER) during daytime (C), mean RER during night-time (D) and mean daytime energy expenditure (EE) (F) and mean night time EE, in female offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) at 30 days of age (n=7-15)

Moreover, there was no difference in glucose metabolism between the groups of offspring at 30 days old, as measured by glucose response during a glucose tolerance test, in both male and female offspring (Figure 3.4).
Figure 3.4 Glucose tolerance test at 30 days of age. Response to a glucose tolerance test (GTT) and the respective area under the curve (AUC) in male (A, C) and female offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) (B, D), at 30 days of age (n=6).
3.4.2 Offspring 3 months of age

3.4.2.1 Bodyweight

At 3 months of age, the bodyweights of the offspring were not significantly different (Figure 3.5); Males, OffCon, 31.38±0.75 g (n=15), OffOb 33.18 ± 3.71 g (n=15) and OffObP 32.90 ± 0.93 g (n=5). Females, OffCon, 23.60±0.57 g (n=15), OffOb 24.58 ± 1.26 g (n=15) and OffObP 23.54 ± 0.54 g (n=5).

Figure 3.5. Bodyweight at 3 months of age. Bodyweights in male (A) and female (B) offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) at 3 months of age, n=7-15
3.4.2.2 Body Composition

Bioelectrical impedance did not show any significant differences in body composition between groups at this age (Figure 3.6).

Figure 3.6. **Body composition at 3 months of age.** Percentage fat mass (FM %) in male (A) and female (B) offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) at 3 months of age, (n=5-6)

3.4.2.3 Daily Calorific Intake

There was no difference in the mean calorific intake at 3 months of age between the control and the experimental groups in either male or female offspring (Figure 3.7).

Figure 3.7. **Calorific intake at 3 months of age** Mean daily calorific intake in male (A) and female (B) offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) at 3 months, (n=7-15)
### 3.4.2.4 Glucose metabolism

At 3 months of age intraperitoneal glucose tolerance tests were performed in the offspring. There was no significant difference between the male groups. However, in the females, 15 min after glucose injection, the OffOb demonstrated an increased blood glucose concentration compared to the control (OffOb 31.65 ± 1.26 mmol/L (n=5) versus OffCon 21.98 ± 0.42 mmol/L (n=5); P<0.001), which was reflected in an increase in the AUC (OffOb 78.66 ± 5.76 versus OffCon 59.87±0.75 mmol.min/l; P<0.01). Whilst administration of PDX to the dams was associated with lower 15 min blood glucose in OffObP (25.98±1.68 mmol/L compared to the OffOb; P<0.05); this was not reflected in the AUC, which was similar to OffOb (OffObP; 75.86±3.03 mmol.min/L; Figure 3.8).

![Figure 3.8. Glucose tolerance test at 3 months of age.](image)

Response to a glucose tolerance test (GTT) and the respective area under the curve (AUC) in male (A, C) and female offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) (B, D), at 3 months (n=6). For GTT 3 months female: * P<0.05 OffOb vs OffObP; *** P<0.001 OffCon vs OffOb. For AUC, *p<0.05; **P<0.01.
3.4.2.5 Respiratory Exchange Ratio (RER)

At 3 months of age there was a reduction in RER compared to controls in the male offspring during the night-time (Figure 3.9 B) (OffCon: 0.94 ± 0.02 versus OffOb: 0.88 ± 0.02, n=15) and in the females (Figure 3.9 C) during the daytime (OffCon: 0.92 ± 0.01 OffOb: 0.86 ± 0.01, n=15; P<0.05). Supplementation of the dams with PDX resulted in no difference in RER compared to OffCon or OffOb.

Figure 3.9. RER at 3 months of age. RER in male during day (A) and night-time (B); and in RER of female offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) during day (C) and during night-time (D) at 3 months, n=5-15, * P<0.05
3.4.2.6 Energy Expenditure (EE)

There was no effect of maternal obesity on male or female 3 month old offspring EE when compared to controls. However administration of PDX to the obese dams was associated with an increase in EE in male OffObP compared to OffOb during both day and night-time (Figure 3.10 A, B) (day time EE, OffObP 13.68 ± 0.59 kcal/h/kg versus OffOb 11.85 ± 0.29 kcal/h/kg, P<0.05. Night-time EE, OffObP 17.32 ± 1.09 kcal/h/kg versus OffOb 14.04 ± 0.58 kcal/h/kg; OffObP: n=7 OffOb: n=15. There was no effect of PDX on EE in the female offspring of obese dams.

Figure 3.10. Energy Expenditure at 3 months of age. Mean energy expenditure (EE) in male during daytime (A) and night-time (B) and in female during daytime (C) and night-time (D) offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) at 3 months, n=5-15 **P<0.01.
3.4.3 Offspring at 6 months of age

3.4.3.1 Bodyweight

At 6 months of age bodyweight of the female mice did not differ. However, in male OffOb bodyweight was increased compared to OffCon (OffOb 40.69 ± 0.70 g, n=5 versus OffCon: 32.24 ±0.83g, n=6), P<0.01. Supplementation of the dams with PDX was associated with a reduction in body weight in male OffObP (35.88±1.33g, n=5), P<0.05 versus OffOb (Figure 3.11). No significant differences were observed amongst female offspring.

Figure 3.11 Bodyweight at 6 months of age. Bodyweight male (A) and female (B) offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP), at 6 months of age (n=5-6); * P<0.05, ** P<0.01
3.4.3.2 Daily Calorific Intake

There was no statistically significant difference in calorific intake between groups, in either males and females (Figure 3.12).

![Graph showing daily calorific intake](image)

**Figure 3.12. Calorific Intake at 6 months of age.** Mean daily calorific intake of male (A) and female (B) offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) at 6 months of age. (n=5-6)

3.4.3.3 Glucose Tolerance Tests

Both male and female OffOb (Figure 3.13) demonstrated a greater AUC compared with OffCon, P<0.05 (male OffOb: 101±4.72 mmol.min/l, n=6 versus OffCon: 77±2.45 mmol.min/l, n=6; female OffOb: 86.38±2.56 mmol.min/l, n=5 versus OffCon: 70±4.95 mmol.min/l, n=6). Supplementation of the obese dams with PDX, in contrast, was associated with an AUC no different from OffCon in male and female offspring (male OffObP 86.75±6.30 mmol.min/l, n=5; female OffObP 75±7.97 mmol.min/l, n=5).
Figure 3.13 Glucose tolerance test 3 months of age. Glucose tolerance test and the respective area under the curve (AUC) in male (A, C) and female offspring (B, D) of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP), at 6 months of age (n=4-5). * P<0.05.

3.4.4 Respiratory Exchange Ratio

Maternal obesity was associated with a lower RER (OffOb 0.73±0.02, n=5 versus OffCon 0.81±0.01, n=6) in the male 6 month offspring during the daytime (Figure 3.14). Offspring of obese dams supplemented with PDX were not statistically different from controls (OffObP 0.73±0.02, n=5). There was no difference among the female offspring.
Figure 3.14. **RER at 6 months of age.** RER in male offspring during day (A) and night-time (B) and female RER offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) for day (C) and night-time (D) at 6 months. (n=5); * represents P < 0.05

### 3.4.5 Energy Expenditure

Maternal obesity resulted in lower EE in male OffOb during the daytime (OffCon: 15.07 ± 0.79 kcal/h/kg, n=6; OffOb 10.98 ± 0.41 kcal/h/kg, n=5); and the night-time compared with OffCon. Maternal PDX supplementation led to a significant reversal (p<0.05) of the lower EE associated with maternal obesity during the night-time (OffCon: 15.07 ± 0.79 kcal/h/kg, n=6; OffOb 10.98 ± 0.41 kcal/h/kg, n=5; OffObP: 13.56 ± 0.76 kcal/h/kg, n=5). However, daytime EE (OffCon: 12.70 ± 0.28 kcal/h/kg, n=6; OffOb 10.17 ± 0.26 kcal/h/kg, n=5; OffObP: 10.82 ± 0.42 kcal/h/kg, n=5 was not affected by maternal PDX supplementation. There was no difference between the female groups (Figure 3.15).
Figure 3.15. **Energy Expenditure at 6 months of age** Mean energy expenditure (EE) in male during daytime (A) and night-time (B) and female daytime (C) and night-time (D) offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) 6 months, n=4-5; * P<0.05; ** P<0.01. *** P<0.001.

### 3.4.6 Body composition by assessment of fat pad weight

Male offspring of obese dams demonstrated a significant increase in the mesenteric fat mass. No other fat pads were significantly different between groups.

Supplementation with PDX resulted in reduced mesenteric fat mass (Figure 3.16 C) among the male offspring at 6 months of age compared to the offspring of the obese dams (OffObP: 0.58 ± 0.03 g, n=5 *versus* OffOb 0.92 ± 0.09 g, n=5 and OffCon 0.6550 ± 0.03403, n=6; P<0.05). Despite a trend towards lower total fat mass (White Adipose Tissue, WAT) among OffCon and OffObP males, there was no statistical difference (Figure 3.16 A). No difference in fat pad masses was observed among
female offspring (Figure 3.16 B, D). Total visceral fat was also calculated by adding together gonadal, retroperitoneal and mesenteric fat for all groups, but no differences were detected.

**Figure 3.16 Body composition at 6 months of age.** Mean weight of white adipose tissue (WAT) and fat mass distribution as assessed by weight in male (A, C) and female (B, D) offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) at 6 months of age, (n=5-6), * represents P<0.05.

### 3.5 Discussion

The data presented in this chapter have shown for the first time that maternal diet-induced obesity in mice is associated with reduced EE in the offspring in association with increased body weight and increased abdominal mesenteric fat pad mass. This suggests a potential mechanism, which may contribute to the developmental programming of obesity and offers new avenues for exploration in the search for causal pathways. Furthermore, we present data to suggest a
beneficial effect of maternal supplementation with PDX in adult male offspring of the obese mice dams. Specifically, male offspring of supplemented dams had increased EE at 3 and 6 months of age, and decreased bodyweight at 6 months of age compared to the offspring of untreated obese dams.

To my knowledge this is the first study to have accurately measured RER, and EE in a developmental programming study in the mouse. As discussed in the pilot study (Alton et al., 2007), the values in the control animals would seem to faithfully reflect RER and energy expenditure as they compare favourably with the previous literature (Pfluger et al., 2008; Longo et al., 2010; Van Klinken et al., 2012).

3.5.1 Offspring at 30 days old

At 30 days of age, no difference in body weight was observed between OffCon and OffOb as previously observed ourselves (Samuelsson et al., 2008) and others (Liang et al., 2009). At this age there was also no difference observed in calorific intake as we have reported previously (Samuelsson et al., 2008; Kirk et al., 2009; Nivoit et al., 2009). Similar to other studies, there was also no difference in glucose metabolism, as determined by the GTT at 30 days of age (Bayol et al., 2008; Samuelsson et al., 2008; Liang et al., 2009; Nivoit et al., 2009; Rajia et al., 2010).

3.5.2 Calorific Intake

There was also no change in calorific intake at 3 or 6 months of age in this study, which contrasts with previous studies including our own (Samuelsson et al., 2008; Kirk et al., 2009; Nivoit et al., 2009). Differences in methods of data collection could explain these differences, as previously mentioned in the pilot study (section 2.3).
Moreover, in this protocol there have shown no differences in birthweight but reduced litter size while in our previous study there was significant increase in the birthweight and no change in litter size (details in chapter 5). It is unlikely that the latter has affected our results, as small litters have not been included in the study. However, the differences in birthweight may explain these changes. Interestingly, another study showed reduction in EE but not in calorific intake due to maternal obesity (Borengasser et al., 2011). Others have shown that exposure to an obesogenic diet later in life in offspring of obese dams unmasks differences in appetite and/or food preference (Bayol et al., 2007). This has been considered in Chapter 4.

### 3.5.3 Energy Expenditure

At 3 months of age, males did not differ in bodyweight and there was no difference between the EE of offspring of the obese and their control counterparts.

There has been no previous study to suggest that maternal obesity influences energy expenditure in the offspring. The novelty of this study lies in the suggestion that the observed changes in bodyweight, at 6 months, can be attributed to the reduced EE in relation to the male offspring from 3 months of age, as there no change in calorific intake.

Importantly, supplementation with PDX increased EE of the offspring of obese dams. The increase EE at 3 months of age in the offspring of obese PDX-treated dams could explain why these offspring did not have increased bodyweight at 6 months of age despite significantly lower daytime EE compared to the control offspring at this time point.
There have been very few attempts previously to explore the relationship between maternal obesity/overnutrition and EE in offspring. One study measuring resting metabolic rate using an ‘enhanced metabolic testing’ chamber, showed that 6 month old infants (n=21) born to overweight and obese mothers had a lower 24 hour EE than those of lean mothers (P<0.05) (Rising & Lifshitz, 2008). In addition, although only indirectly relevant, a study in genetically modified mice, showed that intrauterine exposure to gestational diabetes results in lower EE in offspring (Lau et al., 2011). Most relevant to this study, in a model of diet-induced obesity in the rats were shown have reduced EE, secondary to overcalorific consumption before mating and during gestation only (Borengasser et al., 2011).

It is difficult to compare this reduction in EE with previous models of obesity investigating reduction in EE because the age of the animals, the sex and the way EE is presented differs. Similar trends, though, were noticed in melanocortin 4 receptors (MC4) knock-out animals but the authors only measured VO₂ (Balthasar et al., 2005). Moreover, the study in rats mentioned above also showed similar reduction in the offspring of obese dams (Borengasser et al., 2011). It is very important to notice that these changes in EE (approximately 5 kcal/h/kg) could have a great impact on weight gain as small changes in energy imbalance accumulate in time. Therefore, following up offspring up to 1 year of age could amplify these changes.

Several potential mechanisms may contribute to reduced EE in the offspring of the obese dams. These include differences in diet-induced thermogenesis, in physical activity and in BMR. BMR is the biggest component of EE, however in this study, a greater effect was observed during the night-time when the animals are active and the EE is higher, making this an unlikely explanation. A potential mechanism of particular relevance to PDX, could be a persistent change in the offspring gut
microbiota, as it has been suggested that PDX plays a significant role in nutrient acquisition and energy regulation (Davis & Milner, 2009). Specifically, the two dominant bacterial divisions in the gut, the Bacteroidetes and the Firmicutes influence the ability of the host to harvest energy from the diet, thus leading to less energy expended and subsequent adipogenesis (Turnbaugh et al., 2006).

3.5.4 Glucose metabolism

The female offspring demonstrated glucose intolerance from 3 months of age, which interestingly does not appear to reflect any other outcomes of their energy balance. Insulin resistance in the female offspring only has been previously reported in a model of maternal fructose feeding in pregnancy and lactation (Vickers et al., 2011). Since there were no differences in females adiposity, glucose intolerance appears to be a primary programmed effect. Progressive impairments of glucose homeostasis attributed to persistent changes in insulin-signaling molecules in insulin responsive tissues like the liver or muscle have been demonstrated in models of developmental programming of obesity before (Ozanne, 1999; Oben et al., 2010a; Alfaradhi & Ozanne, 2011). It is possible that such mechanism is mediating the changes observed in the animals later in life in this study.

The impaired glucose homeostasis among the offspring of the obese dams from 3 months of age onwards is, also, supported by a simultaneous reduction in RER. Reduced RER has been previously been associated with insulin resistance, as glucose oxidation reaches a plateau and glucose is thought to be metabolised via a non-oxidative pathway and stored as glycogen (Thiebaud et al., 1982; Thiebaud et al., 1983; Simonson & DeFronzo, 1990) (as discussed in section 2.3). In accordance with our results a study using FVB mice (a transgenic inbred strain), diabetes was significantly associated with lower RER (Zhang et al., 2012).
Acute effects of PDX on insulin sensitivity have been demonstrated previously (Shimomura et al., 2005). In this study I also present some evidence that maternal supplementation in obese dams with PDX improves offspring glucose homeostasis (Figure 3.13). Evidence from one previous study supports our findings, as it was reported that offspring from rat dams fed a high fibre diet, had improved glycaemic control and decreased fatty acid synthase, suggesting improved insulin sensitivity, compared to offspring of high fat fed dams (Maurer et al., 2010).

Altered glucose homeostasis has been previously associated with reduced glucose uptake in brown adipose tissue and subsequently a reduction in diet-induced thermogenesis, thereby linking glucose tolerance with total EE (Storlien et al., 1986). This association could explain the greater reduction in EE during the night-time in male offspring, when the animals are active and eating. Measuring UCP gene expression, and protein expression could determine whether this is a feasible explanation. Therefore, the improvement among the OffObP in EE and glucose metabolism could be explained by the improved glucose homeostasis mentioned above. However, it is possible that differences in energy expenditure during the night-time could be attributed to decreased locomotor activity, as previously shown by our group (Samuelsson et al., 2008).

3.5.5 Body Composition

I did not observe a difference in body composition at 3 months of age, but mesenteric fat was increased among the male offspring at 6 months of age due to maternal obesity, which was reversed by maternal administration of PDX (Figure 3.16). At 3 months of age, as the animals were not sacrificed, body composition was measured by bioimpedance, which only allows measurement of total fat mass (Figure 3.6). Identification of differences between the different fat pads at 3 months of age was therefore not possible.
Mesenteric fat is a component of the visceral fat, which has been previously associated with insulin resistance (Wajchenberg, 2000; Neeland et al., 2013). This has been shown in a study using a transgenic mouse model of visceral obesity, associating the development of obesity with hyperlipidaemia and impaired insulin sensitivity (Masuzaki et al., 2001). Moreover, it has been demonstrated that calorie restriction resulted in a reduction in visceral adiposity, including mesenteric fat. The strongest predictor of improved insulin sensitivity was the reduction of mesenteric fat (Catalano et al., 2010). Therefore, it could be suggested that the improvements in glucose tolerance among the offspring of the PDX group were a result of the reduction in mesenteric fat mass. In addition to the above, mesenteric fat has been associated with an increase in serum inflammatory markers when compared increases in other fat depots in high fat fed rats, suggested to be a consequence of the ‘leakage’ of gut luminal content (Lam et al., 2012). Evidence showing that Firmicutes/Bacteroidetes ratio, previously associated with obesity, was also increased in association with greater mesenteric fat mass suggests that there is a link between gut microbiota, mesenteric fat and glucose intolerance (Lam et al., 2012). Assuming that similar changes occurred in offspring of obese dams, this pathway could provide an explanation for the differences observed and why maternal PDX supplementation prevented the negative maternal influences on offspring glucose tolerance.
3.5.6 Sex specific maternal influences

It is important to acknowledge the differences between male and female offspring. In relation to EE, it was mentioned previously that BMR is influenced by sex even when adjusting for body size and composition (Arciero et al., 1993; Poehlman et al., 1997). Moreover, evidence from the Helsinki Birth Cohort study (Sandboge et al., 2011), have demonstrated a U-shaped correlation of birthweight with RMR among men, but not in women, after adjustment for FFM and FM. This evidence is in accordance with our data, suggesting sex specific maternal influences on offspring EE.

Despite sex specific differences, the origins of altered EE have not been previously reported. The sex differences in developmental programming have been well documented, as recently reviewed (Aiken & Ozanne, 2013). Sex differences in offspring from models investigating the developmental origins of cardio-metabolic disease have been previously reported in offspring of rats (Ozaki et al., 2001; Khan et al., 2003) and mice (Gallou-Kabani et al., 2010; Vickers et al., 2011). Similarly to our findings, male offspring are frequently reported to be more susceptible to adverse maternal exposures in pregnancy (Alexander, 2003; Maloney et al., 2011; Reverte et al., 2011). However, glucose intolerance has been associated with maternal exposures in both sexes (Seckl, 2004; Ozanne et al., 2005), but also specifically only in male (Nivoit et al., 2009) and female offspring (Vickers et al., 2011). Several mechanisms have been considered to underlie these differences including, genetic and morphological differences in development, the effect of oestrogen levels in females, the timing of sexual development, the influence of steroid hormone exposure during life and sex specific epigenetic regulations in utero and postnatally (Aiken & Ozanne, 2013). Further investigations are required to explore these differences in this model.
3.5.7 Limitations and Future Studies

Problems with breeding led to marked differences in the numbers of animals available for study, and to the discrepancies in numbers between the three experimental groups. Originally, it was planned to do two discrete protocols, the first to assess the influence of maternal obesity on offspring pathways of energy balance and a second to address the effect of maternal PDX supplementation. As these protocols dovetailed one another in time, and because the failure rate in breeding was so high, it became necessary to merge the two protocols to achieve adequate numbers of offspring for all end points. Thus there are fewer animals in the PDX group.

In addition, because in this study, mice were studied at both 3 and 6 months, and because of inadequate numbers to allow sacrifice at both time points, tissues were not available from 3 month old animals, nor organs or fat pads. A shorter study where the offspring would be sacrificed at 3 months of age would provide information regarding offspring metabolism earlier in life. Furthermore, exposure to the obesogenic diet in adulthood would help identify whether the offspring of obese dams are more susceptible to it and if maternal supplementation with PDX plays a protective role.

Several mechanisms could contribute to the changes observed in the offspring of the obese dams and the influence of PDX. Expression of UCP genes and gut microbiota analysis will be described in Chapter 6. Moreover, mitochondrial dysfunction could also be associated with altered RER (Galgani et al., 2008). Since differences were observed at 3 months of age, delineation of any differences in mitochondria function earlier in life would provide evidence as to whether this is a fundamental mechanism rather than a consequence of the metabolic defects observed.
Finally, since we have identified differences in EE, it would be interesting to identify which of the components of EE are altered. Therefore, in future studies simultaneous measurement of diet-induced thermogenesis and physical activity will also provide us with a better estimate of the changes in BMR.

### 3.5.8 Conclusions

In summary, it was presented for the first time evidence of influences of maternal obesity on male offspring EE. In addition, it was demonstrated that maternal supplementation with PDX prevents the increase in offspring body weight and adiposity associated with maternal obesity. In addition, supplementation with PDX prevented impaired glucose metabolism and glucose utilization. Further investigation in relation to the underlying mechanisms of these changes will be presented in the following chapters. Finally, investigation of the obesity-associated changes in the metabolic profile of the dams is required in order to assess how EE is influenced by maternal obesity and whether it can be improved by maternal supplementation with PDX.
Chapter 4

Effect of an obesogenic dietary challenge in adult offspring of obese dams and the potential protective role of maternal supplementation with soluble prebiotic fibre.
Chapter 4: Effect of an obesogenic dietary challenge in adult offspring of obese dams and the potential protective role of maternal supplementation with soluble prebiotic fibre.

4.1 Introduction

Whilst independent associations between maternal obesity and offspring obesity have been reported in mother-child cohort studies (Yu et al., 2013), there is also suggestion that there is a significant interaction between offspring exposure to maternal obesity and the postnatal dietary environment.

As described previously, bariatric surgery for weight loss in women has been associated with a markedly reduced incidence of macrosomia and obesity in the offspring (Smith et al., 2009). The 3 fold reduction in prevalence of severe obesity compared to siblings born prior to weight loss surgery, whilst providing evidence for the importance of pre-pregnancy BMI and the intrauterine environment on risk of obesity, may also indicate a component of a shared postnatal environment contributing to the association between maternal and childhood BMI (Smith et al., 2009).

It has been shown in rodent models of maternal obesity that continued overfeeding of the obesogenic diet after weaning results in exaggeration of the metabolic and cardiovascular abnormalities observed in the offspring of obese dams, and that these effects are greater than the consequences of this diet fed to controls which had not been prenatally exposed to maternal obesity (Bayol et al., 2007; Elahi et al., 2009; Bayol et al., 2010; Oben et al., 2010b; Muralidaran et al., 2013). These observations have sometimes been inferred to demonstrate a ‘second hit’ effect of the dietary insult at the earliest stages of life. There is also evidence suggesting that
the effect of maternal obesity only becomes apparent when the animals are exposed to overfeeding at weaning (Page et al., 2009).

Whilst the majority of studies have focused on the effect of overnutrition introduced early in postnatal life; the effect of a nutritional ‘challenge’ during adult life has not been rigorously investigated. A postnatal challenge introduced in the weaning period has the potential to persistently change pathways of energy balance during vulnerable periods of development in the young animal, whilst a dietary challenge in adulthood directly questions whether the capacity of the animal to respond to the challenge has been compromised by exposure to maternal obesity in the in utero and lactation period. A dietary challenge, which is maintained over many weeks, could also mask more subtle effects of exposures to maternal obesity. One relevant study determined the effect of a high fat challenge in young adult rats. In this report, the authors assessed the effect of either low or high fat diet exposure in utero. The offspring of high fat fed diet dams exhibited increased bodyweight and greater adiposity compared to controls when they were exposed to a high fat diet in adulthood at 8-weeks of age (White et al., 2009). This implied that exposure to the maternal high fat diet in utero and during weaning had permanently compromised the animal’s physiological response to a high fat diet.

In this Chapter the hypothesis that maternal obesity persistently influences the response of the adult offspring to a dietary challenge has therefore been addressed. Specifically, experiments are described to address the influence on energy expenditure of a postnatal obesogenic dietary challenge in adult offspring of obese dams. Furthermore, as administration of polydextrose (PDX) has been shown to have a beneficial effect on energy expenditure (chapter 3), this study investigated whether offspring of PDX-supplemented obese dams are less susceptible to the adverse effects of an obesogeneic environment in adulthood.
4.2 Hypothesis

Maternal obesity exacerbates the effects of obesogenic dietary challenge in adult offspring; while maternal PDX supplementation in obese pregnancy will confer protection against the consequences of the dietary challenge.

4.2.1.1 Aim

To determine whether the dietary challenge in adult offspring has greater adverse outcomes in offspring of obese dams with offspring of control and of dams supplemented with PDX in relation to:

- Susceptibility to bodyweight gain
- Increased calorific consumption
- Changes in EE
- Changes in RER and glucose metabolism

4.3 Methodology

Details of the methods are given in Chapter 3.

4.3.1 Experimental design

Briefly, Female C57BL/6J mice were fed either standard chow (RM1; n=18) or an obesogenic diet (n=46) for 6 weeks prior to mating and during gestation and lactation. Following successful mating a sub group of the obesogenic-fed dams were assigned to an obesogenic diet supplemented with 5% PDX (n=12) during gestation and lactation, while the others remained on the obesogenic diet (n=34). The three resultant groups were: control (Con), obese (Ob) and obese +5% PDX (OffObP).

The litters were reduced to 3 males and 3 females from each dam (when possible) 48 hours post-partum. Litters < 4 were excluded. At weaning, all offspring were assigned to standard chow. At 3 months of age, the energy expenditure (EE) and
RER of all offspring (OffCon, OffOb and OffObP) were measured using the Labmaster apparatus (see section 2.1.4.2). A subgroup of the animals were exposed to an obesogenic dietary challenge for three-weeks ($n_{\text{OffCon}}=6$, $n_{\text{OffOb}}=6$, $n_{\text{OffObP}}=5$). After three-weeks exposure to the obesogenic diet, EE, RER, calorific intake and bodyweights were measured again.

### 4.3.2 TSE Labmaster

EE, RER and food intake in the offspring were measured in the Labmaster at 3 months (used as baseline) and following the three-week exposure to the obesogenic diet. The animals were weighed before placing them in the CaloCages for 48 hours. The first 24 hours was a period of acclimatisation; only data from the last 24 hours were used in analysis. Data was recorded by the Labmaster approximately every 20 minutes from which hourly averages were calculated.

### 4.3.3 Glucose Tolerance Test

A glucose tolerance test was performed on the offspring following the three-weeks exposure to the obesogenic diet, as described in chapter 3.1.5.

### 4.3.4 Statistical Analysis

Data are expressed as mean ± SEM. 3 months data before the exposure to the obesogenic diet are presented for comparisons. Two-way ANOVA was used to investigate the source of variation of the changes in the offspring metabolic parameters examined. Maternal diet and exposure to the obesogenic diet in adulthood were the variations used for the analysis. One-way ANOVA analysis followed by Bonferroni post-hoc tests was performed for the comparison of the three offspring groups following the exposure to the obesogenic dietary challenge. Statistical significance was assumed at the level of $P<0.05$
4.4 Results

Dietary challenge with the obesogenic diet in both male and female offspring led to an increase in bodyweight. In male offspring (Figure 4.1A) 4.18% of the variance was due to maternal diet (P<0.05) while 55.24% (P<0.001) was due to exposure to the obesogenic diet. Moreover, the interaction of maternal diet and exposure to obesogenic diet accounted for 4.14% of the variance in offspring body weight (P<0.05). Exposure to maternal obesity led to an increase in bodyweight (OffCon 40.61g ± 1.19 vs OffOb 44.67±1.2g, P<0.05) but was prevented with dietary supplementation of PDX (OffObP=39.14±2.25g). Among female offspring (Figure 4.1B), maternal diet and exposure to the obesogenic diet accounted for 4.73% (P<0.05) and 66.96% (P<0.001) of total variation respectively. Offspring of obese dams gained more weight during the three-week obesogenic diet exposure compared to their control counterparts (OffCon 32.09 ± 1.9 g vs OffOb 35.79 ± 0.66 g). Offspring of obese dams supplemented with PDX did not weigh significantly more than controls.

4.4.1 Bodyweight

**Figure 4.1 Bodyweight before and after 3 weeks on the dietary challenge.** Bodyweights in male (A) and female (B) offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) at 3 months of age and after three-weeks exposure to obesogenic diet. Exposure to obesogenic diet and maternal diet significantly accounted for variation (investigated by two-way ANOVA shown by straight line on top of the graph), *** represents P<0.001 and $ represents the effect of maternal diet P<0.05, $$ represents P<0.01. 2way ANOVA showed a significant effect of the interaction of maternal diet with exposure to obesogenic diet § represents P<0.05, n=5-6
4.4.2 Daily Calorific Intake

When female offspring of obese dams (Figure 6.2 B) were exposed to the obesogenic diet, their calorific intake was increased compared to controls (OffCon 18.65 ± 1.48 kcal, n=6 vs OffOb 27.65 ± 3.24 kcal, n=6). Administration with PDX in obese pregnancy prevented increased calorific consumption (OffObP 22.29 ± 1.9 kcal, n=5) in the offspring. The obesogenic dietary challenge accounted for 58.82% of the total variation while the interaction between maternal obesity and the obesogenic dietary challenge accounted for 10.44% of the variation.

There was no difference in calorific consumption among male offspring secondary to maternal obesity but the dietary challenge accounted for 50.68% of the variation.

Figure 4.2 Calorific Intake before and after 3 weeks on the dietary challenge. Average daily calorific intake of male (A) and female (B) offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) at 3 months of age and after three-weeks exposure to obesogenic diet, n=5-6. Exposure to obesogenic diet and maternal diet significantly accounted for variation (investigated by two-way ANOVA shown by straight line on top of the graph), *** represents P<0.001. 2way ANOVA showed a significant effect of the interaction of maternal diet with exposure to obesogenic diet §§ represents P<0.01, n=5-6
4.4.3 Respiratory Exchange Ratio

Maternal obesity did not influence change in the phenotype of the offspring. Male offspring displayed an increase in RER (figure 4.3 A) during the daytime and 27.92% of variance in RER was attributed to the obesogenic diet. No significant difference was observed among female offspring.

Figure 4.3 RER before and after 3 weeks on the dietary challenge RER during daytime (A and C) and night-time (B and D) cycles in male (A and B) and female (C and D) offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) at 3 months and after three-weeks exposure to obesogenic diet, the effect of exposure to the obesogenic diet was significantly accounted for the variation (investigated by two-way ANOVA and shown by straight line on top of the graph) ** (represents P<0.01 relevant to (n=5-6)
4.4.4 Glucose tolerance test

Following exposure to the obesogenic diet, offspring insulin sensitivity did not significantly differ (Figure 4.4).

Figure 4.4 Response to glucose before and after 3 weeks on the dietary challenge. Glucose tolerance test (GTT) response (A to D) and the respective area under the curve (AUC) (E and F) in male (A, C) and female (B and D) offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP), following exposure to obesogenic diet for three-weeks (C and D) at 3 months (A and B), (n=5-6), * represents P<0.05, *** represents P<0.001.
4.4.5 Energy Expenditure

Male offspring showed decreased EE during the night-time following three-weeks exposure to the obesogenic diet (Figure 4.5 B). Moreover, the average EE among the groups significantly differed following the obesogenic dietary challenge. Maternal diet and exposure to the obesogenic diet accounted for 13.30 % (P<0.01) for 31.90 % (P<0.001) respectively of total variation among male offspring. Moreover, maternal obesity resulted in lower EE after exposure to obesogenic diet in adulthood (OffOb 10.76 ± 0.31 kcal/h/kg vs 13.40 ± 0.72 kcal/h/kg, P<0.05); whilst administration of PDX prevented it.

Similarly, when female offspring were exposed to the obesogenic diet, energy expenditure during night-time was decreased and group averages significantly differed (Figure 6.5 D). The differences were 15.77% (P<0.001) due to maternal diet and 36.15 % due to the exposure to the diet (P<0.001). Maternal obesity was associated with reduced EE, by 3 kcal/h/kg, when offspring were “challenged” with an obesogenic diet compared to controls (OffCon 14.78 ± 0.52 kcal/h/kg, OffOb 11.49 ± 0.41 kcal/h/kg, P<0.01). However, the effect of maternal obesity on EE was prevented by administration of PDX during obese pregnancy and lactation (OffObP 15.70±0.79 kcal/h/kg, P<0.001).
Figure 4.5 Energy expenditure before and after 3 weeks on the dietary challenge. Mean energy expenditure (EE) during (A and C) daytime and night-time (B and D) in male (A and B) and female (C and D) offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) at 3 months, n=5-6. *represents P<0.05, **represents P<0.01, *** represents P<0.001. Exposure to obesogenic diet and maternal diet were significantly accounted for variation (investigated by two-way ANOVA shown by straight line on top of the graph), *** represents P<0.001 due to exposure to obesogenic diet and $$ represents the effect of maternal diet P<0.01, $$$ represents P<0.001.
4.5 Discussion

To date, the majority of studies have examined the exacerbation of detrimental effects of a secondary exposure of obesogenic diet in early rodent life (Bayol et al., 2007; Bayol et al., 2009; Elahi et al., 2009; Bayol et al., 2010). Only the study by White et al explored the interaction of maternal overnutrition with postnatal exposure to obesogenic diet at 8 weeks of age in rats (White et al., 2009).

In this study, offspring were exposed to an obesogenic diet in adulthood and it was found that the metabolic changes associated with high fat diet exposure were exaggerated by early life exposure to maternal obesity. When previously the obesogenic diet was introduced from weaning (Pilot study, section 2.3), the marked increase in fat mass following a prolonged postnatal obesogenic diet may have overwhelmed any underlying consequences of the exposure to maternal obesity in utero and lactation. In this Chapter, it was demonstrated that by delaying the postnatal dietary challenge to adulthood and restricting the challenge to a shorter period of time, it is possible to demonstrate the independent influence of maternal obesity on offspring susceptibility to increased weight gain when exposed to an obesogenic environment in adult life.

The observation that, PDX supplementation in the dams during pregnancy and lactation, led to a reversal of the influence of maternal obesity on offspring weight gain and associated metabolic parameters may have important relevance to the search for safe interventions to prevent the consequences of maternal obesity in human pregnancy.
4.5.1 Bodyweights

At 3 months of age, there was no significant difference in offspring bodyweight between the groups studied. However, when exposed to an obesogenic diet, offspring of the obese dams had increased bodyweight compared to their control counterparts and the offspring of obese dams supplemented with PDX. This is in accordance with other studies (White et al., 2009; Oben et al., 2010b). The increase in bodyweight was likely to be attributable in part to the decrease of 3 kcal/h/kg in EE, but predominantly to an increase of 10-kcal/h/kg calorific intake amongst the females. Interestingly, female offspring did not show any differences in weight when they were fed a control diet up to 6 months. Thus, the influences of maternal obesity on female offspring metabolism are apparent only after a secondary challenge, supporting the “second hit” hypothesis. Males’ bodyweight gain can only be attributed to differences in EE. The increase in bodyweight in response to the dietary challenge is similar between male and females (approximately 8-10 grams among the groups). As females offspring of obese dams ate more and had similar EE indicating a protective mechanism among females during the exposure to the high fat diet. It has been previously demonstrated that oestrogen had strong inhibitory effect on adipogenic genes in ovarioctemised females supplemented oestrogen compared to ovarioctemised mice; suggesting a protective role of oestrogen on adipogenesis (Stubbins et al., 2012). A change in adiposity is not presented in this here but this mechanism may explain the differences between male and female offspring.
4.5.2 Calorific Intake

There was an increase in calorific intake among the female offspring when they were fed an obesogenic diet. The fact that these animals showed no hyperphagia due to maternal obesity until they were given a high fat/high sugar diet suggests preference for this diet. Unpublished data from our laboratory (South et al, 2014) revealed that offspring of obese rat dams showed a preference for sugar water, which may be explained by altered reward pathways (Ong & Muhlhausler, 2011). Similar findings have been demonstrated when dams were exposed to a junk food diet (Bayol et al., 2007). The preference on the diet, among all the offspring, could be explained by the palatability of the diet, which has been shown to play an important role in appetite regulation (Erlanson-Albertsson, 2005). However, it is shown here that OffOb have a heightened preference for a highly palatable obesogenic diet, particularly in females. This is consistent with unpublished observations from our laboratory in a model of experimental hyperleptinaemia in which female offspring demonstrate increased preference for sugar water associated with changes in dopaminergic reward-related receptor density in the mesolimbic dopamine pathway (South et al, 2014 unpublished). Increased perinatal leptin exposure secondary to maternal obesity may therefore predispose to increased appetite, altered food preference and risk of obesity.

Alternatively the effect of maternal obesity could be due to either fetal and/or maternal hyperglycaemia, which would lead to increase levels of circulating insulin. As discussed in section 1.1.2, insulin and leptin play an important role in the development of the hypothalamus, which is an important regulator of appetite and energy expenditure.
Enrichment of the maternal diet with PDX prevented increased calorific intake among the female offspring. PDX is known to have an effect on glucose tolerance and subsequently on insulin concentrations. Therefore, alterations in the circulating levels of insulin in utero, could have affected the development of fetal hypothalamus (Nataf & Monier, 1992). Male offspring, though, did not demonstrate differences in appetite during the period of overnutrition. Male offspring may have a higher susceptibility to consume increased quantities of a high fat/high sugar diet, independently of maternal diet.

4.5.3 RER-Glucose metabolism

Differences in RER between all groups were no longer apparent following the exposure to an obesogenic diet. Males during the daytime demonstrated increased RER due to postnatal diet, suggesting increased sensitivity to the changes in the substrate availability. The diet had a high content of both fat and sugar in the diet, the RER remained high (approximately 0.9), reflecting increased oxidation of glucose, which is the substrate that is usually being oxidized first. Similar to data presented later, the glucose oxidation rate is reduced, which could indicate impaired glucose metabolism in all the groups. Furthermore, the increased fat intake together with the decreased fat oxidation, could subsequently lead to fat storage and increased adiposity in the animals. Even though the RER of offspring of dams supplemented with PDX appears lower, there was no significant difference.

The GTTs following the exposure to the obesogenic dietary challenge conducted in this study did not reveal any differences amongst all study groups. Paradoxically, there were no differences between the area under curves before and after the dietary challenge despite the high consumption of sugar during that period. For all groups, it was apparent though, that there was an increase in the return to baseline of the blood glucose concentration compared to the GTT response reported in
Chapter 3 (as shown in Figure 4.4 A and B). This may reflect reduced insulin sensitivity induced by the three-week exposure to the high levels of saturated fat and sugars. It was shown before that rat offspring of dams fed a high fat rich diet had a reduction in glucose stimulated insulin secretion, implying reduced insulin secretory capacity (Taylor et al., 2005). Nevertheless, GTT performed weekly during the 3 week period would determine whether the offspring of obese dams exhibit impaired glucose tolerance earlier in that period and whether prolonged exposure to the diet impairs the insulin secretory capacity. According to this data, however, the offspring of obese dams did not demonstrate differences in glucose metabolism following exposure to the obesogenic diet for three weeks.

4.5.4 Energy Expenditure

In the present study, it was shown for the first time that maternal obesity following an obesogenic dietary challenge in adulthood, results in reduced EE in both males and females. Even though, these differences were subtle, less than 3 kcal/h/kg per day, but it has been demonstrated previously that even modest changes have great effect over time on weight gain (Ferrannini, 1988).

Because the changes in EE observed occurred during the night-time, differences in BMR are unlikely to account for the differences observed between the groups. It has been suggested by Neumann that during periods of overfeeding, EE increases in order to maintain bodyweight (Norgan & Durnin, 1980). An increase in EE due to overfeeding is known as “luxukonsumption” and has been attributed by some to activation of the sympathetic nervous system. However, more recent studies have challenged this theory by showing no increase in EE due to overfeeding (Welle & Campbell, 1983; Ravussin et al., 1985) and no differences between obese and non-obese individuals (Bandini et al., 1989).
In the present study, a reduction in night-time EE following a three-week challenge with an obesogenic diet was observed in all three groups. This contradicts previous evidence showing an increase in total EE due to overfeeding by Borengasser et al. (Borengasser et al., 2011). However, in their study, 24h EE was presented and bodyweights were not been taken into account in the measurement of EE. It is well established that increases in EE can be attributed to the mechanical effort of carrying excess weight (Prentice et al., 1986; White & Seymour, 2005), thus, measurement of EE could be overestimated if bodyweight is not taken into account. However, similar to the data of Borengasser et al, it was observed that offspring of obese dams had lower EE compared to controls. Maternal administration of PDX also resulted in higher EE in offspring from obese dams suggesting an influence on pathways of energy balance in early life, in a direction, which decreases the predisposition to a lower EE.

In accordance with the “luxukonsumption” theory, it has been previously demonstrated that diet-induced thermogenesis following glucose consumption was reduced in obese individuals, suggesting that there may be increased susceptibility to obesity when consuming diets rich in sugars (Schutz et al., 1984; Bogardus et al., 1985; Golay et al., 1989). Therefore, reduced diet-induced thermogenesis could be related to the reduction in EE during night-time when the animals are alert and eating. However, reduced physical activity could also play a role.

Since there were differences in EE but not in glucose metabolism following the exposure to the obesogenic dietary challenge, the improvement observed in the PDX group could theoretically be attributable to another mechanism, possibly a persistent influence of the prebiotic properties of PDX ‘carried over’ from mother to the offspring. As previously mentioned, specific gut microbiota populations have been associated with a predisposition to obesity and influence energy harvest in the
host (Sefcikova et al., 2010). Recently, it was demonstrated that consumption of a high fat diet by mice not only affects the microbial diversity but also their composition. In particular, proteins involved in carbohydrate metabolism were decreased following exposure to high fat diet (Daniel et al., 2014). Nevertheless, germ-free mice were resistant to Western type diets, highlighting the role of the microbiota to the development of obesity (Backhed et al., 2007). Therefore, exposure to the obesogenic diet could affect the gut microbiota and subsequently influence energy harvest in the host. Based on the evidence from the studies above, it could be hypothesised that sustainable changes due to the effect of maternal supplementation with PDX on microbiota early in life could not only predispose to obesity but also play a protective role when exposed to an obesogenic diet. Whether the offspring microbiota had been altered due to maternal supplementation with PDX will be investigated in the next chapter.

4.5.5 Limitation and future studies

One male and one female of each litter had been included in this study but due to smaller litters, this was not always feasible. Thus, the total number of mice included in this study was smaller than expected. However, due to the exacerbation of the effects of the obesogenic diet challenge, variation in the outcomes was still identified. Increased numbers, though, would likely reduce the variation related to RER.

Moreover, as the changes noticed in EE were during night-time, the influences of maternal obesity could be accounted for by impaired diet-induced thermogenesis. Rectal temperatures following meal consumption were not taken during the experiments, and should be included in future experiments. As described in the next chapter, changes in UCP genes have been explored as an indication of diet-induced thermogenesis. In order to determine whether changes in diet-induced
thermogenesis are mediated by sympathetic nervous activity, measurement of plasma catecholamine (Tappy, 1996) and norepinephrine (Bandini et al., 1989) could also have provided an insight into mechanism. Also, it would be interesting to incorporate the Inframot system, which allows measurement of physical activity, in order to examine whether the offspring move less when they are consuming a high fat diet.

In addition, analysis of the microbiota before and after the exposure to the obesogenic diet would show how gut microbiota alters due to overnutrition. In particular, this would delineate whether maternal influences alter offspring gut microbiota in such a way that would play a protective or detrimental role to the development of obesity in adult life during periods of overnutrition.

Furthermore, as part of this study was conducted before purchasing the Bio-ImpediMed, fat measurement by bioimpedance was not performed at this stage. The animals were not sacrificed at the end of 3 months because they will also be studied at 6 months of age. Thus, fat pad weights following the three weeks exposure to the obesogenic diet were not collected. In future studies, animals could be sacrificed at the end of the three weeks period in order to allow investigation of differences in body composition and fat deposition.

Moreover, as suggested earlier weekly GTT would provide us with a better insight in relation to any changes in glucose homeostasis. Moreover, in order to assess whether these animals were less insulin sensitive whole body insulin sensitivity could be measured by euglycemic-hyperinsulinemic clamp, as described previously (Taylor et al., 2005).
Finally, it would be interesting to follow up these offspring during the exposure to the obesogenic diet in order identify whether the changes in EE precede the bodyweight gain or *vice versa*. Exposing the animals to the obesogenic diet in the CaloCages and obtaining constant measurements for 3 weeks would provide valuable information regarding the adaptation of the offspring to the obesogenic diet and gradual changes in metabolic parameters. It should also be acknowledged that the animals would remain single-housed for longer periods of time, potentially leading to increased stress.

### 4.5.6 Conclusions

These data in the present study are the first to report that maternal obesity predisposes the offspring during consumption of Western-type diet in adulthood to increased weight gain possibly due to reduced EE. Moreover, this effect of maternal obesity can be prevented if the mothers are supplemented with a prebiotic soluble fibre, PDX, during gestation and lactation. Further investigation is required in order to determine the potential underlying mechanisms.
Chapter 5

Maternal Supplementation with Polydextrose in Obese Pregnant Mice; effects on Maternal Metabolic Function, Energy Intake and Reproductive Function
Chapter 5: Maternal Supplementation with Polydextrose in Obese Pregnant Mice; effects on Maternal Metabolic Function, Energy Intake and Reproductive Function

5.1 Introduction

The prevalence of maternal obesity is increasing worldwide resulting in an increased risk of pregnancy complications such as pre-eclampsia, gestational diabetes, early pregnancy loss, premature delivery and stillbirth among others (Waller & Dawson, 2005; Poston, 2012). As previously discussed, maternal obesity is also implicated in the developmental origins of cardio-metabolic disease in the offspring (Symonds et al., 2009). In Chapter 3 it was shown that maternal obesity influences EE and glucose metabolism in the offspring and that maternal dietary intervention with a soluble prebiotic fibre prevented these metabolic defects. Our current understanding of how maternal obesity influences offspring metabolism is increasing rapidly. Pathophysiological concentrations of lipids (Okereke et al., 2004; Nelson et al., 2010), leptin (Kirk et al., 2009), glucose (Catalano et al., 2003; Catalano et al., 2009; Catalano, 2010) and inflammatory mediators (Dahlgren et al., 2001) in the maternal metabolic milieu as well as altered gut microbiota (Santacruz et al., 2010), placental dysfunction (Sferruzzi-Perri et al., 2013) in obese dams are amongst the proposed mechanisms (further discussed in section 1.4). Identifying which of these parameters mediate the beneficial affects due to maternal intervention with PDX could provide important insight into identification of the underlying mechanisms involved in developmental programming secondary to maternal obesity.
5.2 Hypothesis

Supplementation of obese dams with the soluble fibre, polydextrose, during the critical periods of gestation and lactation will improve the maternal metabolic profile and subsequently benefit the offspring.

5.2.1 Aim

The specific aim of this study was to determine metabolic changes in obese pregnant mice due to supplementation with polydextrose in pregnancy. In particular:

- To examine whether supplementation with PDX improves reproductive success in obese dams
- To examine whether maternal glucose metabolism is influenced by supplementation with PDX
- To examine whether PDX reduces energy intake in the obese dams
- To investigate any beneficial changes in the adipokine profile due to supplementation with PDX

5.3 Methods

5.3.1 Animals husbandry and diets

C57BL/6J mice (n=12), known to be proven breeders (one previous litter) at approximately 100 days of age (Charles River Laboratories, UK) were housed under controlled conditions (25°C, 12-hour light/dark cycle). Dams were fed an obesogenic diet for 6 weeks before mating, during lactation and gestation. Following successful mating, half (n=6) of the dams were randomly assigned to an obesogenic diet supplemented with 5% (w/v) solution of PDX in the drinking water during gestation and lactation. The other half (n=6) remained on the obesogenic diet. The
two resultant groups were: obese (Ob) and obese +5% PDX (ObP). Details of the diet composition can be found in the general methods (section 2.1.1).

At day 18 of pregnancy, following an overnight fast the animals were sacrificed; fetuses, placentas and maternal bloods were collected.

5.3.2 Glucose tolerance test
The pregnant dams were fasted overnight and a GTT (i.p.) was performed on day 18. Further details of the GTT protocol can be found in chapter 2.

5.3.3 Calorific and water intake
Following confirmation of pregnancy, the dams were housed individually and their bodyweights were monitored as well as weekly calorific intake. Water intake was also measured.

5.3.4 Pilot study of Maternal Serum Adipocytokines
Following an overnight fast, dams were sacrificed (day 18) and blood samples were collected and allowed to clot for 2 hours at room temperature before centrifugation. Serum aliquoted and stored at ≤ -80 °C. Adipocytokine protein expression was assessed in 2 samples of pooled serum (n= 5 animals per group) using a Proteome Profiler Mouse Adipokine Array kit (R&D Systems) as per manufacturer’s guidelines. The density of the proteins was analysed with Image J.

5.3.5 Birth related outcomes
Because reproductive function is influenced by maternal obesity and associated with disorders of metabolism, data from offspring of the dams (Con n=18, Ob=34 and ObP=12) in Chapter 3 are presented here. Forty-eight hours after birth, the litters were weighed. The litter size was recorded in order to investigate whether administration of PDX to dams affected the litter size, and specifically the number of
litters with fewer than 4 pups as a litter size of less than 4 has been associated with susceptibility to the development of obesity (Chen et al., 2009). It follows that a PDX induced increase in litter size could directly influence adiposity in the offspring. Cannibalisation was noted when missing pups were recorded during the first postnatal days. A female mouse was considered infertile after being mated twice without a successful pregnancy.

5.3.6 Statistical Analysis

The unpaired student's t test was used to compare the mean differences between two independent data sets.

In order to test whether reproductive outcomes were associated with maternal diet, \( \chi^2 \) test was used.

All data are expressed as mean ± SEM with P< 0.05 assigned statistical significance. Data regarding the expression of adipocytokines were expressed as mean ± SD as the samples were pooled.
5.4 Results

5.4.1 Birth related outcomes

5.4.1.1 Reproductive rates

Assessment by $\chi^2$ test demonstrated that maternal diet was significantly associated with cannibalisation and infertility ($P<0.05$). There was a significant reduction of both outcomes due to maternal obesity as control dams had 88% of successful pregnancies and only 5% of cannibalisation compared to 44% and 17% for obese dams (table 5.1). Administration of PDX in obese pregnant dams improved fertility by 14% ($P=0.05$) and slightly but significantly reduced cannibalisation of the newborn pups by 1% ($P=0.05$).

<table>
<thead>
<tr>
<th></th>
<th>Control (n=18)</th>
<th>Obese (n=34)</th>
<th>Obese +PDX (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Success %</td>
<td>88.8</td>
<td>44.1§</td>
<td>58.3§</td>
</tr>
<tr>
<td>Cannibalisation %</td>
<td>5.5</td>
<td>17.6§</td>
<td>16.6§</td>
</tr>
<tr>
<td>Infertility %</td>
<td>5.5</td>
<td>38.2</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 5.1 Reproductive Rates. Summary of percentages of control (Con), obese (Ob) or obese dams supplemented with PDX (ObP) that had a successful pregnancy, were infertile or cannibalised their offspring presented as n%. § represents $P<0.05$ in relation to improved fertility and reduced cannibalisation as assessed by $\chi^2$ test. (n=12-34)
5.4.2 Litter size and birthweight

There was no significant influence of maternal obesity or maternal obesity with PDX on the birthweight of offspring (Figure 5.1 A). There was, however, a significant reduction of the number of the litter size due to maternal obesity, which was partially reversed by administration of PDX during gestation (Con 7.93± 0.52 (n=15), Ob 5.93± 0.53 (n=15) ObP 6.714 ±0.68 (n=7); Figure 5.1 B).

**Figure 5.1 Birthweight and litter size.** A. Mean birthweight of newborn viable offspring; B. Mean number of pups of control (con), obese (ob) or PDX supplemented obese dams (ObP) measured 48h after birth, n=7-15. * represents P<0.05
5.4.3 Maternal outcomes related to maternal obesity and influence of PDX supplementation

5.4.3.1 Bodyweight during gestation

There was no difference in gestational weight gain following maternal supplementation with PDX during pregnancy (figure 5.2).

Figure 5.2 Gestational bodyweights. Gestational bodyweights of obese (Ob) and obese supplemented with PDX (ObP) dams during measured during gestation (n=6)
5.4.4 Calorific Intake during pregnancy

There was no statistical difference between the total weekly calorific intake as a result of maternal supplementation with PDX.

Figure 5.3 Calorific Intake during gestation. Mean of total calorific consumption of obese (Ob; black bars) and obese supplemented with PDX (ObP; hashed bars) dams during the 3 weeks of gestation, n=6

5.4.5 Water and PDX intake during pregnancy

As the intervention was delivered to dams in the water, the weekly water consumption was measured (figure 5.4). During gestation, the dams administered PDX shoed significantly increased water intake. Week 1 Ob 15.8 ±1.75 ml versus ObP 25.7 ±1.81 ml, Week 2 Ob 17.05 ± 2.36 versus ObP 28.85 ± 2.66, Week 3 Ob 16.18 ±1.18 ml versus ObP 27.95 ± 2.43ml, P<0.01.

Based on the average water consumption and the average weight of the mice the PDX consumption can be calculated. The average weekly consumption of water in the PDX group in three weeks was 26.5 ml, thus, 3.78 ml/day. Since, there was a 5% solution of PDX in drinking water, the daily intake of PDX was 0.19g.
**Figure 5.4 Water Intake during gestation**. Mean of daily water intake of obese (Ob) and obese supplemented with PDX (ObP) dams during the 3 weeks of gestation, n=6

**5.4.6 Glucose tolerance test**

No differences in the glucose response to an i.p. glucose load was observed between the two groups (Figure 5.5).

**Figure 5.5 Glucose tolerance test at day 18 of pregnancy** in obese (Ob) and obese supplemented with PDX (ObP) dams, n=6
5.4.6.1 Serum Adipocytokines

The relative protein expression of tnty-four adipocytokines from 2 samples of pooled serum samples (n=5 per pool) from dams at day 18 was assessed in a pilot study. The majority shod little or no obvious differences between the 2 pooled samples whereas TNF and CSF-1 (Cerebrospinal fluid protein) showed a 4 and 3–fold decrease respectively (Figure 5.6).

![Graph showing serum adipocytokines expression](image)

**Figure 5.6. Serum adipocytokines.** Expression of serum adipocytokines in pooled serum samples from obese dams and obese dams supplemented with PDX (n=5). Data expressed as mean ± SD.
5.5 Discussion

Despite accumulating evidence regarding the influences of maternal exposures on offspring metabolism, the causative relationships remain to be fully elucidated. This chapter investigated the effect of supplementation with PDX in obese pregnancy on maternal metabolic phenotype and putative biochemical mediators in the developmental programming of altered energy balance and metabolism in the offspring, which could potentially explain the beneficial effect of PDX observed in the offspring.

5.5.1 Reproductive rates

There was a lower fertility and pregnancy success rate amongst the obese dams. The association of obesity with infertility is well known; increased androgen, insulin resistance and polycystic ovary syndrome are probable causes (Huda et al., 2010). Obesity has also been associated with miscarriage (Mewally et al., 2008) and in this study, occasionally, despite increasing bodyweight by day 14; the dams suddenly demonstrated a reduction in bodyweight. These pregnancies were not followed further as the weight reduction is indicative of reabsorption of foetuses.

Overall reproductive success is also dependent on successful labour, which may be compromised by obesity in pregnancy. Caesarean section rates are much higher (Fyfe et al., 2013) in obese pregnancy and are associated with reduced contractility of myometrium, as previously shown in obese women (Zhang et al., 2007) and rats on high fat diet (Elmes et al., 2011). In our group we have some experience of obstructed labour in rodent models of high fat feeding in pregnancy, and complicated delivery and stillbirth may have contributed to fetal loss, although without 24hr surveillance this is difficult to quantify accurately.
Rates of cannibalisation were increased in obese dams compared to controls. A possible explanation could be failure of lactation, which has been previously reported in obese women, due to reduced secretion of prolactin (Rasmussen & Kjolhede, 2004). Thus the dams could be sacrificing their pups due to their inability to feed them, or, perhaps through an evolutionary developed behaviour, in an attempt to reduce litter size in order to ensure the survival of the rest. Measuring prolactin levels in future studies would confirm whether the increased cannibalisation rates were ultimately a protective evolutionary mechanism in these animals.

5.5.2 Birthweight and litter size

There was no difference in birthweight among the groups. Contradicting results have been observed in regard to birthweight amongst reported studies. Some have shown no effect on birthweight (Samuelsson et al., 2008; Giraudo et al., 2010; Bringhenti et al., 2013), while others have demonstrated increased (Samuelsson et al., 2008; Ashino et al., 2012) or decreased litter weight (King et al., 2013a) due to maternal overnutrition. High birthweight, which is regarded as a marker of intrauterine function, has been identified as an independent risk factor of childhood obesity (Cnattingius et al., 2012). Since no difference was observed in this study, it is suggested that the differences in adiposity observed between the offspring (chapter 3) are independent of birthweight.

Despite no differences in birthweight, there was a significant difference in the number of pups at birth due to maternal obesity. The current study apparently contradicts previous animal studies, including a previous study from our own laboratory (Samuelsson et al., 2008), which showed no effect of maternal obesity on litter size, but either an increase (Rolls & Rowe, 1982; Heng & Kliegman, 1990) or a decrease in birthweight (King et al., 2013b). An inverse association between
birthweight and litter size has been previously demonstrated from others (Romero et al., 1992; Freetly & Leymaster, 2004; Ishikawa et al., 2006) and from our group in rats (Nivoit et al., 2009). However, another study has demonstrated a decrease in litter size together with a decrease in birthweight (Hayes et al., 2012). No such association was observed in the present study, suggesting that the previously described association of birthweight with litter size may not be relevant in this study.

Decreased litter size is associated with an increased number of defective blastocysts, due to abnormal embryonic development, and resulting to a failure of implantation. Thus, impaired pre- and peri-implantation could explain the decreased reproductive success of the obese dams (Day et al., 1991; Huang et al., 1997). Whether, stress induced changes occur during the period of implantation in the blastocysts and subsequently affect the reproductive rate should be investigated further. Moreover, altered placental vascular function and blood flow may result in reduced fetal oxygenation and subsequently premature demise, and fetal loss (Hayes et al., 2012). As previously stated increased cannibalisation among the obese dams, may reflect increased fetal complications and/or poor neonatal survival.

In future studies a cohort of obese pregnant dams could be monitored continuously with infra-red cameras in order to measure the exact pup number and at which point cannibalisation occurs.
5.5.3 Effects of Maternal PDX Supplementation on Reproductive Function

Despite the poor pregnancy success among obese dams, supplementation with PDX had a positive effect on fertility, pregnancy success rate and litter size. In one previous study, pregnant rats fed a high fat diet supplemented with fibre also demonstrated improvements in reproductive success. More specifically in both maternal serum and placentae, an increase in antioxidant markers were observed among the samples from dams supplemented with fibre compared with dams on only a high fat diet during pregnancy (Lin et al., 2011). Since oxidative stress has been previously associated with poor reproductive outcome, poor oocyte quality in obese dams due to oxidative stress resulting in reduced reproductive success (Poston et al., 2011b). In future studies, the quality of the oocytes can be assessed by markers covering a range of intraovarian and post fertilization parameters including oocyte differentiation, developmental competence and fetal development, covering a range of intraovarian and post fertilization parameters (Combelles & Albertini, 2003).

The improved fertility and pregnancy success rates may be an early indication of the improved outcomes observed in the offspring (Chapter 3). In agreement with our study, supplementation with fibre also prevented reduction in pup number, an effect, which has been attributed to improved placental free radical scavenging properties placentas, suggesting, reduced oxidative stress (Bellver et al., 2007). Whether the changes can be exclusively attributed to oxidative stress and how oxidative stress influences the blastocysts, specifically remains to be elucidated in future studies by measurements of oxidative stress markers.
5.5.4 Maternal physiological changes due to supplementation with PDX

Similar to previous results (Bellver et al., 2007), no differences were observed during gestation in relation to calorific intake and gestational weight gain (GWG). Despite the reported effects of PDX on satiety (Shimomura et al., 2005), pregnant mice supplementation with PDX did demonstrate any evidence of reduced calorific intake. When the dams were given PDX, they were already pregnant and obese; thus, any beneficial effect of the prebiotic fibre could possibly not overcome the detrimental effects of prepregnancy obesity on satiety, or, more likely, the high palatability of the diet. Nevertheless, the aforementioned evidence suggests that the differences observed in the offspring were independent of weight loss or calorific restriction in the dams.

In addition, no differences in glucose homeostasis were observed between obese dams and controls based on the response to glucose. These results are in agreement with other studies showing that obese dams are not glucose intolerant in the last days of pregnancy (Samuelsson et al., 2008; Petry et al., 2010). It has been suggested that mice show improved glucose metabolism in the last days of pregnancy. Thus, an earlier GTT might potentially identify differences in glucose homeostasis associated with obesity, and measurement of insulin during the GTT would determine whether insulin sensitivity is reduced, providing a more complete picture of glucose homeostasis. Investigation of glucose homeostasis during suckling would also be of interest, as we have previously reported hyperglycaemia in obese dams at weaning (Samuelsson et al, 2008).

Dams supplemented with PDX drank significantly more water compared to the obese dams. PDX tastes subtly sweet (1%), thus the mice could have been drinking more water due to it being more palatable than PDX-free water. PDX may also indirectly increase thirst through increasing plasma osmolarity as it can facilitate
faecal excretion (Nielsen et al., 2011) and shorten transit time. Subsequently, less water is reabsorbed in the gut, resulting in water retention in the faeces and higher plasma osmolarity (Spiller, 2001). There is little previous evidence regarding water intake during pregnancy; a study in humans, reported no effect of water intake on fetal growth (Wright et al., 2010). However, it had been observed that water restriction during lactation results in reduced milk production and growth impairment in the offspring (Baverstock & Watts, 1975). If water intake during gestation, and possibly during lactation influences milk production, the reduced prevalence of cannibalisation among the ObP compared to Ob could be explained by the fact that the mothers were better able to feed their offspring. Future studies could assess milk production by recording change in pup weight after timed separation (Hernandez et al., 2012).

5.5.5 The effect of the intervention on serum adipocytokines

In a pilot study, administration of PDX resulted in substantively decreased expression of the inflammatory mediators TNF, but these data require confirmation by quantitative ELISA assay. Inflammatory mediators have been previously shown to increase in obese pregnancy (Huda et al., 2010) and gestational diabetes (Ategbo et al., 2006). The influence of inflammation on developmental origins of health and disease has been previously documented; others have shown that administration of TNF in obese pregnant rats resulted in increased adiposity among the offspring (Dahlgren et al., 2001). CSF-1, a pro-inflammatory marker, was also down-regulated in the dams supplemented with PDX and it has been previously shown that administration of high levels of recombinant CSF-1 is associated with fetal resorption and implantation failure in mice (Tartakovsky et al., 1991). A reduction in CSF-1 provides a strong candidate to explain the improved reproductive rates observed in the obese dams with PDX. Furthermore, a study in human primates concluded that widespread activation of proinflammatory cytokines due to
overfeeding influenced the development of the offspring hypothalamus and subsequently early onset weight gain (Grayson et al., 2010). Similarly, an investigation of the association of dietary fibre intake with inflammatory mediators has shown that higher fibre intake is correlated with reduced inflammation (Qi et al., 2006).

In summary this study suggests a beneficial effect of supplementation with PDX through reduced the expression of inflammatory markers.

**5.5.6 Study limitations and future suggestions**

The present study investigated whether the intervention results in changes in the maternal profiles during pregnancy. Because of the poor pregnancy outcomes previously observed, continuous experiments during pregnancy were avoided in order to be able to obtain serum samples at the end of pregnancy. The volume of the serum samples, however were not enough to allow measurements of multiple parameters. Therefore, a further study specifically designed around sample collection would enable investigation of the many relevant parameters that have not been explored in the current study. For example, measurements of fasting insulin would allow us to investigate whether insulin sensitivity improved in the last days of pregnancy (Petry et al., 2010). Moreover, if samples from the tail vein proved insufficient, subset of dams could be sacrificed earlier to examine the effects of PDX on maternal glucose homeostasis during early and middle pregnancy.

In addition, maternal lactation deserves exploration. Thus, a separate study including supplementation of the dams during the period of lactation only, would delineate whether the influences observed in the offspring can be attributed to changes during lactation.
Considering the evidence of an antioxidant effect of maternal fibre (Lin et al., 2011) supplementation effects on placenta and fetuses, and the reduction in the inflammation in obese dams supplemented with PDX, measuring oxidative status would delineate the possible antioxidant effect of the PDX in obese pregnant mice e.g. lipid hydroperoxides which can be quantified in maternal serum and placentas (TBARS assay kit; Cayman Chemical, USA) could be measured. In addition, superoxide anion scavenging capacity and hydroxyl radical scavenging of maternal serum and the placenta could be evaluated, as previously described (Lin et al., 2011).

Finally it is important to consider the daily intake of PDX in relation to humans. The 5% of PDX solution in the drinking water of the mice was proven effective and safe in the current animal study; however, the design would need to be re-evaluated in a human study in respect to the physiological differences between humans and mice. Assuming that an adult is drinking in average 2lt of water 5% of PDX in the drinking water would be 100g of PDX per day, which greatly exceeds the 18 g of recommended daily intake of fibre per day in UK. Therefore, if this study were replicated to humans the dosage would need to be adjusted accordingly.

5.5.7 Conclusions

Many of the adverse outcomes of obesity in non-pregnant population are thought to be mediated by inflammation and its sequelae. Moreover, adverse outcome in obese pregnancy may be partly attributed to increased inflammatory response in obese pregnancy (Denison et al., 2010). In the present study, reduced expression of inflammatory markers may have occurred due to an intervention with fibre supplementation in obese pregnancy. Moreover, the increased water intake of the dams could also influence the health of the offspring.
Chapter 6

Potential underlying mechanisms for developmental programming of energy expenditure and glucose homeostasis in offspring of obese dams.
Chapter 6: Potential underlying mechanisms for developmental programming of energy expenditure and glucose homeostasis in offspring of obese dams.

6.1 Introduction

Maternal gut microbiota are known to have an influence on the metabolic and immunologic profiles of pregnant women but have also been implicated in the risk of developing disease later in life (Barker, 2007; Myles et al., 2013). In the non-pregnant state, alterations in microbiota composition have been associated with altered energy harvest from food and increased weight gain (Turnbaugh et al., 2006), insulin resistance (Shen et al., 2013) and visceral adiposity (Stolarczyk et al., 2013). The male offspring of obese dams demonstrate all the above. Therefore, alteration in the composition of the offspring gut microbiota a consequence of exposure to the influences of maternal obesity offers a potential mechanism underlying these metabolic changes. Moreover, since PDX also acts as a prebiotic, the observed improvement in adult metabolic phenotype in the offspring of obese dams after maternal supplementation with PDX could be mediated through changes in the gut microbiota.

Mitochondria play a central role in energy production and constitute a major site of reactive oxygen species (ROS) production. Metabolic diseases associated with mitochondrial dysfunction and increased oxidative stress includes obesity, cancer and type 2 diabetes (Mancuso et al., 2007; Debray et al., 2008; Malik & Czajka, 2013). In chapters 3 and 4, the offspring of obese dams were shown to demonstrate reduced energy expenditure and glucose intolerance. Mitochondrial
dysfunction, and reduced mitochondrial copy number have been previously demonstrated in insulin resistant offspring of fat fed, obese dams ((Taylor et al., 2005; Shelley et al., 2007; Samuelsson et al., 2008; Bruce et al., 2009; Shelley et al., 2009; Igosheva et al., 2010). Therefore, it is theoretically possible that mitochondrial dysfunction could contribute to altered energy expenditure in the offspring of the obese dams.

Mitochondria copy number is a biomarker of mitochondrial dysfunction. Mitochondrial DNA is unmethylated, similarly to bacteria DNA, it could cause directly cause disease. Moreover, mitochondrial copy number is affected by ageing (Peterson et al., 2012). Therefore, mitochondria copy number was assessed prior to the development of the observed changes in EE in order to investigate whether developmental programming of mitochondrial dysfunction could play a causative role in the observed adverse health outcomes in offspring of obese mice later in life. Moreover, as mitochondrial dysfunction can be caused by oxidative stress I have also investigated whether supplementation with PDX, known for its antioxidant properties, had any influence on mitochondrial function secondary to obese pregnancy.

There was a significant increase in the energy expenditure of male offspring of obese dams at 6 months of age during the night-time i.e. during the period of active feeding compared to offspring of lean controls. Moreover, when the offspring were exposed to high fat/ high sugar diet, there was a further reduction in EE during the night-time in both male and female offspring. Therefore, impaired feeding-induced thermogenesis may mediate these observed changes in EE. In previous studies UCP proteins have been implicated in adaptive thermogenesis and in diet-induced thermogenesis in particular (Lowell & Spiegelman, 2000). UCP1 is downregulated during starvation due to reduced sympathetic nerve activity to brown fat. However,
UCP1 knockout mice do not become obese while transgenic mice with reduced brown fat (BAT) do become obese. Therefore, an alternative role for BAT is suggested, possibly involving UCP2 and UCP3 proteins or an appetitive regulator of brown fat (Lowell & Spiegelman, 2000). Indeed it has been previously demonstrated that obesity resistant mice had increased levels of UCP2 in BAT, suggesting a role for this protein in diet-induced thermogenesis (Ricquier & Bouillaud, 2000). Progressively decreasing levels of UCP3 have been associated with the development of insulin resistance during high fat feeding (Senese et al., 2011). Moreover, ablation of UCP3, in a transgenic mouse model, was associated lipid induced mitochondria damage resulting from prolonged high fat feeding (Nabben et al., 2011). However, during high fat feeding UCP1 expression is also increased but the effect size greatly differs between studies (Fromme & Klingenspor, 2011). The mRNA expression of these UCP proteins was investigated in offspring BAT and skeletal muscle in order to determine the differential expression, associated with the various maternal exposures, including PDX supplementation in obese pregnancy, which may underlie differences in diet-induced thermogenesis and therefore energy expenditure in the offspring.

6.2 Hypothesis

Maternal obesity results in altered mechanistic pathways involved in the regulation of energy balance. Supplementation of the obese dams with a soluble fibre will reverse the changes in the mechanistic pathways induced by maternal obesity.

6.2.1 Aim

To investigate the potential pathways affected by maternal obesity and by the supplementation with soluble fibre in obese pregnancy, with particular focus on:

ii. Mitochondrial copy number.

iii. Uncoupling Protein (UCP) gene expression.
iv. Changes in microbiota

6.3 Methods

The methods are described in detail in chapter 2.2.

6.3.1 Tissue collection

At 30 days and 6 months of age offspring of control dams (OffCon, \( n=6 \)), obese dams (OffOb, \( n=6 \)) and obese dams supplemented with PDX (OffObP, \( n=6 \)) were sacrificed by exposure to a rising \( \text{CO}_2 \) concentration. Brown fat (BAT) and skeletal muscle was dissected and snap frozen in liquid nitrogen. Samples were stored at -80°C until required for further analysis.

6.3.2 Microbiota composition in faecal samples assessed by fluorescent in situ hybridisation and flow cytometry

At weaning, 3 months and 6 months of age faecal samples from the animals were collected in 2 ml Eppendorf tubes (\( n=4-6 \)). Following collection, samples were snap frozen and kept at -80°C until required for analysis. The samples were then quantified by fluorescent in situ hybridisation and analysed with flow cytometry as described by Rigottier-Gois and colleagues (Rigottier-Gois et al., 2003). Details in relation to these methods and the probes used can be found in section 2.2.8.

6.3.3 Mitochondria DNA content in skeletal muscle

Genomic DNA was extracted from skeletal muscle (\( n=5-6 \)) from offspring of control (\( n=6 \)), obese (\( n=6 \)) and obese supplemented with PDX dams (\( n=5 \)) at 30 days of age. Real time PCR was performed and the ratio of mitochondrial versus nuclear genome was measured. More details regarding the methods and the primers used can be found in section 2.2.6.
6.3.4 Real Time Quantitative PCR

Gene expression profiles of UCP1, UCP2 and UCP3 measured by qPCR were examined in females and male 30 days and 6 months old mice. Data for qPCR were expressed relative to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

6.4 Results

6.4.1 Microbiota Composition

6.4.1.1 Microbiota composition at weaning

At 30 days of age the samples from the OffObP males demonstrated an increased percentage of Bacteroides in the total bacterial population compared to OffCon (OffObP: 6.11 ± 0.31 % versus OffCon: 49.38±7.79 %, n=6, P<0.05) (Figure 6.1 A). There were no significant differences among the percentages of the other microbiota populations.
Weaning males

A

\[
\begin{align*}
\text{Bac}^+ &\quad \text{OffCon} & \text{OffOb} & \text{OffObP} \\
0 & \quad 20 & \quad 40 & \quad 60 & \quad 80
\end{align*}
\]

B

\[
\begin{align*}
\text{Erec}^+ &\quad \text{OffCon} & \text{OffOb} & \text{OffObP} \\
0 & \quad 10 & \quad 15
\end{align*}
\]

C

\[
\begin{align*}
\text{Bif}^+ &\quad \text{OffCon} & \text{OffOb} & \text{OffObP} \\
0 & \quad 5 & \quad 10 & \quad 15
\end{align*}
\]

D

\[
\begin{align*}
\text{Lab}^+ &\quad \text{OffCon} & \text{OffOb} & \text{OffObP} \\
0 & \quad 2 & \quad 4 & \quad 6 & \quad 8
\end{align*}
\]

Figure 6.1 Microbiota composition weaning males. Percentage of A. \textit{bacteroides} (Bac+) B. \textit{Eubacterium rectale-Clostridium coccoides} (Erec+) C. \textit{Bifidobacterium} (Bif+) D. \textit{Lactobacillus-Enterococcus} (Lab+) in bacteria cells (EUB+) identified in faecal samples from weaning male offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP), * represents P<0.05. (n=6)

There were no significant differences in the percentages of these bacterial species in the faecal samples from the different groups of females at weaning (Figure 6.2).
Figure 6.2 Microbiota composition weaning females. Percentage of A. Bacteroides (Bac+) B. Eubacterium rectale - Clostridium coccoides (Erec+) C. Bifidobacterium (Bif+) D. Lactobacillus-Enterococcus (Lab+) in bacteria cells (EUB+) identified in faecal samples from weaning female offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP). (n=6)
6.4.1.2 Microbiota composition at 3 months

There was no difference in microbiota populations among either male or female offspring at 3 months of age (Figures 6.3, 6.4).

**Figure 6.3 Microbiota composition 3 months males** Percentage of A. *Bacteroides* (Bac+) B. *Eubacterium rectale-Clostridium coccoides* (Erec+) C. *Bifidobacterium* (Bif+) D. *Lactobacillus-Enterococcus* (Lab+) in bacteria cells (EUB+) identified in faecal samples from 3 months male offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) (n=4-6).
Figure 6.4 Microbiota composition 3 months females. Percentage of A. bacteroides-Prevotella (Bac+) B. Eubacterium rectale-Clostridium cocoides (Erec+) C. Bifidobacterium (Bif+) D. Lactobacillus-Enterococcus (Lab+) in bacteria cells (EUB+) identified in faecal samples from 3 months female offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) (n=4-6).
6.4.1.3 Microbiota composition at 6 months

At 6 months of age OffOb males had a significantly increased percentage of Eubacterium rectale- Clostridium coccoides group compared to OffCon (OffOb: 18.67± 1.78 % versus 3.72± 0.34 %, n=4-6, P<0.01; Figure 6.5). Maternal dietary intervention with PDX partially reversed this change. There was no statistical difference among the other bacteria populations in any of the groups.

Figure 6.5 Microbiota Composition 6 months males. Percentage of A. bacteroides-Prevotella (Bac+) B. Eubacterium rectale-Clostridium coccoides (Erec+) C. Bifidobacterium (Bif+) D. Lactobacillus-Enterococcus (Lab+) identified in faecal samples from 6 months male offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP), (n=4-5)** represents P<0.01.
The similar increase in number of *Eubacterium rectale-Clostridium coccoides* among female OffOb was borderline significant (Figure 6.6;) compared to controls (OffOb: 5.88 ± 1.7 % vs OffCon 5.19 ± 5.88 %, n= 4-5, P=0.07).

**Figure 6.6 Microbiota Composition in Faecal Samples from 6 months females.** Percentage of A. bacteroides-Prevotella (Bac+) B. Eubacterium rectale-Clostridium coccoides (Erec+) C. Bifidobacterium (Bif+) D. Lactobacillus-Enterococcus (Lab+) identified in faecal samples from 6 months female offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP), (n=4-5).
6.4.2 Mitochondria Copy number in skeletal muscle

Among males at 30 days of age (Figure 7.8 A) Mt/N ratio in skeletal muscle was significantly increased due to maternal obesity compared to controls (OffOb: 358.1 ±89.93 vs OffCon: 675.6± 47.16, n=6, P<0.01). Supplementation with PDX in obese pregnancy prevented this increase (OffObP: 174.5 ± 25.73, n=6; P<0.001). There was no significant effect of maternal diet on mitochondrial copy number ratio in female offspring, although the pattern was similar.

Figure 6.7. MtDNA copy number ratio in skeletal muscle from A. male and B. female 30 days old offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP). (n= 5-6)
6.4.3 UCP mRNA expression

6.4.3.1 UCP mRNA expression in skeletal muscle at 30 days

At 30 days of age there was no apparent effect of maternal obesity on UCP mRNA expression. There was a significant increase in UCP1 (OffObP 0.40± 0.07 vs OffCon 0.11± 0.06) and UCP3 (OffObP: 0.001917 ± 0.0017 vs 0.02 ± 0.02) expression in male offspring of obese dams supplemented with PDX compared with the offspring of controls (Figure 6.8).

Figure 6.8 UCP mRNA expression in skeletal muscle at 30 days of age. UCP1 (A&D), UCP2 (B&E), UCP3 (C&F) mRNA expression corrected by GAPDH expression in male (A-D) and female (D-F) offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP) (n= 5-6)
6.4.3.2 UCP mRNA expression in skeletal muscle at 6 months of age

There was no change in UCP mRNA expression in skeletal muscle among the offspring of obese dams or in offspring of obese dams supplemented with PDX dams at 6 months of age (Figure 6.9).

Figure 6.9 UCP mRNA expression in skeletal muscle at 6 months of age. UCP1 (A&D), UCP2 (B&E), UCP3 (C&F) mRNA expression corrected by GAPDH expression in male (A-D) and female (D-F) offspring of control (OffCon), obese (OffOb) and obese dams supplemented with PDX (OffObP). (n= 5-6)
6.5 Discussion

6.5.1 Microbiota Composition

In this chapter the effect of maternal obesity on the gut microbiota population at 30 days, 3 months and 6 months of age was addressed. An effect of maternal obesity is demonstrated at 6 months of age in male offspring. Specifically, the population of the groups *Eubacterium rectale* /*Clostridium coccoides* was significantly increased and a similar trend was also observed in 6 month females. However, when the dams were supplemented with PDX the increase in *Eubacterium rectale*/*Clostridium coccoides* in the male offspring was prevented. Previously, it has been shown that the population of *Eubacterium rectale* surges with increased carbohydrate consumption (Duncan *et al.*, 2007). There is little evidence regarding the effect of the *Eubacterium rectale*/*Clostridium Coccoides* in obesity and existing studies are relatively contradictory. One study, among obese pregnant women, reported a borderline increase (P = 0.088) in *C. coccoides* compared with lean control pregnant women (Santacruz *et al.*, 2010), while another observed no change in the *C. coccoides* population due to increased weight gain (Schwiertz *et al.*, 2010).

Whilst there is little direct evidence for the effect of obesity on *C. coccoides*, populations, prebiotics (Cani *et al.*, 2009) and short chain fructooligosaccharides (Respondek *et al.*, 2013) are reported to increase numbers of *C coccoides* in mice; this increase was associated with increased insulin sensitivity. If it is assumed that gut flora is directly transmitted from dam to pup, evidence from the aforementioned studies would appear to contrast with the data reported here, since a reduction in populations of *Eubacterium rectale*/*Clostridium coccoides* would have been expected among the OffOb and an increase among the OffObP. However, both previous studies were performed in much younger animals (4 and 8 weeks, respectively) suggesting that changes could occur in the population of these
bacteria with time. Since the evidence on the exact association of *Eubacterium rectale*/*Clostridium coccoides* with obesity are not conclusive (Santacruz *et al*., 2010; Schwiertz *et al*., 2010), the exact significance of these results remains to be determined. Nevertheless, offspring of obese dams demonstrate significantly different microbiota composition at 6 months along with decreased energy expenditure and glucose.

It was interesting to observe that supplementation with PDX in obese pregnancy resulted in increased numbers of *Bacteroides* compared to controls. As discussed previously, administration of prebiotics improved pregnancy outcomes (Laitinen *et al*., 2009) and administration of L. rhamnosus induced specific changes in the transfer and initial establishment of bifidobacteria in the infant (Collado *et al*., 2012). A beneficial effect of breastfeeding in infant microbiota, including increase in *Bacteroides* has been demonstrated (Mevissen-Verhage *et al*., 1987). Similarly inclusion of prebiotics in formula milk mimics the effect of breastmilk (Boehm & Moro, 2008). Therefore, it is possible that maternal supplementation with PDX in obese mice during pregnancy and lactation influenced the gut microbiota early in life leading to an increase in *Bacteroides*. Reduced number of *Bacteroides* has been previously associated with obesity (Sanz *et al*., 2010). *Bacteroides* utilize available nutrients very effectively. In the large intestine, they induce the utilization of simple and complex sugars and polysaccharides in order to promote growth (Hooper *et al*., 2002). Moreover, supplementation with *Bacteroides* in high fat fed mice resulted in reduced weight gain, liver steatosis and liver cholesterol and triglyceride concentrations and increased small adipocyte numbers (Gauffin Cano *et al*., 2012). These results are of particular relevance to our model since we have demonstrated all the above adverse health outcomes in offspring of obese dams (Samuelsson *et al*., 2008; Mourtadarane *et al*., 2013). It has been previously suggested that decreased number of Bifidobacteria in infancy could play a role in the susceptibility
to obesity in later life (Kalliomaki et al., 2008). As discussed above, increased numbers of Bacteroides to play a protective role against obesity. Therefore, in the model described in the this thesis the early increase of the population of Bacteroides which could be associated with the improved glucose metabolism of these offspring, as Bacteroides are involved in the effective utilization of nutrients. Finally, by improving glucose metabolism and energy harvest, the EE of the offspring could have been increased by improved diet-induced thermogenesis.

Increased permeability of the gut has been reported following exposure to a high fat diet (Watson & Duckworth, 2010) and is thought to be mediated through immune dysfuction and a resultant metabolic endotoxemia (Cani et al., 2008). An influence of maternal overnutrition on the offspring gut and specifically on its barrier function has been previously demonstrated and recently reviewed (Zhu et al., 2013). In addition, maternal overnutrition influences macrobiota composition, and subsequently plays a role in the maturation of the immune system (Myles et al., 2013). Moreover, it has been shown that visceral adiposity (as demonstrated in Chapter 5) is associated with insulin sensitivity via changes in the immune system which also correlate with energy expenditure (Stolarczyk et al., 2013). In our model we have already described evidence to suggest that the development of fatty liver disease secondary to maternal obesity and a postnatal obesogenic envornment is mediated at least in part by via impaired innate immunity in the liver, and by an increase in proinflammatory markers (Mouralidarane et al., 2013). In this study evidence of the effect of maternal obesity on offspring microbiota composition is presented. Whilst the inter-sample variation was high amongst some of the bacterial species, and therefore, for some, inevitably underpowered, the data for others, with less variability, warrants further investigation on the influences of maternal obesity on offspring gut flora, the immune system and its impact on energy metabolism. This is
essential for our understanding of the interplay between these dynamic systems and the consequences of maternal obesity in pregnancy.

6.5.2 Mitochondria DNA content

Male offspring of obese dams had lower EE, lower RER and increased bodyweight in adult life but not at 30 days of age. In this chapter, evidence of altered mitochondrial DNA copy number in skeletal muscle at 30 days of age are presented, suggesting influences of maternal obesity on male mitochondria prior to the development of other metabolic defects.

The mtDNA/nuclear DNA ratio (Mt/N) reflects the tissue concentration of mtDNA per cell. Increased reactive oxygen species resulting from a developmental insult such as hyperglycaemia or increased fat intake under conditions of oxidative stress could lead to enhanced mitochondrial biogenesis, and increased Mt/N. Altered MtDNA levels may contribute to enhanced oxidative stress and inflammation and could play a pathogenic role in mitochondrial dysfunction and disease (Malik & Czajka, 2013). Damaged mitochondria undergoes fission and is degraded via mitophagy (Kim et al., 2007a; Hirota et al., 2011), however under oxidative stress damaged mitochondria can be accumulated in the cell (Scherz-Shouval & Elazar, 2007). The electron transport chain in the damaged DNA may be blocked resulting in accumulation of free radicals (Giacco & Brownlee, 2010). Exposure of MtDNA ROS may damage the DNA itself resulting in accumulation of deletions and mutations (Indo et al., 2007). Accumulation of damaged DNA would cause chronic innate inflammatory response in the cell. Therefore, it has been suggested that oxidative stress could result in increased mitochondrial biogenesis (an adaptive mechanism) resulting in increased Mt/N, which would subsequently contributes to further oxidative damage, mitochondria dysfunction (Malik & Czajka, 2013).
Mitochondria DNA (MtDNA) could directly lead to pathology because it is unmethylated (Malik & Czajka, 2013), similar to bacterial DNA. MtDNA fragments following DNA damage are known to cause inflammation; in a mouse model of impaired degradation of damaged mitochondrial DNA, increased numbers of MtDNA in the cytosol of cardiomyocytes resulted in heart failure (Oka et al., 2012). Therefore, in the current study investigated the MtDNA concentration in skeletal muscle prior to the metabolic changes observed in the offspring, at 30 days of age.

Changes in MtDNA content have been associated with a number of diseases, including diabetes and its complications, obesity, cancer, HIV complications, and ageing (Malik & Czajka, 2013). Increased MtDNA content precedes the development of complications in patients with type 2 diabetes mellitus (Song et al., 2001). Reduced Peripheral blood mitochondrial DNA content was positively correlated and considered the main predictor of insulin resistance in offspring [of type 2 diabetes (Song et al., 2001). In another study, increased MtDNA content was found in adipose tissue from obese patients but was not correlated with changes in BMR (Lindinger et al., 2010). Similarly, in a group of 148 healthy volunteers with a large inter-individual variation in BMI, mtDNA copy number was enriched in adipocytes and decreased with ageing (p = 0.015) and increasing BMI (p = 0.004). Decreased MtDNA content was not associated with resting energy expenditure but it was strongly linked with lipogenesis (p < 0.0001) in adipocytes, and expression of several genes involved in mitochondrial oxidative capacity, suggesting the importance of adipocyte mitochondria as local regulators of metabolism (Kaaman et al., 2007). Surprisingly, despite the role of mitochondria in energy production there is very little evidence directly associating BMR with mitochondrial function. It has been shown, though, it was shown that mitochondrial oxygen efficiency, measured
in isolated skeletal muscle mitochondria from healthy individuals, strongly correlate with BMR (adjusted for body mass) (Larsen et al., 2011).

Animal studies from our group have also demonstrated altered mitochondrial copy number due to fat feeding in pregnant rats (Taylor et al., 2005). More specifically, offspring of high fat fed rats had reduced mitochondrial copy number and higher plasma insulin. Investigating the link between reduced MtDNA content and subsequent risk of type 2 DM, a maternal diet rich in animal fat resulted in whole body insulin resistance and pancreatic beta-cell dysfunction in adult offspring, which was preceded by reduced tissue (aorta) mtDNA content and altered mitochondrial gene expression. The results from this study differ from the present one, as the offspring were one year old. As mtDNA damage is associated with age, the difference in age between the two studies could explain the differences observed in the findings. In another rat study high fat feeding resulted in increased Mt/DNA in the muscle and subsequent insulin resistance (Hancock et al., 2008). More specifically, it was show that findings that 5 week consumption of a high fat diet-induced increases in PGC-1α protein, a number of mitochondrial proteins and fat oxidative capacity in skeletal muscle of rats.

Intervention with PDX in the obese dams prevented increased Mt/N in their offspring. It was previously shown that TNF and CSF-1 (Chapter 5) were reduced in dams supplemented with PDX, The changes in these inflammatory mediators could explain the changes observed in offspring Mt/N at 30 days of age and as suggested precede the changes observed in later life.

Overall, in the present study maternal obesity results in increased Mt/N at 30 days of age males, which may be indicative of oxidative stress and damaged mitochondrial DNA. This observed increase in Mt/N in day 30 juvenile animals precedes
subsequent metabolic changes. In addition, supplementation with PDX in obese dams prevents mitochondrial damage, which could explain the improved glucose metabolism and the increase EE, potentially by increases in BMR.

6.5.3 UCP mRNA expression

I have examined UCP mRNA expression because of the observed differences in offspring energy expenditure during the ‘active feeding’ night-time phase. Moreover, these changes were exacerbated when the animals were challenged with the obesogenic diet in adulthood. Therefore, impaired diet-induced thermogenesis was considered a potential mechanism underlying changes in energy expenditure. However, there were no apparent changes in UCP mRNA expression at either 30 days or 3 months of age associated with maternal obesity.

A role for maternal diet in the expression of UCP mRNA and proteins has been previously suggested in animal models. In sheep, UCP1 mRNA expression was increased in offspring of dams calorie restricted before and during gestation (Budge et al., 2004). Moreover, dams consuming 125% compared to 85% of total energy requirements during gestation showed increased UCP1 protein expression, increased thermogenesis and subsequently increased offspring viability (Budge et al., 2000). The UCP2 protein expression was also increased in male offspring of rat dams fed a junk food diet and subsequently exposed to a postnatal junk food diet as well (Bayol et al., 2010).

In this study it was observed that exposure to overnutrition in utero had no effect on UCP mRNA expression. Whether mRNA expression would differ following the exposure to the obesogenic diet remains to be investigated. However, there is not an indication, based on this evidence, that differences observed in EE among the
male offspring of obese dams can be explained by differences in diet-induced thermogenesis mediated by UCP genes.

There was, interestingly, an effect of maternal supplementation with PDX on offspring UCP mRNA expression at 30 days of age. Specifically, there was an approximately 2-fold increase in UCP1 and UCP3 mRNA expression compared to controls. Similarly, in rats a maternal diet high in prebiotic fibre (inulin and oligofructose) resulted in increased expression of BAT genes involved in glucose and lipid metabolism at approximately 30 days of age (Maurer & Reimer, 2011). In a subsequent study from the same group, these offspring appeared to be protected from diet-induced obesity when exposed to a high fat diet at 8 weeks of age. The effect of a maternal high fibre diet on UCP3 has not been previously examined. However, increased UCP3 protein expression has been shown to prevent mitochondrial damage during prolonged high fat feeding. Therefore abundance in UCP3 mRNA expression and therefore increased expression of UCP3 protein may explain the reduced mitochondria copy number observed in the offspring of PDX supplemented obese dams (Hallam & Reimer, 2013).

6.6 Limitations and future studies

6.6.1 Microbiota Composition

Inter-individual genetic variability is reduced in laboratory mice, but some environmental variability is still present, which is likely to have led to the substantial variability observed between animals for some bacterial species (Thompson et al., 2010). Moreover, due to poor reproductive rate the total number of samples collected for the microbiota analysis resulted in smaller number of samples than anticipated. An increased sample number would lower the observed variability and enable better definition of changes in gut microbiota composition due to maternal
obesity. Microbiota populations are reported to be influenced by high fat/high sugar diet in humans (David et al., 2014) and mice (Daniel et al., 2014). Therefore, investigating the effects on the maternal diet on gut microflora in pregnancy, and also defining the changing gut flora in the offspring over time and before and after exposure to obesogenic dietary challenge would be of great interest, and provide valuable information on the transmission of microflora from dam to neonate.

Increased serum concentrations of short chain fatty acids (SCFAs) have been found in obese and overweight people (Schwiertz et al., 2010) compared to lean, and SCFAs have been implicated in the immune status of the colon (Smith et al., 2013) and in insulin metabolism and subsequent fat storage (Esteve et al., 2011; Shen et al., 2013). In future studies, measurement of SCFAs could be performed using gas chromatography-mass spectrometry (Garcia-Villalba et al., 2012), which is more time efficient and cost effective than the traditional gas chromatography and flame ionization detection (F. Pietro, 2002).

### 6.6.2 Mitochondrial dysfunction

In the current study increased Mt/N due to maternal obesity was observed, which was prevented by maternal supplementation with PDX. This increase is indicative of and precedes further mitochondrial dysfunction. Several investigations could be performed in the future in order to fully assess mitochondria function in this model. Firstly, mitochondrial electron transport chain enzyme activity could be measured using a spectrophotomic activity as previously described (Bruce et al., 2009). Secondly, respiration measurements could be performed in an incubation chamber using a Clarke-type $O_2$ electrode. This approach would confirm dysfunction and provide an indication as to whether reduced $O_2$ consumption in mitochondria precedes changes in offspring EE (Mourmoura et al., 2011).
Thirdly, having established that mitochondrial dysfunction precedes future metabolic impairments in the offspring, it would be interesting to examine whether mitochondria function continues to deteriorate with age. Therefore, analysis of samples at 3 and 6 months of age would help further our understanding of the role of mitochondria in developmental programming.

6.6.3 UCP expression

Based on mRNA expression it cannot be concluded that the differences in EE among male offspring of obese dams at 6 months are explained by reduced diet-induced thermogenesis mediated by UCP mRNA expression. Transcriptional changes could occur resulting in differences in the protein expression, which were not investigated in this study. Such differences have been noticed in previous studies (Budge et al., 2004; Fromme & Klingenspor, 2011). Therefore, measurement of UCP protein expression via immuno-blotting would be essential to undertake before reaching conclusions about the role of UCP.

In addition, further mRNA expression and protein expression from animals exposed to the obesogenic diet in adulthood would reveal whether there were changes in the expression of UCP in the BAT as a result of the interaction between the maternal diet and a postnatal obesogenic environment.

Moreover, administration of adrenergic receptor β-3 would reveal whether offspring of obese dams have an altered sympathetic drive for energy expenditure compared to controls, and differences in adaptive thermogenesis (Lowell & Spiegelman, 2000).
6.7 Conclusions

In this study preliminary studies are reported to examine potential mechanisms that may underlie the differences observed in the offspring shown in chapters 3 and 4. The effect of maternal obesity was, in all of the different approaches studied limited to male offspring which is in agreement with the data previously presented in this thesis.

Mitochondrial dysfunction as assessed by mitochondrial content preceded any physiological changes in the offspring of the obese dams and was prevented by maternal supplementation with PDX. This evidence suggests a causative role of mitochondrial dysfunction in the development of metabolic dysfunction later in life. Further investigation would provide us with an insight in the exact mechanisms involved in the mitochondrial dysfunction.

Maternal obesity results in increased populations of *Eubacterium rectale/ Clostridium coccoides* at 6 months, the exact role of these species requires further investigation. However, an early increase in *Bacteroides* among the offspring of dams supplemented with PDX could partly explain the reduced susceptibility of these offspring to weight gain later in life. Overall, there seemed to be an effect of maternal obesity on the composition of offspring microbiota, but its exact significance requires elucidation in the future.

Finally, a conclusion in regard to reduced energy expenditure and changes in UCP mRNA expression due to maternal obesity cannot be substantiated without further study. A protective effect of maternal supplementation with PDX towards reduced thermogenesis, though, is implied by an increase in UCP1 and UCP3 mRNA expression at 30 days of age.
Chapter 7

General Discussion
Chapter 7: General Discussion

Maternal obesity is a major public health problem, not only because it is associated with increased risk of a variety of complications of pregnancy, but also because accumulating evidence highlights its impact on the offspring susceptibility to cardio-metabolic disease.

Maternal obesity (prepregnancy BMI) and excessive gestational weight gain (GWG) constitute the most common obstetric risk factors and have direct implications for neonatal and maternal morbidity and mortality (Nelson et al.; Heslehurst et al., 2008; Poston et al., 2011a) but also increased risk of obesity in the next generation (Mingrone et al., 2008; Oken et al., 2008a; Norman & Reynolds, 2011). Contemporary epidemiology studies now suggest the ‘transgenerational acceleration’ of obesity via an as yet undefined association between maternal body mass index (BMI) in pregnancy and risk of obesity in childhood and beyond. With approximately 1 in 5 UK pregnant women now obese (Heslehurst et al., 2008; Heslehurst et al., 2010), there is now increasing acceptance that exposure to obesity in utero and in the perinatal period may beget obesity and related disorders in childhood (Drake & Reynolds, 2010; Poston, 2012; O'Reilly & Reynolds, 2013).

Mounting evidence from human cohort studies are supported by compelling evidence from animal models in rodents, sheep, and non-human primates, which clearly demonstrate a persistent influence of prenatal exposure to maternal obesity on offspring metabolic function. Animal models also have been invaluable in elucidating the mechanisms involved in the early life origins of metabolic disease associated with maternal obesity, and have provided hypotheses for direct translation to humans (Taylor & Poston, 2007).
Despite the great variety in the experimental models of maternal obesity, the adverse health outcomes observed in the offspring appear similar, suggesting some commonality of mechanism. Our rodent model of maternal obesity promotes diet-induced obesity in the dam by the consumption of a highly palatable diet high in fat and sugar, designed to mimicking the typical Western diet. The dams present hyperlipidaemia, hyperinsulinaemia and increased pro-inflammatory markers in pregnancy. In our previous studies, several adverse outcomes of maternal obesity in the offspring have been demonstrated including obesity, hyperphagia, cardiovascular dysfunction, non-alcoholic fatty liver disease and insulin resistance in mice (Samuelsson et al., 2008; Oben et al., 2010a; Oben et al., 2010b; Muralidarane et al., 2013). However, experimental evidence relating to the influence of maternal obesity on offspring energy expenditure is limited, and very few studies have addressed this aspect of the energy balance equation. Moreover, in our model, a maternal intervention targeted at the prevention of the aforementioned defects, has not been previously investigated.

During the period that obesity rates have increased, the consumption of dietary fibre has also been reported to be decreased (Ford & Frost, 2010). Polydextrose (PDX) is now widely accepted as a dietary fibre with a calorific value of 1 kcal/g, a neutral taste, low glycaemic impact and prebiotic properties. The introduction of PDX to the diet in previous studies in adult humans and animals has been proven to be beneficial as it can increase satiety; lower insulin levels and decrease cholesterol and triglycerides concentrations (Shimomura et al., 2005). Therefore, PDX supplementation in obese women (and mice) offers a potential means to improve metabolic profiling during pregnancy and to positively impact on the health of the offspring.
Therefore, the rationale of the present study originated from the need to further explore the role of maternal obesity on energy expenditure and to investigate whether an intervention with a soluble fibre may prevent potential detrimental effects.

7.1 New insights into the murine model of developmental programming of obesity

The studies presented in this thesis further expand on our knowledge of this model by presenting, for the first time, evidence of altered EE due to maternal obesity in the mouse. Specifically, at 6 months of age, male offspring of obese dams had reduced EE compared to controls. This evidence is in accordance with previous studies in rodents, however the protocols greatly differ. In a genetically modified model of GDM, offspring of GDM had decreased EE and increased RER prior to development of obesity compared to controls at approximately 4 months of age (Lau et al., 2011). In a more relevant rat study (Borengasser et al., 2011), dams were fed either overcalorific or a control liquid diet to induce obesity prior to mating. During gestation all dams were provided with the overcalorific diet. After birth the offspring were crossfostered between the lean and the obese dams. Maternal obesity before conception resulted in reduced offspring 24 EE (EC50 values of EE were shown) at approximately 30 days of age. These offspring also demonstrated markers of mitochondrial dysfunction and impaired fatty acid oxidation. Following exposure to a high fat diet the EE of the offspring of dams did not differ. The findings from this study support our findings as they show an effect of maternal of obesity on both EE and mitochondrial function. However, the possible independent effect of cross-fostering in offspring phenotype should be taken into account.
In this thesis, an effect of maternal obesity in mice is shown in the adult male EE prior to dietary challenge. To my knowledge this is the first study in mice showing a decrease of EE due to maternal-induced maternal obesity with a Western type diet. In addition, the offspring demonstrated reduced RER, together with glucose intolerance, suggesting impaired glucose oxidation. Moreover, following a three weeks obesogenic dietary challenge at 3 months of age, both male and female offspring of obese dams demonstrated reduced EE at night-time compared to controls. Females also, demonstrated increased energy intake during the obesogenic dietary challenge. These changes resulted in increased weight gain in the offspring. This evidence suggests susceptibility of the offspring of obese dams to the adverse effects of an obesogenic environment in adulthood.

The present study demonstrates for the first time in the murine model of diet-induced obesity a beneficial effect of maternal dietary intervention on energy balance. The intervention was a supplementation with a soluble prebiotic fibre, PDX (5% w/v in drinking water) that did not significantly change the composition or the calorific value of the diet. It was observed that intervention with the PDX prevented reduced EE, reduced RER, impaired glucose metabolism and prevented the development of hyperphagia and further reduction in EE that resulted in weight gain in the offspring following the exposure to obesogenic diet. Previous interventions with fibre have also proven effective, but the changes in dietary composition could have confounded these results (Lin et al., 2011; Lin et al., 2012; Hallam & Reimer, 2013). Investigation of the maternal profiles suggested a reduction of pro-inflammatory cytokines in the obese dams supplemented with PDX.

The mechanisms that have been identified to possibly contribute to the observed changes in EE and glucose metabolism, secondary to maternal obesity were:
1. mitochondrial dysfunction; Mt/N ratio in skeletal muscle was significantly increased in male OffOb at 30 days which was associated with reduced EE and insulin resistance,

2. changes in the microbiota composition, Specifically, increased percentage of *Eubacterium rectale- Clostridium coccoides* in male OffOb at 6 months

Increased mitochondrial DNA copy number and altered microbiota composition are influenced by maternal obesity and may play a causative role in the reduced energy expenditure and the development of obesity in this model.

Supplementation with PDX in obese pregnancy had a beneficial effect on offspring food intake, body weight, RER and Energy expenditure from 3 months of age. In addition:

1. Maternal PDX supplementation improved mitochondrial dysfunction, which correlates with increased EE and improved glucose tolerance.
2. PDX prevented changes in the microbiota composition, specifically, increasing populations of *Bacteroides* in male OffOb at 30 days and reducing number of *Eubacterium rectale- Clostridium coccoides* in male OffOb at 6 months, which are relevant to the prebiotic features of PDX and
3. Maternal PDX supplementation increased expression of UCP 1 and UCP 3 gene in OffOb, indicative of improved adaptive thermogenesis.

In conclusion, this study provides evidence on the changes in energy balance and specifically in EE due to exposure to maternal obesity during gestation and lactation. Moreover, a beneficial effect of a supplementation with a soluble fibre in obese pregnancy was identified. However, there are several questions that arise from this thesis and understanding these will form the target of future studies.
7.2 Understanding the effect of maternal obesity on the different components of energy expenditure

Indirect calorimetry for small laboratory animals is a valuable tool, but has certain disadvantages. The main disadvantage is that measurement of BMR is difficult; the animals ideally need to be at post-absorptive state, at complete rest and in a thermoneutral environment. The first two conditions are impossible to ensure when working with laboratory animals. Daytime EE can be used as crude measurement of BMR, as the mice are nocturnal and therefore usually rest during the light phase. However, if they are sleeping, the metabolic rate will be lower than in the awake phase, and BMR should to be measured when the animal is awake. Despite these difficulties, simultaneous measurement, should it become technically possible of the different components of EE would help us better understand which parameter is being altered.

Based on UCP mRNA expression, the reduced EE at 6 months could not be attributed to diet-induced thermogenesis. However, since protein expression was not measured, and because the measurement of UCPs is only indicative of diet-induced thermogenesis, in future experiments in addition to protein measurement by western blot, any changes in diet-induced thermogenesis need to be measured accurately. Therefore, telemetric monitoring of core temperature or measuring rectal temperature following a meal would provide confirmatory evidence of altered diet-induced thermogenesis (Levin et al., 1983; Rothwell & Stock, 1997; Ueta et al., 2012).

Moreover, even though we have indications of reduced locomotor activity (Samuelsson et al., 2008), these only provided a crude measure of physical activity. Using a more accurate method, the study by Fernandes et al, (Fernandes et al.,
2012) published in Molecular Psychiatry employing the same murine model, reported that prenatal exposure to maternal obesity leads to hyperactivity in offspring, with increased distance run and increased speed as assessed by 24 hour video recording by cameras mounted on their homecages. Hyperactivity in the offspring of the obese dams would argue against a contribution of physical activity to the observed decline in energy expenditure in these animals and instead implicates programmed changes in BMR or diet-induced thermogenesis. In future studies, the use of InfraMot™ sensors (TSE, Labmaster) incorporated into the Labmaster system would allow accurate measurement of physical activity and simultaneous recording of energy expenditure (TSE, 2014).

Despite having identified a greater reduction in EE during night-time, differences in EE were also apparent during the daytime. These results also support changes in BMR. The most important determinant of BMR after adjustment for FM is the FFM, which includes the organs. The heart, kidneys, liver, brain and resting muscles are all of high metabolic activity and despite representing only 5.5% of the total body mass, 60% of total EE can be attributed to their function (Geissler, 2005; Javed et al., 2010). Therefore, variability in the metabolic activity of the organs can influence BMR variability between individuals.

The sympathetic nervous system has been previously suggested to mediate changes in resting metabolic rate. Our previous studies in offspring of obese rats and mice have reported that hypertension (Samuelsson et al., 2010; Samuelsson et al., 2013) and non-alcoholic fatty liver disease (Oben et al., 2010a) appear to be mediated by increased sympathetic drive to the kidney and liver respectively. However, a reduction in BMR would require reduced sympathetic drive to other organs and metabolically active tissues, suggesting divergence of sympathetic drive to the different tissues perhaps due to regionally distinct programming of the central
sympathetic pathways originating in different regions of the brain. Recent work in our laboratory, employing region specific reactivation MC4, has identified divergent sympathetic responses for the regulation of blood pressure and energy expenditure in MC4R Null mice [Samuelsson et al, 2013, IUPS abstract]. Intracerebroventricular (ICV) injection of insulin in obese agouti mice revealed a variety of responses in different tissues. Multifiber recording could also be used in our model in order to measure regional sympathetic nervous system activity, as previously described (Morgan & Rahmouni, 2010). Other methods include mictoneurography (Hagbarth & Vallbo, 1968; Sundlof & Wallin, 1977) and estimation of noradrenaline spillover by isotope dilution (Esler, 1993), which have mainly been used in human studies, but could be modified for mice.

![Figure 7.1](image.png)

Figure 7.1 The role of MC4 neurons in the regulation of energy balance

Elucidating the changes in the different components of EE will help us focus in on the underlying mechanisms and subsequently design effective interventions to increase EE in offspring of obese.
7.3 The influences of maternal obesity on brown adipose tissue

Brown adipose tissue (BAT) is a highly oxidative tissue, which contains multilocular fat cells with high concentration of mitochondria that oxidize fatty acids and generate heat via UCP1 (Lee et al., 2014). BAT is heavily innervated by sympathetic nerves, and plays a major role in thermoregulatory thermogenesis in rodents (Liew et al., 2013). Adaptive thermogenesis to cold has little relevance in today’s Western society as the majority of the population live in temperature controlled environment. Changes in the calories expended due to diet-induced thermogenesis may also be relevant to the current obesity epidemic. Due to the increased consumption of Western type diet worldwide even small changes in calorific excess, due to impaired diet-induced thermogenesis, could accumulate and lead to weight gain. Whether BAT plays an important and/or direct role in energy imbalance during period of dietary changes is still ambiguous (Ma et al., 1988; Nedergaard & Cannon, 2010; Liew et al., 2013).

BAT has been shown to exhibit reduced sympathetic nerve activity in many models of obesity, including leptin-deficient ob/ob mice (Himms-Hagen, 1989). Moreover, genetic ablation of BAT in mice resulted in the development of obesity, without increases in food intake (Lowell et al., 1993). Furthermore, activation of BAT appears to be beneficial on glucose homeostasis in models of type 2 diabetes in rodents (Arch, 2002). Since the discovery of BAT in adult humans, BAT has gained great attention and is now considered a potential therapeutic target (Ravussin & Galgani, 2011).

Considering the above, further investigation on the development of BAT in the offspring of obese mice would determine whether BAT development is affected by this early life exposure. Parameters that could be examined would include:
adipocyte size distribution, adipose tissue oxygen consumption and extracellular acidification (Yehuda-Shnaidman et al., 2010). Furthermore, levels of BAT catecholamines could be measured in order to assess BAT activity (Symonds, 2013). Finally, BAT denervation could be performed in mice before exposure to an obesogenic diet.

### 7.4 The influences of maternal obesity on the offspring immune system

In the present study, an effect of maternal obesity on offspring microbiota was observed. Microbiota transferred from the mother at birth are also considered to play a role in immunologic development and colonic inflammation. Mechanistically this may occur through microbiota induced changes in abundance of SCFAs, which are thought to regulate the function and size of T regulatory cells that are critical for intestinal inflammation (Smith et al., 2013). Moreover, maternal overfeeding negatively influences the offspring immune system and subsequent susceptibility to disease; effects which could be regulated by changes in the offspring microbiota (Myles et al., 2013). Furthermore, altered immune function arising from changes in the microbiota could give rise to insulin resistance, as it is known that T-beta deficient mice (transcription factor involved in the regulation of the differentiation and function of immune cells) have increased adiposity, reduced EE, but increased insulin sensitivity (Stolarczyk et al., 2013). Moreover, an association of EE with the immune system is implied. This evidence highlights the importance of immune function and warrants further investigation in this model.

We have already shown that non alcoholic fatty liver disease in the offspring of obese dams is associated with evidence of impaired immune function in the liver (Mouralidarane et al., 2013). We have however, not addressed these changes in
relation to the gut microbiota composition. In order to assess whether maternal obesity persistently alters immune function in the offspring, inflammatory markers should be measured prior to the development of obesity in offspring. Moreover, since gut permeability has also been associated with several autoimmune diseases, including diabetes (Arrieta et al., 2006), examining the effect of maternal obesity on gut permeability would also expand our knowledge in this area. Gut permeability can be tested in vivo by housing mice fasted in metabolic cages following gavage with a sugar probe; and analysing the collected urine (Arrieta et al., 2009).

7.5 The influences of maternal obesity on offspring longevity
As mentioned in chapter 3, I initially set out to study only the effect of maternal obesity on energy expenditure. The offspring of this study were challenged, as described in chapter 4 with an obesogenic diet. During that study unexpected deaths were observed among the offspring, especially among the offspring of obese dams. After post-mortem biopsy the animals appeared to have suffered myocardial infarction. There was a significant association between premature offspring death and maternal obesity (P<0.05 Fisher’s exact test; 95% O.R. 5.03, C.I. 1.71-21.64). This evidence is in accordance with recently published data associating increased mortality (hazard ratio 1.35, 95% CI1.17 to 1.55) with maternal obesity (BMI >30)(Reynolds et al., 2013). In addition, maternal protein restriction in rats has been shown to result in decreased longevity, being associated with telomere shortening in the kidneys (Jennings et al., 1999). Future work should further address the role of maternal obesity on offspring longevity, and on telomere shortening, and examine the role of maternal supplementation with PDX in obese pregnancy on these outcomes.
7.6 Oxidative stress

Indirect evidence of oxidative stress is apparent throughout the study. PDX fibre has antioxidant properties and has been proven beneficial therapeutically in this and previous studies (Lin et al., 2011; Lin et al., 2012; Hallam & Reimer, 2013). The suggested decrease in inflammation, as shown by decreased inflammatory markers, in the obese dams supplemented with PDX could have resulted in reduced oxidative stress. Moreover, the offspring of obese mothers, supplemented with PDX had reduced mitochondrial dysfunction compared to the offspring of obese dams, indicative of reduced oxidative stress. Despite the indirect evidence, time limitations precluded assessment of markers of oxidative stress. As previously mentioned oxidative stress could be measured by quantification of lipid hydroperoxides in maternal serum, placentas and fetuses (TBARS assay kit; Cayman Chemical, USA). In addition, superoxide anion scavenging capacity and hydroxyl radical scavenging of maternal serum and the placenta could be measured, as previously described (Lin et al., 2011). In order to assess, whether oxidative stress has a persistent effect on the offspring of obese dams, measurement of oxidative stress could be taken at 30 days, 3 months and 6 months of age. Such measurement would also elucidate whether there is an exacerbating effect of a postnatal obesogenic diet in the offspring exposed to maternal obesity in utero.

7.7 Lactation, as a critical “developmental window” for intervention

In this study a maternal intervention has been introduced in our model of developmental programming in the form of PDX supplementation. The intervention was proven beneficial for the offspring by preventing metabolic defects, secondary to maternal obesity. The intervention was introduced during gestation and lactation. By examining the maternal serum profiles at the end of gestation some pilot data
was obtained to suggest mechanistic pathways, which could have contributed to the improved phenotype observed in the offspring. However, as discussed in section 1.3.2.1 the period of lactation is very important especially for rodents, since neurotrophic development of the hypothalamic neural circuits that regulates appetite and energy expenditure occurs during this critical period (Bouret et al., 2004; Taylor & Poston, 2007). Exposure to high fat diet during lactation resulted in increases susceptibility to obesity in male mice (Tsuduki et al., 2013) and impaired the development of neuronal projections between the hypothalamic nuclei involved in energy balance (Vogt et al., 2014). Therefore, it would be important to examine the period of lactation, independently by introducing PDX at the day of birth. Confounding factors associated with cross-fostering prevent this experimental approach (Matthews et al., 2011) however supplementation of PDX in the drinking water could be achieved without significant stress to the mother. This intervention would define the magnitude of the effect of intervention at each critical window in offspring development.

7.8 Application of the current findings on a clinical study

There is limited evidence for the adverse effect of maternal obesity on offspring EE in human infants (Roberts et al., 1988; Rising & Lifshitz, 2008). Small reductions in EE at 3 month old infants, were associated with risk of overweight by 1 year of age (Roberts et al., 1988). Even small reductions in EE could predispose children towards a positive energy balance. Based on this evidence, and the findings of this study, an epidemiologic correlate of this study could be designed to investigate EE in infants born to obese versus lean mothers. Resting EE in the infants could be measured in an enhanced metabolic testing activity chamber, as previously described (Rising & Lifshitz, 2008). In childhood, an indirect calorimetry metabolic monitor could be used, such the Deltatrac II (Datex, Helsinki, Finland) as previously
described (Jackson et al., 2007). There are currently two large RCTs underway to evaluate the efficacy of dietary and lifestyle interventions on pregnancy outcome in obese pregnancies; the UPBEAT study in the UK (Poston PI, NIHR programme; ISRCTN89971375) and the LIMIT trial in Adelaide, Australia (Dodd J PI; ACTRN12607000161426). Both studies will follow up the children, and offer a unique opportunity to investigate the effect of diet and lifestyle intervention on the relationship between maternal metabolic profile and the metabolic health of the child. Studies of neonatal cardiovascular parameters are currently on-going as part of the UPBEAT follow up study, and the advanced methods for EE estimation in infants described above could also be applied to the UPBEAT follow up study to examine whether the children display altered energy expenditure.

PDX has been approved by the FDA and is currently used in numerous food products including health drinks. Some beneficial effects of PDX have already been reported (Achour et al., 1994; Yoshioka et al., 1994; Shimomura et al., 2005; Beards et al., 2010). The purpose of the present study was to examine whether supplementation with PDX would be beneficial, without posing any hazard to the mother or the offspring. Even though there have been no studies specifically investigating PDX supplementation of pregnant women, both prebiotics (Wacha & Szijarto, 2011) and fibre supplementation (Qiu et al., 2008) have been previously tested in pregnancy without adverse outcomes. Initially this intervention should be repeated in precocial species, for example in the sheep, which has been extensively used as a model of maternal obesity, or in non-human primates. Providing that such studies show similar efficacy as reported here, a pilot nested study with adjusted PDX dosage could be designed in order to assess the effect of PDX in obese pregnant women and subsequently the influence of obesity in their children.
7.9 Conclusions

In this thesis evidence has been presented that diet-induced maternal obesity in the mouse results in reduced EE, reduced RER, glucose intolerance and increased bodyweight in 6 month male offspring compared to controls. Moreover, following a 3 week obesogenic dietary challenge, offspring of obese dams had reduced energy expenditure, increased calorific intake an increased weight gain compared to controls. These changes correlate with early mitochondrial damage and changes in the gut microbiota.

Maternal supplementation with a soluble fibre protected the offspring from developing all the above adverse health outcomes. These changes can be attributed to a great degree to improved maternal profile during pregnancy. From 30 days old the offspring had decreased mitochondrial function, increased expression of UCP1 and increased numbers of beneficial population of microbiota.

Further investigation into the mechanisms involved will expand and consolidate our knowledge on the developmental programming model of obesity. This will enable future translation to the human clinical setting and potentially contribute to a reduction in the transgenerational acceleration of obesity.
Appendix: List of publications arising from this thesis and relevant work

Research Papers


Abstracts

X. Maragkoudaki, L. Poston, P. D. Taylor Maternal Obesity followed by High fat/High sugar Postnatal diet programs Sexual Dimorphism in the Development of Offspring Obesity via both hyperphagia and basal metabolic rate. School of Medicine Graduate Showcase 2012, King’s College London, UK. March 2012. Poster presentation


X. Maragkoudaki, L. Poston, P. D. Taylor Maternal obesity results in lower energy expenditure in adult offspring. Joint symposium of Centre for Fetal programming and Early Nutrition Consortium, Copenhagen, March 2013. Poster Presentation-Poster Award winner

X. Maragkoudaki, L. Poston1, P. D. Taylor Maternal obesity results in lower energy expenditure in adult offspring when challenged with an obesogenic diet. The 37th Congress of the International Union of Physiological Sciences, Birmingham, UK. July 2013. Poster Presentation

X. Maragkoudaki, M. Naylor, T. South, J. Pombo L. Poston1, P. D. Taylor Maternal obesity results in impaired glucose tolerance and lower energy expenditure in adult offspring. 8th World Congress on developmental origins of health and disease, Singapore, November 2013 - Poster Presentation, Travel Award winner
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