

Metabolic alterations induced by high maternal BMI and gestational diabetes in maternal, placental and neonatal outcomes

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Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy

December 2013

Abstract

Maternal obesity and diabetes increase the risk of delivering large for gestational age infants (LGA), who have higher risk of long term obesity or metabolic syndrome [1]. As the underpinning mechanisms of how fetal growth is regulated by the placenta remain unclear [2], this thesis has investigated placental responses to high maternal BMI and gestational diabetes.

Spanish pregnant women recruited at 20 gestational weeks were classified according to pre-pregnancy BMI as control (BMI<25kg/m²; n=59), overweight (BMI=25-30kg/m²; n=29) or obese (BMI>30kg/m²; n=22), and gestational diabetes status (GDM) classified at 28 weeks. Maternal anthropometry and gestational weight gain (GWG) were measured during pregnancy. Placenta, cord blood, newborn anthropometry and infant weight were sampled or measured. Expression of genes involved in placental energy sensing pathways, folate transporters and DNA methylation was determined using real-time PCR, and placental triglyceride concentrations, lipid peroxidation and genomic DNA methylation patterns measured. Data were analysed according to their parametric distribution by Kruskal-Wallis or 1-way ANOVA.

Despite lower GWG, a greater proportion of obese women exceeded recommended weight gain [3], had higher placental weight and increased numbers of LGA infants. Maternal hyperinsulinaemia and hyperglycaemia with obesity were accompanied by unchanged placental IGFR1 and ISR1 expression, similar cord blood glucose and triglyceride concentrations. Placental mTOR was halved with obesity, whilst SIRT1 and UCP2 gene expression were 1.8 and 1.6 fold upregulated respectively with no differences in TBARS concentrations. Hyperleptinaemia in obese women resulted in unchanged placental leptin and leptin receptor expression, but higher cord blood leptin and monocyte concentrations with placental hypermethylation of genes involved in the immune response. Lower folate concentrations in obese mothers led to similar cord blood folate, and decreased placental FRα, but raised DNMT1, mRNA expression. No major differences were observed with GDM, probably due to small sample size.

In conclusion, it appears that the placenta can protect the fetus of obese women by increasing antioxidant capacity, compensating for maternal hyperglycaemia and lower folate. However, maternal obesity was associated with enhanced cord blood leptin and monocyte concentrations, increased placental weight and more LGA delivery, leaving infants at ongoing risk of increased adiposity and inflammation. Therefore, ongoing studies are currently exploring these interacting aspects.

Acknowledgements

Firstly, I would like to thank my funding organisations, Abbott Laboratories for funding the sample collection in Granada, the Nottingham Respiratory Biomedical Research Unit, the Nottingham University Hospitals Charity and the Rowett Research Institute for funding my PhD in Nottingham. I am grateful to Dr Cristina Campoy for giving me the opportunity to take part in this project and further investigations in Nottingham. My sincerest thanks go to Professor Michael Symonds and Dr Helen Budge for providing excellent supervision and direction throughout this period, enabling me to interpret my research within a professional and clinical setting. Special thanks go to Professor Harry McArdle for his advice and scientific support.

I am sincerely thankful to Sylvain Sebert for his directions during the first part of my PhD, and for his friendship and genuine dedication to science. I really appreciated the support and friendship of all the staff within the Clinical Hospital in Granada, who shared with me the Preobe experience, and the Academic Division of Child Health in Nottingham. Special thanks go to Dr Andrew Prayle for being always helpful with my statistical enquiries, and to Vicky Wilson and Mark Pope for their excellent technical support and expertise. I am truly grateful to Dr Ian Bloor, Dr Shalini Ojha, Dr Neele Dellschaft, Stuart Astbury and Dr Mathias Droescher for their support to reach the end of this thesis. I would like to thank all my friends in Nottingham, especially Dr Marta Larraona, Marta Cigna, Angeles Muñoz and Bruno Rodriguez, for their friendship and moral support both on the good and hard days.

Finally, sincere thanks go to my family for their continuous encouragement and for being always there.

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Declaration

The work in this thesis was performed at the Granada Clinical Hospital, University of Granada between 2008 and 2009 for the collection of samples and within the Academic Child Health Division, School of Clinical Sciences, University of Nottingham between November 2009 and October 2012.

This thesis illustrates my own work, completed under the supervision of Professor Michael Symonds and Dr Helen Budge in Nottingham and Dr Cristina Campoy and Professor Harry McArdle in Granada and Aberdeen respectively.

This report is an accurate representation of the work performed and no other study reproducing this work, to my knowledge, has been carried out within the the University of Nottingham or University of Granada.

Jole Martino

May 2013

Abbreviations

11 β HSD	11-beta hydroxysteroid dehydrogenase
11 β HSD1	11-beta hydroxysteroid dehydrogenase type-1
11 β HSD2	11-beta hydroxysteroid dehydrogenase type-2
5-MTHF	5-methyl tetrahydrofolate
Akt	v-akt murine thymoma viral oncogene homologue
AMPK	AMP-activated protein kinase
BHMT	betaine-homocysteine methyltransferase
BPM	basal plasma membrane
BMI	body mass index
cAMP	cyclic adenosine monophosphate
COX2	cyclooxygenase-2
CpG	cytosine-phosphate-guanine
CRP	C-reactive protein
CVD	cardiovascular disease
C β S	cystathionine beta-synthase
DMG	dimethylglycine
DMRs	differentially methylated regions
DNMT1	DNA methyltransferase-1
DNMT3 α	DNA methyltransferases-3-alpha
DNMT3 β	DNA methyltransferases -3-beta
DNMTs	DNA methyltransferases
FABP	fatty acid binding protein
FATB	fatty acid transport protein
FFA	free fatty acids
FR α	folate receptor-alpha
GC	glucocorticoids
GDM	gestational diabetes mellitus
GH	growth hormone
GLUT1	glucose transporter-1
GLUT4	glucose transporter-4
GLUTs	glucose transporters
GR	glucocorticoid receptor
GR α	glucocorticoid receptor-alpha
GWG	gestational weight gain

HAPO	Hyperglycaemia and Adverse Pregnancy Outcome
hCG	human chorionic gonadotropin
HFD	high fat diet
hGF	human growth factor
HPA	hypothalamic–pituitary-adrenal
hPGH	human placental growth hormone
hPL	human placental lactogen
HSL	hormone-sensitive lipase
Hcy	homocysteine
IGF1	insulin-like growth factor-1
IGF1R	insulin-like growth factor receptor-1
IGF2	insulin-like growth factor-2
IGF2R	insulin-like growth factor receptor-2
IGFs	insulin-like growth factors
IL6	interleukin-6
IOM	Institute of Medicine
IR	insulin receptor
IRS	insulin receptor substrate
IRS1	insulin receptor substrate-1
IRS2	insulin receptor substrate-2
IUGR	intrauterine growth restriction
JNK	c-Jun N-terminal kinase
LGA	large for gestational age
LPL	lipoprotein lipase
MAPK	mitogen-activated protein kinase
MCP1	monocyte chemotactic protein-1
MDA	malondialdehyde
MS	methionine synthase
MTHFR	5,10-methylene tetrahydrofolate reductase
mTOR	mammalian target of rapamycin
MVPM	microvillous plasma membrane
NADPH	nicotinamide adenine dinucleotide phosphate
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
P70S6K	ribosomal protein S6 Kinase
PAI1	plasminogen activator inhibitor-1
PCFT	proton-coupled high-affinity folate transporter
PI3K/PKB	phosphatidylinositol 3-kinase/protein kinase B

PPAR α	peroxisome proliferator-activated receptor-alpha
PPAR γ	peroxysome-proliferator activated receptor-gamma
RFC	reduced folate carrier
ROS	reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SGA	small for gestational age
SIRT1	sirtuin-1
SNAT2	sodium-dependent neutral amino acid transporter 2
SOCS	suppressor of cytokine signalling
T1DM	type 1 diabetes mellitus
T2DM	type 2 Diabetes Mellitus
TAE	tris-acetate-EDTA
TBARS	thiobarbituric acid reactive substances
TG	triglycerides
THF	tetrahydrofolate
TLR4	toll-like receptor-4
TNF α	tumour necrosis factor-alpha
UCP2	uncoupling protein-2
WHO	World Health Organization

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Chapter 1 - Introduction

1.1. General study overview

This thesis investigates the influence of differences in maternal body mass index (BMI) and glucose tolerance during pregnancy on specific aspects of placental function relating to energy metabolism. In the first study, the metabolic status of pregnant women with a high BMI, gestational diabetes or with both conditions was assessed through analysis of maternal dietary intake, weight gain and physiological characteristics during pregnancy. The relative influences of these maternal conditions on placental, fetal and newborn growth were further examined by analysing anthropometry of the placenta, fetus and infant during the first year of postnatal age, and fetal physiological characteristics at the time of delivery as reflected in cord blood. In the second study, the crucial role of the placenta for maternal-fetal exchange of nutrients and hormones was assessed by analysing placental gene expression of some of the genes involved in key molecular pathways, including placental insulin signalling, oxidative stress and inflammation.

Finally, the third study investigated folate status in the mother, placenta and fetus through analysis of maternal and cord blood concentrations of folate, and placental gene expression of key folate transporters. Placental DNA methylation was also assessed in order to further elucidate the contribution of maternal high BMI and/or gestational diabetes on epigenetic modifications within the placenta and potential critical pathways.

In this Chapter, current knowledge on the relative and combined contributions of obesity and diabetes in pregnancy to maternal, placental and fetal physiopathological adaptations will be summarised and potential metabolic pathways and molecular mechanisms within the placenta will be highlighted.

1.2. Early life programming

In recent decades, the rapid increase in metabolic disorders worldwide has centered attention towards potential critical factors in early stages of development [4, 5]. Since the “Thrifty Phenotype” hypothesis by Hales and Barker was published in 1991 [6], new insights on metabolic adaptations in a poor nutritional fetal environment started to be investigated. Such adaptations include both an enhanced capacity to store fat through alteration in insulin sensitivity and an adaptive response to optimize the growth of key organs at a detriment to growth of less vital organs. The hypothesis suggested that these adaptations are strategies for survival in similarly poor nutritional post-natal environments; however, when exposed to over-nutrition, maternal nutritional restricted offspring become more at risk to develop metabolic disorders like obesity and type 2 diabetes mellitus (T2DM). Epidemiological and animal studies following Barker’s theory have confirmed a link between birth weight and increased risk of metabolic diseases later in life [7, 8], highlighting the crucial role of the in utero environment. The phenotype of an individual is determined both in utero (maternal genetics and nutritional state) and from early postnatal environmental factors (lifestyle choices and habits) [9] (Figure 1.1). Poor nutrition in utero followed by normal postnatal growth is a risk factor for the increased incidence of cardiovascular disease (CVD) [10], glucose intolerance and T2DM [6].

In the last twenty years, the dramatic surge in obesity and maternal obesity have opened intriguing new insights focused on the potential detrimental effect of these conditions on maternal and fetal health. Current evidence links obesity and diabetes in pregnancy to the onset of the metabolic syndrome, including obesity and diabetes, in the offspring [11-13]. However, the number of studies investigating pregnancy outcomes among obese women is still limited and needs further investigation.

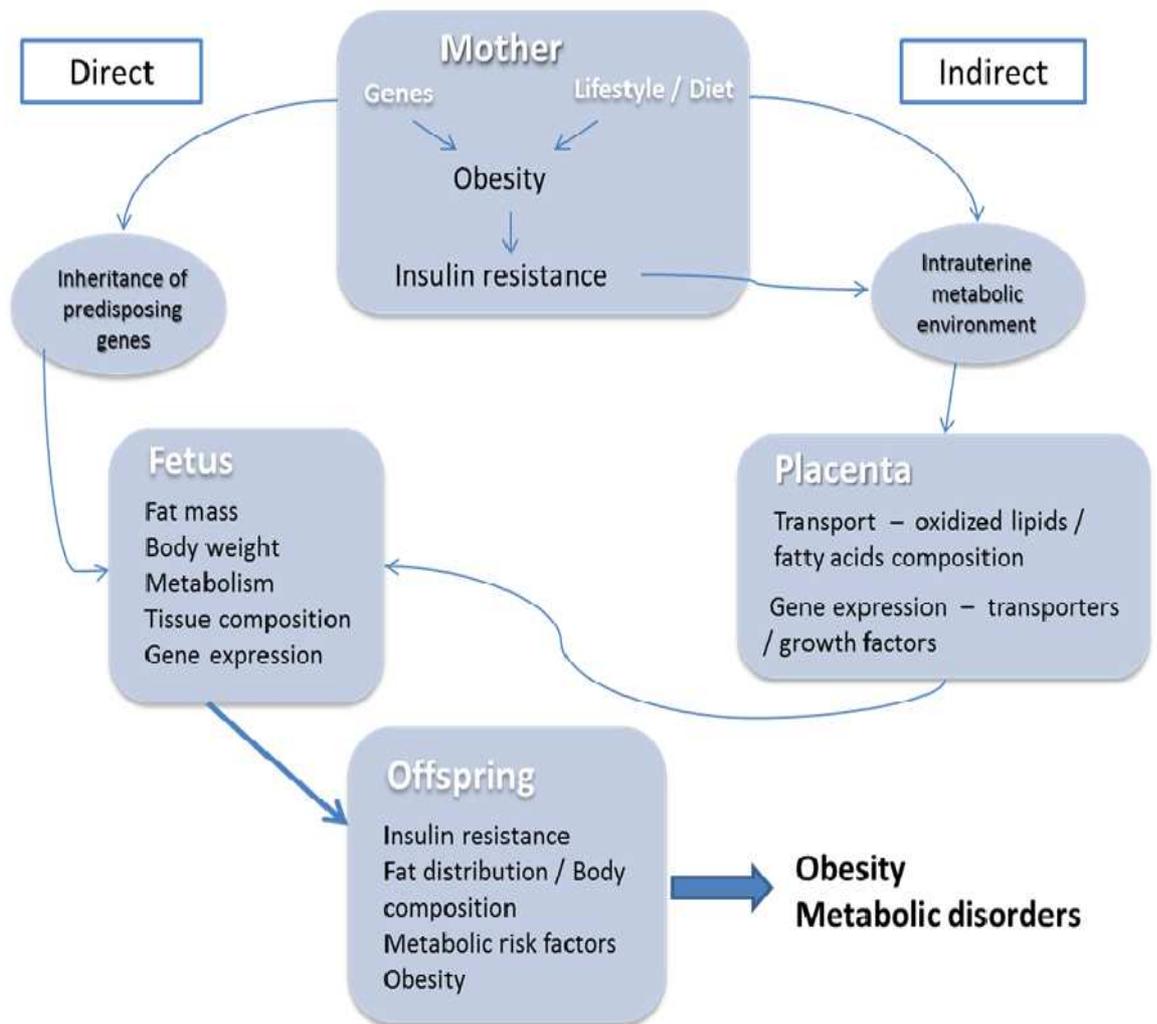


Figure 1.1: Programming of offspring obesity.

Fetal body composition, metabolism and gene expression can be directly or indirectly influenced by the mother to develop patterns resulting in future obesity or other metabolic disorders. The placenta can respond to the in utero metabolic environment by altering transport processes, gene expression and metabolism. Adapted from [14].

1.3. The obesity pandemic

The past twenty years have seen a steep increase in being overweight and obesity worldwide along with associated health risks. In 2005 the World Health Organization (WHO) declared obesity as a “worldwide pandemic” and estimated more than 1.4 billion adults as overweight with a body mass index (BMI) range between 25 - 29.9 kg/m² [15, 16]. Amongst these, over 200 million men and nearly 300 million women were clinically obese (BMI ≥ 30kg/m²). The BMI represents an efficient and widely used measure to estimate adiposity and increased health risks related to excess body fat [17] and it is defined as:

$$BMI = \text{Body weight (kg)} / \text{Height}^2 \text{ (m}^2\text{)}$$

As recently discussed in the Lancet, changes in the global food system, including reduction in time spent preparing food, excess food intake, dietary imbalance, low physical activity, are the major drivers of this staggering rise in obesity. Also, substantial differences in socio-cultural and economic environment contribute to a wide variation in the incidence of obesity across populations [15]. Global interventions have been undertaken although our genetics is emerging to be critical in the present obesity epidemic [18]. The continuous growth of obesity within the next two decades estimate 65 million more obese adults in the USA and 11 million more obese adults in the UK by 2030 [19].

This will probably get worse as there is a parallel rise in childhood obesity as reported by the European Environment and Health Information system (ENHIS) in 2009 [20]. Greater body weight in infancy and childhood significantly enhances the risk of becoming obese as an adult [21] with up to 70% of obese adolescents remaining obese as adults [22]. The consequent increase of being overweight and obesity among pregnant women is also of particular concern due to its additional risks for both the mother and newborn [23, 24].

The potential transgenerational transmission of obesity highlights the urgency to further understand the impact of high BMI in pregnancy in order to improve public health interventions.

1.4. Obesity in pregnancy

The National Health and Nutrition Examination Survey (NHANES) reported that in the USA, the incidence of obesity in women between 20 and 39 years of age tripled between 1960 and 2000 [25]. More recently in the UK, the mean BMI of pregnant women at their first prenatal booking increased by 1.37 kg/m² in 2004 compared to 1990 [26]. Obesity among American pregnant women ranges from 18.5% to 38.3%, depending on the study-cohort and cut-off point used [27]. A review from Guelinckx et al. summarised the prevalence of prepregnancy overweight and obesity among pregnant women from different countries [28, 29], showing that the incidence of obesity ranges from 1.8% (China) to 25.3% (USA, New York), using WHO criteria (Figure 1.2).

Maternal obesity may be related to the onset of metabolic syndrome in the offspring through the association between maternal BMI with offspring adiposity [12, 23, 30]. Obese gravidae experienced higher incidence of obstetric complications, miscarriage [24], stillbirth as well as neonatal death [31]. Furthermore, common complications seen in obese mothers include gestational diabetes mellitus (GDM) and preeclampsia [32], conditions associated with different birth outcomes. A large proportion of studies link high pre-pregnancy BMI with GDM and fetal macrosomia, which results in large for gestational age (LGA) offspring [33, 34]. Those neonates are at increased risk of obesity and insulin resistance when they become adults [35]. On the other hand, a greater proportion of obese women develop preeclampsia and give birth to small for gestational age (SGA) offspring [36, 37]. Whether these neonatal outcomes are caused by increased [38] or reduced energy intake during pregnancy [39], is still under investigation [40].

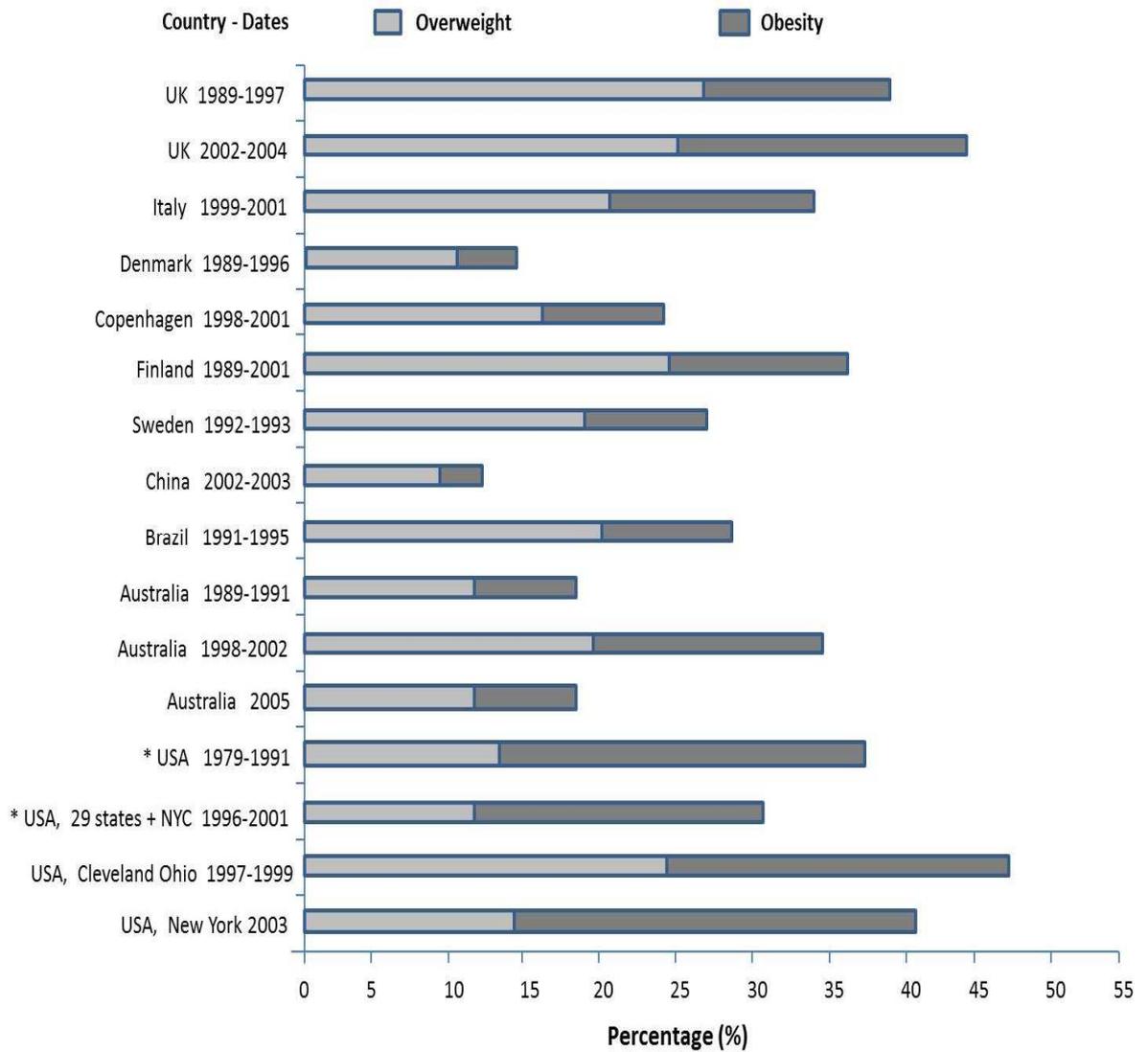


Figure 1.2: Prevalence (%) of overweight and obesity in pregnant women.

Overweight and obesity in pregnant women using data from the World Health Organization (WHO) and the *Institute of Medicine (IOM). Adapted from [27].

Overall, it is clear that the underlying cause of maternal obesity is multifactorial, taking into account caloric intake, physical activity and potential genetic components. However, the exact contribution of each factor to offspring health is still unclear and will be examined in more detail in the following sections.

1.4.1. Maternal diet and nutrient intake

Maternal diet during pregnancy may influence outcomes of pregnancy and childhood. Whilst diet in the first trimester may be more important for organs' development and differentiation, diet later in pregnancy may be important for overall fetal growth and brain development [41]. Beneficial changes in diet, if continued after pregnancy, may have long term benefits for women's health. In 2010 the NICE published public health guidelines on dietary and physical activity interventions for weight management before, during and after pregnancy [42]. However, data on determinants of dietary quality during pregnancy are scarce. Recent studies emphasize the importance of specific aspects of a healthy maternal dietary pattern, such as the association of folic acid intake in a Mediterranean diet compared with that in a Western diet and spina bifida [43], and the association of high calcium intake with greater bone mass in the offspring at the age of 9 years [41]. In obese pregnancies, inadequate intake of vitamin D, calcium, folate and iron can partly explain the occurrence of complications such as neural tube defects [41, 44].

Overweight and obesity have been related to diet of poor quality, unbalanced [45], rich in fat and low in dietary fibre and essential micronutrients [41, 46]. This is, in turn, associated with increased maternal weight gain with important implications for long term maternal [47] and offspring obesity [48, 49]. Both animal [46] and epidemiological studies [50] have shown that prolonged reduction in maternal food intake or consuming an imbalanced diet at critical stages of development compromise offspring's health. Given the adverse consequences of obesity, these women might be encouraged to diet during pregnancy with the aim to reduce or minimise the negative outcomes [39]. One of the major factors that influence nutrient intake, dietary restriction and its health awareness is socioeconomic status [22]. Women consuming poorly balanced diets, with high sugar and fat content and inadequate micronutrient status, are usually younger, have lower educational attainment and have higher pre-pregnancy BMI [41].

Women who diet in pregnancy are characterised as having a low annual income, pre-existing T2DM and/or mental stress and current smoking [51]. Successful intervention strategies are hence directed to those mothers of lower socioeconomic status at risk of poor diet, obesity or hyperglycaemia [52, 53]. Nutritional interventions have indicated that a health conscious diet leads to less total energy intake, reflected by less dietary fat and more fruit [54]. Conversely, a 'Western' diet, based on red and processed meat with high fat products, increases the risk of having a SGA infant [54].

In addition, the type and amount of dietary carbohydrate also have highly significant effects on fetal growth and size at birth. Women consuming a low glycaemic index diet were found to have reduced levels of triglycerides (TG), total cholesterol and C-reactive protein (CRP) and a lower cardiovascular risk [55]. As reviewed by Clapp these women deliver normal sized infants within the 25th and 75th body weight centiles whereas a high glycaemic diet during pregnancy is associated with overgrown infants [56]. A large epidemiological study confirmed these findings by directly linking glycaemic index of the diet, plasma glucose, fetal growth and birth weight [57].

As such, the need to optimise dietary intake as well as lifestyle habits before, during and after pregnancy is becoming essential, especially in pregnant women with high BMI.

1.4.2. Maternal weight gain

The consumption of an unhealthy diet in pregnancy is often associated with increased gestational weight gain (GWG) with adverse consequences for mother, fetus and the newborn [47, 58]. Longitudinal studies link maternal weight gain in pregnancy with offspring birth weight and their BMI later in life [59], although some others have not observed differences [60].

In 1990 the Institute of Medicine (IOM) recommended target weight gain ranges for pregnant women and, recently, acknowledged the need to consider additional factors for each individual, including the severity of obesity [61].

Therefore, in 2009, new IOM guidelines were published based on the WHO cut-off point for the BMI categories [61], in which a specific narrow range of recommended weight gain for obese women was also included (Table 1.1). However, the NICE has recently reported that there are no evidence-based UK guidelines on recommended GWG [42] as the amount of weight a woman may gain in pregnancy can vary a great deal with only some of being due to increased body fat.

Table 1.1: Recommended gestational weight gain.

World Health Organization (WHO) and Institute of Medicine (IOM) classifications

Pre-pregnancy BMI categories	BMI (kg/m ²)	Total weight gain		Rates of weight gain 2 nd and 3 rd trimester	
		Range in kg	Range in lbs	Range in kg/week	Range in lbs/week
Underweight	< 18.5	12.5 - 18	28 - 40	0.44 - 0.58	1 - 1.3
Normal weight	18.5 - 24.9	11.5 - 16	25 - 35	0.35 - 0.50	0.8 - 1
Overweight	25.0 - 29.9	7 - 11.5	15 - 25	0.23 - 0.33	0.5 - 0.7
Obesity	≥ 30.0	5 - 9	11 - 20	0.17 - 0.27	0.4 - 0.6

Weight gain during pregnancy according to pre-pregnancy BMI categories as recommended by the World Health Organization (WHO) and Institute of Medicine (IOM) in 2009. Calculations assume a 0.5–2 kg (1.1–4.4 lbs) weight gain in the first trimester (based on [61]).

Exceeding the recommended GWG [61] has been associated with greater adiposity and metabolic traits in children as well as greater postpartum weight retention in the mother [59]. In fact, there is strong evidence linking high GWG with subsequent maternal overweight and long term obesity [47, 58]. In addition, increases in BMI before and between pregnancies have been highly associated with enhanced risk of adverse pregnancy outcomes [62, 63] and childhood obesity [64]. Despite greater GWG being associated with high maternal BMI [48] and the development of GDM [65], it is also frequent among mothers with a lean BMI [66].

Prevention of excessive weight gain before, and between pregnancies in women with a healthy BMI is therefore recommended [3].

Finally, current evidence pointed out that the prevalence of excessive gestational weight gain declined, and weight loss increased, as obesity became more severe [32]. The resulting offspring have enhanced risk of being SGA with long term adverse outcome [67, 68]. As such, to avoid such consequences, overweight and obese women are recommended to diet before planning a pregnancy rather than during the gestational period [42].

1.5. Gestational diabetes mellitus

The increasing prevalence of obesity among women of reproductive age is contributing to the development of GDM [69]. There are different types of diabetes mellitus, depending either on beta-cell failure to produce insulin (Type 1 diabetes mellitus (T1DM)) or on a chronic defect in their function, ultimately resulting in beta-cell resistance to insulin's activity (T2DM) [70]. GDM, by definition, occurs only in pregnancy, usually in the second half [71], and shares pathophysiologic similarity with T2DM [72]. This condition is characterised by greater reduction in insulin sensitivity and insufficient insulin production to meet pregnancy demands [33]. The increased insulin resistance of GDM women, together with decreased pancreatic beta-cell reserve, triggers impaired glucose tolerance, ultimately resulting in a state of hyperglycaemia and hyperinsulinaemia in the maternal circulation [73].

The diagnosis of GDM worldwide is made by using 75 g glucose load. However, different countries have recommended various glucose loads (50, 75 or 100 g) in non pregnant individuals [74]. It is controversial whether maternal hyperglycemia less severe than that required for a diagnosis of diabetes is associated with increased risks of adverse pregnancy outcomes.

As such, based on the results of the "Hyperglycaemia and Adverse Pregnancy Outcome" (HAPO) study [75], an international group of experts has developed recommendations for diagnostic criteria for GDM.

Essentially, pregnant women at high risk of GDM are screened by taking a glucose challenge test (GCT) between 16 and 20 weeks of gestation and a timely oral glucose tolerance test (OGTT) (usually within a week) if the plasma glucose concentration is above 7.5 mmol/l. An early negative or positive GCT followed by a negative OGTT results in a second screening test, which is undertaken in the later period of pregnancy (26-30 gestational weeks) [76].

Clinical factors reported to predict the development of T2DM in women with a history of GDM include high pre-pregnancy BMI as well as elevated fasting glucose and hyperglycaemia in pregnancy [74]. It is currently estimated that about half of women of childbearing age are either overweight or obese [42], and, in England, 15.6% of women at the start of pregnancy are obese [23]. On the other hand, 10-25% of pregnant women develop GDM [77, 78], among which 65-75% are overweight or obese [75, 79], making it difficult to tease apart the relative contribution of maternal BMI and glycaemia on pregnancy outcomes. Similar to obese pregnant women, those with GDM are at increased risk for perinatal morbidity as well as impaired glucose tolerance and T2DM in the years post-pregnancy [72, 80]. In addition, their children have an increased risk of developing obesity, insulin resistance and hypertension in childhood and early adulthood [70, 81]. Therefore, considerable attention has been focused on the pathological effects of obesity and how they may be related to insulin resistance, beta-cell dysfunction, and the clinical development of GDM and T2DM. The emerging risk factors linking maternal obesity with GDM will be discussed in the following section.

1.6. Metabolic alterations with obesity and diabetes

Glucose and macronutrient intake have been associated with oxidative stress and inflammatory changes through an increase in fat mass [82]. Chronic overnutrition, high adiposity and hyperlipidaemia are common features of obesity and represent a pro-inflammatory state associated with oxidative stress [83]. Pregnancies complicated by obesity are characterised by a greater degree of insulin resistance, hyperlipidaemia, exaggerated inflammatory response and poor microvascular function [22].

A potential role of visceral fat [84] in contributing to increased lipid response in obese individuals with greater degree of oxidative stress has been highlighted [31, 83]. The resulting lipotoxicity seems to be responsible for the observed endothelial abnormality of obese women and can influence gene expression by acting as ligands for nuclear receptors. Oxidised lipids have been shown to induce plasminogen activator inhibitor-1 (PAI1) expression and reduce insulin-mediated vasodilatation [85]. Increased levels of plasma cytokines induced by oxysterols reflect the tight correlation between obesity, oxidative stress and inflammation [86]. This has been confirmed by laser Doppler imaging showing impaired endothelial function, dyslipidaemia and inflammatory up-regulation in obese pregnant women [83].

Furthermore, enhanced circulating levels of CRP, tumour necrosis factor- α (TNF α), interleukin-6 (IL6) and leptin are reported in obese women as a result of higher visceral adiposity [87, 88].

These generate an acute inflammatory response among obese subjects leading to the recruitment of macrophages. Increased monocyte chemoattractant protein-1 (MCP1) in adipose tissue [89, 90] as well as in the placenta [91-93] contribute to the chronic inflammatory status of obesity. Similarly, as a result of GDM, an increase in a wide range of cytokines, growth factors and hormones is observed in the maternal circulation of GDM women.

Paradisi et al. provided evidence that the endothelial dysfunction found in diabetic pregnant women is strongly correlated with elevated plasma glucose independent of the degree of adiposity [94]. Women who develop diabetes show higher incidence of obesity, abnormal insulin secretory function and insulin resistance [34]. The chronic inflammation and adipokine dysregulation caused by obesity is related to insulin resistance and beta-cell dysfunction contributing to the development of T2DM [95] (Figure 1.3).

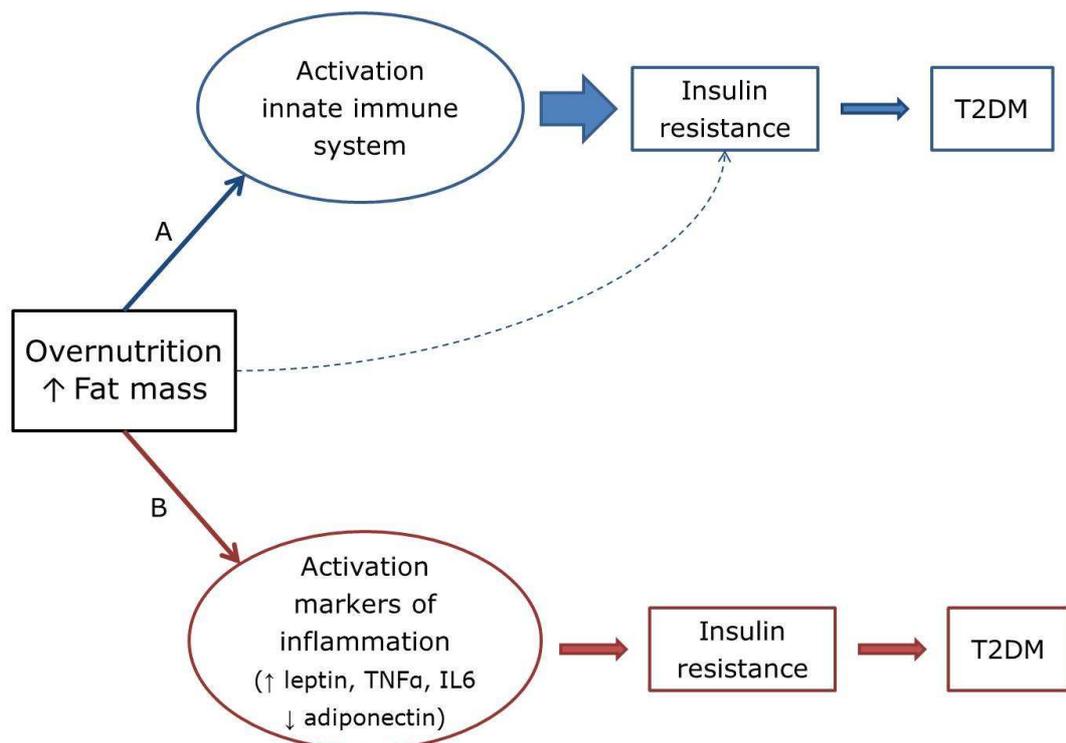


Figure 1.3: Overnutrition and obesity activate the immune system.

(A) Overnutrition and obesity cause activation of the immune system, which in turn promotes insulin resistance, ultimately, leading to type 2 diabetes mellitus (T2DM).

(B) Inflammatory markers resulting from obesity can be indirectly associated with the development of T2DM. TNF α : tumor necrosis factor-alpha; IL6: interleukin-6.

Obesity, T2DM and insulin resistance are similarly associated with raised concentrations of non-specific markers of the immune system including total gamma-globulin concentration, white blood cell count, and CRP. Excess of free fatty acids (FFA), reactive oxygen species (ROS) and pro-inflammatory cytokines activate c-Jun N-terminal kinase (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) signalling [96, 97].

Accordingly, it seems that inflammation critically links obesity, insulin resistance and obesity-related diseases through modulation of insulin signalling. However, the mechanisms underlying the inflammatory state of obesity and T2DM are still under investigation.

As will be discussed in section 1.7, post-receptor defects in the insulin signalling cascade seem to be responsible for this condition [98].

1.7. Insulin signalling in obese and GDM pregnancies

Obesity is a common antecedent of GDM and many of the biochemical mediators of insulin resistance associated with obesity have been identified in small studies of women with GDM or a history of diabetes [99, 100]. These mediators include increased circulating leptin [101] and the inflammatory markers TNF α [102] and CRP [103]. In addition, decreased levels of adiponectin [104] and increased fat in liver and muscle [105] are other markers of insulin resistance. In vitro studies of adipose tissue and skeletal muscle from women with GDM reveal abnormalities in the insulin signalling pathway as a result of altered levels of cytokines and hormones [73, 106]. These aberrations in insulin signalling are similar to the ones reported with maternal obesity and cause changes in the expression levels of its components resulting in reduced insulin action. TNF α impairs insulin receptor (IR) signalling by inhibition of IR substrate-1 (IRS1) activity whereas hyperleptinaemia leads to insulin resistance by activation of suppressor of cytokine signalling (SOCS) proteins. These ultimately reduce leptin and IR signalling [107]. Abnormal subcellular localization of glucose transporter 4 (GLUT4) [108] and decreased expression of peroxisome-proliferator activated receptor-gamma (PPAR γ) [109] are also detected in GDM pregnancies and could contribute to the observed reductions in insulin-mediated glucose transport.

Insulin activates two main signalling pathways: the phosphatidylinositol 3-kinase/protein kinase B (PI3K/PKB) and the mitogen-activated protein kinase (MAPK) pathway [110] (Figure 1.4). The PI3K/PKB pathway induces the metabolic effect of insulin resulting in translocation of GLUT4 together with glycogen and protein synthesis. On the other hand, the MAPK pathway stimulates proliferation, migration and angiogenesis.

Experiments using knockout mice and isolated cells in vitro suggest that MAPK signalling is mainly activated via IRS1 whereas IR substrate 2 (IRS2) plays a key role in the regulation of carbohydrate metabolism [111]. Therefore, expression changes of any insulin signalling component may affect IR signal transduction and ultimately, insulin's effects.

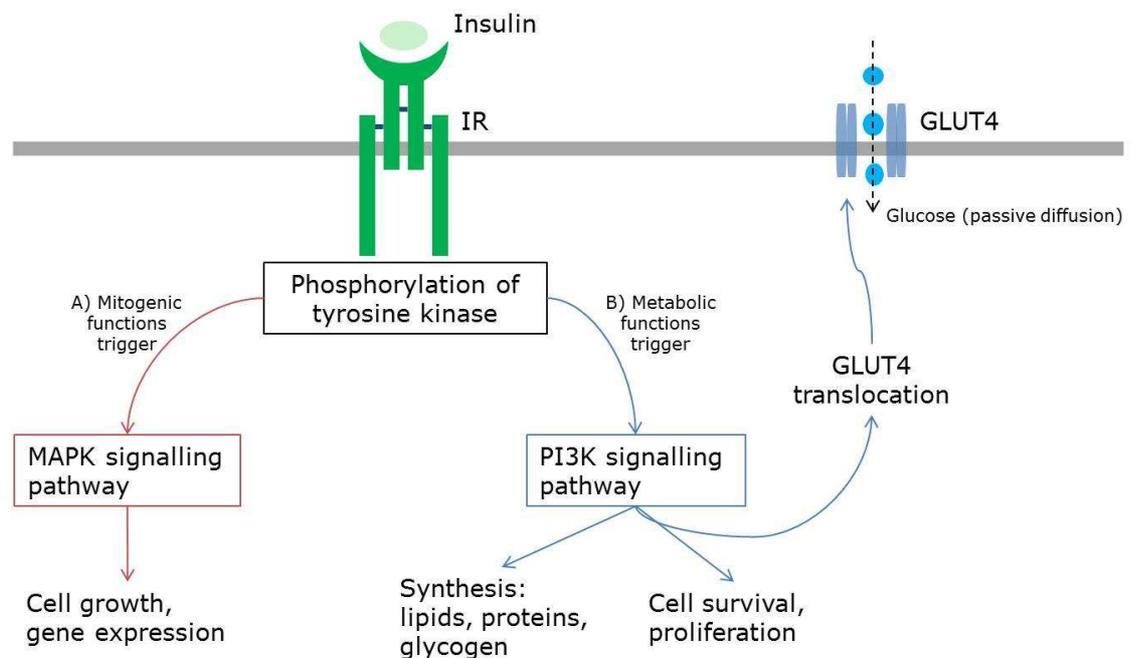


Figure 1.4: Insulin signalling pathways.

Insulin binds to its receptor (IR), triggering the tyrosine kinase activity which causes activation of the enzyme mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/PKB).

A) Activation of MAPK leads to completion of mitogenic functions like cell growth and gene expression. B) Activation of PI-3K leads to crucial metabolic functions (synthesis of lipids, proteins and glycogen) and cell survival and proliferation. Most importantly, the PI3K pathway is responsible for passive diffusion of glucose in cell through translocation of GLUT4.

1.8. Regulation of human fetal growth

The intrauterine environment in which the fetus develops represents a major physiological, hormonal and environmental challenge for its survival and long term health. Embryonic nourishment prior to implantation is initially histiotrophic, with the trophoblast phagocytosing first oviductal and then uterine secretions. Following implantation and establishment of the chorioallantoic placenta, the nourishment of the fetus is provided by exchange between the maternal and fetal circulation (haemotrophic nutrition) [112]. The assumption that in humans the transition to haemotrophic nutrition occurs soon after implantation has been recently questioned. In fact, there is now evidence that the maternal placental circulation is not fully established until the end of the first trimester, with the uterine glands releasing secretions into the intervillous space until at least 8 weeks of pregnancy, which are then taken up by the syncytiotrophoblast [113, 114]. Histiotrophic nutrition provides nutrients under a low oxygen concentration, so reducing the risk of damage from free radical during the sensitive period of organogenesis. Once this is complete, and fetal oxygen requirements rise, transition to haemotrophic nutrition at the start of the second trimester follows, with the complete establishment of the maternal placental circulation.

Thus, multidirectional interactions between mother, placenta and fetus during development are critical to regulate and ensure normal fetal growth. Key gestational adjustments involving circulatory and metabolic changes are necessary to provide adequate nutrition and oxygen to the fetus, which, on the other hand, influences maternal nutrient supply and oxygen via the placenta. This crucial organ represents the maternal fetal exchange barrier and regulates fetal growth through the production and metabolism of growth-regulating hormones such as insulin-like growth factors (IGFs), leptin and glucocorticoids (GC) [115-117]. In addition, the placenta will respond to fetal

endocrine signals via changes in its morphology, activation of transport systems and production of its own hormones.

As such, the placenta not only promotes fetal growth, but also influences maternal physiology and even behaviour. Similarly, maternal hormones such as insulin, leptin, and insulin-like growth factor-1 (IGF1) regulate placental nutrient transport and the maternal side of the placenta expresses numerous hormone receptors, including receptors for insulin [118], IGF1 [119], and leptin [120]. This is consistent with regulation of trophoblast function by maternal hormones.

1.8.1. Maternal metabolic changes in pregnancy

During pregnancy, the mother modifies her metabolism in order to meet the nutritional needs of the developing fetus. The first trimester is an anabolic state characterised by increased maternal body weight through hyperphagia and storage of nutrients in adipose tissue (Figure 1.5). This is facilitated by increasing insulin sensitivity of adipose tissue which promotes de novo lipogenesis [121]. Lean women increase their fat stores more than obese women per kilogram body weight, which is likely be mediated by their higher insulin sensitivity in early pregnancy [122].

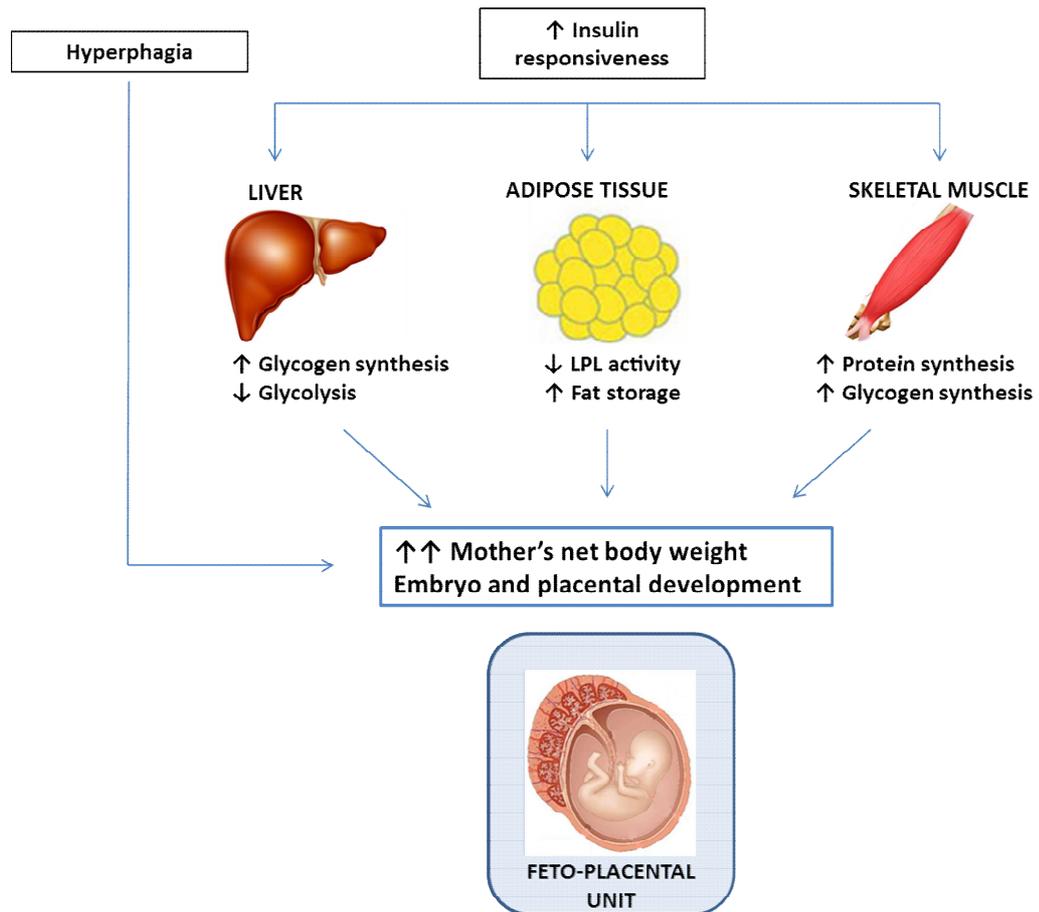


Figure 1.5: Anabolic maternal metabolism in the 1st trimester of pregnancy.

The mother stores lipids, carbohydrates and proteins as a result of the enhanced insulin sensitivity and hyperphagia.

From mid- to late gestation, maternal lipid metabolism switches to a catabolic state as a result of elevated fetal nutrient demand (Figure 1.6). In this stage, maternal insulin sensitivity significantly decreases allowing the mother to provide large amounts of nutrients to the fetal placental unit via diffusion or active transport across the placenta. Adipose tissue lipolytic activity increases under the hormonal control of progesterone, cortisol, prolactin and leptin [123]. Also, high circulating oestrogens cause a decrease in extra-hepatic lipoprotein lipase (LPL) activity [124] while the intestinal absorption of dietary fats increases [125].

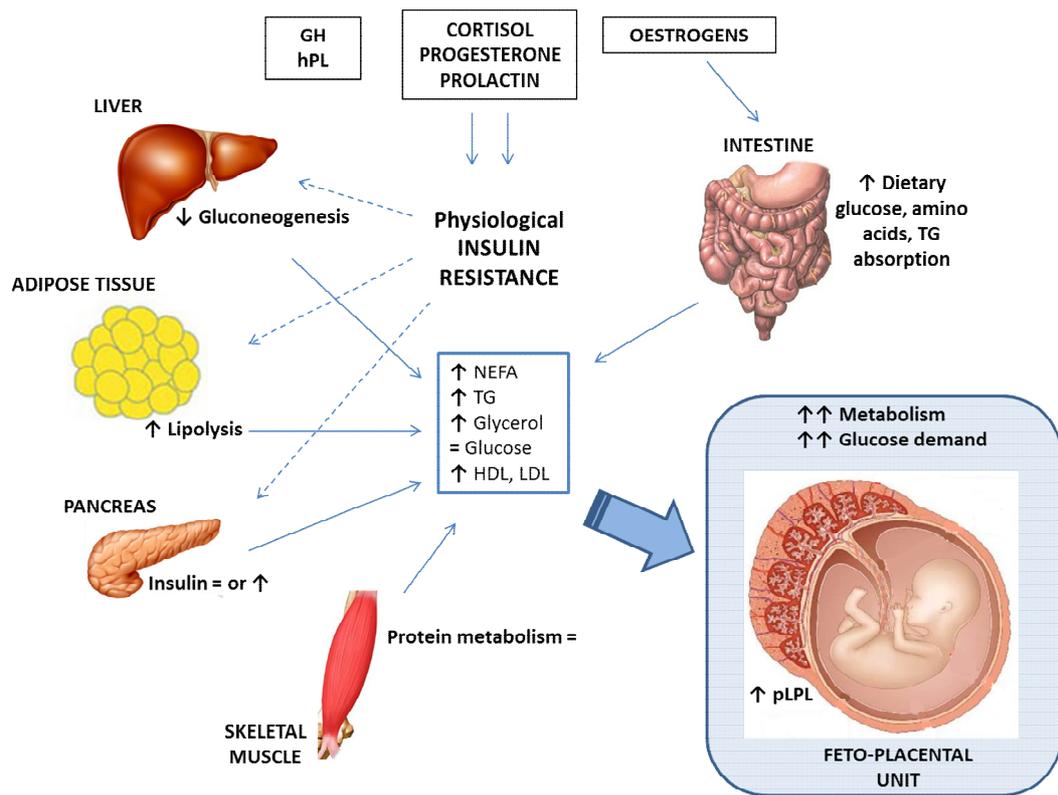


Figure 1.6: Catabolic maternal metabolism in the 3rd trimester of pregnancy.

Catabolic maternal metabolism in the 3rd trimester of pregnancy provides availability of nutrients to sustain fetal growth. GH: Growth hormone; hPL: Human placental lactogen; pLPL: placental lipoprotein lipase; NEFA: Non-esterified fatty acids; TG: Triglycerides; HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

All these events result in elevations of FFA and glycerol plasma concentrations taken up by the liver. Hyperlipidaemia occurs during this phase, representing the increased levels of lipolytic products in the circulation combined with enhanced TG production by the liver. TG do not cross the placenta but are used to release FFA, which are transported through the placenta to provide a source of energy for the fetus. During the last week of gestation, mainly under fasting conditions, FFA and glycerol released from the adipose tissue are also taken up by the liver and undergo beta-oxidation to form ketones [126]. These substrates cross the placenta and provide fuel for the fetus, and can, otherwise, be used by maternal tissues. During the first trimester, protein synthesis remains similar to that of non-pregnant women, whereas in the second and third trimesters this increases by 15% and 25%, respectively [127].

Overall, throughout gestation, protein synthesis in maternal tissues increases whereas a slightly reduction (about 10%) in amino acids oxidation occurs.

Pregnancy is a state of insulin resistance, characterised by a 40-80% decrease in insulin sensitivity throughout gestation [128]. Whilst a majority of women maintain normal glucose tolerance, approximately 10-25% develop GDM [42]. As previously summarised, insulin resistance in GDM cannot be sufficiently compensated by maternal insulin production resulting in hyperglycaemia and hyperinsulinaemia [129]. This significant reduction of insulin sensitivity is also observed in obese women with normal glucose tolerance and GDM during late gestation [130].

All three major insulin target tissues (liver, skeletal muscle and adipose tissue) develop insulin resistance during normal pregnancy and to an even greater extent when associated with obesity and GDM. Insulin stimulated glucose transport in skeletal muscle decreases considerably during pregnancy, becoming worse in GDM subjects [131]. Similar results occur in isolated adipocytes where a more severe decrease in glucose transport occurs in obese women with GDM [132]. Additionally, obese subjects with or without GDM show decreased suppression of hepatic glucose production during insulin infusion with advancing gestation [130, 133].

As discussed in Section 1.8.6, various placental hormones can reprogramme maternal physiology to meet fetal needs. However the cellular mechanisms for this complex transition are still unclear [134].

1.8.2. The placenta: the programming agent

The placenta constitutes the active interface between the maternal and fetal circulation, regulating maternal physiological changes and fetal homeostasis. In fact, this vital organ provides a wide range of functions, including immune-protection and supply of nutrients and oxygen to the growing fetus [135], and receives and transmits endocrine signals between the mother and the fetus [136].

The slowing in placental growth at 35-36 gestational weeks reflects the major structural changes in this organ allowing a considerable increase in its function. The rise in maternal-placental blood flow throughout pregnancy is reflected by a 40 fold increase in the fetal/placental weight ratio from 6 weeks to term [137]. This higher efficiency results from vascular adaptations during development, which ensure the transport of nutrients across the placenta. Any perturbations in the maternal compartment influence placental function and trigger adaptive responses in the growing fetus.

Placental insufficiency, which reflects a reduced ability of the placenta to exchange nutrients and waste products between mother and fetus, is the main cause of IUGR in the absence of maternal undernutrition or fetal genetic anomaly [138]. For many years placental insufficiency has been associated exclusively with reduced blood flow through the uterine and/or fetoplacental circulations [139, 140] as a result of abnormal vascular development [141] or dysregulation of uterine and/or fetoplacental vessels [114]. However, recent evidence pointed out that placental insufficiency can additionally involve physical abnormalities, including decreased surface area and increased thickness of the exchange barrier, and molecular abnormalities [138]. Among these, reduced activity of key plasma membrane nutrient transporters in the syncytiotrophoblast, such as glucose and System A amino acid transporter have been associated to IUGR [142, 143].

In diabetes, the placenta undergoes a variety of structural and functional changes, which depend on a range of variables. Significant factors are the quality of glycaemic control during placental development, the modality of treatment, and the period of severe hyperglycaemia. Diabetes at the beginning of pregnancy may have long term effects on placental development resulting in adaptive responses to the diabetic environment [144]. Placental action to compensate excess of maternal glucose may limit fetal growth. However, if the duration or extent of the diabetic insult exceeds the placental ability to adequately respond, excessive fetal growth may follow [145].

Conversely, diabetes experienced at the later stages in gestation, such as in GDM, will mainly lead to short term changes in a variety of molecules for key functions including gene expression [133].

The effect of maternal obesity on placental structure at the microscopic level has not been examined in detail. Given the abnormalities observed in placental morphology from women with gestational diabetic (i.e. higher presence of villous immaturity, chorangiosis and ischemia) [146], it is plausible that obesity may also affect placental structure in a similar way. A study by Challier et al. supports this hypothesis by demonstrating a 2 to 3 fold increase in placental macrophages in obese women compared with non-obese women [92]. These observations, together with previous findings, have demonstrated the critical role of the placenta in fetal programming [147]. Accordingly, the placental phenotype is proposed as a better representation of the intrauterine environment than birth weight [116].

The following sections will therefore examine aspects of placental function critical for fetal growth and development with particular emphasis on placental vascular development and blood flow, nutrient transfer capacity and regulation of hormone production.

1.8.3. Placental blood flow and vascular development

As pregnancy progresses, a rise in blood flow from the maternal circulation is required to compensate the increased metabolic demand from the growing uterus. Throughout gestation, total maternal blood volume and cardiac output increase by approximately 40%, and the total utero placental blood flow represents 25% of cardiac output [148]. The uterine artery volume flow rate is enhanced by more than 3 fold during pregnancy, partly reflecting an increased artery diameter and reduced flow resistance [149], which results from the establishment of the utero-placental circulation.

Other maternal adaptations during normal pregnancy include the development of new blood vessels in the uterus, possibly promoted by the placental hormones human chorionic gonadotropin (hCG) [150] and insulin-like growth factor-2 (IGF2) [151]. Decreased rates of placental blood flow, reflecting reduced fetal oxygen and nutrient uptake, are associated with lower fetal and placental growth [139, 152]. Obese gravidae with impaired endothelial vasodilatation often show increased incidence of preeclampsia and placental dysfunction [86], and a high fat diet (HFD) in pregnancy alters placental haemodynamics and structure [153]. Furthermore, when hyperinsulinaemia is associated with obesity in pregnancy, these alterations appear to be even more severe with reduced blood flow to the fetus increasing susceptibility to stillbirth [39].

The term human placenta consists of a tree-like, villous structure covered by terminally differentiated syncytiotrophoblast. This syncytium is formed by the fusion of subjacent cytotrophoblast cells, which are mitotically active during fetoplacental development, contributing to the establishment of the uteroplacental circulation [154]. Throughout the first trimester, the intervillous space of the developing placenta is separated from the uterine circulation by plugs of trophoblast cells that occlude the tips of the uteroplacental arteries [141]. In order for maternal placental blood flow to increase normally, it is critical that invasive trophoblast cells migrate into the maternal decidua, resulting in the remodelling and opening of the maternal spiral arteries. This implicates the degeneration of elastic and smooth muscle tissue in spiral artery walls and the replacement of endothelial cells by trophoblasts [155]. At the end of the first trimester, these plugs are dislocated, allowing maternal blood to flow freely and continuously in the intervillous space. As a result, maternal blood enters the placenta via the spiral arteries, which delivers blood directly to the intervillous space (Figure 1.7).

These ramify into smaller vessels resulting in a capillary network at the tips of the villi for then merging again to form the vein of the umbilical cord. Thus, the maternal and the fetal compartment are in contact with different surfaces of the placenta. Severe reduction in trophoblast invasion results in decreased intervillous blood flow and IUGR fetuses [139].

A large body of evidence indicate that increased placental angiogenesis and feto-placental vessels are features of maternal diabetes. Higher fetal levels of fibroblast growth factor-2, which stimulate placental angiogenesis, are associated with T1D diabetes leading to the hypercapillarisation [157]. Increased capillary length, branching and surface area have been reported for both T1D [158, 159] and GDM [160] although reports on the latter are still conflicting [161, 162]. Higher levels of circulating glucose and insulin have profound effects on the fetal vascular system and can be responsible of these observed changes [163].

Avagliano found that maternal BMI was the major maternal risk factor of abnormal spiral artery remodelling during pregnancy [164]. This endothelial dysfunction may result from chronic hypertriglyceridemia and hyperlipidemia observed in obese subjects. In addition, hyperglycaemia has been shown to have a direct effect on vascular development [165]. The inflammatory response generated by obesity is accompanied by impaired vascular and endothelial function with increase production of angiogenic and factors. However, comparative studies of placental morphological changes in obese pregnant women are currently scarcely available in the literature indicating the need of further research [166].

1.8.4. Placental size and morphology

Placental weight and ratio to birthweight are predictors of the long term outcome for the offspring as reported in epidemiological data [167]. Increased placental to fetal weight ratio seems to represent an adaptive process by the feto-placental unit to an unfavourable maternal environment. Changes in placental growth have been shown to influence fetal development.

Whilst intrauterine growth restricted (IUGR) newborns are associated with smaller placental volume, GDM, even with optimal glycaemic control [168], leads to greater placental weights and birth weight ratios [169].

One mechanism by which placental size influences nutrient transfer is via changes in its morphology. In fact, alterations in the placental surface area, vascularity and cell composition affect placental function. A growing body of evidence confirms a link between maternal diabetes and changes in placental morphometry [170], changes in the villous surface area [171] and deposition of perivillous fibrinoid at sites of epithelial damage [172]. These alterations reflected a perturbed placental haemodynamic and transport [172]. Interestingly, placental morphology has been shown to be preserved in diabetic subjects with good glycaemic control [173]. Further studies have confirmed the presence of morphological abnormalities associated with GDM, including villous immaturity and chorangiosis [174], degenerative lesions and abnormal distribution of fibronectin [175].

Obese women show an increased systemic pro-inflammatory status similar to that of preeclampsia and diabetes, suggesting obesity could be linked to similar changes in placental morphology. Roberts et al. were the first to describe structural changes in the placenta of obese women [176]. Unexpectedly, his findings showed only a generalised increased in vessel muscularisation without any major placental alterations. Additional data is needed however to explain the link between placental inefficiency and altered structure in pregnancies complicated by obesity.

1.8.5. Placental nutrient transport

In physiological conditions, the placenta is able to use 40-60% of the total glucose and oxygen supplied by the uterine circulation [177]. The remaining nutrients and metabolites are transferred by different mechanisms through the two polarized plasma membranes of the syncytiotrophoblast, which are in contact with both the maternal and the fetal compartment (Figure 1.8). The microvillous plasma membrane (MVM) of the syncytiotrophoblast faces the maternal blood whereas the endothelium of the basal plasma membrane (BM) is adjacent to the fetal capillary [178]. As in other epithelial cells, differences in type, number and activity of transporters in these two plasma membranes provide the basis for transplacental transport of primary nutrients [145, 179-181].

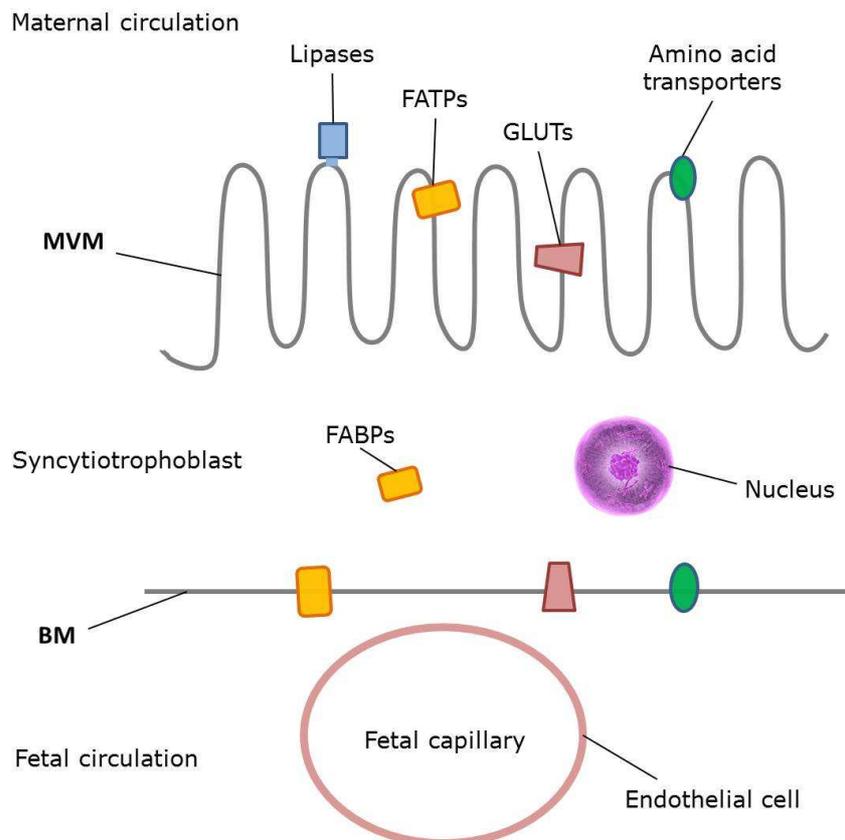


Figure 1.8: Placental nutrient transport is regulated by maternal, placental and fetal factors.

MVM: microvillous plasma membrane; BM: basal plasma membrane; FATPs: fatty acid transporter proteins; FABPs: fatty acid binding proteins; GLUTs: glucose transporters. Adapted from [180].

Changes in the expression or activity of placental transporters have been associated to altered fetal growth [182, 183]. Evidences from human data report that IUGR placentas show alterations in placental vasculogenesis [184], trophoblast transporters expression [183], trophoblast enzyme activity and hormone production [185]. Specific upregulation of placental transporters seems to explain the occurrence of fetal overgrowth in GDM and T1DM [186]. As suggested by Cetin et al., changes in placental nutrient transport resulting from increased exchange surface area or increased transporter densities, could further enhance substrate levels and fetal growth in diabetic pregnancies [187]. On the other hand, no data with respect to placental transport function are currently available in high BMI pregnancies, making it a novel and intriguing field of investigation.

1.8.5.1. Glucose

Glucose represents the primary source of energy for the human fetus and the placenta during development. Fetal glucose production is minimal and, therefore, it is entirely dependent on placental supply from the maternal circulation [188]. Net transport of glucose from mother to fetus across the placenta requires a net maternal-fetal plasma glucose concentration gradient, which is maintained via facilitated diffusion involving transporters on both maternal and fetal side of the trophoblast [189].

Glucose transporters (GLUTs) are highly expressed in the MVM allowing rapid glucose uptake into the placenta for its own use while maintaining a gradient between the syncytiotrophoblast cell and fetal capillary [188]. The lower GLUTs expression in the BM, which is in contact with the fetal surface, has pointed out that this placental membrane represents the rate limiting step in placental glucose transport [180, 190, 191].

Glucose transporter-1 (GLUT1) is the primary placental glucose transporter in humans at term and is constantly expressed throughout pregnancy [192].

Its expression in both MVM and BM increases in the first half of pregnancy and remains constant thereafter [193], suggesting that this placental glucose transporter is regulated early in pregnancy, but not at term.

Furthermore, the increase in placental surface area in the latter part of pregnancy as well as the maintenance of a high GLUT1 density can explain the increase in placental glucose transport at term [189, 193]. Glucose transporter 4 (GLUT4), on the other hand, is primarily expressed during the first trimester [194] and is affected by defective insulin signalling [195] (see Section 1.7).

It has been hypothesised that excess maternal glucose in pregnancies complicated by diabetes increases placental glucose transfer to the fetus [196]. This, in turn, results in fetal hyperglycaemia and hyperinsulinaemia [197] leading to the delivery of LGA infants [186]. However, macrosomia occurs even under strict glycaemic control [198], suggesting that hyperglycaemia alone is not driving fetal overgrowth but other molecules are involved in the metabolic alterations of diabetes.

In patients with T1DM, hyperglycaemia is detected prior to gestation, whereas GDM is diagnosed during the second half of pregnancy. Human studies have demonstrated that patients with GDM controlled by diet had reduced placental glucose transport compared with insulin treated GDM [199]. Powell et al. found higher glucose transport activity and enhanced GLUT1 expression in the BM of placentas from T1DM pregnancies with LGA infants [198]. This was not observed in pregnant women with GDM delivering LGA offspring [200]. Therefore, the authors suggested that hyperglycaemia upregulates this transporter in the first trimester leading to accelerated fetal growth in late gestation. As the activity of GLUT1 is influenced by glucose concentration, and not by insulin, high glucose levels leads to its saturation [201], pointing out that this transporter may not be the main cause of macrosomia in diabetic pregnancies. Although the role of insulin in regulating placental glucose at term is controversial [117, 202], its effect on placental glucose transport has been demonstrated in the first trimester [203].

This has highlighted a potential role of GLUT4 in T1DM pregnancies [195]. Conversely, the incidence of macrosomia in pregnancies complicated by GDM, even under optimal glycaemic control, suggests that different nutrients other than glucose can contribute to fetal overgrowth in these complicated pregnancies (see section 1.8.5.2).

The short- and long term outcomes of maternal obesity for both mother and fetus have been recently reviewed by Catalano and Ehrenberg [204]. Jones et al. has demonstrated that mice fed with a HFD (32% energy from fat) are associated with upregulation of GLUT1 and sodium-dependent neutral amino acid transporter 2 (SNAT2) in the MVM [205]. This suggests that maternal HFD and obesity are linked to fetal overgrowth via increasing specific placental transporters. Ericsson et al. reported that hormones, such as leptin, growth hormone (GH), IGF1, insulin and cortisol, did not affect glucose transporter activity at term [117]. In contrast, insulin stimulated glucose uptake was enhanced in primary villous fragments obtained at 6–8 weeks of gestation [203]. This may be related to the presence of the insulin-sensitive GLUT4 in the cytosol and syncytiotrophoblast MVM in the first trimester.

1.8.5.2. Amino acid transport

The active transport of amino acids across the human placenta is suggested by a higher concentration of these metabolites in the fetus compared to that in the mother [206]. Their transport across the polarized syncytiotrophoblast membranes occurs via proteins located in both the BM and MVM [178, 207]. Accumulative amino acids transporters mediate net uptake of specific amino acids into the syncytiotrophoblast, which are exchanged for those in the maternal or fetal circulation by exchange transporters [181]. The function of the exchangers is dependent on the activity of accumulative transporters on the MVM and facilitated transporters on the BM, which directs the critical passage of amino acids from the placenta to the fetus [178]. Thus, impairment in rate limiting activity of efflux transporters on the BM [190] or MVM [191] compromises fetal growth.

As such, *in vitro* studies of isolated MVM and BM represent a valuable experimental system to elucidate the cellular mechanisms of this selective transport through the placenta. Five different transport systems for neutral amino acids have been identified in the human syncytiotrophoblast using villous tissue fragments, *in vitro* perfused placenta, cultured trophoblast cells and cell lines [207]. Sodium-dependent transporter for alanine, proline, glycine, and serine (System A) is found mostly active in on the MVM whereas system ASC, seems to be localized mostly on the BM. The placenta can also metabolize and process amino acids itself, such as leucine, which are then transferred to the fetus [207].

Hypoxia, NO, and ROS have been shown to reduce the activity of several placental amino acid transporters, and a decrease in essential amino acid transport has been associated with IUGR [208, 209]. Jansson et al. described higher activity of system A transporter in both T1DM and GDM diabetes [183], and increased activity of L and ASC transporters in diabetes associated with fetal macrosomia [179]. In contrast, Kuruvilla et al. described reduced system A activity in macrosomic babies of diabetic mothers without changes in the activity of system L in MVM isolated vesicles [210]. In addition, inverse relationship between placental system A activity and size at birth was reported by Godfrey et al. [208]. This increased activity with smaller size may be a compensatory mechanism in smaller neonates.

The findings by Jansson et al. on glucose and amino acid transport have indicated that GDM is associated with different adaptations than those of T1DM [198, 200]. They also suggest that fetal macrosomia observed in GDM pregnancies may depend on higher uptake of neutral amino acids rather than glucose [183]. On the other hand, the effect of obesity on amino acid transport has not been studied during obese pregnancy. However, Jones found that mice fed with a HFD had increased expression of specific placental amino acid transporters associated with significantly higher fetal growth [205].

1.8.5.3. Triglyceride transport

In the third trimester, the increased FA demand is accompanied by a rise in placental FFA transporters. FFA transfer across the placenta occurs via passive diffusion as well as by fatty acid binding protein (FABP) and fatty acid transport protein (FATP) in the MVM and BM [211]. It has been proposed that high local levels of IL6 stimulate trophoblast FFA accumulation and may contribute to excessive FFA transfer in pregnancies complicated by high BMI [212].

GDM is consistently associated with elevated triglyceride levels and this has been found to be independent of maternal obesity [213]. Recent evidence links the higher risk for macrosomia in GDM pregnancies to altered maternal lipid metabolism rather than maternal hyperglycaemia [214]. Radaelli et al. reported studies in which the placental gene expression profile of women with normal glucose tolerance and GDM was investigated using oligonucleotide microarray analysis [214, 215]. A pronounced modification of fetoplacental lipid pathways and selective activation of transplacental lipid fluxes were observed in pregnancies complicated by GDM.

These findings were confirmed recently by Catalano who reported enhanced genes for fetoplacental lipid metabolism in fetuses of obese mothers [216]. As a result, increases in lipid transporters, angiogenic factors and inflammatory markers may explain the onset of complications in these pregnancies [217, 218].

1.8.6. Placental hormonal regulation

Placental endocrine activity during pregnancy also plays a crucial role in the regulation of fetal growth. Hormones secreted by the placental syncytiotrophoblast include human placental lactogen (hPL), oestrogen, progesterone, hCG, and human growth factor (hGF) variants.

Placental hormones interfere with insulin-stimulated glucose uptake into skeletal muscle and adipocytes [219] and with insulin's ability to suppress lipolysis through the enzyme hormone-sensitive lipase (HSL) [220]. In late pregnancy, failure to fully inhibit lipolysis results in increased release of FFA, which are significantly higher in obese individuals with GDM [220]. Therefore, insulin resistance in adipose tissue leads to a rise in circulating FFA, which can accumulate in non-adipose depots.

In the following sub-sections, the main hormones and metabolic factors known to influence fetal growth and development will be reviewed along with the contribution of maternal obesity and diabetes in altering these mechanisms.

1.8.6.1. Insulin

Maternal insulin cannot cross the placenta [221]. Therefore, the fetus has to meet its own needs and begins to secrete its own insulin after the tenth week of gestation [222]. This hormone acts as a signal of nutrient availability through its action on the IR, thus promoting fetal growth [136]. Insulin deficiency causes a reduction in fetal growth as fetal tissues decrease their uptake and utilisation of nutrients [223].

Venous cord blood concentrations of insulin are significantly lower in SGA neonates and positively correlate with birth weight, birth length, and placental weight. Conversely, insulin levels in maternal serum or amniotic fluid do not correlate with birth weight [224]. Furthermore, increased insulin production is associated with increased fetal growth [169, 186].

As discussed in Section 1.5, maternal hyperglycaemia in diabetic pregnancies results in fetal hyperinsulinaemia, which is thought to be the cause of macrosomia [225] and increased adiposity in infants of diabetic mothers [226]. However, severe uncontrolled diabetes can also lead to SGA newborns [224] due to compromised uterine blood flow resulting in endothelial dysfunction and atherosclerosis [94, 225].

Catalano et al. have demonstrated that the chronic depression in insulin sensitivity found in obese pregnant women results in basal hyperinsulinaemia and impaired glucose uptake exposing the fetus to hyperglycaemia [216, 227]. In addition, fetal hyperinsulinaemia will follow, resulting in elevated levels of fetal TG and ultimately to fetal overgrowth [228, 229]. Obese women in whom GDM develops have a significant increase in insulin response but decreased insulin sensitivity. Studies in adipose tissue and skeletal muscle indicate that impaired insulin action is due to defects in the insulin signalling pathways [106, 230, 231] (see Section 1.7).

1.8.6.2. Insulin-like growth factors

Like insulin, IGF1 and IGF2 are other important growth factors in fetal development and they are both synthesised in the placenta and fetus. Whilst IGF1 is present in trophoblast cells at all gestational stages, placental IGF2 expression in cytotrophoblasts becomes undetectable at term [232, 233]. Therefore, IGF2 is thought to be a primary growth factor required for early development, whereas IGF1 expression is essential for achieving maximal growth.

Evidence of a link between intrauterine growth and IGFs come from studies in knockout mice of IGF2 gene. Using a mouse model in which placental-specific IGF2 had been deleted (P0), Sibley et al. measured nutrient transfer in vivo. diffusional exchange characteristics of the placenta [234]. The outcome of this study showed that the diffusing capacity in P0 knockout placentas was dramatically reduced to 40% of that of wild-type placentas.

Therefore, placental IGF2 seems to regulate the development of the diffusional exchange characteristics of the mouse placenta, controlling placental nutrient supply and fetal growth. In humans, reduced levels of IGF2 are seen in IUGR fetuses [235], whereas LGA fetuses show higher IGF2 levels [236]. It has been suggested that maternal and placental, rather than fetal IGFs, regulate syncytiotrophoblast transport function. Reduction in maternal levels of IGF1 is associated with decreased glucose and amino acid transport across the placenta, resulting in IUGR offspring [237]. In contrast, increased maternal levels of IGFs associated with diabetes may influence fetoplacental growth by increasing nutrients transfer across the placenta [238].

At physiological concentrations, both IGF1 and IGF2 bind the IGF1 receptor (IGF1R), which activates intracellular signalling pathways [237]. In addition, in embryonic and cancer cells IGF2 binds to an IR isoform with slightly lower affinity than that of insulin [239]. Mutations of IGF1R gene are associated with profoundly lower fetal weight, while overexpression of this gene leads to heavier and longer neonates [237]. Alterations in insulin and IGFs levels found in diabetic pregnancies can result from the spatial-temporal change in the expression of their receptors within the placenta. Whilst in the first trimester, both IR and IGF1R are predominantly expressed on the MVM, which are directed to the maternal circulation, at term their expression is mainly found on placental endothelial cells, which are facing fetal blood [118, 240]. This is accompanied by a change in gene expression via activation of insulin downstream signalling [240]. As such, the shift in IR expression from trophoblast to endothelium reflects the different regulation of insulin effects from the mother to the fetus. The morphological and functional placental changes in T1DM may have their origin early in pregnancy due to the insulin regulatory effect on trophoblast invasion and migration. Conversely, insulin involvement in vascular growth can explain the enhanced angiogenesis characteristic of placentas from GDM pregnancies.

1.8.6.3. Leptin

The adipokine leptin also influences fetal growth as its concentrations in both maternal [241] and fetal cord blood are positively correlated with birth weight [242]. The role of leptin, a plasma protein secreted by adipocytes, is to regulate food intake and energy expenditure via the hypothalamus [243]. In addition, its production by placental and fetal tissues has suggested a key role of leptin in regulating fetal and placental growth [242]. Its regulation differs from that of adipose tissue, therefore, this hormone may have a different role during pregnancy [244].

Leptin synthesis and secretion by the trophoblast is likely to be induced by hCG via activation of MAPK and cAMP signalling pathways [245], and leptin action through its receptor, promotes protein synthesis and trophoblast proliferation [246], whilst inhibiting apoptosis [247]. Mucci et al. found an association between maternal progesterone and cortisol with placental leptin expression [248] and Smith et al. observed that transplacental leptin transfer is reduced in association with excess maternal GC leading to IUGR fetuses [249]. Insulin has been proposed as an important modulator of leptin expression in the placenta [250, 251], with an increase in leptin concentrations been reported in obese and insulin-treated diabetic pregnancies associated with macrosomia [252, 253]. It has been proposed that high concentrations of insulin in cord blood are correlated with hyperleptinaemia in the placenta which, in turn, stimulates fetal growth [254]. Newborns of diabetic [255] and obese [256] mothers show impairment of hypothalamic metabolic regulation associated with leptin resistance during adulthood. Conversely, leptin inhibits glucose-stimulated insulin secretion suggesting the existence of a negative feedback system between leptin and insulin. In contrast, adiponectin and ghrelin have opposite functions to leptin as they activate the cAMP signalling ensuring trophoblast differentiation [257].

As leptin is co-localised to both syncytiotrophoblast and villous endothelial cells, it is likely to be released into both the maternal and fetal blood circulation [244, 252] (Figure 1.9). The transmembrane isoforms of leptin receptor (ObRa, ObRb) are expressed only on the MVM indicating a possible autocrine role of leptin at the maternal side [244, 252]. In addition, the peak in maternal circulating leptin seen in late pregnancy can reflect its binding to the soluble receptor (ObRe) released by the placenta [258]. This may contribute to leptin resistance in the mother at the end of pregnancy suggesting an endocrine activity of this hormone in maternal appetite, energy metabolism and fat deposition. Placental lactogen and prolactin have been shown to increase maternal food intake by induction of central leptin resistance, as well as promote maternal insulin production by beta cells [155].

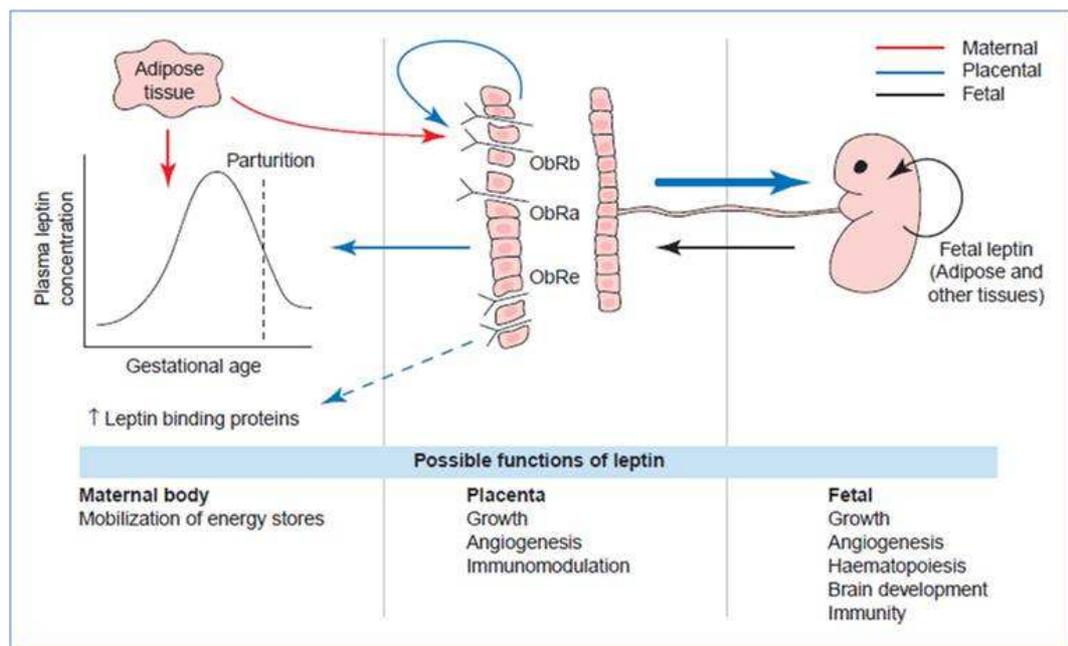


Figure 1.9: Proposed roles of placental leptin at the maternal-fetal interface. ObR: leptin receptor. From [244].

Leptin also promotes angiogenesis in human endothelial cells and it may exhibit an indirect angiogenic role in the placenta through stimulation of angiogenic factors [244]. Its potential autocrine activity at the maternal-fetal interface reflects local immunomodulation of pro-inflammatory cytokines such as TNF α [244].

Hence increased concentrations of leptin in preeclamptic and diabetic pregnancies may indicate a protective response of this hormone against higher levels of pro-inflammatory cytokines [259, 260]. Recent evidence links this adipokine to the expression of key enzymes for lipid synthesis confirming their interplay between inflammatory stress and intracellular lipid content within the placenta [261, 262]. In obese individuals, circulating leptin levels are usually very high and this chronic hyperleptinaemia seems to desensitise leptin signal [263]. Maternal hyperleptinaemia and placental leptin resistance were observed in a group of obese pregnant women [253]. However, it is unclear the extent to which maternal hyperleptinaemia can influence the fetus.

1.8.6.4. Glucocorticoids and the placental 11 β HSD2 barrier

Maternal cortisol increases about 2.5 fold during pregnancy and represents another potentially important hormone [264]. Under conditions of GC excess, there is a decrease in peripheral insulin sensitivity, leading to hyperglycaemia [265]. These hormones are well known to interfere with insulin signalling in skeletal muscle by post-receptor mechanisms [266]. Chronic GC treatment decreases insulin-stimulated glucose uptake and GLUT4 translocation in skeletal muscle, without reducing the total content of GLUT4 [267, 268]. Furthermore, GC exposure reduces insulin stimulated protein kinase B/v-akt murine thymoma viral oncogene homolog (PKB/Akt) phosphorylation [269], although this is not a consistent finding [270]. Similarly, IRS1 protein content and tyrosine phosphorylation appear to be reduced in skeletal muscle following GC treatment [271], as is IRS1-associated PI3K [272, 273].

GC are primarily synthesised and secreted by the adrenal cortex, but are also produced by other tissues, including the placenta [274]. They are essential for the development and maturation of fetal organs before birth playing a crucial role in fetal development [275, 276]. Furthermore, GC regulate the production of hormones associated with the onset of labour such as prostaglandins [277].

In fact, in human pregnancies, these hormones are used in the treatment of preterm deliveries and their administration, especially during late gestation, promotes maturation of lungs and other organs [177], saving the lives of infants when born preterm. Nonetheless, administration of excess GC to pregnant animals and humans is associated with IUGR fetuses [278]. The resulting offspring have altered activity of the the hypothalamic–pituitary–adrenal (HPA) axis and are susceptible to develop hypertension and hyperglycaemia in adult life [279, 280]. This has been confirmed also in obese sheep where moderate energy restriction results in changes of the development of HPA axis as well as enhanced cortisol response to stress [281].

Fetal exposure to GC is finely regulated by the family of 11-beta hydroxysteroid dehydrogenase (11β HSD) enzymes, which have different biological characteristics and tissue distribution within the placenta [185]. The isoform 11β HSD type-1 (11β HSD1) is expressed in the decidua, placental extravillous trophoblast, chorion trophoblast, amnion epithelial cells and generates active cortisol from cortisone. Conversely, 11β HSD type-2 (11β HSD2), which represents the placental barrier to maternal GC, is localised in the placental syncytiotrophoblast and limits the passage of cortisol to the fetal circulation by converting this active metabolite to inactive cortisone (Figure 1.10). Physiological variations in placental 11β HSD2 activity can cause impaired placental inactivation of cortisol leading to IUGR [282], with enhanced plasma cortisol levels in adulthood and activated HPA axis [283]. The latter has been shown to ultimately result in altered metabolic profile and behavioural phenotype.

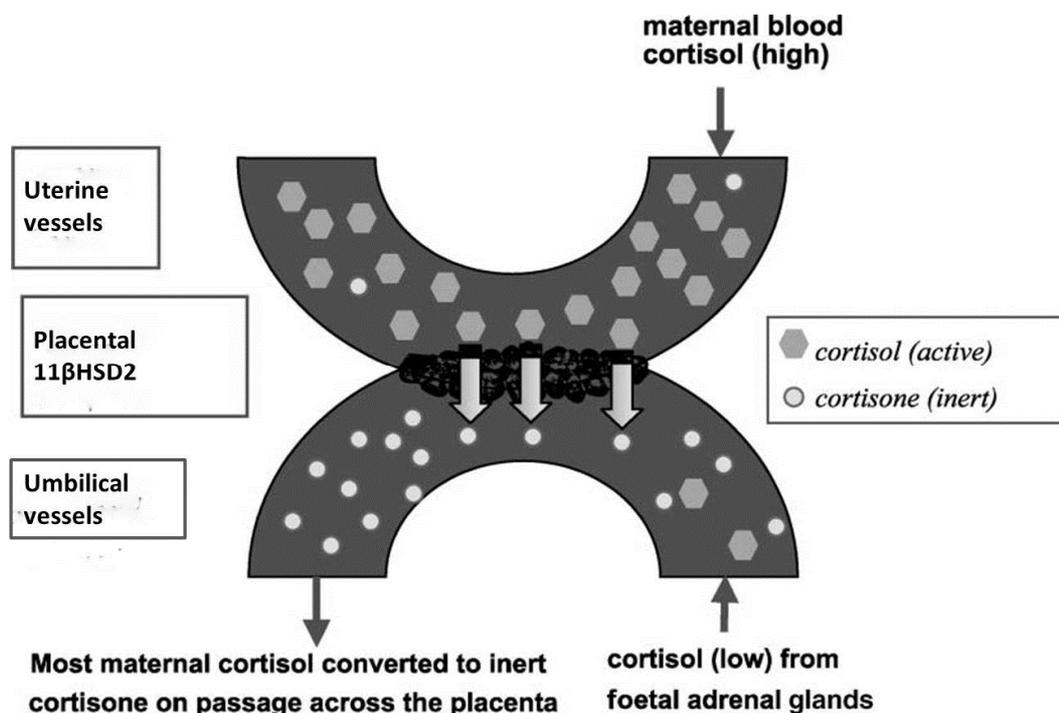


Figure 1.10: Glucocorticoids regulation of fetal growth and tissue maturation through placental 11β-hydroxysteroid dehydrogenase type-2 (11βHSD2). From [284].

At present, there is a lack of knowledge regarding the effect of maternal BMI and glucose tolerance on GC levels and 11βHSD enzymes [285]. In patients with Cushing's syndrome, central obesity is a prominent characteristic [286]. This disease can be caused by disorders of the adrenal glands resulting in excess level of endogenous GC in the body, mainly cortisol [285]. Evidence from human and rodent studies shows the existence of higher intracellular tissue concentrations of cortisol in obese subjects due to increased activity of 11βHSD1 [286, 287]. Changes in this enzyme expression result in the development of insulin resistance, hypertension and associated metabolic syndrome [285]. Experimental studies reported that offspring of mothers treated with carbenoxolone, an inhibitor of placental 11βHSD, have lower birth weights as well as hypertension and glucose intolerance in later life [288, 289].

The action of GC is mediated by the GC receptor (GR), a nuclear receptor located in the cellular cytoplasm of almost all tissues [290, 291].

The human placenta expresses a variety of GR mRNA transcripts, among which the majority is represented by GC receptor alpha (GR α) [292]. The binding of GC to their receptor primarily initiates transcription of target genes and factors involved in metabolic homeostasis. Also, it can contribute to an anti-inflammatory status through immunosuppressive action on monocyte and macrophage differentiation [293]. In response to obesity, a downregulation of GR has been suggested in subcutaneous adipose tissue but not in visceral adipose depots, which are still responsive to local GC synthesis [294].

1.9. Placental metabolic regulation

1.9.1. Placental insulin signalling

Insulin signalling is crucial for the regulation of intracellular and blood glucose levels and its alteration may play a role in the pathophysiology of insulin resistance associated with obesity and GDM [106, 195]. As illustrated in Figure 1.11 activation of insulin signalling involves insulin binding to the IR, which results in receptor autophosphorylation as well as tyrosine phosphorylation of the insulin substrate receptor (IRS). The subsequent association of IRS with downstream activators including PI3K allows regulation of glucose uptake via GLUT4 translocation [272] (Figure 1.11-A). Alterations in the number of insulin binding sites reflecting placental IR expression have been documented in various forms of diabetes mellitus [108]. However, a scarcity of data is available on insulin signalling protein expression in placental tissue [295]. Previous studies in skeletal muscle [131] and adipose tissue [109] of GDM pregnant women indicated alterations in insulin signalling resulting in decreased protein expression of IRS1 along with increased IRS2. Increased basal serine phosphorylation of IRS1 in skeletal muscle from mouse and humans has recently been associated with GDM [231, 296, 297] (Figure 1.11-B).

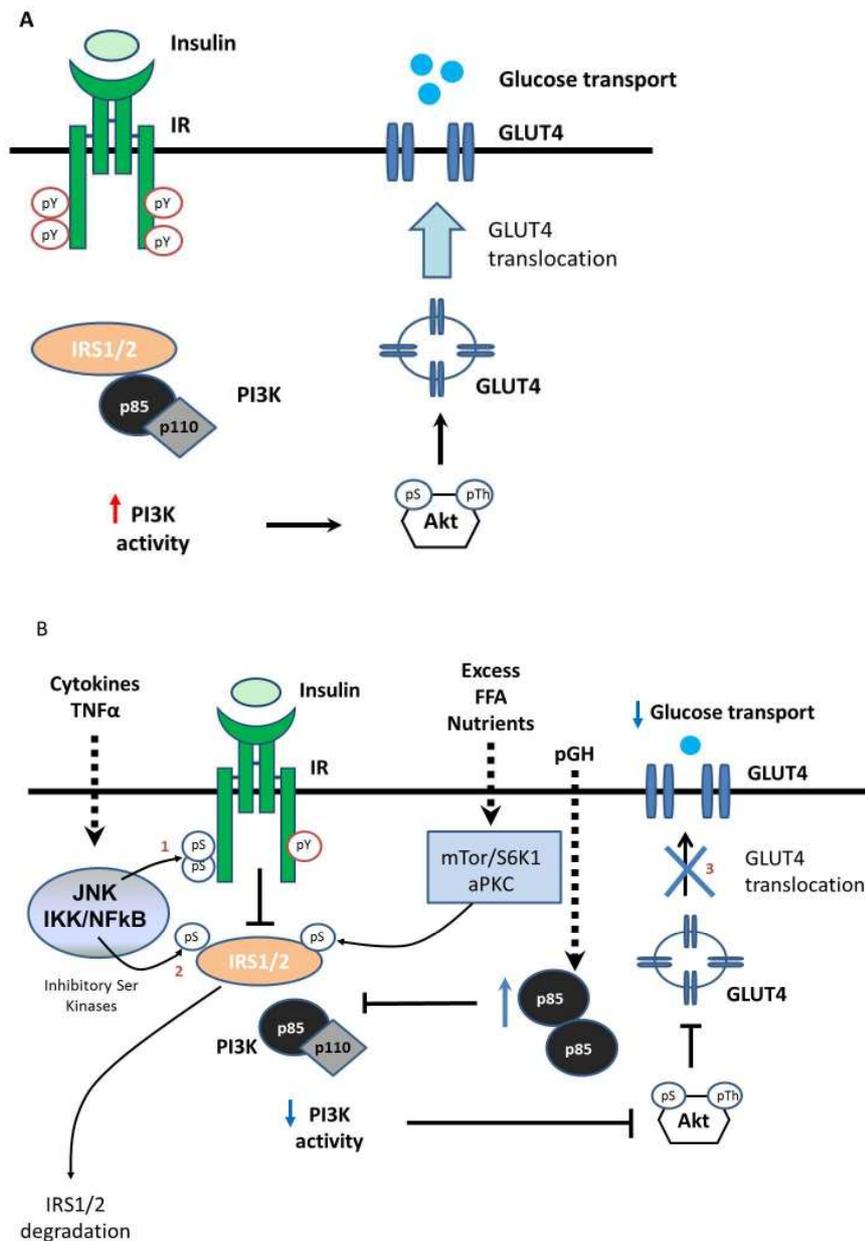


Figure 1.11: Insulin signalling for glucose uptake.

A) Insulin binds to its receptor causing a conformational change in the receptor that activates its intrinsic tyrosine kinase activity. This leads to autophosphorylation of the insulin receptor (IR) resulting in the translocation of glucose transporter-4 (GLUT4) to the plasma membrane allowing glucose to enter the cells. (B) Gestational diabetes mellitus (GDM) results in the suppression of several key steps in insulin signalling mainly by negative regulatory mechanisms: (1,2) Serine phosphorylation of both IR and IR substrate (IRS) blocks tyrosine phosphorylation on IRS1/2 leading to decreased recruitment of Phosphatidylinositol 3-kinase (PI3K). Upregulation of cytokines, like tumour necrosis factor-alpha (TNF α), or increased nutrient flux into the cell can cause activation of serine kinases; (2) increased serine phosphorylation on IRS1/2 promotes its degradation; (3) upregulated placental growth hormone (pGH) increases the expression of p85 subunit resulting in inhibition of PI3K activity and decreased glucose uptake.

These findings have been confirmed by Colomiere et al. in diabetic and obese placentas suggesting a maternal regulation of these two proteins in GDM [195]. There is support for a diminished glucose uptake in placental tissue from GDM individuals [201] reflecting reduced GLUT4 and increased GLUT1 expression [195, 198]. Although the specific serine kinase responsible for increased IR/IRS1 serine phosphorylation during pregnancy is not known, several possible candidates have been identified, including increased activation of JNK1/NFkB and ribosomal protein S6 Kinase (p70S6K) [106].

1.9.2. Placental nutrient sensing

The syncytiotrophoblast plays a unique role in determining fetal growth and development by functioning as a placental nutrient sensor. Recently, the mammalian target of rapamycin (mTOR) pathway has been discovered to be central in trophoblast development and placental nutrient-sensing [298]. In vitro studies using immortalised human trophoblasts and human choriocarcinoma cell lines highlighted its role in inducing trophoblast proliferation and differentiation through activation of the PI-3 kinase signaling pathway [299, 300]. Furthermore, Busch et al. demonstrated that this cytoplasmic molecule contributes to the invasiveness of human trophoblast cells by secretion of extra cellular matrix remodelling enzymes and its dysregulation may lead to pregnancy-related pathologies [301]. Placental insulin/IGF1 signalling as well as fetal glucose and amino acids, act as upstream regulators of mTOR and are altered in pregnancies complicated by maternal obesity and GDM [302]. This central molecule also regulates the transcription of key transporters and enzymes involved in energy intake and metabolism [303, 304]. It has been suggested that mTOR inactivation can result by either mTOR autophosphorylation or a mechanism involving the AMP-activated protein kinase (AMPK) pathway [305, 306]. Constitutive activation of this Ser/Thr kinase also results in inhibition of IR response to insulin [307].

A central role of PPAR γ in supporting mammalian fetoplacental development has been suggested from both animal [308] and human studies [309, 310]. The pleiotropic function of this nuclear protein during placentation was reported in human cultured villous trophoblastic cells isolated from term placenta [311]. Suwaki et al. showed that hyperglycaemia causes increased PPAR γ expression accompanied by high hCG secretion and decreased cell proliferation, suggesting that this pathway might be involved in the impairment of placental development [312]. PPAR γ signalling also regulates FFA transport and metabolism, lipid storage, and insulin modulation within the trophoblast [313].

1.9.3. Placental inflammation

During pregnancy, the placenta [86] and adipose tissue [314] become important sources of many cytokines and adipokines, the expression of which is exacerbated by maternal diabetes and obesity. Hauguel-de-Mouzon et al. found striking homologies between the expression and regulation of cytokines and inflammatory genes in both term placenta and white adipose tissue, suggesting that the abnormal metabolic environment generated by obesity can lead to the propagation of the inflammatory response [315]. In addition, studies of transcriptional profiling have shown that maternal hyperglycaemia promotes overexpression of inflammatory genes as well as a range of adipose tissue related proteins within the placenta [214].

Trophoblast cells are the major source of placental TNF α , leptin and IL6 [316]. Integrins and cytokines expressed by the proliferating trophoblast allow it to switch from a proliferating state to an invasive one [140]. Changes in insulin sensitivity from early to late gestation are correlated with plasma TNF α . Infusion of TNF α in rat [317] and human skeletal muscle cells [318] results in enhanced insulin resistance and its levels are positively correlated with BMI and hyperinsulinaemia in obesity [319]. Obese women with GDM appear to have a local 5 fold increase in TNF α mRNA in skeletal muscle, which persists post-partum.

Adiponectin, which highly correlates with whole body-insulin sensitivity [320], can increase glucose uptake in skeletal muscle and suppress glucose production of the liver through AMPK stimulation [321]. This adipokine has also been associated with FFA oxidation through activation of peroxisome proliferator-activated receptor-alpha (PPAR α) [322]. Adiponectin reduction in obesity and GDM could contribute to the decreased insulin sensitivity in those subjects [323, 324].

As described in Section 1.6, inflammation links obesity with insulin resistance with potential implications for fetal development and related diseases during adulthood [93]. In fact, the chronic inflammatory state of obesity is extending from the mother to the placenta with accumulation of inflammatory markers such as raised cluster of differentiation 14 and 68 (CD14, CD68), leading to macrophage infiltration [90, 92]. This may result in increased inflammatory response in the newborn. In the placenta, leptin can influence the expression of key enzymes for lipid synthesis confirming the interplay between inflammatory stress and intracellular lipid content [261, 262]. This hormone has been shown to regulate lipid metabolism through direct activation of AMPK and it has been implicated in diabetes associated with obesity [250]. However, results have been inconsistent due to the intricate functions of this hormone, which acts both centrally and peripherally [325].

Molecular interplays between immune function and metabolism have been proposed to take place within the placenta with mTOR regulating the expression of NF κ B signalling, thereby promoting pro-inflammatory and pro-oxidative pathways within the cell [326-328].

In contrast, PPAR γ has been proposed as an anti-inflammatory factor in both human and animal studies [308, 313]. Reciprocal expression of PPAR γ and cyclooxygenase-2 (COX2) in human term placenta suggests a role of the former in the initiation of labour [310]. High levels of circulating FFA and oxidised lipids have been associated with decreased PPAR γ expression and subsequent cytokine activation [309, 329].

1.9.4. Placental oxidative stress

Along with cytokine production, inflammation is also linked to oxidative stress in the placenta [113]. This condition is defined as an imbalance between the production of reactive oxygen species (ROS), such as superoxide, and the ability of antioxidant enzymes to scavenge ROS. Pregnancy is a state of oxidative stress due to the high metabolic activity of placental mitochondria that generate ROS. Intensified ROS production occurs physiologically during certain stages of placental development as well as under certain pathological conditions such as in preeclampsia, diabetes and IUGR [37]. Measurements of partial pressure of oxygen (pO_2) within the placenta from women with a diagnosis of missed miscarriage have associated the establishment of the maternal-placental circulation with increased oxygen tension [114]. Oxidised lipids, resulting from the combination of excess fatty acids and oxidative stress, have been proposed to inhibit trophoblast invasion and influence placental development by acting as ligands for nuclear receptors [84]. A surplus of lipid nutrient supply from the mother can therefore, alter placental gene expression with potential negative impact for the offspring [216, 330].

In addition, growing evidence in overweight and obese adults associates endothelial oxidative stress with increased expression of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) and selective upregulation of antioxidative enzymes and NO synthase [331, 332]. Expression of the antioxidant gene sirtuin-1 (SIRT1) can reduce mTOR pro-inflammatory effect enhancing cell anti-oxidative capacity [333, 334]. SIRT-1 overexpression in neurons promotes neurite outgrowth and cell survival through inhibition of the mTOR signalling [335]. SIRT-1 maintains insulin sensitivity and glucose homeostasis and its inhibition impairs mTOR signalling resulting in hyperglycaemia, oxidative damage and insulin resistance [333]. This pleiotropic protein is involved in the regulation of intracellular metabolism, cell growth and survival and has been identified as a novel upstream regulator for AMPK signalling.

Its stimulation by AMPK via elevation in the cellular NAD(+)/NADH attenuates ROS-induced apoptosis leading to longevity and improving cellular stress resistance [336].

Uncoupling protein-2 (UCP2) has been proposed to uncouple beta-oxidation with energy production and protect the cell from the production of ROS contributing to the generation of an antioxidant response. There is evidence that FFA could upregulate UCP2 expression by acting as ligands for PPARs [308, 337, 338]. Although the complete mechanism is not fully understood, UCP2 has been found to regulate the production of ROS following mitochondrial respiration and may influence glucose uptake [339, 340]. In normal conditions, the expression and activity of this protein is low.

However, alterations in cell homeostasis can induce an increase in UCP2 and thus decrease the ability of the cell to produce ATP and be protected from oxidative stress. Maternal obesity and insulin resistance result in enhanced glucose and FFA availability to the fetus, whose oxidation in the mitochondria might lead to higher production of ROS [339, 341], activating UCP2 expression as well as NFkB signalling [342].

A proposed mechanism linking oxidative stress and inflammation is presented in Figure 1.12. Circulating FFA have been also demonstrated to activate myeloid pro-inflammatory cells through toll-like receptor-4 (TLR4) [343] whose upregulation has been linked to enhanced adipogenesis and insulin resistance in several tissues [344, 345]. TLR4 has been proposed as the main placental receptor which stimulates the inflammatory response against bacterial infection through NFkB and JNK signalling and may play a major role in the low grade inflammatory response frequently observed in obese individuals [346].

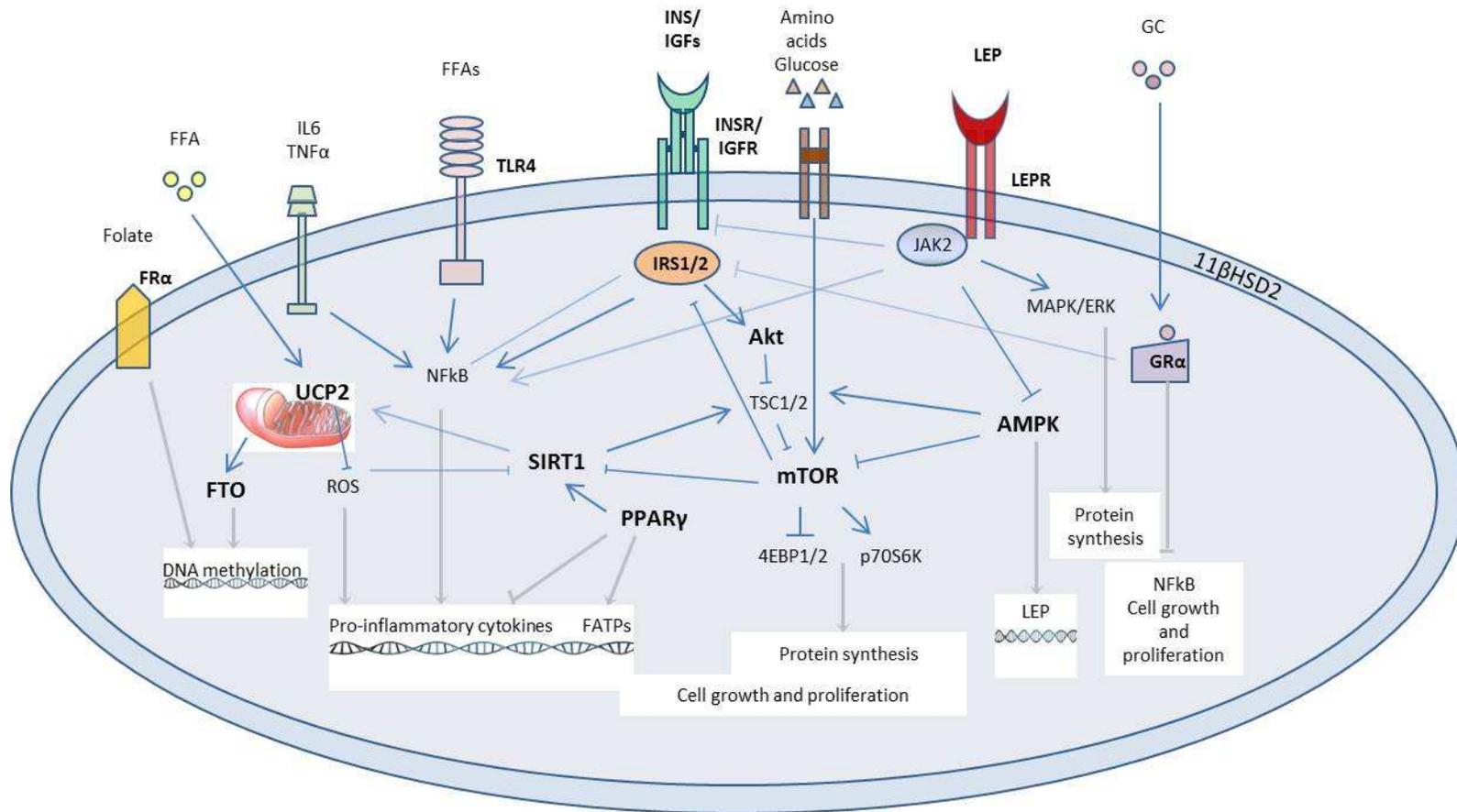


Figure 1.12 : Proposed mechanism of placental metabolism.

FR α : folate receptor alpha; FFAs: free fatty acids; UCP2: uncoupling protein-2; ROS: reactive oxygen species; IL6: interleukin-6; TNF α : tumour necrosis factor- α ; NF κ B: nuclear factor kappa B; TLR4: toll like receptor-4; IGF: insulin-like growth factors; IR: insulin receptor; IGFR: IGF receptor; IRS1/2: insulin substrate receptor-1/2; Akt:v-akt murine thymoma viral oncogene homolog; TSC1/2: tuberous sclerosis 1/2; mTOR: mammalian target of rapamycin; p70S6K: ribosomal protein S6 kinase 70kDa polypeptide; 4EBP1/2: initiation factor 4E binding protein 1/2; LEP: leptin; LEPR: leptin receptor; GC: glucocorticoids; 11 β HSD2: 11-beta hydroxysteroid dehydrogenase-2; GR α : glucocorticoid receptor-alpha; MAPK/ERK: mitogen-activated protein kinase/extracellular regulated kinase; JAK2: janus kinase-2; AMPK: AMP-activated protein kinase; PPAR γ : peroxisome proliferator-activated receptor gamma; FATS: fatty acid transporters; SIRT1: sirtuin-1. Arrows and bars represent activation and inhibition respectively (see text for details).

1.9.5. Folate metabolism and placental transport

Among the nutrients required during gestation, folate is critically important for nucleic acid synthesis and cell proliferation, contributing to adequate fetal growth and development [347]. This water-soluble vitamin is a member of the B9 family of vitamins (Fig 1.13) involved in several coenzymatic reactions essential for the one-carbon metabolism of key amino acids including homocysteine and methionine.

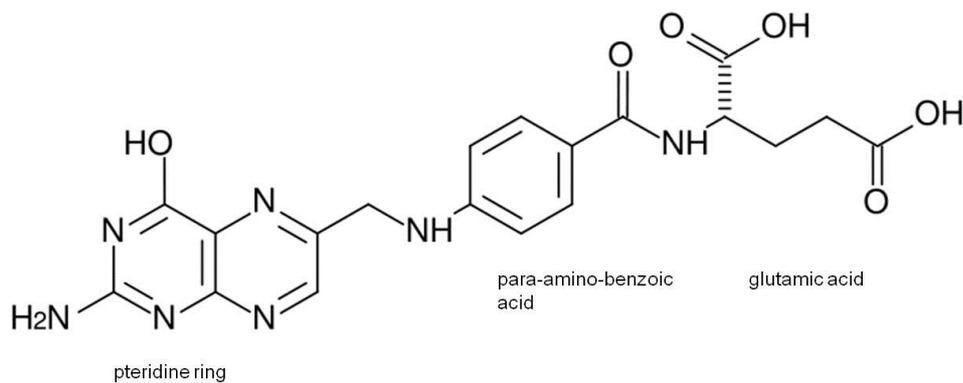


Figure 1.13: The chemical structure of folate.

Folate requirements are estimated to be increased 5- to 10 fold during pregnancy, driven by its critical importance for growth of the fetus and the placenta [348, 349]. Maternal folate deficiency has been implicated in a number of pregnancy complications including increased risk of premature delivery, neural tube defects (NTDs) and reduced birthweight [44]. The incidence of such outcomes has been reduced by adequate maternal folate intake via folic acid supplementation or food fortification [350]. In the USA, Canada and Chile, mandatory folic acid fortification of food has already been introduced. However, in most other countries where food is unfortified, the population do not consume the recommended 0.4 mg of food folate per day [350]. The bioavailability of folate from ingested dietary sources is the only way of meeting the high demand for folate as mammals are unable to synthesise this vitamin and body stores are very limited.

This emphasises the critical importance of placental folate transfer for fetal development [351]. Overweight and obese pregnant women normally consume a poor diet lacking in vital micronutrients including iron and folate [38, 45]. These pregnancy complications have been associated with higher risk of delivering a child with NTDs [44], although some studies have shown that this risk is not modified by folic acid food fortification [352] nor is linked to maternal T2DM [353].

Folate transport within the placenta is dependent on maternal folate concentrations, as reflected by the positive association between maternal plasma, umbilical cord plasma and placental folate concentrations. Reduced placental transport of folate in obese mothers would thus result in fetal folate deficiency. One of the few studies in the literature investigating placental folate transport in obese pregnancies, hypothesised an association between obesity and lower fetal folate concentrations because of impaired placental folate transport [354]. However, the authors found that obesity did not alter fetal serum folate and placental folate transport at term.

As proposed by Solanky et al. (Figure 1.14), placental transport of folate from the mother to the fetus is mediated by the action of polarised folate transporters [351], which generate a higher folate concentration in the fetus relative to the mother [355]. First, folate binds to folate receptor-alpha (FR α) localised to the maternal-facing MVM of the syncytiotrophoblast which transports the vitamin by receptor-mediated endocytosis. Proton-coupled high-affinity folate transporter (PCFT) mediates the co-transport of folate and protons, whereas folate efflux across the fetal-facing BM is ensured via the reduced folate carrier (RFC) [356]. Expression and localisation of these three folate transporters within the placenta in early and late pregnancy have been also demonstrated [351, 357, 358].

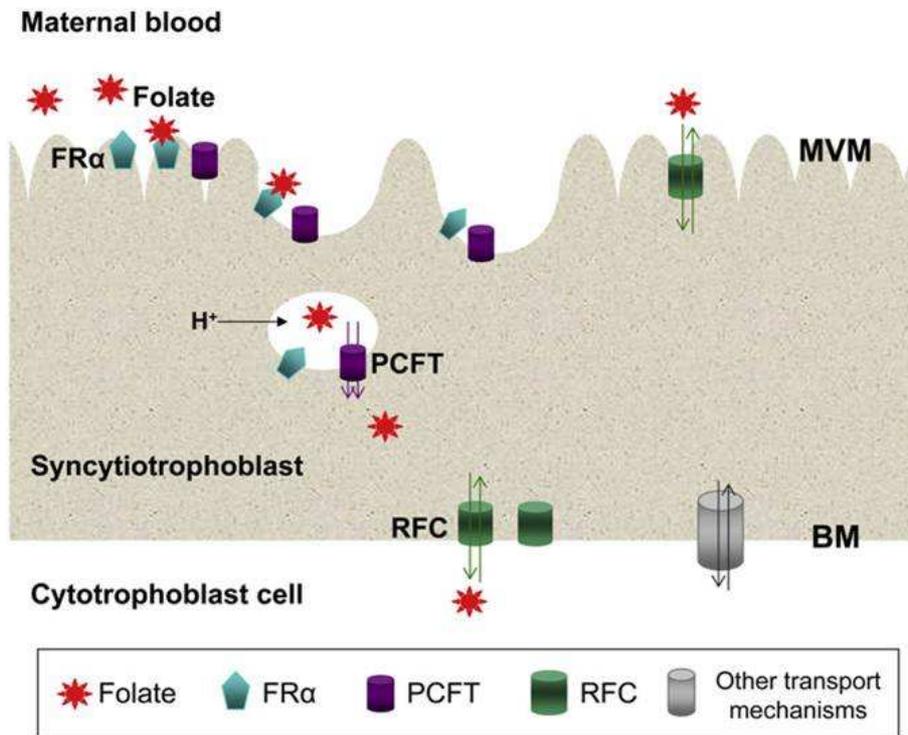


Figure 1.14: Proposed model for folate transport across syncytiotrophoblast in human placenta. From [351].

FR α localised to the MVM surface binds folate, and co-localisation of FR α and PCFT to MVM allows internalisation of both transporters into an endosomal structure. Acidification of the endosome occurs by vacuolar proton ATPase and a favourable H $^+$ gradient allows the H $^+$ -coupled movement of folate by PCFT into the cytoplasm. Whilst FR α and PCFT are re-cycled back to the MVM surface, RFC at the MVM surface provides an alternative folate uptake mechanism. Folate is transported across BM via an exchange mechanism by RFC and does not involve FR α or PCFT. Other transport mechanisms localised to BM may also play a role in folate efflux across this membrane. FR α : folate receptor alpha; MVM: microvillous plasma membrane; PCFT: proton-coupled high-affinity folate transporter; RFC: reduced folate carrier; BM: plasma basal membrane.

Folic acid is not biologically active itself but its biological importance is due to tetrahydrofolate (THF) and other metabolites obtained after its conversion to dihydrofolic acid in the liver [359]. This nutrient is crucial to the methionine cycle (Figure 1.15) by serving as a single carbon donor for 5-methyl tetrahydrofolate (5-MTHF) to convert homocysteine (Hcy) into methionine by methionine synthase (MS) [359, 360]. Methionine then acts as the precursor for the ATP-dependent synthesis of S-adenosylmethionine (SAM), a primary methyl donor involved in the methylation of DNA, RNA, proteins and phospholipids [361]. This reaction generates S-adenosylhomocysteine (SAH) as well as the methylated substrate.

As efficient cellular metabolism of SAH is crucial to prevent its inhibition of most SAM-dependent methyltransferases [362], rapid metabolism of Hcy is required to ensure sustained and optimal function of the methionine cycle in vivo [361, 362].

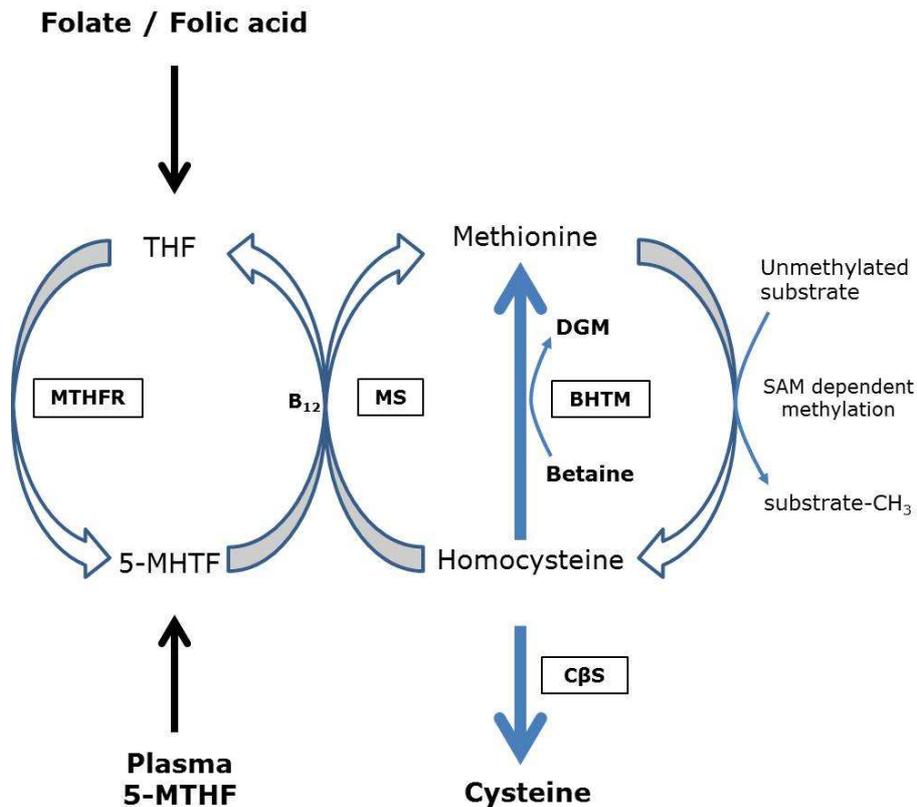


Figure 1.15: Homocysteine metabolic pathways.

Folate/folic acid is transported into the cell and converted to tetrahydrofolate (THF) or may enter the cell as 5-methyltetrahydrofolate (5-MTHF). Homocysteine can be metabolised by three metabolic pathways as described in text.

MTHFR: 5,10-methylene tetrahydrofolate reductase; MS: methionine synthase; DGM: dimethylglycine; BHTM: betaine-homocysteine methyltransferase; CBS: cystathionine beta-synthase; SAM: S-adenosylmethionine. Adapted from [351].

Experimental evidence has revealed that moderately elevated homocysteine concentrations (16–24 μM) may induce cytotoxic and oxidative stress, resulting in endothelial cell impairment [363]. Furthermore, exposure of trophoblast cells to Hcy (20 μM) causes increased cell apoptosis leading to inhibition of trophoblastic function [364].

Enhanced Hcy and lower folate concentrations in early pregnancy are associated with lower placental weight and birthweight with higher risk of adverse pregnancy outcomes [365].

Hcy can be rapidly metabolised in three different ways as illustrated in Figure 1.15 [359, 360, 362]. It can be remethylated to methionine using 5-MTHF as methyl donor catalysed by the vitamin B₁₂-dependent action of MS. THF is then re-cycled to form 5-MTHF catalysed by 5,10-methylene tetrahydrofolate reductase (MTHFR). Otherwise, betaine can be utilised as an alternative methyl donor for Hcy to generate methionine and dimethylglycine (DMG) catalysed by betaine-homocysteine methyltransferase (BHMT). Hcy can enter the trans-sulphuration pathway where it is converted to cysteine through the action of cystathionine beta-synthase (CβS). The irreversibility of this reaction ensures removal of Hcy from the methionine cycle. Consequently, cellular folate availability and the relative contribution of the Hcy-dependent metabolic pathways will influence cellular Hcy metabolism. Constitutive expression of the methionine cycle highlights the pivotal role of folate and one carbon unit metabolism in maintaining cellular homeostasis, with SAM levels directing the cellular utilisation of folate metabolites. MTHFR deficiency results in hypomethylation of DNA [366-368], reflecting a role for folate in DNA methylation [369] with the potential of programming metabolic dysfunction and CVD [366].

The importance of maternal folate as a methyl donor during embryogenesis [347] has been demonstrated in animal studies and will be discussed in more detail in Section 1.10.

1.10. Epigenetics and imprinted genes

There are three main processes that are tightly linked to gene expression: DNA methylation, post-translational histone modifications (e.g. methylation or acetylation) and nucleosome remodelling. The study of these three modifications is commonly known as epigenetics, indicating changes in gene expression or cellular phenotype that do not include alterations in DNA sequence. Epigenetic modifications within the placenta have been linked to alterations in fetoplacental lipid metabolism [370-372]. DNA methylation refers to the covalent addition of a methyl group to cytosine residues in specific genomic regions containing a high frequency of cytosine-phosphate-guanine (CpG) sites, known as CpG islands [373]. These sites, mostly associated with promoter regions of genes, are generally not methylated or have very low levels of CpG methylation throughout the genome [374]. Methylation of CpG islands in the promoter region, termed "hypermethylation", commonly leads to transcriptional repression. Conversely, no methylation of these CpG sites is known as "hypomethylation" and does not silence the gene [375]. Nonetheless, it is not the methylation of DNA per se that most greatly contributes to the transcriptional repression as the binding of various elements to methylated stretches of DNA has been shown to be the main factor [376]. Throughout embryo development important resetting of methylation patterns of germline and somatic lineage occurs. Whilst genome methylation of the zygote is almost completely removed, in between implantation and gastrulation [377], de novo methylation re-establishes the developing organism's methylation patterns [378]. Under normal conditions, these patterns are maintained throughout the rest of the organism's life (Figure 1.16). These finely regulated processes are ensured by a complex interplay of DNA methyltransferases (DNMTs), which use SAM as the methyl donor [371, 379]. De novo DNA methyltransferases-3-alpha and -3-beta (DNMT3 α , DNMT3 β), expressed mainly during early embryo development, contribute to set up the pattern of methylation [376].

DNA methyltransferase-1 (DNMT1) adds patterns of methylation to a newly synthesised DNA strand which is already methylated [380]. Altered levels of DNMTs can cause hyper- and hypo-methylation leading to down- and up-regulation of gene expression, respectively [381, 382].

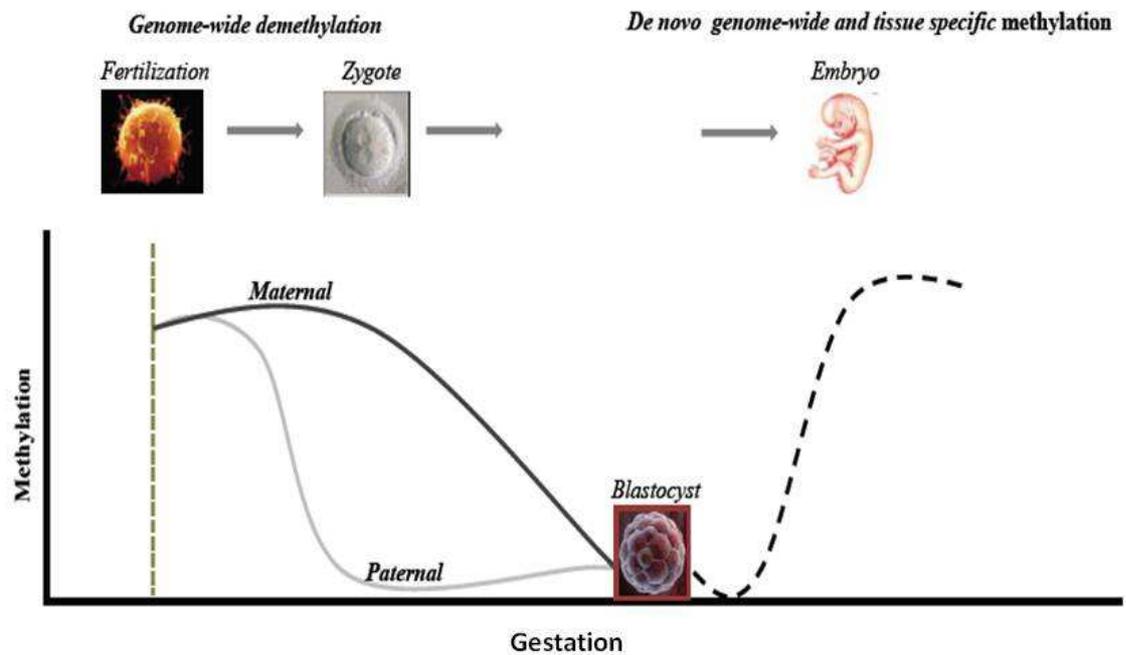


Figure 1.16: DNA methylation changes throughout embryo development.

From [383]. Epigenetic DNA methylation patterns markedly increased during early embryonic development. De novo DNA methylation patterns are made soon after early embryogenesis, whereas tissue-specific epigenetic patterns are placed at later developmental stages. After fertilization, there is active demethylation of the paternal genome (grey line) and passive demethylation of the maternal genome (black line), whilst de novo methylation occurs at the blastocyst stage (dotted line).

The process of DNA methylation is dependent on dietary methyl group donors and cofactors, which are involved in methionine and folate metabolism [347, 366, 367]. Maternal nutritional status before or at the time of conception can alter the methylation status of genes involved in trophoblast development as well as in placental transport of folate and other methyl donors [347, 351]. This will result in permanent changes in placental structure and function providing a direct link between placental function, gene methylation and fetal programming [347].

It has been shown that higher maternal insulin resistance is associated with lower methylation levels in the promoter region of placental adiponectin [384], suggesting that this epigenetic adaptation might induce persistent glucose metabolism changes in the mother and offspring later in life.

One class of genes regulated by epigenetic mechanisms are imprinted genes, the expression of which is dependent on their parent of origin. Imprinted genes are thought to be essential in the control of embryonic and placental development [385] as well as in functions later in life such as behaviours and metabolism [379]. Modifications to normal imprinting pattern have been associated with human syndromes of fetal overgrowth [386, 387], IUGR [388] and altered neonatal glucose homeostasis [389]. Consistent with the growth phenotypes observed, many of the imprinted genes currently known are expressed in the human placenta [379, 390], highlighting their role in controlling both fetal demand and placental supply. Thus, disease pathologies resulting from inappropriate imprinted gene expression may be due to an abnormal placental function [390]. DNA methylation controls the expression of the specific allele at differentially methylated regions (DMRs) [368, 391] by altering the binding of specific transcription factor and enhancer elements [385, 392]. It has been proposed that paternally expressed genes, such as IGF2, promote fetal growth while maternally expressed genes, like insulin-like growth factor receptor-2 (IGF2R), function to better ensure equal access to maternal resources for each offspring [387, 393].

The evidence linking human DNA methylation and periconceptional nutrition is limited and does not control for postnatal effects on the epigenome. It has been reported that two placental imprinted genes (H19 and IGF2), crucial for placental development and fetal growth, are not altered by diabetic pregnancies associated with fetal macrosomia [394, 395]. Another recent report showed that offspring DNA methylation profiling at imprinted loci is altered by maternal nutrient supplementation [368]. However, no large body of data is currently available, raising common interest in this epigenetic phenomenon during critical developmental process.

Advances in technology have allowed researchers to measure changes in DNA methylation patterns. The early use of restriction enzymes and Southern blotting with site-specific probes only consented the evaluation of methylation at specific restriction sites and required large quantities of DNA each time [379]. Pretreatment of the DNA with sodium bisulphite is one of the most widely used techniques to detect cytosine methylation of a specific DNA sequence in a genomic region [396]. This chemical introduces differences between methylated and unmethylated sites allowing single-base discrimination [397]. Methylation-specific PCR uses oligonucleotide primers, which bind and amplify sequences of template DNA modified by sodium bisulphite in a methylation- specific manner [398]. These procedures enable researchers to determine changes in single site DNA methylation.

Novel techniques such as quantitative real-time PCR and pyrosequencing now allow the relative quantification of the methylation status at multiple CpG sites within a region reflecting the prevalence of methylated alleles within the template DNA [379, 399]. Infinium array from Illumina allows quantitative measurements of DNA methylation levels at specific single-base resolution [400-402]. The correlation coefficient between DNA sequence and microarrays is generally consistent. However, very low or very highly expressed genes can show variable values causing inaccuracy [403, 404]. This discordance in microarray data suggests that some array probes do not capture the levels of the target and therefore, caution should be used when interpreting their output.

1.11. Hypothesis and Aims:

The primary hypothesis behind this thesis is that entering pregnancy with a BMI > 25 kg/m², diabetes or a combination of the two, promotes the development of metabolic alterations in the placenta leading to decreased placental nutrient transport efficiency and physiological adaptations in the mother and fetus. These potentially compromise newborn outcomes by increasing oxidative stress and amplifying the inflammatory milieu within the placenta, which can result in structural placental impairment, dysfunction and damage. Also, maternal obesity and gestational diabetes can influence folate status in the newborn by changing placental folate transport and DNA methylation leading to altered DNA methylation profile within the placenta.

In order to answer these questions, the following work will be undertaken:

1. Measurements of anthropometry, circulating hormones and metabolic factors, as well as placental and newborn outcomes, in a cohort of women delivering between 2008 and 2011 at the San Cecilio Clinical Hospital, Granada. Specifically, data was gathered on:
 - Maternal body weight, height and parity
 - Maternal 7 day food intake of glucose, TG, cholesterol, folate
 - Maternal plasma hormones (insulin, leptin), metabolites (glucose, TG, cholesterol, folate) and immune cells (monocytes, neutrophils, lymphocytes)
 - Placental weight and efficiency
 - Cord blood hormones and metabolites (glucose, TG, cholesterol, folate, monocytes, neutrophils, lymphocytes)
 - Newborn weight and growth (up to 12 months of age)

2. Determination of placental energy homeostasis, oxidative stress and inflammation on the placental metabolic phenotype through the analysis of:
 - Energy metabolism (leptin and insulin signalling)
 - mTOR signalling (mTOR, Akt, AMPK, p70S6K)
 - Oxidative stress (UCP2, SIRT1)
 - Glucocorticoid metabolism (GR, 11 β HSD2)
 - Inflammatory signalling (PPAR γ , TLR4, placental TG concentrations)

3. Determination of gene regulation of placental folate metabolism and analysis of DNA methylation in the placenta by identification of differentially methylated genes:
 - Folate transport (FR α , PCFT, RFC) and metabolism (MTHF)
 - Placental DNA methylation (DNMT1, -3)
 - Profile of differentially methylated genes
 - mRNA expression of key target genes identified as differentially methylated by methylation analysis (Illumina Infinium array)

Chapter 2 - General materials and methods

2.1. Ethical Approval

The proposed research was conducted according to the Declaration of Helsinki's guidelines and all experimental procedures were approved by the Medical Ethic Review Committee of Granada University, San Cecilio University Hospital Ethics Committee and the University of Nottingham Medical School Ethics Committee. Written informed consent was obtained from all subjects before their inclusion in the study and participants were assured of anonymity and confidentiality. After obtaining consent, each participant was allocated a study number, which was used thereafter for all sample labelling. The signed consent forms, with study allocation numbers, were held separately from all other study data. Patient clinical data was retrieved from the clinical medical records as soon as possible. Anthropometric assessments of mother, fetus and newborn were undertaken following the standards established by the Spanish Society of Gynaecology and Obstetrics, the Fetal Foundation and the Spanish Association of Paediatrics.

2.2. Participants

The subjects were participants in a longitudinal study on fetal adiposity programming by maternal genetics and nutrition (Preobe study P06-CTS-02341) undertaken between 2007 and 2010 and funded by Consejería de Innovación, Ciencia y Empresa de la Junta de Andalucía, Spain.

A total of 350 pregnant women aged between 18 and 45, with singleton pregnancies, were recruited between 12 and 20 weeks of gestation at three different primary health care settings (Clinical University Hospital "San Cecilio", Ambulatorio Zaidín-Vergeles, and Hospital "Materno-Infantil") in Granada, Spain. Among these, underweight women (self-reported pre-pregnancy BMI < 18.5 kg/m², n=4) were excluded as they did not fulfil inclusion criteria. In addition, 61 women did not complete the study, attending only 1 or 2 visits, and were not included.

Consequently, only 285 of 350 women recruited were included in this study despite some of them (control n=6; obese, n=2) failed to attend the visit at 34th gestational week (Figure 2.1).

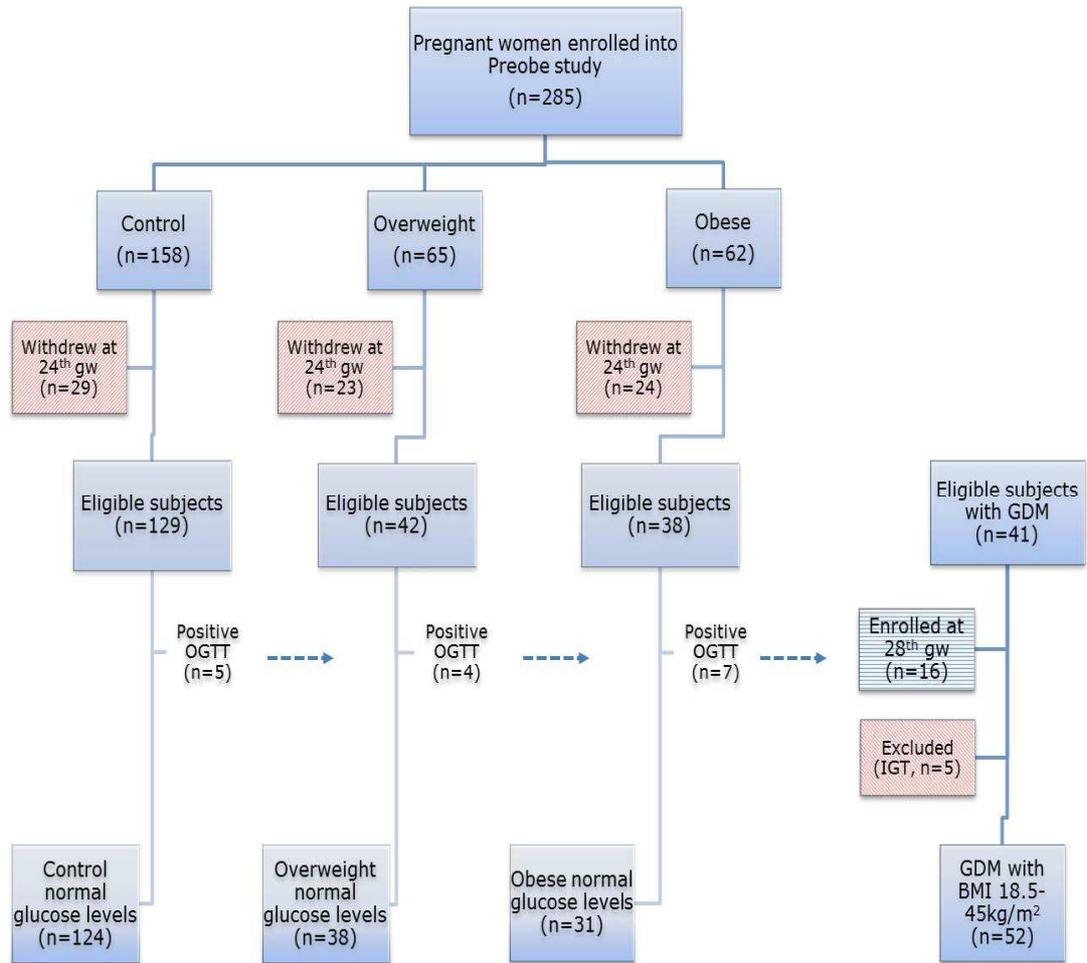


Figure 2.1: Participants in Preobe study and classification following BMI and gestational diabetes criteria.

n=number; gw: gestational weeks; OGTT: oral glucose tolerance test; GDM: gestational diabetes mellitus; IGT: impaired glucose tolerance.

Several mothers (n=76) dropped out of the study due to incapacity at attending one of the visits (work and/or family contingencies) and a substantial number of samples (n=24) were lost during delivery due to circumstances beyond our control (e.g. clinical complications during labour, fetal death, among others).

Of the 285 eligible participants, 16 were diagnosed with GDM, hence, they were added to the GDM group. Additional participants (n=41) diagnosed with GDM were recruited between 24th-28th weeks of pregnancy and classified as GDM. Since 5 women initially diagnosed with GDM were subsequently classified as having impaired glucose intolerance, they were excluded from the study. Finally, the total number of women who accomplished the study following the Preobe criteria was 245 out of 350. Among this, a subpopulation of 149 subjects was selected according to their BMI, and analysed in the Nottingham Early Life Nutrition Unit. Here, a further classification of women with GDM was performed according to their BMI (Figure 2.2).

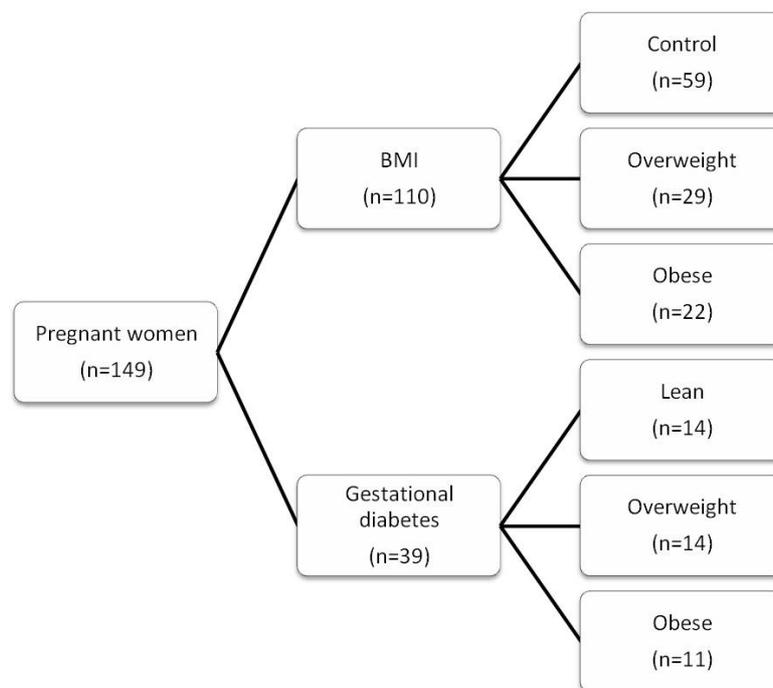


Figure 2.2: Preobe study.

Final classification of the Preobe participants into 6 groups according to BMI and gestational diabetes criteria as analysed in the Nottingham Early Life Nutrition Unit.

Other criteria for participant exclusion were receiving any drug treatments, having type 1 diabetes mellitus (T1DM) or any other diseases different from those included in the study. Maternal and fetal anthropometry was evaluated at 24 and 34 weeks of pregnancy and at birth. Gestational age was calculated as from the last menstrual period and through ultrasound scan considering a gestational age below 37 weeks as preterm delivery.

Large (LGA), average (AGA) and small for gestational age (SGA), defined as >90th, 10-90th, <10th birthweight population centile respectively [25], were calculated using the Lubchenco growth curves [405] adjusted for newborn weight and gestational age. Ponderal index (PI) was obtained from the formula:

$$PI = \text{Birth weight (g)} \times 100 / \text{length (cm}^3)$$

2.2.1. Pre-pregnancy BMI classification

Maternal pre-pregnancy BMI was calculated as weight in kilograms divided by height in meters squared. Maternal height was measured at the first antenatal clinic visit. As maternal weight was not possible to measure before pregnancy, self-reported weight was used in the calculation of BMI, despite there is concern about its reliability [42] due to incorrect classification, especially in women with high BMI [406-408]. At 20 weeks of gestation, the subjects were divided into three different groups based on their self-reported pre-gestational BMI. Mothers were either classified as control or comparison group (pre-pregnancy BMI ≥ 18.5 kg/ m² but <25 kg/m²; normal glucose tolerance; n = 59), overweight (pre-pregnancy BMI ≥ 25 but <30 kg/m²; normal glucose tolerance; n = 29), and obese (pre-pregnancy BMI ≥ 30 kg/m²; normal glucose tolerance; n = 22). Since maternal weight at delivery was not recorded, weight gain during pregnancy was calculated using clinical data only recorded at gestational week 24 and 34. To minimise estimation errors and increase accuracy, total gestational weight gain was calculated from the last recorded weight during pregnancy (34 gestational weeks) and self-reported pre-gestational weight. Each BMI category was then categorized as having gained inadequate, adequate, or excess weight based on 2009 IOM guidelines [61] until 34 gestational weeks. According to this, total maternal weight gain at delivery was divided by 40 (gestational weeks), and then multiplied by 34 (gestational weeks) as shown in Table 2.1.

Specifically, adequate GWG recommended by IOM was defined for each BMI category as 9.8-13.6 kg for women of normal BMI, 5.9-9.8 kg for overweight women and 4.2-7.6 kg for obese women. Inadequate and excessive GWG were defined as less and more than adequate GWG, respectively.

Table 2.1: Recommendation for weight gain during 40 and 34 gestational weeks in each pre-pregnancy BMI category according to 2009 Institute of Medicine (IOM) guidelines.

IOM classification	Pre-pregnancy IOM BMI (kg/m²)	Total IOM GWG (kg)	Equivalent GWG to 34 gw (kg)
Adequate	< 18.5	11.7-18.1	9.9-15.4
	18.5-24.9	11.5-16	9.8-13.6
	25-29.9	7-11.5	5.9-9.8
	> 30	5-9	4.2-7.6
Inadequate	< 18.5	< 11.7	< 9.9
	18.5-24.9	< 11.5	< 9.8
	25-29.9	< 7	< 5.9
	> 30	< 5	< 4.2
Excess	< 18.5	> 18.1	> 15.4
	18.5-24.9	> 16	> 13.6
	25-29.9	> 11.5	> 9.8
	> 30	> 9	> 7.6

Total gestational weight gain (GWG) corresponds to IOM weight gain until 40 gestational weeks (gw) [61], whereas GWG within the 2nd-3rd trimester was calculated by dividing IOM total gestational weight gain by 40gw and multiplying it by 34 gw.

2.2.2. Gestational diabetes mellitus

High risk women, including maternal age ≥ 35 years, BMI ≥ 30 kg/m², relevant past obstetric and family history and impaired glucose metabolism, had been previously screened and diagnosed for GDM. This was obtained by measuring fasting plasma glucose concentration or performing an oral glucose tolerance test (OGTT) at 16 to 18 weeks of gestation.

An OGTT involves a blood test before breakfast, then again 2 hours after a 75 g oral glucose drink. The results were interpreted according to the National Diabetes Data Group (NDDG) criteria [409] and the Third International Workshop-Conference on Gestational Diabetes Mellitus (GDM) [410]. If the first OGTT test was normal an additional 100 g OGTT was given at 24 to 28 weeks (Table 2.2).

Table 2.2: Criteria for diagnosis of gestational diabetes mellitus (GDM).

100g oral glucose (OGTT)	mg/dl	mmol/l
Fasting	105	5.8
1h	190	10.5
2h	165	9.1
3h	145	8

GDM was defined when two or more of the following glucose concentrations (fasting value and values at times after 100g oral glucose) were met or exceed; OGTT: Oral glucose tolerance test.

Persons whose fasting plasma glucose concentration was less than that required for a diagnosis of diabetes (<140 mg/dl), and whose plasma glucose value at 2 hours after a OGTT was intermediate between normal and diabetic (140-199 mg/dl), were classified as impaired glucose tolerance (IGT). In Nottingham, IGT women were excluded from additional analysis.

In addition, the BMI in GDM participants was considered in order to divide this group into gestational diabetic lean or normal weight (pre-pregnancy BMI ≥ 18.5 kg/m² but < 25 kg/m²; hyperglycaemic; n=14), gestational diabetic overweight (pre-pregnancy BMI ≥ 25 kg/m² but < 30 kg/m²; hyperglycaemic; n=14), gestational diabetic obese (pre-pregnancy BMI ≥ 30 kg/m²; hyperglycaemic; n=11). This allowed the effect of gestational diabetes per se and associated with an increase in body mass to be considered separately.

2.3. Maternal questionnaires

At the time of recruitment, each participant was asked whether she received multivitamin and mineral tablet supplements, such as iron, iodine and folate, during the first trimester.

Pre-pregnancy diet was evaluated with food frequency questionnaires, which were completed by each mother after instruction were given, and returned at the first antenatal visit at 24th week of pregnancy. However, in this study dietary intake was estimated using a 7 day food records (SDDR) given to the participants during the second visit at 34 gestational weeks, allowing information about folate and nutrient intake before delivery to be collected. Despite food-frequency questionnaires having been validated for determining food intake in a variety of populations, food records are considered the gold standard for dietary intake assessment in measuring actual food intake due to their higher accuracy and minor estimation errors [411].

Each subject was given verbal and written instructions by the investigator on how to record everything she consumed during the 7 days recording period (week 34-36). At the time of delivery or after a week of giving birth, food records were reviewed with each mother by a dietician for completeness and accuracy of food description and amount. For this purpose, a booklet of common food items and mixed dishes was used to facilitate the estimation of portion sizes.

Nutritional data were then analyzed for nutrient intake by using a nutritional software program (CESNID 1.0, Barcelona University, Spain) based on validated Spanish food tables (“Tablas de composición de alimentos del CESNID”) [412, 413].

2.4. Collection of biological samples

A chronological diagram of the study reflecting collection of biological samples from the mother as well as anthropometry of both pregnant women and newborns is illustrated in Figure 2.3.

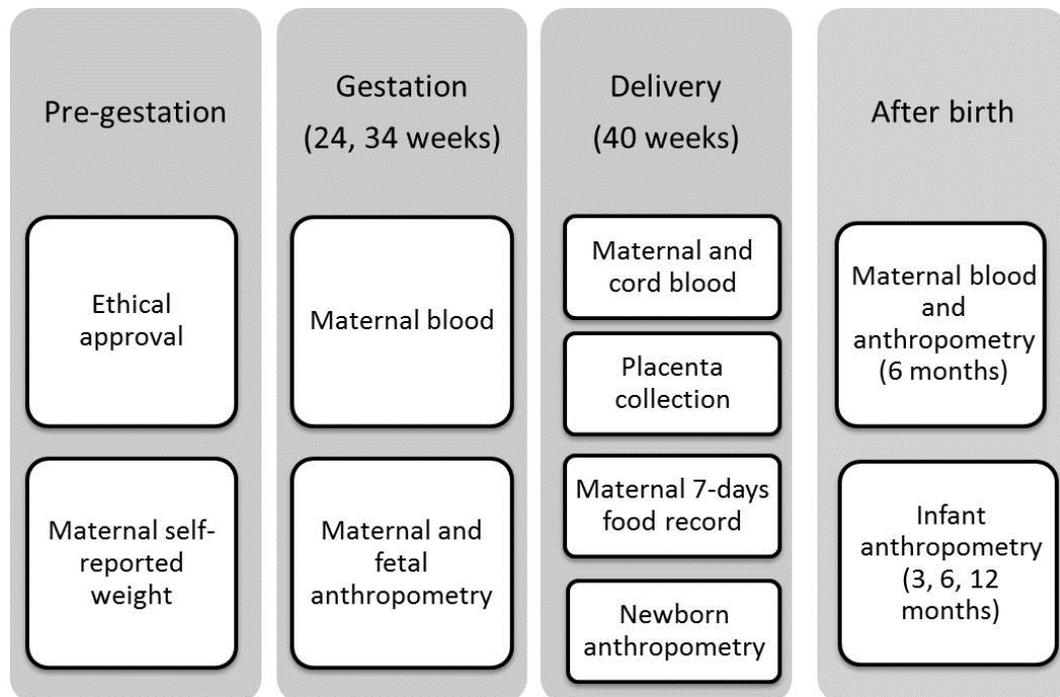


Figure 2.3: Programme of evaluations in the Preobe study.

Maternal examinations during gestation and after delivery. Measurements and collection of samples are reported in white boxes whereas greyed out boxes represent time before, during and after pregnancy.

Maternal pre-gestational weight was self-reported by the participants at the day of recruitment (approximately 20 weeks of gestation). During gestation, each mother attended two medical examinations, at 24 and 34 gestational weeks respectively, and anthropometry measurements were undertaken by a gynaecologist as well as maternal blood collected by a nurse.

At delivery, additional maternal biological samples as well as the placenta and cord blood were collected. At this stage, self-reported maternal weight was annotated, whereas newborn weight and height measured by a midwife.

Maternal anthropometry was evaluated at 6 months post-delivery and infant anthropometry was measured at 3, 6 and 12 months of age respectively by a paediatrician.

Food records questionnaires were handed to every subject at 34 weeks of gestation in order to be completed approximately one week before delivery and returned to the investigator at the next visit.

2.4.1. Blood sampling

All blood samples were collected after written consent was obtained from the mother. Maternal venous blood samples (25 ml) were collected at 24, 34 weeks of pregnancy and during labour for haematological and biochemical analysis, which was performed by the Clinical Analysis Service of San Cecilio Clinical Hospital. As mothers with gestational diabetes were recruited after 24 gestational weeks, their samples were collected by the second revision at 34 weeks. After birth, umbilical venous blood samples were taken (where possible) from cord double-clamped section, immediately after a small aliquot was used for blood gas analysis to record the fetal pH at birth.

An aliquot of each blood sample was immediately transferred into 7.5% EDTA, 3.0 ml chilled collection tubes (ref. 368857, Vacutainer®) for hematologic assessment and 8.5 ml chilled heparin collection tubes (ref. 367953, Vacutainer®) for biochemical analysis. Once collected, the heparinised plasma samples were centrifuged at 900 g for 10 minutes at 4°C and the plasma fraction was then transferred into sterile blood collection tubes (Vacutainer®) and stored at 4°C until performing biochemical analysis the following day.

The serum samples were left at 4°C for 15 minutes to allow the blood to clot fully and then they were centrifuged at 900 g for 10 minutes at 4°C. The resulting serum fraction was carefully transferred into fresh sterile blood collection tubes and stored at 4°C until use.

Haematological parameters were analyzed using a haematologic counter (Roche Diagnostic, model and serial number: Sysmex XE-2100 Sysmex XE=5000) and citometers BAYER (model and serial number: ADVIA 120-160858. ADVIA 120-160529. ADVIA 120- 162136).

Serum folate was evaluated by using the automatic analyser Elecsys 2010 with modular analytics E170 (Roche, Neuilly sur Seine Cedex, France). Serum leptin concentrations were measured using a sensitive ELISA kit (Biosource Kap 2281, Denmark), whereas cytokines were quantified by using eBioscience human Th1/Th2 ELISA Ready-Set-Go kit, according to the manufacturer's instructions.

2.4.2. Tissue sampling

All placentas samples were collected within 20-30 minutes of delivery, placed in a sterile tray, and carefully inspected for any visible abnormalities. Wet placental weights and diameter were immediately recorded before a triangular segment of the placenta was excised using a sterile scalpel and re-inspected for any visible abnormalities. Both the decidual layer along the basal plate as well as the chorionic surface and membranes were removed by sharp dissection and the remaining tissue was then divided into two portions along the long axis. A set of three samples (200 mg) of approximately two cm² in length was excised from the medial to the lateral edge of the placenta (Figure 2.4), rinsed twice in physiological saline (NaCl 0.9%) and then divided in two 0.5-1 cm² specimens. Those samples were immediately collected into labelled sterile 1.5 ml tube (Greiner®) containing RNeasy lysis solution (Qiagen®, Cat.Nr.76106), a reagent which immediately stabilizes RNA in tissues to preserve the gene expression profile eliminating the need for dry ice or liquid nitrogen. The remaining portions of the placental tissue were also sampled and, like the ones in RNA later, kept frozen under RNase free conditions first at -20°C and then at -80°C awaiting analysis.

Where delivery occurred overnight, the placentas were stored in the same way as described above although the time of collection sometimes was delayed by up to 1 hour due to circumstances beyond our control.

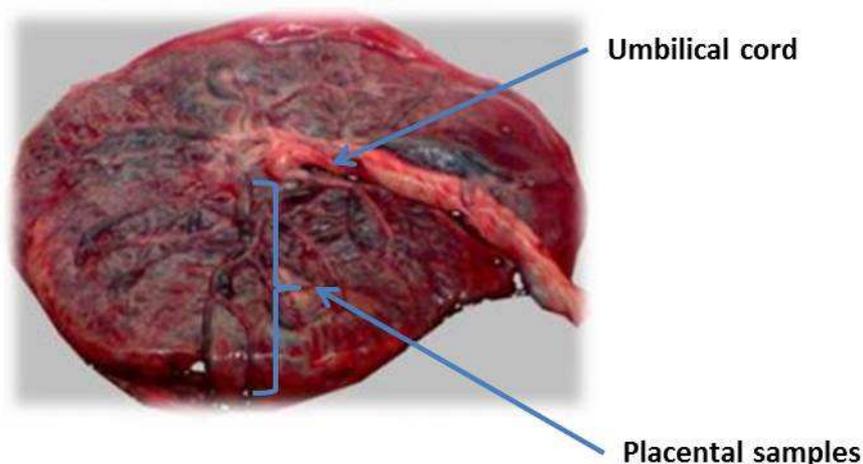


Figure 2.4: Diagram of placental sections taken after delivery.

One set of three samples was taken and kept frozen in RNAlater solution at -80°C .

2.5. Tissue analysis

Frozen placental samples and samples stored in RNAlater solution were shipped in dry ice to the Early Life Nutritional Academy department in the Queen's Medical Centre (University of Nottingham) where all laboratory-based techniques and protocols for tissue analysis were conducted. The selected tissues were immediately placed at -80°C while an accurate record of location and usage was created using validated software database (Pro-curo software Ltd, Bethesda, MD 20814-3993, USA). In order to minimise the risks of possible contamination and degradation of RNA, all equipment was autoclaved and sterilized prior to all experimental work as well as working areas cleansed of ribonucleases using RNaseZap® (Ambion, California, USA), 70% denatured ethanol (Ecolabs, Surrey, UK) and 1% Virkon disinfectant (Antec International, Suffolk, UK). In addition, all reagents were RNase and ribonuclease free while special care was taken to set up reactions and maintaining samples on ice wearing gloves.

To further avoid RNA contaminations by handling human samples, all tissue and molecular work was carefully performed under a UV cabinet (UV4PCR, Scientific Laboratories Supplies, UK) and a laminar flow cabinet was used when working with hazardous biological agents. All placental tissues and RNA samples were stored at -80°C until needed whereas DNA samples and some reagents were kept at -20°C.

2.6. Ribonucleic acid analysis

The number of copies of a gene transcript, expressed as messenger ribonucleic acid (mRNA), in a cell or tissue is determined by the rates of its expression and degradation. A common method for amplifying DNA is the polymerase chain reaction (PCR) [414], which allows specific detection and quantification of gene expression from small amounts of RNA. According to this, the RNA sample is first extracted from cells or tissues and then reverse transcribed into cDNA through a reverse transcription (RT) reaction in order to be measured and amplified during PCR at each cycle. The data thus generated can be analysed by computer software to calculate relative gene expression (or mRNA copy number) in several samples.

2.6.1. RNA extraction

Isolation of RNA of extremely high integrity must be performed cautiously as RNA can rapidly be degraded by the ubiquitous presence of ribonuclease enzymes [415]. Total RNA was extracted from placental tissues following an adapted version of the Chomczynski and Sacchi method [416] before further purification using RNeasy Plus Mini kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's instructions [417]. A single step using Tri Reagent® (Sigma Chemical Co. Poole, UK), which contains guanidinium thiocyanate and phenol, ensures both tissue lysis and protein denaturation, thus allowing inhibition of any RNase activity [418].

Subsequent addition of chloroform to the lysate leads to three phase separation by centrifugation resulting in an upper aqueous phase containing total RNA, an interphase with most of the dissolved DNA and a lower organic phase with proteins. In the final step, total RNA ($\geq 100 \mu\text{g}$) is recovered from the aqueous phase by precipitation with either isopropanol or ethanol while passing to an RNAeasy mini column. After appropriate binding of total RNA, the RNAeasy membrane is washed with buffers of ethanol and guanidium salt-based to remove any contaminants and RNA is finally eluted using nuclease-free water.

Extracted RNA yields are unknown and can contain proteinaceous impurities; therefore, the concentration and purity of extracted samples was determined spectrophotometrically (Nanodrop[®] Technologies, Wilmington, USA). Since nucleic acids absorb UV light at 260 nm, the concentration and purity of extracted RNA yields was estimated through relative wavelength absorption (1 OD unit = 40 μg RNA/ml) [419]. In addition, protein absorbs UV light at 280nm therefore the presence of protein contamination in the sample was measured through a ratio of absorption between wavelengths 260nm:280nm; optimal absorbance between 1.8 and 2 indicated good quality RNA [420].

2.6.1.1. RNA extraction procedure

Total RNA was extracted from 100 mg placental samples with 1 ml TRI Reagent[®] and then homogenised at 800 g using a Dispomix homogeniser (Medic Tools, Zurich, Switzerland) and centrifuged for 1 minute at 800 g. Samples were left at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes and the supernatant was transferred to a sterile 1.5 ml eppendorf tube.

Next, 200 μl analytical-grade chloroform (Fisher Scientific, Leicestershire, UK) was added followed by vortex for 15 seconds. Incubation at room temperature for 15 minutes then allowed phase separation.

After centrifugation at 12500 g for 15 minutes at 4°C the upper aqueous phase ($\approx 600 \mu\text{l}$) was carefully transferred to specially designed genomic deoxyribonucleic acid (gDNA) columns and centrifuged at 8000 g for 30 seconds at room temperature to allow efficient removal of any remaining gDNA contamination. The supernatants were then centrifuged at room temperature for 1 minute at 8000 g and the gDNA columns discarded before one volume ($\approx 700 \mu\text{l}$) of ice cold 70% ethanol was added to the flow-through providing appropriate binding conditions for RNA. RNeasy Plus Mini extraction kit (Qiagen, West Sussex, UK) was used to extract RNA from each individual sample according to manufacturer's instructions.

The solution was pipetted to an RNeasy spin mini column allowing total RNA binding to the membrane after centrifugation at 8000 g for 15 seconds at room temperature. All contaminants were washed away by discarding the flow-through. RW1 buffer (700 μl) was used to wash the column which was centrifuged at 8000 g for 15 seconds at room temperature before discarding the flow-through. RPE buffer was then added (500 μl) to the column followed by centrifugation at 8000 g for 2 minutes at room temperature and discard of the flow-through before transferring the minispin column to a sterile 2 ml collection tube. Further centrifugation at 12500 g for 1 minute ensured elimination of any RPE buffer carry-over, which could interfere with future RNA applications. The minispin column was transferred to a sterile 1.5 ml collection tube and high-quality RNA was then eluted in 50 μl RNase-free water after centrifugation at 8000 g for 1 minute. RNA concentration and purity were determined using a Nanodrop®ND-1000 (Nanodrop Technologies, Wilmington, USA) spectrophotometer by pipetting 1.5 μl each RNA preparation. In order to be used in the quantitative RT-PCR analysis, 10 μl aliquots from each extracted RNA stock were then diluted with RNase-free water to 1 $\mu\text{g}/\mu\text{l}$ in a final volume of 20 μl . All extracted RNA samples were labelled with an individual sample identification code (ID) and stored in 0.3 ml microcentrifuge tube at -80°C until further analysis.

2.6.2. RNA integrity

The integrity of total RNA was assessed by running an aliquot of 2 μ l from 13 random samples stored in RNA later solution on a denaturing agarose gel stained with 0.01 % ethidium bromide. Visualization of sharp and clear 28S and 18S rRNA bands on the gel as shown in Figure 2.5 (samples 1-5, 7-8, 10-13, lane 1 and lane 2 respectively) confirmed the integrity of the sample. A 2:1 ratio (28S should be twice as intense as 18S) is a good indication that the RNA is completely intact [419], whereas a partially or completely degraded RNA would show a smeared appearance or lacking in sharp rRNA bands (partial degradation, Figure 2.5, sample 6), or it would appear as a very low molecular weight smear (complete degradation, Figure 2.5, sample 9).

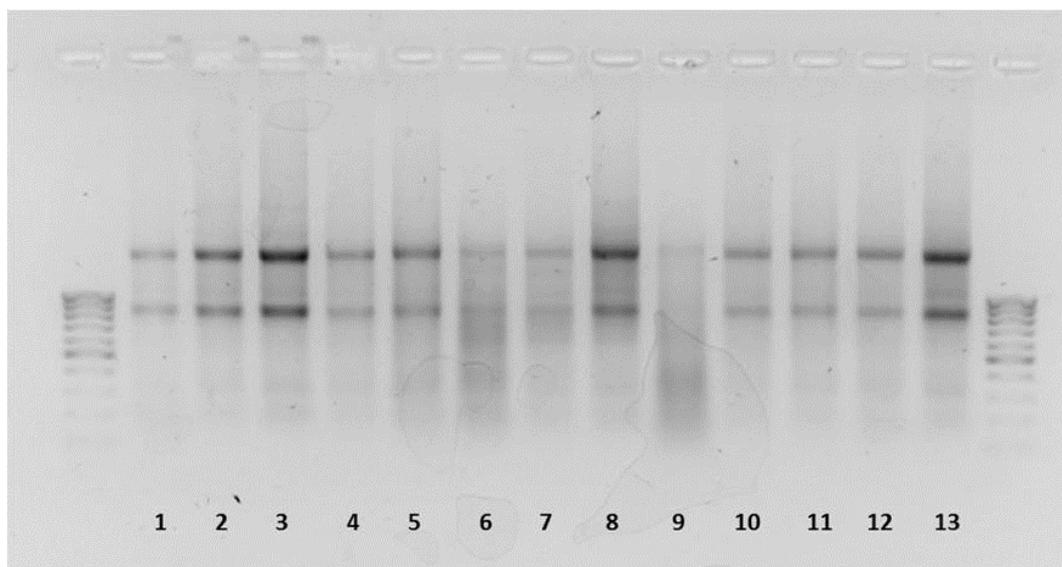


Figure 2.5: Purity of RNA samples.

2 μ l total RNA were run beside DNA marker ladder on a 2% denaturing agarose gel. From top to bottom: two intense bands corresponding to 28S and 18S ribosomal RNA bands respectively are clearly visible in all intact RNA samples (1-5, 7-8, 10-13) whereas partially degraded (6) and completely degraded samples (9) do not show well-defined bands.

In the case of RNA, a sample containing no protein would be expected to have a 260nm/280nm ratio of approximately 2 [421]; however in the two samples above mentioned, a 260/280 ratio of 1.65 (sample 6) and 1.52 (sample 9) was detected, indicating sample contamination. Therefore, all samples with a 260nm/280nm ratio below 1.8 were excluded from further analysis.

2.6.3. Reverse transcription polymerase chain reaction

Following extraction, the single stranded RNA template must be first reverse transcribed into a double stranded complementary DNA (cDNA). This molecular process is known as reverse transcription PCR (RT-PCR) which allows the detection of a low copy number of mRNA molecules by using a DNA primer sequence and a reverse transcriptase. This enzyme is provided with both RNA and DNA polymerase activity ensuring efficient transcription, elongation and synthesis of the single stranded RNA template into a double stranded cDNA which is then ready for standard PCR and further amplification. RT-PCR is widely used in the diagnosis of genetic diseases and in the cellular determination of the abundance of specific mRNA molecules as a measure of gene expression [422].

In this work, cDNA was created using the High Capacity RNA-to-cDNA kit (Applied Biosystems, CA 94404, USA) which has the advantage to require fewer pipetting steps thus reducing reverse transcription time since it consolidates all the components of the reaction into two tubes, one RT buffer containing deoxyribonucleotide triphosphates (dNTPs) and an enzyme mix containing MuLV reverse transcriptase and RNase-free inhibitor protein.

2.6.3.1. RT-PCR procedure

A set of RNase-free 0.2 ml eppendorf tubes were labeled for every sample, which was run concurrently with a no-reverse transcription enzyme control (NRTC) to ensure transcription efficiency and a negative control without RNA template (NTC), replaced with nuclease-free water. The reaction consists of pipetting 2 μ l the 1 μ g/ μ l RNA samples (or nuclease-free water) into the RT and controls labelled tubes placed on Eppendorf PCR Cooler (Sigma Aldrich) before adding 18 μ l each master mixes to reach a final volume of 20 μ l as described below:

Volume (µl)	Reagent
2	RNA 1µg/µl (or water)
10	2x RT buffer mix
1	20x RT enzyme mix
7	RNase-free water

After centrifugation for 1 minute at 12500 g, the tubes were then transferred to the PCR machine (Techne Thermal Cycler, Stone, Staffordshire, UK) using the RT for the the RT reaction to proceed for 60 minutes at 37°C. After the reaction was stopped and cooled at 8°C the samples were stored in a freezer at -20°C.

2.6.4. Classical polymerase chain reaction

The PCR technique allows isolation and amplification of a specific region of a small chosen DNA sequence using a pair of complementary primers in an enzymatic reaction. The method relies on thermal cycling which uses repeated cycles of heating and cooling to denature and replicate the two strands of DNA. During the DNA melting step the doubled stranded DNA is physically separated subsequently allowing primers to anneal to the DNA sequence at lower temperature. These short DNA oligonucleotides (primers) are complementary to the target DNA and ensure selectivity of the PCR process enabling its amplification under specific thermal cycling conditions [423]. Adequate design of primers, by considering their length and sequence, annealing and melting temperatures, is critical for a successful PCR reaction as poorly designed primers can result in a decreased product yield and/or formation of primer dimers. The sequence that is amplified during PCR is called amplicon or PCR product and corresponds to the part of the gene between the two primers.

The bound primer is elongated by binding free phosphonucleotides in the 5' to 3' direction by a thermostable DNA polymerase (usually Taq Polymerase extracted from the bacteria *Thermus aquaticus*) which maintains its activity at high temperature (>105°C) for a long period of time [414]. Subsequently, both DNA strands – the original template and the newly synthesised strand including the primer – are denatured allowing the primers to bind to both of them again. Following each PCR cycle, the DNA product is doubled, leading to an exponential amplification of the target sequence for the number of cycles (2^n , n = number of cycles). In the case of cDNA, the final amount of amplicon copies is representative of the number of intact mRNA molecules for this gene present in the target tissue at the time of RNA isolation.

2.6.4.1. Classical PCR procedure

Conventional PCR method was performed to amplify target cDNA, which was then used to prepare standards dilutions for the Q-PCR reaction. A set of sterile 0.2 ml eppendorf tubes was labelled on ice for each sample and reaction mixture added per tube as follows:

Volume (µl)	Reagent
2	cDNA
10	Thermo start PCR master mix
1	Forward primer (1:10)
1	Reverse primer (1:10)
6	RNA-free water

Negative controls containing no template cDNA and no primers were run concomitantly to ensure the integrity of the prior RT-PCR reaction, and to confirm quality controls against genomic DNA contamination.

As shown above, 2 µl cDNA or nuclease free water was then pipetted into the each 0.2 ml tube respectively before 18 µl PCR master mix was added to reach a final volume of 20 µl.

Tubes were then centrifuged for 1 minute at 12500 g before being loaded into the Techne thermal cycler (Scientific Laboratories Supplies Ltd, Nottingham, UK) and run on a 60°C hot start PCR program (Table 2.3). The thermo-start master mix (Thermo Scientific, Abgene, Epsom, UK) includes the thermo-start Taq DNA polymerase and all the components to perform a rapid and reproducible PCR. The enzyme is completely inactive at room temperature, preventing the formation and subsequent amplification of non-specific products, and requires an activation step at 96°C for 15 minutes.

Table 2.3: Hot start PCR program conditions.

Step	Temperature (°C)	Duration
Initiation	105	4 min
Enzyme activation	96	15 min
PCR cycles (n=35)		
Denaturation	94	30 sec
Primer annealing	60	30 sec
Primer Extension	72	60 sec
Final Extension	72	7 min
Hold	4	Indefinitely

2.6.5. Design of primers for RT-PCR

Primer sequences for RT-PCR were designed with Beacon Designer 4.0 software (Premier Biosoft, Palo Alto, USA) and Primer3 Input 4.0 software (available online <http://frodo.wi.mit.edu/primer3/>) which automatically interpret the homology by using the human genome sequence from the National Centre for Biotechnology Information (NCBI) online database. To avoid amplification of contaminating genomic DNA, primers were designed ensuring that one half of the primer hybridizes to the 3' end of one exon and the other half to the 5' end of the adjacent exon with an annealing site that is at least 300–400 bp apart. The software also guarantees greater specificity to the target gene by creating primers that flank exon boundaries containing at least one intron sequence, resulting in products amplified from cDNA (no introns) being smaller than those amplified from genomic DNA (with introns). Similar optimum annealing temperatures, guanine-cytosine base pair content and length of the primers designed conferred ideal reaction conditions. The target sequence was then tested using the NCBI basic nucleotide BLAST search engine (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) ensuring that each primer was amplifying the correct sequence without cross-hybridising with other gene sequences within the genome.

All oligonucleotides were developed and purchased from Sigma-Aldrich UK, resuspended in an appropriate volume of RNase-free H₂O and stored at -20°C at a stock concentration of 100 µmol/l. Primers details for each gene analysed in this thesis are reported in Table 2.4.

Table 2.4: Oligonucleotide primer sequences used for quantification of target and endogenous control gene cDNA by real time PCR.

NCBI sequence	Target Gene	Sense primer sequence (5'-3')	Anti-sense primer sequence (5'-3')
NM_001014432	Akt1	TCACCATCACACCACCTGACCAAG	CGCCTCTCCATCCCTCCAAGC
NM_001130823.1	DNMT1	TTCTTCGCAGAGCAAATTGA	CCATGAAGGAGGCAGATACG
NM_022552.4	DNMT3a	AAGCCTCAAGAGCAGTGGAA	AAGCAGACCTTTAGCCACGA
NM_058186.3	FAM3B	GCCAAAATCTGCTTTGAGGA	CACCATGAAGAGCAGGGATT
NM_016725.2	FRa	CACTCCCTGCCTGTCTCC	TCTGCTCTGCTCTACTCC
NM_001080432	FTO	TGGAAGCACTGTGGAAGAAGATGG	TGTTCGGGCAATTCGTGACTGG
NM_001134945	GHRL	GGCAGAGGATGAACTGGAAG	GAGGTGAGTAAGGCTTGTGG
NM_000176	GRa	GGAATAGGTGCCAAGGATCTG	GCTTACATCTGGTCTCATGCT
NM_000196	HSD11B2	ACTCATGGACACATTCAGCT	CCACGTTTCTCACTGACTCT
NM_000875	IGF1R	CCTTGCTGCTGCTCCATCC	GACTCTGCTCCTGCGTGTG
NM_005544	IRS1	CAGTCCTAACCGCAACCAGAGTG	CGCTGCTGCTGCTGCTACC
NM_000230	LEP	TCCTGAGACCTGACAAGCACTG	CCCAAACCTCCTTTTCATCTTCC
NM_002303	LEPR	GCTAAGGTCGGAGTTCTGC	CTGAAGGATAATGCCACGAGAG
NM_017898.3	MOSC2	GACAGGTTTTGGCTGGTGAT	TTTGTTTGAGGAAGGCTGCT
NM_005957	MTHFR	TCCCGTCAGCTTCATGTTCT	TGTCGTGGATGTACTGGATGA
NM_004958	mTOR	CGAGGGCAGCAACAGTGAGAG	ACAAGGAGATGGAACGGAAGAAGC
NM_003161	p70S6KB1	GACGCACTGAGCCTAAGC	CCAGGTCTATGTCAAACACTCC
NM_015869.4	*PPAR γ	TTCAGAAATGCCTTGCAAGTG	CCAACAGCTTCTCCTTCTCG
NM_022551.2	18S	GATGGGCGGCGGAAAATAG	GCGTGGATTCTGCATAATGGT
NM_001142498	SIRT1	TACCGAGATAACCTTCTGTTCCG	GTTTCGAGGATCTGTGCCAAT
NM_006996.2	RFC	CAGCATCTGGCTGTGCTATG	TGATGGTCTTGACGATGGTG
NM_080669.4	PCFT	ATGCAGCTTTCTGCTTTGGT	GGAGCCACATAGAGCTGGAC
NM_001135930.1	TLR4	AAGCCGAAAGGTGATTGTTG	CTGAGCAGGGTCTTCTCCAC
NM_001033611.1	*UCP2	CCCGAAGCCTCTACAATGGG	CTGAGCTTGAATCGGACCTT
NM_003391.2	Wnt2	GTGGATGCAAAGGAAAGGAA	AGCCAGCATGTCCTGAGAGT

*Human primers were used for all genes except for UCP2 and TLR4 which were analysed using sheep primers after homology was tested with the BLAST database [424].

2.6.6. Agarose gel electrophoresis and DNA extraction

PCR products can be analysed and resolved using a matrix gel (either agarose or polyacrylamide) by electrophoresis. DNA molecules are negatively charged and can be separated according to their size by applying an electric field through the gel which will make the smaller fragments migrate further and quicker towards the positive electrode [425]. A DNA dye, ethidium bromide, is added to the gel matrix to visualise the DNA fragments using ultra-violet (UV) fluorescence. The unknown DNA band size can be estimated by running a DNA marker ladder alongside the samples before proceeding with their extraction from the agarose gel using a commercial gel extraction kit.

In this study, the DNA gel fragments were isolated with the QIAquick® Spin extraction kit (QIAGEN) which combines spin-column technology with the high DNA affinity of a silica membrane which selectively binds DNA in the presence of a high concentration of salt. Washing steps with different buffers provided by the kit also ensure the elimination of any possible contamination or residual agarose before eluting the purified DNA using either nuclease-free water or elution buffer.

2.6.6.1. Agarose gel electrophoresis & DNA extraction procedure

A 2% (w/v) agarose gel was prepared by dissolving 2% agarose electrophoresis grade (Invitrogen Life Technologies) in 50ml of 1x Tris-acetate-EDTA (TAE) buffer, from a 50x stock solution of 2M tris (hydroxymethyl) aminomethane base (tris), 1M glacial acetic acid (Fisher Scientific), 50mM Na₂ EDTA buffer. After heating, the solution was mixed with 2 µl 10mg/ml ethidium bromide for visualization and then cooled to form a gel.

Following, each PCR product (20 µl) was mixed with 5 µl glycerol blue dye (2.5% Ficoll® - 400 11mM, EDTA 3.3mM, Tris-HCl 0.017%, SDS 0.015%, bromophenol blue) and was loaded into separate lanes together with a DNA marker ladder (100 bp Blue eXtendec, Bioron, Ludwigshafen, Germany). Electrophoresis was run at constant voltage (100 V) for about 45 minutes for small gels (V=50 ml), 60 minutes for medium gels (V=100 ml) or 75 minutes for large gels (V=150 ml). After DNA separation, the PCR product was visualised under a UV trans-illuminator CCD camera (Fuji film luminescent image analyzer LAS-1000 V1.01) with an ultra violet lens for imaging and its size estimated by comparison to the ladder. Fluorescent target bands were cut out of the gel, transferred into sterile 1.5 ml eppendorf tubes and weighed before proceeding to DNA purification using the QIAquick® gel extraction kit (Qiagen). The dissected DNA bands were re-dissolved in 300 µl GQ buffer per 100 µg of gel using a thermoblock at 50°C for 10 minutes and this was followed by addition of 100 µl isopropanol (Fisher Scientific) per 100 µg of gel to ensure DNA precipitation. The mixture was then pipetted into a sterile QIAquick spin column and centrifuged at 12500 g for 1 minute in order to discard the GQ buffer and isopropanol filtrate. To remove any remaining trace of agarose, another 500 µl GQ buffer was added to the spin column and centrifuged at 12500 g for 1 minute before discarding the filtrate. An additional wash was performed with 700 µl PE buffer containing ethanol followed by 5 minutes of incubation and 1 minute of centrifugation at 12500 g with final discard of the filtrate. The empty column was centrifuged again at full speed for 1 minute to ensure removal of any residual PE buffer and the spin column was then transferred to a fresh 1.5 ml eppendorf tube. Elution of DNA was obtained by carefully pipetting 30 µl elution buffer (EB) directly onto the column membrane which was centrifuged at 12500 g for 1 minute and then discarded. Before storing all purified DNA samples at -20°C until needed, their concentration and purity were measured using the Nanodrop spectrophotometer and the resulting values were used to set the standard of the Q-PCR reaction.

Newly designed primer products were sent for sequencing at the University of Nottingham's Centre for Genetics and Genomics (Queen's Medical Centre, Nottingham UK) and the resulting sequences were cross-referenced using the NCBI online database (BLAST).

2.6.7. Real-Time polymerase chain reaction

Quantification of a target DNA sequence can now be achieved in real time through advanced PCR technologies which by including additional analysis steps ensure a reduction of time expenditure and the chance of having contamination [426]. Quantitative PCR (Q-PCR) detects and quantifies a PCR product by using a fluorescent signal whose intensity is correlated to the amount of DNA template (the greater the intensity, the higher the DNA concentration detected). The level of fluorescence emitted by the dye is normally detected at a crossing point (Cp) or threshold point (Ct) that reflects the number of cycles after which the signal has crossed certain level of background fluorescent [427]. As the cycle threshold is correlated to the initial concentration of the DNA template this parameter is used for the quantification of the targeted gene expression.

The increase in the signal fluorescence is graphically represented by the instrument as a sigmoidal curve reflecting the fact that the amplification of the targeted sequence within the PCR reaction is exponential. The linear section of the curve can be compared to signals from other samples run within the same experiment. Figure 2.6 shows the magnitude of normalised fluorescence generated by the software at each PCR cycle that is expressed as ΔR_n , where R_n is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye (i.e. ROX).

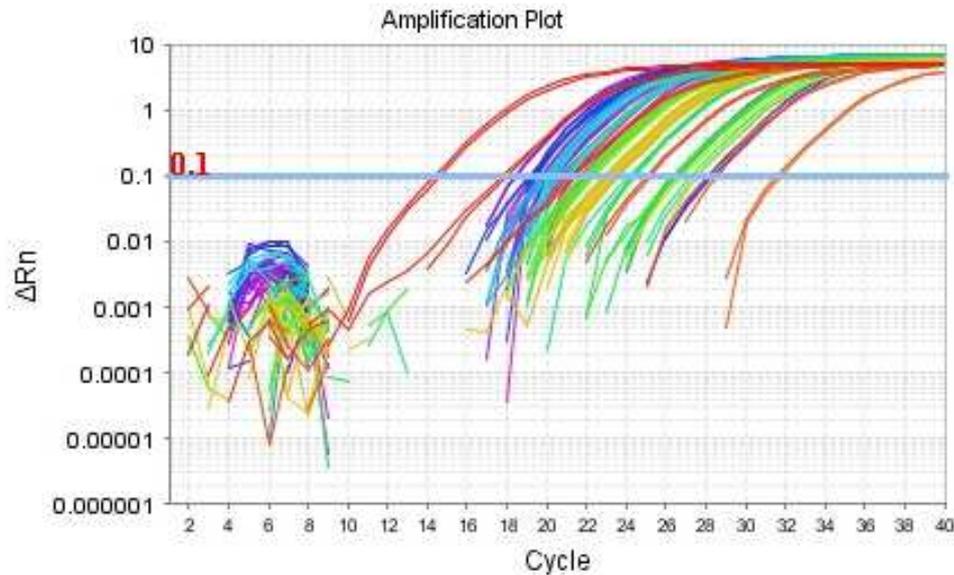


Figure 2.6: Example of amplification curves for a gene of interest in Q-PCR.

Fluorescent signal crosses a threshold and increases exponentially until a plateau from expenditure of reagents or cycle number. Amplification plot shows the variation of log (ΔR_n) with PCR cycle number calculated at each cycle as $\Delta R_n(\text{cycle}) = R_n(\text{cycle}) - R_n(\text{baseline})$. R_n is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye (i.e. ROX).

Red lines represent external standard dilutions (10^{-3} to 10^{-8}) while colored lines correspond to the unknown samples, which fall within the standard curve. Samples that fell outside the last standard (10^{-8}) were run again using a more diluted standard (10^{-9} or 10^{-10}) to avoid false estimation of gene expression.

The unknown cDNA concentration is calculated by interpolation using a standard curve. Gene standards are prepared from serial dilutions of cDNA amplicon of the measured gene (i.e. 10^{-3} to 10^{-8} ng/ml or 10^{-4} to 10^{-9} ng/ml if the gene is low expressed) and are run with the samples into a 96-well plate. A standard curve is produced by plotting the log concentration of each standard against their Ct value resulting in a straight line equation.

This reflects the coefficient of correlation (R^2) and the efficiency of the reaction (E), which is calculated by the following formula derived from the basic equation describing PCR amplification [428]:

$$E = 10^{(-1/\text{slope})} - 1$$

Measurements are only considered successful if the standard curve shows an R^2 of >0.95 and an efficiency of between 1.95 and 2.05.

An efficiency of 2 indicates the ideal amplification (i.e. a doubling of the number of amplicon molecules with each thermocycle).

Normalisation of target gene expression levels must be performed to compensate intra- and inter-kinetic variations of RT-PCR reactions (sample-to-sample and run-to-run variations) [429]. To quantify any variations in the abundance of a target gene between samples, mRNA expression levels are normalised to a reference gene, which is highly expressed in all cells or tissues at relatively constant levels [430]. This gene is used to compare the expression of the target gene in the same sample allowing quantification of its expression (see section 2.6.8 for details of housekeeping genes used in this study). Results can then be analysed assuming an efficiency of 2 based on the $\Delta\Delta C_t$ method developed by Pfaffl [431]. As illustrated in the formula below, the amount of a target gene (QT) is normalised to a reference control, where ΔC_t is the difference between C_t of target gene and C_t of reference gene (of the same sample):

$$QT = 2^{-(C_t \text{ target} - C_t \text{ reference})}$$

In this mathematical model, the relative expression ratio of a target gene is calculated based on the efficiency (E) of the reaction and on the difference in threshold cycles (C_t) between an unknown and a control sample in comparison to a reference gene. The method assumes that the efficiency of the PCR reaction is 100%, indicating that primer concentrations have been properly optimized and cDNA strands replicate entirely within each cycle, hence $2^{\Delta C_t}$. This assumption of efficiency is verified by the standard curve, whereby general accepted efficiency range from 90-105% [431].

Q-PCR reaction can be affected by a number of variables including length of the amplification, secondary structure of the product and primer quality [432]. As with standard PCR, primer design is a key component to amplification of specific region of a sequence. One disadvantage of using fluorescent dyes, such as SYBR Green, is the generation of false positive signals which can be originated from non-specific DNA bindings of the dye.

Therefore, to ensure that the amplified PCR product specifically corresponds to the targeted sequence, a melt curve stage is performed following the PCR cycles. The plot of data collected generates a melt curve (or dissociation curve) for every sample where the peaks display the melting temperature (T_m) at which 50% of the cDNA is double-stranded and 50% of the cDNA is dissociated into single-stranded DNA. A specific PCR amplification should be identified by only one peak in the melt curve whereas a double- or multi-peaks melt curve would reflect a primer dimer or non-specific double stranded DNA bindings. Further qualitative analysis can be performed by DNA sequencing after the product has been extracted by gel electrophoresis.

2.6.7.1. Q-PCR procedure

Following DNA gel extraction, serial dilutions (1:10) up to and including 1×10^{-9} were prepared from 1 ng/ μ l cDNA standard template for all target genes (housekeeping genes included). A standard curve was first tested in the Q-PCR instrument together with two samples and a no-reverse transcription enzyme control to ensure transcription efficiency. In addition, a negative RNA template control, replaced with nuclease-free water, was also run in the same reaction. When primers annealing was not optimal, different conditions of primer concentrations (pilot concentration=250nM), i.e. 100, 300 and 500 nM, or primer annealing temperature (pilot temperature=60°C), i.e. 58, 62 and 64°C, were modified to improve reaction conditions.

For gene expression analysis, all unknown samples were measured with housekeeping gene 18S to normalise all unknown cDNA samples. Following primers optimisation, samples, standards and negative controls were run for each optimised gene in a total volume of 15 μ l per Q-PCR reaction. 4.5 μ l samples, standards and negative controls were loaded into a 96 well PCR plate (Abgene) and, subsequently, 10.5 μ l master mix containing the following reagents was added:

Volume (µl)	Reagent
4.5	cDNA (1:10)
7.5	SYBR® green (Thermo Scientific)
1.5	Forward primer (250 nM)
1.5	Reverse primer (250 nM)

Samples and standards were run in duplicate to calculate the coefficient of variation within each experiment. For each gene analysed, negative controls for no template cDNA and no primers were also run. The enzyme SYBR® green (Thermo Scientific, ABgene Ltd. Epsom, UK) contains Taq DNA polymerase, magnesium chloride and dNTP mix in optimised buffer.

After sealing the Q-PCR plate using an Abgene plate sealer and thermal seals (Alpha Laboratories, Hampshire, UK), samples were placed in the Quantica® Q-PCR instrument (Techne) or the StepOne Plus Real Time PCR System (Applied Biosystems). Q-PCR relies on the same reactive principles as a regular PCR, although the reactions are carried out without an extra temperature step for primers extension because the polymerases used in Q-PCRs are less temperature-sensitive than the polymerase used in regular PCR (Figure 2.7). In the sample mix, the extension of short amplicons occurs in the time the machine needs to heat up from the annealing temperature (60°C) to 72°C. During the cycling, fluorescence is measured at the end of each annealing step. After completion of the cycling, a "ramp" is carried out with the temperature steadily rising in 1°C from 65°C to 95°C with a 10 second hold and a measurement of fluorescence on each step.

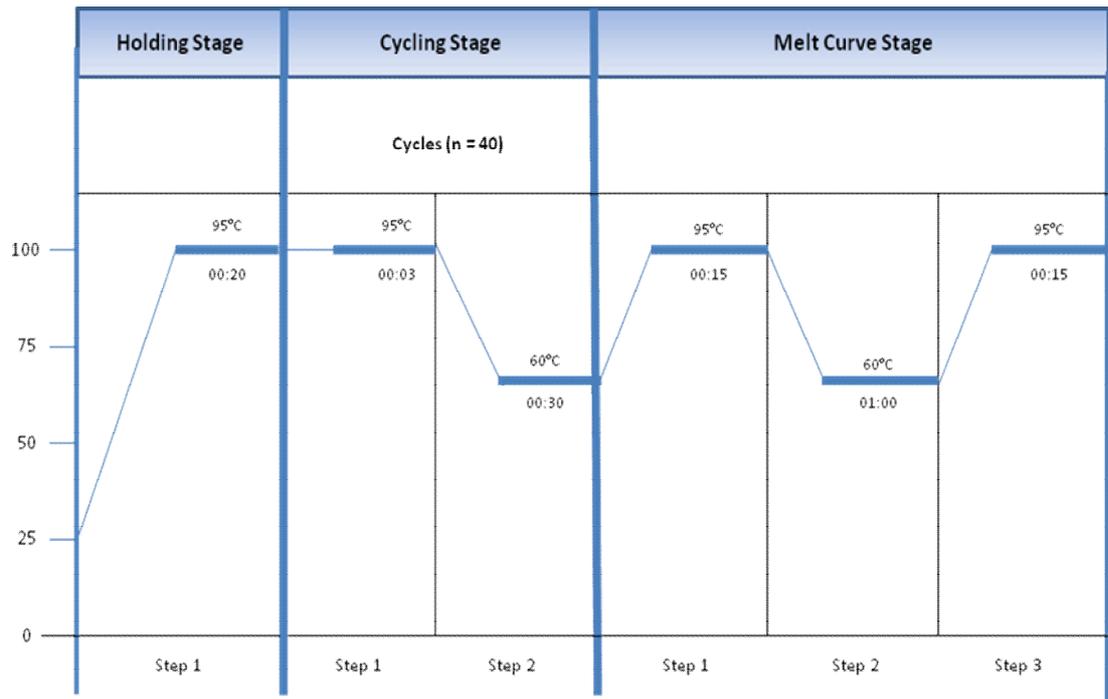


Figure 2.7: Q-PCR program conditions.

The programme starts with a denaturation at 95°C for 20 minutes followed by 40 cycles of 95°C for 3 seconds and a gene-specific annealing temperature of 60°C for 30 seconds. During the cycling, fluorescence is measured at the end of each annealing step, and, after the cycling is completed, a melt curve stage is carried out to test product specificity. During this step, the temperature steadily rises in 1°C increases from 65°C to 95°C with a 15 second hold and a measurement of fluorescence on each step is performed. Range of optimal temperatures for genes analysed were used according to specific primers binding.

Q-PCR analysis was performed by using the Quansoft® (Techne) or the StepOne™ v2.2 (Applied Biosystems) software packages respectively and gene expression of each target gene was calculated as described above (section 2.6.7).

2.6.8. Housekeeping genes

The reliability of any relative RT-PCR experiment is improved by including an invariant endogenous control (reference gene) in the assay to correct for sample-to-sample variations as well as errors in sample quantification which can result in misinterpretation of the derived target genes expression profile [433]. Therefore the abundance of each gene of interest was normalised relative to endogenous reference genes, known as housekeeping genes, which by definition have a relatively higher expression than other genes of interest maintaining a constant level of expression across samples, physiological states, and treatments [434].

However, reference genes are not stably expressed in all scenarios as numerous studies have been shown as certain experimental conditions can influence their expression leading to incorrect findings if inappropriate housekeeping gene is used [435]. This discovery pointed out the critical importance of validating them within the specific tissue and biological conditions.

To address this issue, seven candidate housekeeping genes (18S, ACTB, B2M, GAPDH, YWHAZ, SF3A1, UCB), previously validated as reference genes within the placenta [436], were investigated using RT-PCR for their expression stability in placentas from pregnancies complicated by overweight (n=10), obesity (n=10), gestational diabetes (n=10) and control pregnancies (n=10). While the primers set for 18S was designed as previously described (section 2.6.5), endogenous control genes provided and already optimised by Primer Design (geNorm™ reference gene selection kit) were used as additional genes of reference (Table 2.5).

Table 2.5: Human GeNorm housekeeping genes (Primer Design geNorm™ reference gene Selection).

NCBI sequence	Target Gene	GeNorm Reference Gene
NM_001135702.1	YWHAZ	Homo sapiens phospholipase A2
NM_031144.3	ACTB2	Homo sapiens actin, beta
NM_004048	B2M	Homo sapiens beta-2-microglobulin
AF261085.1	GAPDH	Homo sapiens glyceraldehyde-3-phosphate dehydrogenase
NM_005877.4	SF3A1	Homo sapiens splicing factor 3a, subunit 1
NM_021009.5	UBC	Homo sapiens ubiquitin C

These genes were used to identify the most stable reference/s gene/s in a random selection of human placental samples.

The most stable reference genes were determined from an experimental database (Cotton Est Database) using a web-based comprehensive tool (RefFinder), available at <http://www.leonxie.com/referencegene.php>, which integrates the data from four of the currently major statistical approaches (geNorm, Normfinder, BestKeeper and the comparative Δ Ct method) [437].

2.6.8.1. Housekeeping genes analysis procedure

After re-suspending the lyophilised primer mix in 200 μ l of RNA-free water, a reaction mix using the PrimerDesign 2xqPCR Mastermix also provided by the kit, was made up for each reference gene according to the protocol below.

3 μ l of cDNA from each sample was pipetted onto a 96-well plate before adding 17 μ l of the reaction mix to reach a final volume 20 μ l.

Volume (μ l)	Reagent
3	cDNA (1:10)
1	Resuspended primer mix *
10	PrimerDesign 2X Precision™ Mastermix
6	RNA-free water

* using RNA-free water

All 40 placental samples were run in duplicate for each reference on the same plate. In this analysis, no standard curve was used to compare samples as all the genes have been already optimised either by the company or in the course of this work. The most stable housekeeping genes (ACTB, 18S, GADPH, B2M) were validated with RefFinder (Figure 2.8 A-B).

Method	Ranking Order (Better–Good–Average)						
	1	2	3	4	5	6	7
Delta CT	ACTB	GADPH	18S	SF3A1	B2M	YWHA2	UBC
BestKeeper	YWHA2	18S	B2M	GADPH	ACTB	SF3A1	UBC
Normfinder	ACTB	GADPH	18S	SF3A1	B2M	YWHA2	UBC
Genorm	18S B2M		ACTB	GADPH	SF3A1	YWHA2	UBC
Recommended comprehensive ranking	ACTB	18S	GADPH	B2M	YWHA2	SF3A1	UBC

A)

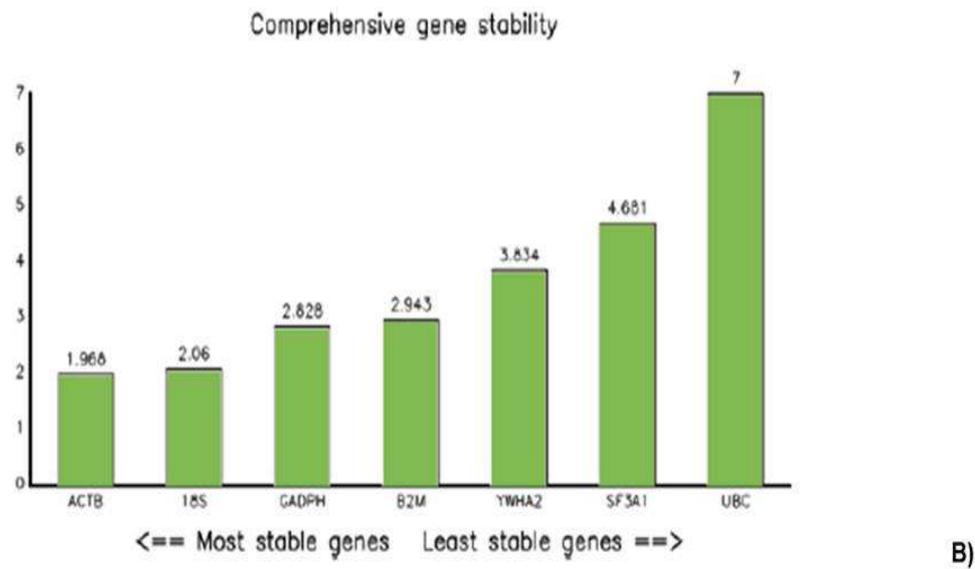


Figure 2.8: Evaluation and screening of seven reference genes expression from Cotton Est Database using RefFinder software, a user-friendly web-based comprehensive tool which is fully available online from <http://www.leonxie.com/referencegene.php>

A) Ranking order of the tested reference genes according to each computational program. The first and second entries provided by Genorm are equally good and correspond to 18S and B2M. B) Graph of the comprehensive gene stability for each of the tested reference genes, the most stable of which resulted to be ACTB and 18S.

This database integrates data from the geometric mean calculated for each individual gene using the algorithms from four of the major computational programs; a lower geometrical mean value reflects higher stability.

Gene expression levels were first normalised relative to 18S whereas, subsequently, the arithmetic mean of the best housekeeping genes above reported was used. However, using different housekeeping genes did not influence the outcome of gene expression analysis in this study, hence, gene expression levels were finally reported only relative to 18S, which represents a good reliable control gene [436].

2.7. Triglycerides extraction

Higher triglycerides have been associated with pregnancies complicated by maternal high BMI and diabetes [438] so lipids were isolated and extracted from human placental samples using a modified method previously validated by Folch et al. in different animal tissues [439]. This procedure is based on partitioning chloroform-methanol organic solvents with water or salt solutions (8:4:3) to separate the lipid and no-lipid components by dissolution, centrifugation and filtration. Tissue homogenisation with 2:1 chloroform-methanol and gravity filtration result in lipids extraction and solubilisation in the solvent phase whereas washing the filtrate with at least a fifth of either distilled water or 0.9% saline solution allows elimination of non-lipid substances. The pure lipid extract is then obtained from evaporation of the samples under nitrogen steam to ensure the removal of the solvent mix and the triglyceride concentration can be measured with a commercial assay after re-suspending each sample in another solvent. In this study the Randox triglycerides assay kit (Cat. No: TR212, Randox laboratories Ltd, Crumlin, UK) was used to determine triglycerides concentration by spectrophotometric absorbance after converting them into quinoneimine as described in Figure 2.9. Interpolation of measured values from an internal control standard curve allows calculating triglyceride concentrations for unknown samples.

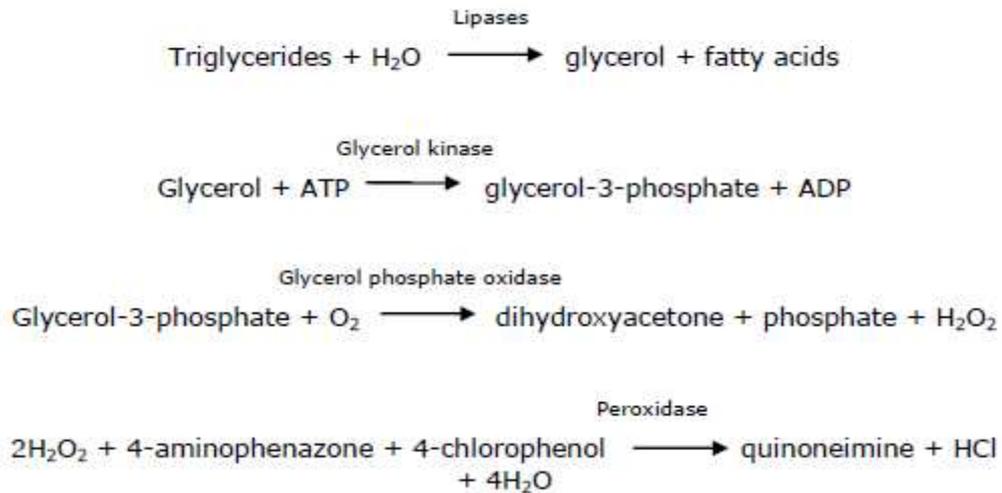


Figure 2.9: Principle enzymatic chemical reactions involved in triglyceride analysis reaction.

2.7.1. Triglycerides extraction procedure

A total of 149 placentas were removed from -80°C , weighed (500mg) and homogenised in 50ml dispomix tube with 2ml cold chloroform-methanol (2:1) solvent previously prepared. Samples were gently centrifuged (200 g) at room temperature for 1 minute to ensure complete exposure of all homogenised tissue to the solvent before being agitated at for 20 minutes at room temperature using an orbital shaker (Shaker incubator SI50, Stuart Technology, Staffordshire ST15 0SA, UK). The mixture was then filtered under gravity through a 150mm Whatman filter paper (Scientific Laboratory Supplies, Nottinghamshire, UK), placed on a funnel, into a 15ml centrifuge tube (Greiner bio-one, Cat.No:188261, Stonehouse, Gloucestershire, UK) to recover the liquid phase. One ml solvent solution was used to wash the inside wall of the dispomix tube whereas additional 8ml same homogenization solution were applied to wash the filter paper ensuring all available lipids in the homogenate were collected. After removing the funnel and filter paper, 2ml distilled water was added to the filtrated samples, which were vortexed for 5 seconds and then centrifuged at low speed (800 g) for 10 minutes at room temperature allowing the separation of the organic and aqueous phase of the solution.

The upper phase (aqueous phase) containing the non-lipid contaminants was removed with a Pasteur pipette by siphoning and 2ml lower phase containing the purified lipid was collected into 2ml eppendorf tube. Then it was vortexed for 5 seconds and centrifuged at low speed (400 g) for 10 minutes at room temperature. If impurities were still present, the top layer and any remaining gel-like interphase were discarded and samples containing the solvent mix were evaporated under nitrogen steam using a Driblock DB-3 (Techne) for 15-20 minutes. After complete evaporation of the aqueous phase samples were re-dissolved in 60 μ l tert-butanol (BDH) and 40 μ l triton X-100 and stored at 4°C in a spark-free fridge until further analysis. Standards were prepared by diluting the provided triglyceride standard (CAL) in a 60:40 ratio of tert-butanol : triton X-100 before pipetting 2 μ l each sample, control and standard was into the a 96 well microplate (Grenier Bio-one, Gloucestershire, UK). The enzyme reagent supplied by the kit was then added (200 μ l) avoiding formation of bubbles and the plate was incubated at 37°C for 5 minutes for color development. Absorbance was measured using a μ Quant plate reader at 500 nm and triglyceride content expressed as a ratio of milligrams (mg) of triglyceride per gram (g) of placental tissue (mg/g). All samples analyzed were performed in triplicate with a 10% intra-assay coefficient of variance (calculated as standard deviation/mean X 100). Analyses were repeated for samples outside this CV% value.

A serial dilution calibration curve was prepared fresh each time whilst the internal standards were used to minimize inter-plate variability and optimize accuracy.

2.8. Thiobarbituric acid reactive substances analysis

The oxidative degradation of lipids, known as lipid peroxidation, has been implicated in the pathogenesis of several diseases and clinical conditions including chronic inflammation, diabetes and premature birth disorders [440]. Progression of lipid peroxidation generates a large number of unstable biproducts which are highly detrimental to the structure of biological membranes thus to cell functioning and survival [441]. Some of these reactive compounds of major toxicological interest include malondialdehyde (MDA) and 4-hydroxynonemal (4-HNE) and measuring their levels in vitro is one of the most widely accepted assays for oxidative damage [442]. The thiobarbituric acid reactive substances (TBARS) method is based on the reaction between MDA and thiobarbituric acid (TBA) at 95°C under acidic conditions with the formation of an adduct (MDA-TBA) which can be measured spectrophotometrically or fluorometrically (Figure 2.10).

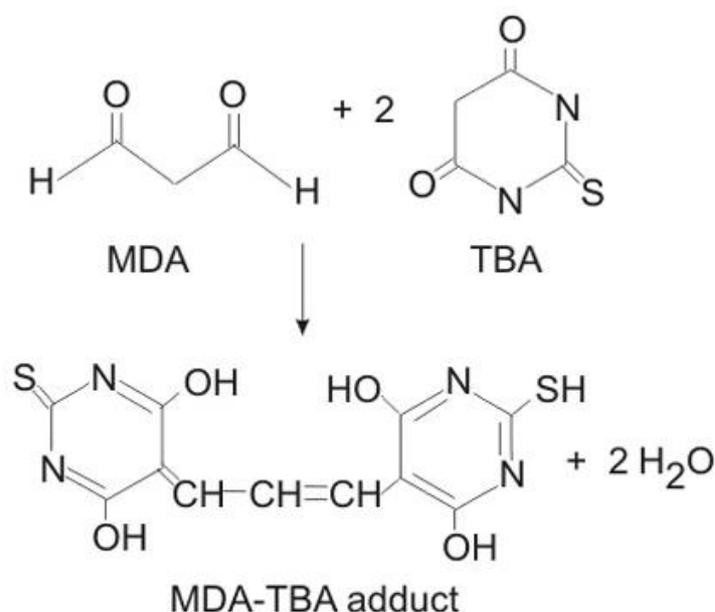


Figure 2.10: Malondialdehyde-thiobarbituric acid (MDA-TBA) adduct formation.

The Oxiselect™ TBARS Assay kit (Cell Biolabs Inc, CA, USA) determines lipid peroxidation through the formation of MDA-TBA adducts, a reaction that produces a pink chromagen which can be measured colorimetrically at 532nm. In recent years, however, the development of a large number of assays for lipid peroxidation measurement have questioned the specificity of TBARS reaction as TBA and MDA can both react with additional compounds such as aldehydes, DNA and amino acids to form similar colorimetric products [443-445]. Hence, it is suggested the reaction measures TBARS rather than MDA concentration. Despite this, the assay is still being used in many laboratories for its rapid and easy protocol and remains one of the most extensively employed format for screening and monitoring secondary products of lipid peroxidation in biological samples [443, 444].

2.8.1. TBARS procedure

Placental tissues of 139 samples, reagents and all experimental tubes were prepared the day before to ensure running the experiment in the most efficient way. Each sample was weighed (approximately 100mg), resuspended and homogenised in 1ml 1X butylated hydrotoluene kit reagent (BHT) dissolved in phosphate buffered saline (PBS). Adding the anti-oxidant BHT to the sample prevents further oxidation of lipid during sample processing and must be performed quickly. Samples were initially centrifuged for 1 minute at 2000 g at room temperature in a 50 ml dispomix tube and the supernatant was transferred to 1.5ml labelled eppendorf tubes. Centrifugation was repeated at 10000g for 5 minutes at 4°C before transferring the supernatant to clean 1.5ml tubes to ensure removal of any remaining cell debris. Samples were then stored at -20°C until performing the TBARS assay or total protein concentration analysis. Oxiselect™ TBARS assay kit was used for TBARS analysis as per the manufacturer's instructions; analysis of both standards and samples were performed in duplicate.

Ten MDA standards (500µl) were prepared ranging from 0mg/dl-195mg/dl using the MDA reagent provided and distilled water. 100µl of standard, negative control or unknown sample was added to a fresh 1.5ml eppendorf tube. Subsequently, 100µl of sodium dodecyl sulphate (SDS) lysis solution was added to each tube and incubated at room temperature for 5 minutes. 250µl of TBA reagent, which must be prepared fresh every time, was next added and tubes were incubated in a water bath at 95°C for 60 minutes. After this, samples were removed and cooled on ice for a further 5 minutes. Centrifugation of samples was performed at 1200g for 15 minutes, with 300µl of supernatant transferred to a fresh eppendorf tube. Next, 300µl of n-butanol was added to the samples to prevent interference of haemoglobin and its derivatives. Samples were vortexed for 1-2 minutes and centrifuged for 5 minutes at 10000g at room temperature to allow for phase separation. The n-butanol layer, which is the top layer of the solution, was then decanted into another eppendorf tube for spectrophotometric analysis and 200µl of each sample and standard was pipetted into a 96 well microplate to measure absorbance at 532nm using the Nanodrop (Nanodrop® Technologies, Wilmington, USA). Unknown sample concentrations of MDA/TBARS were determined against the MDA standard curve and normalised against total protein content using the bicinchoninic acid (BCA) method (sub-section 2.8.2). The protein content of the samples is an indicator of how efficiently the cellular contents were extracted; therefore, normalisation of the data against protein content was performed to compensate for differences in the extraction efficiency. Analysis of each standard and sample in both TBARS and BCA assays was performed in duplicate to ensure the most reliable reading and results were expressed as pmol / µg.

2.8.2. Bicinchoninic acid total protein determination

Total protein concentrations were determined using the BCA assay method, which relies on a colourimetric reaction between proteins, BCA and copper sulphate [446]. Peptide bonds in proteins reduce Cu^{2+} to Cu^{1+} ions when incubated at room temperature, which are then chelated by BCA reagent to form a purple coloured compound [446]. The colour development from green to purple is in proportion to the amount of protein present in the sample, the absorbance of which can be measured at 562nm with the Nanodrop (Nanodrop[®] Technologies, Wilmington, USA). Unknown sample concentrations can then be referenced against a standard curve.

2.8.2.1. BCA assay

Reagent A containing 1% bicinchoninic acid, 2% sodium carbonate, 0.16% sodium tartrate and 0.4% sodium hydroxide was made up to 50 ml with distilled water and 10% sodium bicarbonate was used to reach a pH of 11.25. Reagent B (50 ml) was made up with 4% copper sulphate solution dissolved in 50 ml distilled water. The two reagents (A+B) were mixed well in a ratio of 100ml:2ml to form a third reagent (Reagent C) which was stored at 4°C until needed (no more than a month).

Eight standard concentrations of 1 mg/ml bovine serum albumin (BSA) ranging from 1.0-0.00 mg/ml were made up in 0.9% saline solution and 2.5 μl of each unknown protein sample were diluted by 1:20 in 0.9% saline to make a final volume 50 μl . Next, 10 μl each standard, negative control (RNA-free water) or unknown sample was pipetted into a 96-well microplate and 200 μl reagent C was added to each well. After 30 minutes of incubation at 37°C in an orbital shaker to allow color development, absorbance was measured spectrophotometrically at 562 nm. A 10% coefficient of variance (calculated as standard deviation/mean X 100) was accepted between duplicate samples and analyses for samples outside this CV% value were repeated.

Samples were run with their own calibrators for the standard curve on multiple assay plates. The interassay coefficient of variation, calculated from the mean values for the standards on each plate, was used as an expression of plate-to-plate consistency. A 20x multiplication factor was then applied to all recorded absorbance results to account for the initial 1:20 dilution factor.

2.9. Histological analysis

Microscopic analysis was performed by using histological techniques to study the cellular differences that might contribute to different size and structure of the placenta. Tissues were fixed in neutral-buffered formalin (10%), which introduces cross-links that preserve peptides of the cellular structures from protein degradation [447]. Following tissue fixation, each sample was encased and embedded in a holding matrix (paraffin wax) to maintain its structural and cellular composition before sectioning five μm using a sledge microtome [448]. As paraffin wax is immiscible in water, a dehydration step followed by immersion in ethanol and xylene was performed on any tissue before embedding in the wax. To allow visualization and/or differential identification of microscopic structures, histological stains were applied in each sample section followed by examination under a light microscope.

2.9.1. Histological tissue processing

A subset of 40 frozen placental samples was selected for histological sectioning from each group (10 C, 10 OV, 10 OB, 10 GDL, 10 GDOV and 10 GDOB) after selecting them by maternal age, BMI, parity, smoking during pregnancy and newborn gender. Half a gram of placental tissues was weighed and treated with 10% formalin (10% v/v formaldehyde in 0.9% w/v sodium chloride/distilled water (Fisher Scientific) saline solution) overnight. Each sample segment was loaded into a Histosette II (Simport, Quebec, Canada) 30 mm x 27 mm x 5 mm cassette and processed through six stages of ethanol dehydration.

This was followed by three stages of xylene (Fisher Scientific) for ethanol-clearing. After three stages of paraffin wax using the Shandon Excelsior™ tissue wax processor (Thermo Scientific) at 60°C, samples were then allowed to solidify overnight. This step ensured to fix in paraffin each sample segment which was then sectioned using a sledge microtome (Anglia Scientific, Cambridge, UK) leading to 10 slides at 5 µm / sample.

After rinsing each slide in 70% ethanol and floated in 45°C water to stretch them out by surface tension, sections were transferred to Superfrost™ Plus slides (Menzel-Gläser Inc, Braunschweig, Germany), dried on a heat rack for 15 minutes and stored in a drying oven at 37°C for 24 hours.

2.9.2. Haematoxylin and eosin staining

The application of Haematoxylin and Eosin staining (H&E), a basic aluminium salt dye was used to visualize the main microscopic cellular structures. Haematoxylin is oxidised to haematein by the chemical agent mercuric oxide, which forms a strongly colored complex with the addition of an aluminium salt mordant by binding acidic structures, i.e. nucleic acids. It is generally used as a regressive dye and its intensity is reduced and "blued" off by washing with a weak alkali [449]. The nuclear staining is thus followed by counterstaining with an aqueous solution of eosin, which colors other eosinophilic structures surrounding the nuclei (cytosol, muscle fibres, collagen and erythrocytes) in various shades of red, pink and orange. Differentiation of the eosinophilic formations occurs through washing with water.

2.9.2.1. H&E staining procedure

One slide from each sample was blinded by assignation of a random identifier and placed in a slide rack. The slides were then dewaxed by immersion in two consecutive xylene washes for 3 minutes which was followed by rehydration through two steps of 100% ethanol immersion and one stage of 70% ethanol/distilled water immersion, before a final wash with distilled water.

The sections were nuclear-stained in a trough of Harris' haematoxylin (VWR Ltd, Lutterworth, UK) for 5 minutes and then rinsed in tap water for 5 minutes to remove all excess dye. The haematoxylin stain was reverted by immersion in an acid-alcohol solution (1% conc. hydrochloric acid in 70% ethanol) for 5 seconds, rinsed in tap water and blue stain was hed off in alkaline Scott's tap water (a 0.2% sodium bicarbonate and 20% magnesium sulphate distilled water solution) for 1 minute. This solution performs rapide and precise blueing of nuclear cromatin and nuclear membranes ensuring minimum loss of tissues sections and cells from glass slides. Subsequently, the sections were washed in tap water and transferred to a 1% Eosin Yellowish (VWR Ltd) counter stain for 3 minutes. Tap water was used once more to wash the slides of any excess of staining allowing the differentiation of eosinophilic structures. Sections were then dehydrated in two stages of 100% ethanol immersion for 2 minutes and ethanol cleared in two 3 minute immersions of xylene. Sections were mounted with coverslips (VWR) using DPX mounting medium (Fisher Scientific), and left to dry overnight.

2.9.2.2. Placental H&E analysis

H&E-stained slides were visualised through a Leica DRM microscope (Leica Microsystems, Wetzlar, Germany) at 4x and 10x magnification and photographed for analysis using a Hamamatsu digital camera (Hamamatsu, Hertfordshire, UK). A total of 45 out of 60 frozen placental samples showed poor tissue integrity due to tissue deterioration during collection, and this did not allow a powerful and complete analysis.

2.10. Immunohistochemistry

Immunohistochemistry (IHC) refers to a technique that allows the detection of specific antigens in a histological section by selective antibody binding and also the determination of their morphological localization and distribution in different parts of a biological tissue. The interaction antibody - target antigen produces a signal which can be visualised in a direct or indirect method of staining by using a conjugated signaling marker, commonly an enzyme (e.g. peroxidase) or a fluorophore (e.g. fluorescein or rhodamine) [450]. The most common enzyme conjugated to the secondary antibody used in the indirect IHC method is the horseradish peroxidase (HRP) which through catalytic conversion develops a brown precipitate in the presence of 3,3 diaminobenzidine (DAB), a chromogenic substance.

Prior to antibody exposure, all sections were dewaxed and rehydrated in order to allow access of all reagents to the tissue although many antigen sites can be masked after formalin fixation as a result of the cross-linking of proteins. This can happen, for instance, if the secondary antibody is conjugated to biotin molecules resulting in recruitment of avidin-biotin-enzyme complexes which leads to nonspecific binding and high staining.

Therefore, to break these protein cross-links and unmask all hidden antigen sites samples should be treated with a heat-induced epitope retrieval (HIER) step (usually heating with citrate buffer).

2.10.1. IHC procedure

Immunohistochemical analysis was performed in order to elucidate and quantify the amount of fibronectin deposits and also markers of macrophages accumulation often observed in complicated pregnancies [451]. Recommended primary antibody dilutions for each human antibody used in this study (Table 2.6) were tested for optimisation using both an automated system and hand staining.

Table 2.6: Antibodies used for detection of tissue macrophages (CD68, CD14) and fibronectin.

Antibody	Supplier	Type	Primary antibody dilution factor	Incubation time and temperature
Anti-CD68	Abcam	Mouse monoclonal	1/200 1/400 1/800	30-60 minutes at RT, overnight
Anti-CD14	Millipore Chemicon	Mouse monoclonal	1/200 1/400 1/800 1/1600	30-60 minutes at RT, overnight
Anti-Fibronectin	Abcam	Mouse monoclonal	1/100 1/200 1/400	30-60 minutes at RT, overnight

RT: room temperature

In the automated method, one slide for each tested dilution and a negative control were labelled, placed in a slide rack and loaded in into a Leica BondMax™ IHC slide processor (Leica Microsystems) before being run on an automated software program (Vision Biosystems Bond version 3.4A).

Primary antibody binding to tissue sections were visualized using BOND Polymer Refine Detection (no-vision biosystem antibody, Leica), which contains a peroxide block, post primary, polymer reagent, DAB chromogen and hematoxylin counterstain. Slides were washed twice in xylene and ethanol for one minute each and finally incubated at 95°C with epitope retrieval solution before being treated with peroxide block for 5 minutes at room temperature. Following exposure of all tissue, sections (except the negative control) to 150 µl different primary dilutions for 30 minutes, 150 µl HRP conjugated secondary anti-mouse and rabbit antibody polymer were applied to all samples for 8 minutes. Subsequent exposure to 3,3 DAB (DAB peroxidise substrate kit, Vector Labs, Cat.Num.SK-400) for 10 minutes results in a brown precipitate development, after which all samples were stained with H&E for 5 minutes for nuclear visualisation (Kiermnan, 2000). Bondwash buffer and distilled water were applied to the slides for washing after each Bondmax stage.

Tissue sections were mounted with coverslips using DPX mounting medium and dried overnight before being visualised at 5x, 10x and 20x magnification and photographed for analysis. After visualisation on the microscope the villous structure of both frozen and RNA later placenta were not well delineated and clear probably due to tissue damage during sample collection. Additionally, non-specific brown staining was still detected at higher antibody dilutions therefore it was not possible to proceed to quantification of antigen-antibody complex (Figure 2.11).

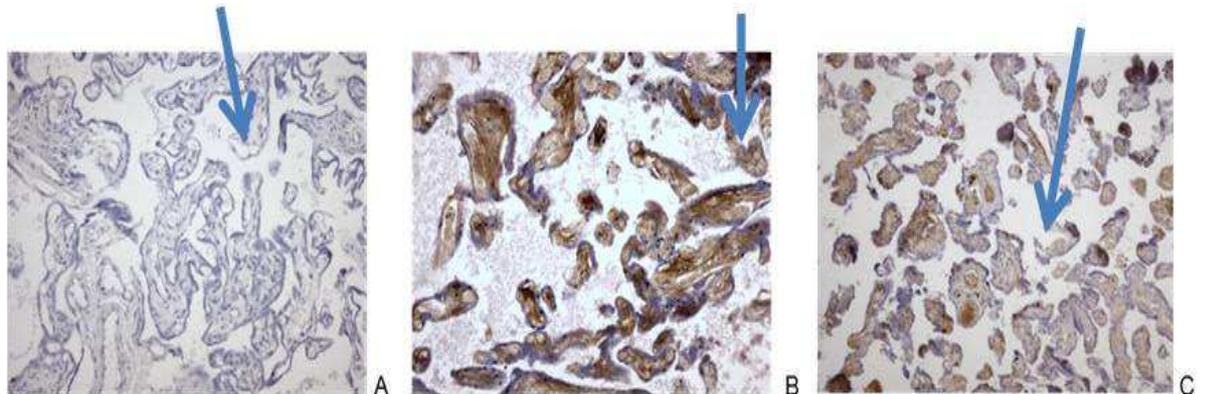


Figure 2.11: Representative CD14 immunohistochemistry 5 μ m stained microscopic sections at 4x magnification in frozen placental sample from obese subject.

A) Negative control; B) 1:400 primary antibody dilution with diffuse brown staining; C) 1:1600 primary antibody dilution still indicating non-specific staining of the antibody. Villous membrane is not well defined (blue arrow) reflecting a partial degradation of those samples which were not suitable for histological analysis. Brown staining represents positively staining of the primary antibody.

A second attempt aiming to eliminate this non-specific staining and eventually detect the correct antigen-antibody binding was undertaken by using the hand staining procedure. After deparaffinization and hydration of all tissues, an antigen retrieval step (Dako Envision^{TM+} system) was performed by microwaving samples for 15 minutes in a citrate buffer solution, pH 6.0 [452]. After immersion in a solution of 0.5% H₂O₂ in water/methanol for 30 minutes to quench endogenous peroxidase activity, samples were washed in Tris-buffer saline (TBS) (containing 20 mM Tris base, 500 mM sodium chloride) for 5 minutes.

Each section was then marked using a PAP pen for immunostaining (Sigma-Aldrich) to prevent the waste of reagents by keeping liquid pooled in a small droplet. Sections were incubated in diluted normal blocking serum using the Vectastain® Universal Elite® ABC kit (Vector Laboratories, Cat. Num. PK-600) for 20 minutes. The excess liquid was blotted from slides, which were then rinsed with TBS 1:10. Incubation with different primary antibody dilutions was carried out overnight at 4°C, following which excess of liquid was blotted and sections rinsed 3 times in TBS for 5 minutes. Slides were then incubated at room temperature for 30 minutes with diluted biotinylated secondary antibody diluted in blocking buffer according to manufacturer's instructions (Vectastain® Universal Elite® ABC kit). An additional wash in TBS for 5 minutes was followed by 30 minutes application of a preformed Avidin and Biotinylated horseradish peroxidase macromolecular Complex (Vectastain® Universal Elite® ABC kit) for peroxidase substrate development. Samples slides were washed 5 minutes in TBS before being exposed to 3,3 DAB for 10 minutes until brown precipitate development. Sections were then counterstained with H&E for 5 minutes for nuclear visualisation, mounted with coverslips using DPX mounting medium and dried overnight at room temperature before being visualised at 5x, 10x and 20x magnification. Once more, non-specific brown staining for each antibody was still diffused all over the sections, despite several attempts with higher antibody dilutions than recommended by manufacturer were performed.

2.11. Western blot analysis

Western blot also known as protein immunoblot is one of the most widely used analytical techniques to detect specific proteins in a tissue [453]. Depending on the source, the protein has to be brought into solution by breaking the tissue (e.g. homogenization and lysis) and can be separated from other cell components (membranes, DNA, etc.) by centrifugation.

Tissue preparation is undertaken at cold temperature to avoid protein denaturation and degradation as well as protease and phosphatase inhibitors added to the lysate to prevent digestion of the sample by its own enzymes. After extraction, the proteins need to be denatured by heating with an ionic detergent in order to be separated by molecular weight through gel electrophoresis. Reducing agents like 2-mercaptoethanol are added to remove the protein's secondary and tertiary structure by cleaving protein's disulfide bonds whereas the addition of SDS maintains the polypeptide in a denatured state and covers it with a negative charge.

Sampled proteins can then migrate to the positively charged electrode through the 2-D polyacrylamide gel according to their isoelectric point (pH at which they have neutral net charge) and molecular weight (the smaller the faster). The use of a greater acrylamide concentration in the gel is recommended for resolution of lower molecular weight proteins whereas decreasing acrylamide concentration ensures resolution of higher molecular weight proteins.

A protein marker ladder (Biorad Precision Plus Protein Standards, Biorad, California, USA) with defined protein molecular weights is run in each gel alongside the samples to enable the estimation of the protein size and monitor the progress of the electrophoresis run. A negative control, i.e. non-immune rabbit serum, known not to express the target protein, is used to check for non-specific binding and false positive results.

A positive control, i.e. a lysate from a cell line (HeLa, COX) known to express the target protein, is also used to demonstrate the efficiency and reliability of the protocol showing that the antibody recognises the target protein, which may not be present in the experimental samples. A positive result from the positive control, even if the samples are negative, will indicate that the procedure is optimized and working. An additional way to test the validity of the protocol and the results is loading an internal control, such as beta-actin [454], which is normally expressed in cells at very high concentrations.

In order to make the protein accessible to antibody detection they are transferred from the gel matrix to an electrostatic membrane using an electroblotting method [455]. This uses an electric current to pull proteins from the gel into the membrane ensuring the maintenance of the same organization they had within the gel. In wet transfer, the gel and membrane are carefully sandwiched between sponge and paper and all are clamped tightly together. The sandwich is then submerged in transfer buffer to which a small electrical field is applied, drawing the negatively-charged proteins from the gel onto the membrane, by a combination of capillary action and electrostatic attraction. After blotting, the membrane is stained using a reversible protein dye (Ponceau red) to ensure completion of protein transfer and each molecular weight is marked with a HRP-conjugated secondary antibody pen in order to be visualised following immunodetection.

Before proceeding to incubation with a primary antibody specific to the target protein the membrane is first incubated in a blocking reagent (usually non-fat milk or BSA) to block any non-specific binding sites of the primary and/or secondary antibodies to the membrane. Optimised dilutions of primary and secondary antibody, and an adequate incubation time will then ensure the formation of the target antigen-antibody protein complex. Detection of the HRP-conjugated secondary antibody occurs by incubation in a chemiluminescent reagent (luminol and peroxide solution), which emits a signal in proportion to the abundance of target antigen present.

Exposing the blot to a suitable X-ray film for an appropriate duration will detect the chemiluminescent signal which can be quantified through analysis of protein band density using densitometry software.

Primary antibodies for folate transporters used in this study are shown in Table 2.7.

Table 2.7: Antibodies used for detection of folate receptor alpha (FR α) and reduced folate carrier (RFC).

Target	Type	Supplier	Catalogue number	Protein size	Primary Ab dilution	Incubation time and temperature
FR α	Rabbit polyclonal	Aviva System Biology	OAAB00710	38 kDa	1/250 1/500 1/1000	3-4h at RT
FR α	Rabbit polyclonal	Santa Cruz Biotech	sc-28997	36-39 kDa	1/200 1/400 1/800	3-4h or overnight at RT
FR α	Rabbit monoclonal	Insight Biotech	5420-1	37-42 kDa	1/1000 1/5000 1/10000	3-4h or overnight at RT
RFC	Rabbit polyclonal	Alpha Diagn Int	RFC11-A	28 kDa	1/150 1/200 1/400	3-4h or overnight at RT

Ab: antibody; RT: room temperature.

2.11.1. Protein extraction procedure

The extraction of protein from frozen placental tissue was performed using CelLytic MT reagent from Sigma-Aldrich which consists of a dialyzable mild detergent, bicine, and 150 mM NaCl, resulting in minimal interference with protein interactions and biological activity. A volume of 20 ml of lysis buffer is sufficient for 1 g of tissue. To increase the yield of proteins in the extracts a protease inhibitor cocktail (Sigma-Aldrich) containing a number of inhibitors with a broad specificity for serine, cysteine, acid proteases, and aminopeptidases was also added to the lysate (0.05 ml per 1g tissue / 20 ml CelLytic MT).

Protein extraction from frozen samples stored in RNA later solution was performed using radio-immunoprecipitation assay (RIPA) buffer.

This solution enables efficient cell lysis and protein solubilization while avoiding protein degradation and interference with the proteins' immunoreactivity and biological activity and also results in low background in immunoprecipitation and molecular pull-down assays.

2.11.1.1. Protein extraction from frozen tissue

Half a gram of frozen tissue was weighed in a dispomix tube and 10 ml of CelLytic MT (C3228) lysis reagent (Sigma-Aldrich) and 0.0025 ml protease inhibitor cocktail P8340 (Sigma-Aldrich) were added. After homogenisation at 800 g for 30 seconds the homogenised samples were transferred to Sorvall tubes and centrifuge in a refrigerated superspeed centrifuge (Sorvall RC-5B, Thermo Life Science) for 10 minutes at 4°C at 12000 - 14000 g to pellet all tissue debris. Protein containing supernatant was then aliquoted in fresh 2 ml eppendorf tubes and lysate stored at -20°C until further analysis. BCA assay (see TBARS section 2.8.1.2, p.33) was used to measure protein concentration of each placental sample.

2.11.1.2. Protein extraction from RNA later tissue

A total of 50 ml RIPA buffer was prepared using 1x TBS and stored at 4°C. The protease inhibitors phenylmethylsulfonyl fluoride (PMSF, 10 µl/ml), apoprotein (30 µl/ml) and 200mM sodium orthovaqnadate (10 µl/ml) were added to the lysis buffer at the time of the extraction. Three frozen RNA later samples were weighed (0.1 g) in dispomix tubes and homogenised 3 times for 30 seconds with 1 ml RIPA buffer mix each until no clumps of tissue were visible. Care was taken to not cause foaming during the process as it can damage proteins. Additional 30 µl PMSF was added to each homogenate, which was then incubated on ice for 30 minutes without vortexing. After centrifugation at 8000 g for 10 minutes at 4°C the supernatant was collected and stored at -20°C until further analysis.

As repeated freeze, thawing is not recommended for RIPA samples small aliquots of each were prepared instead. Concentration of placental extracts was performing using BCA protein assay.

2.11.2. Sample preparation procedure

After measurement at 562 nm, protein concentration was multiplied by the dilution factor used in the BCA assay (1:20) and samples diluted to the desired concentration (4.2 mg/ml) using CellLytic MT diluents. 20 μ l diluted samples was then mixed with 50 μ l protein dissociation buffer (containing 50mM Tris, 10% glycerol, 2% SDS, 5% beta-mercaptoethanol and distilled water pH 6.8) before samples were made up to 84 μ l by adding 14 μ l glycerol-bromophenol dye (containing 0.01 g bromophenol blue, 14.9 g sodium hydroxide and 23.5 ml distilled water). Incubation for 5-10 minutes at 95-100°C in a water bath allows denaturation of sample's proteins without need to re-heat sample after the first time. As freezing can lead to samples degradation, a stock solution of protein mix for each sample was prepared and stored at -20°C until use or ready for electrophoresis (see below).

2.11.3. Protein detection procedure

The detection of the target protein abundance is based on the immunoblotting technique which encompasses two main steps. First, the proteins are denatured and separated according to their molecular weight by gel electrophoresis before being transferred to a membrane (typically nitrocellulose and PVDF) [453]. Next, the membrane is probed for the protein of interest with a modified primary antibody which is linked to an enzyme (either alkaline phosphatase or horseradish peroxidase) - conjugated secondary antibody [456]. The generation of a chemiluminescence signal in proportion to the amount of protein is then generated allowing for protein quantification. To prove the reproducibility of the assay, each Western blot was performed in duplicate.

2.11.4. Gel electrophoresis procedure

After assembling the slab gel unit using the BioRad glass plates 8 cm x 7 cm, spacers and clamp sets (BioRad Lab, Life Science Research, UK), a 12% polyacrylamide-resolving gel containing 3.3 ml 30% polyacrylamide (Severn Biotech, Worcestershire, UK), 4 ml distilled water, 2.5 ml 1.5 M Tris pH 6.8, 0.1 ml 10% SDS, 0.1 ml 10% ammonium persulphate (APS) and 0.004 ml tetramethylethylenediamine (TEMED) (Acros Organics, New Jersey, USA) was prepared. The gel was immediately cast in gel chamber by syringe injection allowing 3 - 4 cm space from the top for stacking gel and loading comb. Before gel polymerization, which usually takes an hour at room temperature, water-saturated butanol was poured onto the gel to level the resolving gel surface and prevent the gel from drying out.

After the butanol was poured off (corresponding to completion of gel polymerization), the resolving gel was washed with distilled water and a 3% stacking gel (containing 1.7 ml polyacrylamide, 6.8 ml distilled water, 1.25 ml 1.0M Tris pH 6.8, 0.1 ml 10% SDS, 0.1 ml 10% APS and 0.01 ml TEMED) was then prepared and injected in the gel chamber on top of the former gel. A loading comb was inserted in the stacking gel and allowed to polymerise for 1 hour at room temperature. After removal of the loading comb, distilled water was used to wash each gel well before filling the gel chamber with 1x running buffer (containing 5 mM tris, 50 mM glycine (Fisher Scientific), 2% SDS and distilled water), ready for electrophoresis. Each sample (20 µl) alongside with a protein marker ladder (5 µl), a negative control (non-immune rabbit serum) (10 µl) and a positive control (HeLa cells lysate) (10 µl) were randomly loaded in duplicate onto the gel ensuring to load any empty wells with 10 µl glycerol-bromophenol dye solution to allow even running during electrophoresis.

The gel chamber was attached to the upper and lower electrophoresis chambers, which were filled with 1x running buffer before electrophoresis was run at 150–200 V (20 mA) for 60 minutes or until the desired band reached the bottom of the gel.

2.11.5. Immunoblotting procedure

Two sheets of 1 mm whatman filter paper and one piece of nitrocellulose membrane (Hybond-C Super, GE Healthcare) were cut for each gel to approximately the same size before soaking them in Towbin blotting buffer (containing 25 mM Tris, 192 mM glycine, 20% v/v methanol, and distilled water, p.H 8.3). Following electrophoresis, the gel apparatus was disassembled and the gel carefully unloaded. The stacking gel was removed and the resolving gel was placed directly on top of the nitrocellulose membrane in order to form a sandwich between the two sheets of soaked whatman paper as shown in Figure 2.12.

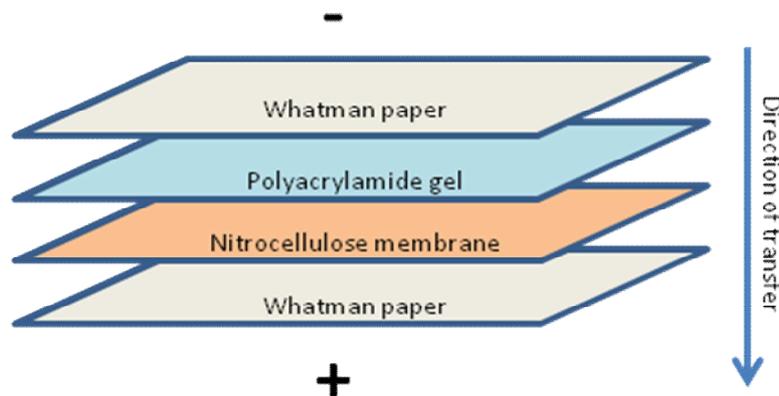


Figure 2.12: Sandwich of polyacrylamide gel and nitrocellulose membrane.

From cathode (-) to anode (+): sponge | sheet of filter paper (whatman) soaked in transfer buffer | polyacrylamide gel | nitrocellulose membrane | sheet of filter paper (whatman) soaked in transfer buffer | sponge.

After trimming the edges of excess of whatman paper and nitrocellulose membrane, the top left corner of the gel was cut off in order to orientate it in the following analysis.

Since current applied and samples move from the negatively-charged cathode to the positively-charged anode, the membrane was located between the gel and the anode to ensure transfer of proteins from gel to membrane. Any excess of liquid and air bubbles were carefully rolled out of the gel sandwich, which was then placed into the transfer system (BioRad 8 cm x 7 cm) for 60 minutes at 80V. When possible, blotting was performed overnight at a lower voltage (25 V) to ensure a more efficient protein transfer from gel to membrane.

2.11.6. Immunodetection procedure

Following protein transfer, the blot was dismantled and the nitrocellulose membrane was briefly washed in 50 ml Tris-tween buffer saline (TTBS) containing 0.2% tween in TBS solution. Efficiency of protein transfer from polyacrylamide gel to nitrocellulose membrane was assessed by washing the gel in Coomassie Brilliant Blue dye for 1 hour. To visualise protein band location 50 ml 1:10 Ponceau S red stain dilution (containing 0.2 g Ponceau S, 3 g trichloroacetic acid (Fisher Scientific), 3 g sulphosalicylic acid (Acros Organics) and distilled water) was applied to the membrane for 10 minutes. After the Ponceau S stain was poured off, excess of staining was washed with distilled water until visualization of the protein bands.

The membrane was then placed on cling film, quickly dried with tissue and wrapped to keep it wet before photographing. A rabbit primary antigen/antibody pen (Alpha Diagnostic Int, San Antonio, TX, USA) was applied to the membrane to highlight the marker bands allowing subsequent visualization of the protein size under chemiluminescence. To prevent non-specific binding and prevent any speckling the membrane was placed overnight at 4°C in 50 ml blocking solution using 10% Marvel dried milk powder and TTBS. The following day the membrane was further blocked for 30 minutes at room temperature with shaking before being rinsed twice in 50 ml TTBS and cut in different strips according to the number of dilutions tested.

FR α primary antibody and a non-immune rabbit serum negative control (1:1000) were diluted in 10 ml of 3% Marvel / TTBS before incubating each sample membrane for 3 hours (or overnight) at room temperature (see Table 2.7 for details). The membranes were then washed 3 times with TTBS for 10 minutes and incubated with 1:2000 HRP conjugated secondary antibody (rabbit to goat polyclonal IgG, Biorad) diluted in 10 ml 3% Marvel / TTBS. After one hour of incubation at room temperature, 15 minute washes in 50 ml TTBS were performed four times followed by two further washes (30 and 45 minutes) in TBS. Chemiluminescent visualisation of the target protein – antibody complex was ensured by applying 1 ml chemiluminescent solution (3 ml of hydrogen peroxide and 3 ml of luminol) onto each membrane (Immobilon Western Chemiluminescent HRP Substrate, Millipore, Massachusetts, USA) which was incubated for 5 minutes before being drained and wrapped in cling film. Protein bands were visualised using a CCD digital camera after exposing the membrane for 1-5-10 minutes under visible light (EPI) and chemiluminescence.

2.11.7. Western blot troubleshooting

Three different antibodies for folate receptor-alpha (FR α) and one for reduced folate carrier (RFC) were used (see table 2.7, page 110). However, none of them detected bands specific for the target antigen showing non-specific binding in the negative control (NRS) samples, whilst a specific band of 37 kDa was detected by the HeLa positive control. At this point, the integrity of frozen samples was questioned and three samples stored in RNA later solution were used for protein extraction using RIPA buffer (instead of cell lytic buffer) in order to investigate whether those tissues were better preserved than the frozen ones. After gel electrophoresis, proteins resolution was better in RNA later samples than in frozen samples as shown in the Ponceau red stain (Figure 2.13). This led to the assumption that the epitope on FR α protein would have been specifically recognised by the antibody.

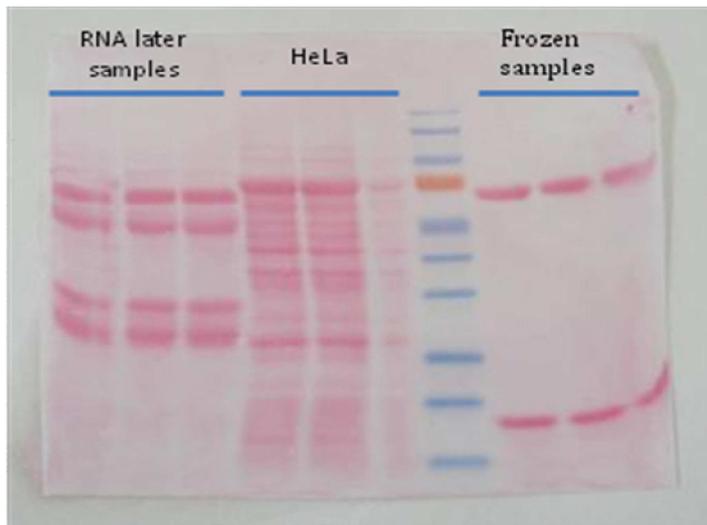


Figure 2.13: Example of Ponceau red staining (1:10) of nitrocellulose membrane.

Left side: protein separation of placental samples from three RNA later samples; middle: protein separation of HeLa cells lysate; right side: protein separation of placental samples from three frozen placental samples. A marker (blue bands) is loaded alongside the samples for estimation of protein size.

This time specific binding for FR α in the three RNA later samples were detected, however the molecular weight of the protein band was almost doubled (68 - 73kDa) compared to the original one (38-42 kDa) and this was once more highlighted by the HeLa positive control.

2.11.8. Protein interactions and urea treatment

The detection of a specific band with a very high molecular weight compared to the expected protein size can have different causes, such as glycosylation, cross reactivity to a contaminant, and protein aggregation. Protein glycosylation can be removed by treating samples with the peptide N-Glycosidase F (PNGase), an amidase which specifically cleaves N-linked oligosaccharides. Albumin (68 kDa) is a typical contaminant of the sample and often can specifically bind the antibody "hiding" the other protein bands. This can be eliminated by immunoprecipitation (RIP) or using a centrifugal filter Centricon with a molecular weight cutoff of 40-50kDa in order to separate soluble protein from the aggregated one.

Finally, if protein aggregation occurred, the interaction can be broken by adding a reducing agent, such as anti-ubiquitin or urea 4-8M, after sample incubation. In this work, it is likely that the 68–73 kDa protein recognised by the antibody results from an interaction of the target protein FR α with another molecule present in the sample and this would impede FR α ability to freely migrate through the polyacrylamide gel. Therefore, a further procedure using urea 4M to separate the aggregate was carried on. Following brief heating of RNA later placental extracts at 95°C, urea 4M was added to each sample which was then incubated at RT for 30 minutes. To remove remaining aggregates centrifugation of the samples was also performed. The resulting protein bands resolution was improved after treatment with urea 4M. However, after immunodetection, the target protein was not recognised by the antibody which once more gave similar non-specific bindings.

2.12. Methylation analysis

Recent experimental advances to detect DNA methylation at genome-scale levels have brought DNA methylation to the vanguard of epigenetics contributing to the development of a large range of experimental techniques [457]. Each of these allows the analysis of DNA methylation at different levels, spanning from genome-wide methylation content to methylation of single residues in specific genes. Global methylation analysis is performed by methods (i.e. chromatography) which can measure the overall level of methyl cytosines in the genome [458]. On the other hand, techniques for gene-specific methylation analysis require the development of computational approaches since they involve the interpretation and comparison of large-scale methylation profiles [459]. While analysis of global DNA methylation content can be determined directly on crude DNA preparations, initial amplification of the target sequence is required when performing the gene-specific analysis.

Because DNA polymerases cannot discriminate between methylated and unmethylated sites, it is necessary to pretreat the DNA with enzymes or chemicals according to the technique applied (i.e. enzyme digestion, affinity enrichment, sodium bisulfite). The most widely used chemical reagent is sodium bisulfite which introduces differences between methylated and unmethylated sites allowing single-base discrimination. Treating the target DNA with sodium bisulfite deaminates unmethylated cytosine to uracil while a lower reactivity with bisulfite protects methylated cytosine from this conversion [460, 461]. DNA pretreatment can then be combined with different detection techniques like high density arrays to detect methylation. Among these, Illumina Infinium Human-Methylation BeadChips [462], have become a popular and reliable high-throughput method to quantitatively measure methylation levels at specific single-base resolution in the human genome [463].

2.12.1. DNA isolation

DNA extraction is a critical first step in the experimental workflow of DNA sequencing and fragment analysis. The overall quality, accuracy and length of the DNA sequence read can be significantly affected by characteristics of the sample itself, and the method chosen for nucleic acid extraction. Efficient genomic DNA isolation was achieved by thorough disruption and digestion of placental tissues which were then purified using QIAamp DNA Mini Kit (50) (QIAGEN). To guarantee homogeneity of the population, a set of 24 placental samples were selected from 5 of the groups (C, n=5; OV, n=4; OB, n=5; GDL, n=5; GDOB, n=5) by maternal age (between 25 and 35 years), BMI (no borderline BMIs), smoking during pregnancy (only no smokers), nulliparity (only first pregnancy) and newborn weight (only AGA).

2.12.1.1. DNA isolation procedure

Each placental sample was chopped from the previously excised samples (see Section 2.4.2, Figure 2.4), and carefully weighed (25 mg) before being cut into small pieces and placed into sterile DispoMix tubes in which 80 ml PBS was added. Samples were then homogenized making sure that any residual deposit was not left in the tube nor any foam produced during the process. Following mechanical disruption 100 µl buffer ATL was added to the homogenate to allow lysis. This buffer cannot be used during the homogenization step as it contains a detergent (for breaking cell membrane). The further addition of 20 µl proteinase K digested the proteins associated with DNA and guaranteed complete digestion of the tissue by vortexing. During incubation at 56°C, to complete the lysis, repeated vortexing was ensured to disperse the sample and after 1-3 hour, the microtube was briefly centrifuged to remove drops from inside the lid. Next, 200 µl buffer AL was directly included into the mixture which was vortexed for 15 seconds and incubated at 70°C for 10 minutes.

Briefly, centrifugation of the microtube to remove any drops from inside the lid was followed by mixing the sample with 200 µl 100% ethanol allowing formation of a white precipitate after vortexing for 15 minutes and pulse-centrifuging. Sample, buffer and alcohol were mixed thoroughly to achieve a homogeneous solution which was carefully applied to a QIAamp Mini spin column placed in a 2 ml microtube. After centrifugation at 5000 g for 1 minute, which was repeated if the solution had not completely passed through the membrane, the spin column was placed in a clean 2 ml collection tube while the filtrate was discarded. Two buffer washes were performed, firstly AW1 (500 µl) followed by centrifugation at 5000 g for 1 minute and filtrate discarding, then AW2 (500 µl) with centrifugation at full speed (12500 g) for 3 minutes. To eliminate the chance of possible buffer AW2 carryover a further step using new 2 ml collection tube was carried out with centrifugation at full speed for 1 minute and discarding of the filtrate.

The QIAamp spin column was immediately placed in a clean 1.5 ml microcentrifuge tube before carefully adding 200 µl buffer EL; the sample was then incubated at room temperature for 5 minute and centrifuge at 5000 g for another minute. This step was repeated to increase DNA yield, which, for 25 mg of tissue, usually corresponds to approximately 10-30 µg of DNA in 400 µl EL buffer. The use of this buffer other than water guaranteed long-term storage of DNA since DNA stored in water is subject to acid hydrolysis. DNA concentration was then measured at the NanoDrop resulting in a range between 25–75 ng/µl with an A260/A280 ratio of 1.7–1.9 and stored at -20°C.

2.12.1.2. DNA methylation detection

Purified DNA extracts were sent to Gen-Probe (Manchester, UK) for quantification of DNA methylation while the data analysis was performed in Nottingham using the Numerical identification of Methylation Biomarker Lists (NIMBL) software developed by Emes and Wessely [402].

DNA methylation levels were detected by using the Infinium array from Illumina (Infinium Human Methylation450 Bead Chip) which features more than 485,000 methylation sites per sample at single-nucleotide resolution [464, 465]. Methylation analysis of all 24 samples was conducted over two arrays since each array can analyse 12 samples in parallel requiring only 500 ng of input (1 µg with automation). Unmethylated and methylated CpG sites were differentiated by pre-treating DNA sample (1 ug) with sodium bisulfite, which converts unmethylated cytosines to uracils allowing single-base discrimination. Following, the bisulfite treated DNA is subjected to whole genome amplification using random hexamer priming and a DNA polymerase with higher proofreading activity compared to the Taq polymerase. The final product is then enzymatically fragmented, purified from dNTPs, primers and enzymes, and ready to apply to the Illumina's high density array [466].

2.12.1.3. Principle of the assay

The molecular principle of the Illumina Infinium assay is based on the immobilization of a single stranded 50 bases DNA oligonucleotides to each bead type and this is followed by specific hybridisation of the probe to the downstream region of the interrogated CpG site. In order to enhance the depth of coverage for methylation analysis, the Infinium HumanMethylation BeadChip450 is provided with two different design types of probe (Infinium I and II) for each CpG (Figure 2.14) [465].

The Infinium I model uses two different probes to differentiate between the unmethylated and methylated bisulfite-converted DNA. Each of the bead types are attached to a single stranded 50 DNA oligonucleotides that differ in sequence only at the free end. One of the bead types corresponds to the methylated cytosine locus whilst the other corresponds to the unmethylated cytosine locus. The latter has been converted into uracil during bisulfite treatment and later amplified as thymine during whole genome amplification. Once the bisulfite converted amplified DNA products are denatured into single strands and hybridized to the chip and allele specific annealing to either the methylation specific probe or the non-methylation probe occurs [400]. Hybridization is followed by single base extension with hapten labelled dideoxynucleotides, among which the ddCTP is labelled with biotin while ddATP, ddUTP and ddGTP are labelled with 2,4-dinitrophenol (DNP) [467].

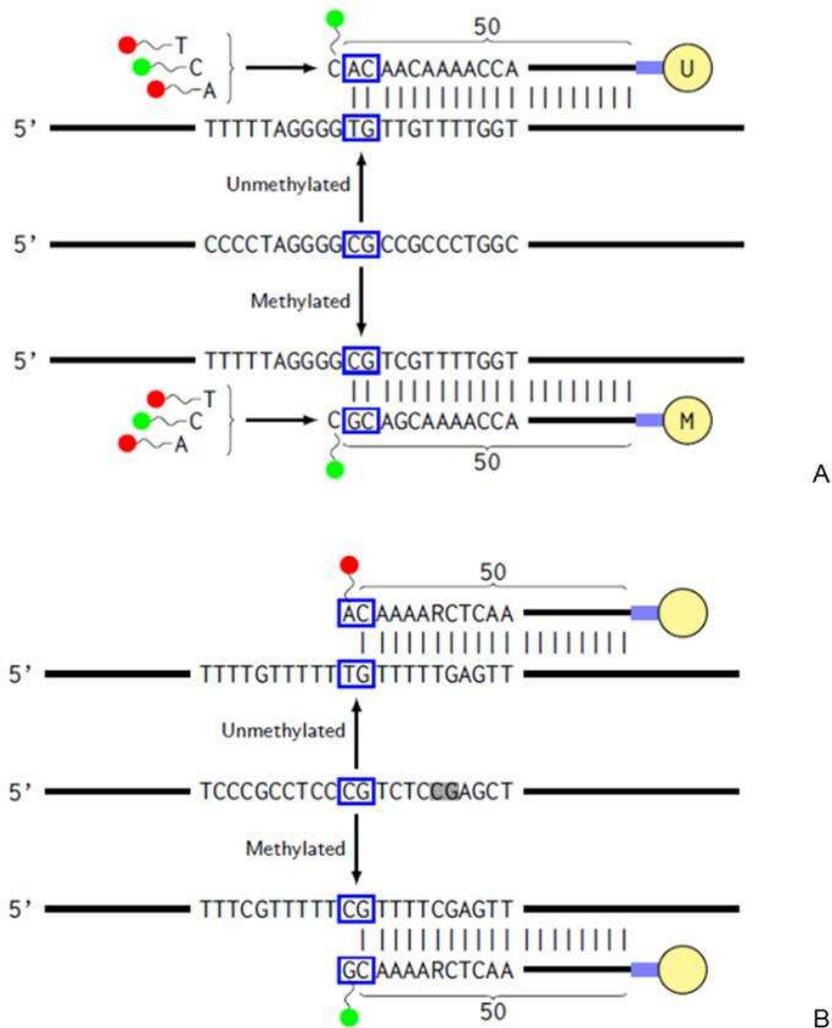


Figure 2.14: Infinium I and II probe design.

Hybridisation and extension of the two different probes in the Infinium I array (A) according to the unmethylated (U) or methylated (M) status of the target DNA (see text for details). Hybridisation and extension of the degenerate probe in the Infinium II array (B) in which the methylation status is unknown (grey color). An 'R' within the probe indicates either a 'G' or an 'A' complementary to the unknown CpG site. Assays were designed using Infinium II chemistry whenever possible.

On the other hand, only one beadtype per locus, which is complementary to both unmethylated and methylated target region, is required in the Infinium II. This is the main beadtype adopted in these assays allowing the use of degenerate oligonucleotides to design for every possible combination of methylation status across up to three CpG sites falling within a probe sequence (for a total of 2^3 possible combinations). For Infinium I assays, all underlying CpG sites were assumed to have the same methylation status as the query base.

After hybridisation, the same single nucleotide labelled with a green fluorophore is used for the extension of both Infinium I probes (2.14-A) at the next position upstream of the interrogated 'C'. On the other hand, single base extension is used to differentiate the methylation state in Infinium II (2.14-B) resulting in labelling an 'A' with a red fluorophore for unmethylated CpG site, whereas a 'G' labelled with a green fluorophore is incorporated at the methylated CpG site.

2.12.2. Quantification of DNA methylation levels

The raw data are analyzed by the software, and the ratios of the fluorescence intensity between the two bead types are calculated allowing quantification of the methylation levels of each CpG site. A ratio of 0 equals to non-methylation of the locus whereas a value of 1 equals to total methylation; a ratio value of 0.5 means that one copy is methylated and the other is not, in the diploid human genome. Since the array contains multiple copies of each bead, a relative methylation level, also known as beta-value (β), is calculated to express the average of the intensity (sum of methylated and unmethylated probe intensities) measured for the same bead type (Equation 2.1). Each beta-values can be intuitively interpreted as the proportion of methylation at a given CpG site with values ranging from 0 (CpG site always found unmethylated in sample DNA) to 1 (CpG site always found methylated in sample DNA).

$$\beta_i = \frac{\max(M_i, 0)}{\max(M_i, 0) + \max(U_i, 0) + \alpha}$$

Equation 2.1: Methylation level expressed as beta-value (β).

M_i and U_i represent the averaged intensities measured for the methylated and unmethylated status of CpG site i , respectively. To avoid negative values after background adjustment, any negative value will be reset to 0. A constant offset (by default $\alpha = 100$) is added to regularise β when both probes intensities are low. α has only a minor effect for the majority of methylation levels because the sum of intensities is generally large compared to this [468].

2.12.3. Analysis of methylation data

A comprehensive annotation file (TAB-delimited), including gene and gene region information, was retrieved by using the HumanMethylation450_15017482_v.1.1 manifest from Illumina [469] and genomic information on DNA sequence and coordinates of gene coding regions were obtained from the UCSC Genome Browser database. Annotation data were then analysed using the NIMBL package, which has been implemented and tested in Matlab release version 7.11 (R2010b) 64-bit [470]. This represents a straightforward approach to identify differential methylation sites and corresponding genes between two groups of samples. The data files includes beta values and corresponding p values ($p > 0.05$), which estimate the quality of the measurement as detected by a Kolmogorov-Smirnov test. This p-value reflects the probability that the resulting CpG site signals are significantly different from the signals of the negative controls on the array, which gives an estimate of the background level. Hence, array sites with one or more of the 24 samples having a p value of less than 0.05, and sites with missing beta values measurements, were excluded from further analysis. The differential methylation settings used for NIMBL are based on a constant level of beta value inter-group distance (i.e. default setting $d=0.2$ corresponding to 20% minimum difference between groups). Therefore, differentially methylated sites were identified as sites with the largest difference in methylation levels between two groups with a minimum beta value distance (d) between non-overlapping groups (Figure 2.15). In each analysis, one sample was allowed to be an "outlier" and was "masked" ($m=0$), whilst the rest were compared to identify those CpGs with the greatest discrimination between groups.

The NIMBL input script for the complete methylation analysis of the test data can be obtained from the example directory within the NIMBL package and are publicly available.

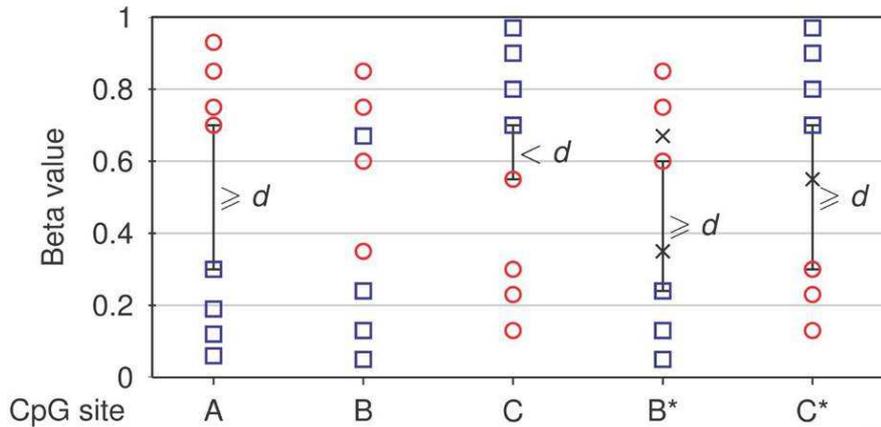


Figure 2.15: Detection of differential methylation by NIMBL.

Each site is tested for a minimum distance of beta values (d) between two groups (corresponding samples are depicted as circles and squares). If no samples are masked ($m=0$), only non-overlapping sites separated by at least a value of d are identified (A). If samples are masked (marked as x), sites with previously overlapping groups (B) or low discrimination (C) are additionally identified and flagged (B*, C*).

Various directories comparing one group to the other were generated within NIMBL, each of them including different files representing differential methylated CpG per gene and the top 50 differential methylated sites. Gene centric plots of the sites of methylation changes relative to the gene were also generated for those genes with the most differential methylation level and their expression determined by QPCR.

2.13. Statistical analysis

Statistical analysis of data was performed using IBM SPSS v20.0 statistical software for Windows package (IBM Corp. Armonk, NY, USA).

Before proceeding to statistical analysis, the distribution of data collected was tested for normality by performing a Kolmogorov-Smirnov normality test. A value of $p \leq 0.05$ indicated that the data was normally distributed and, according to this, appropriate tests (parametric or nonparametric) were used for further analysis [471].

If data were normally distributed, analysis of variance using one-way ANOVA for parametric data was undertaken with a multiple comparison of contrasts between groups (i.e. Bonferroni post-hoc test).

If data were not normally distributed, their logarithm transformation (base 10) was tested for normal distribution. In the case data were still not normally distributed, comparable groups were assessed using Kruskal-Wallis one-way analysis of variance for non-parametric data. To avoid a family-wise error rate, which is the probability of making one or more Type I errors in a family of comparison, a Dunn's test using multiple Mann-Whitney tests was computed for each desired comparison. The resulting p value was then adjusted with the Bonferroni correction, which divides the expected p value (0.05) by the number of paired comparisons to be made (i.e. if $n=3$, $p=0.016$).

The effect of maternal obesity was compared between overweight (OV), obese (OB) and lean control (C) groups. In addition, the influence of gestational diabetes independently and combined with high BMI was assessed between gestational diabetic lean (GDL), gestational diabetic overweight (GDOV), gestational diabetic obese (GDOB) and lean control (C) groups. Association between two continuous variables was assessed using the Pearson correlation, whereas correlation between more than two variables was examined using linear and multiple linear regression analysis.

Categorical data were analysed using Pearson's chi-square test of independence. If the test was statistically significant ($p \leq 0.05$), post-hoc tests using the standardised residual (converted to a z-score) computed for each cell variable, were performed. The residual is the difference between the observed and the expected frequency of the counts. As SPSS does not produce the probability, the size of standardised residuals was compared to the critical values that correspond to an alpha of 0.05 (± 1.96). Statistical difference between two cell variables was detected when standardised residuals resulted ≤ -1.96 or $\geq +1.96$.

For consistency and easier reading, continuous parametric data presented in this study were expressed as mean average \pm standard error of the mean (SEM) whereas for non-parametric data percentages or ratios were used. All statistical graphs produced using Microsoft Office Excel 2010 software (Microsoft Corporation, Berkshire, UK). Further details of any statistical test used are provided in the relevant results Chapters.

Sample size and retrospective power calculations were executed using Minitab v16 statistical software (Minitab Ltd, Warwickshire, UK). This reduced the probability of performing a type I or II error, i.e. by rejecting or accepting the null hypothesis respectively, and enabled to calculate the tests' power based on the sample size and the additional number of samples needed to detect the desired difference.

Chapter 3 - Nutritional and
biochemical influence of
maternal BMI and diabetes on
maternal health and newborn
growth

3.1. Introduction and hypotheses

The incidence of obesity and type-2 diabetes (T2DM) in women of childbearing age is rising dramatically in western countries [472]. Both conditions in pregnancy are associated with adverse metabolic adaptations in the mother compromising fetal and newborn survival and well-being [473]. Obese pregnant women experience an increased risk of miscarriage, preeclampsia, gestational diabetes mellitus (GDM) [24], Caesarean section [474] and stillbirth as well as both intrauterine restriction (IUGR) and macrosomia [475]. Similarly, women who develop diabetes in pregnancy are at increased risk of perinatal morbidity and show higher incidence of obesity, abnormal insulin secretory function [72, 80, 413] as well as large for gestational age (LGA) newborns who have a higher risk of developing obesity, insulin resistance and hypertension in childhood and early adulthood [70, 81, 413]. It is currently estimated that 50-60% of women are overweight or obese at the start of their pregnancies [79, 476] and, overall, 10-25% of pregnant women develop GDM [77, 78]. High pre-pregnancy BMI is also associated with the development of GDM, as 65-75% of women with GDM are overweight or obese [75, 79]. Therefore, the relative impact of pre-pregnancy BMI and maternal hyperglycaemia during gestation on adverse maternal and perinatal outcomes can be difficult to separate.

Overweight and obese women with gestational diabetes are recommended to consume low glycaemic index diets to lower their risk of accelerated fetal growth and LGA [49]. In addition to the adverse effects of hyperglycaemia and obesity, an unbalanced nutrient supply increases the risk of gestational diabetes [477] and a high fat (HF) and low-carbohydrate diet doubles the risk of small for gestational age (SGA) birth [49]. Recently, the beneficial effect of a Mediterranean diet in preventing preterm delivery ensuring adequate fetal development has been proposed [478, 479].

The participants of this study were of Spanish-Caucasian origin consuming a Mediterranean-type diet, which is based on different nutritional components than those routinely consumed in Northern Europe and America [478, 480] and this raises the possibility of evaluating a population's diet and quality by considering specific lifestyle and eating habits [407, 481].

In addition to obesity and GDM, excess gestational weight gain (GWG) complicates a large number of pregnancies and it is highly correlated with high maternal BMI [48] as well as with the development of GDM [65]. Although there is a growing knowledge about increases in the risk of adverse outcomes with increasing GWG [62, 63], many studies examining the effects of maternal obesity and/or gestational diabetes have not accounted for this important factor. The novel approach of my study allows analysis of each condition separately aiming to establish the independent contribution of high BMI, GDM and GWG on pregnancy outcomes.

The consumption of an unhealthy diet in pregnancy is often associated with increased GWG with negative repercussions on maternal, fetal and newborn outcomes [47, 58, 59]. As described in Chapter 1 (Sections 1.4.2), excessive GWG can also occur in women with a lean BMI [66] and has been strongly associated with a higher risk of adverse pregnancy outcomes and childhood obesity. Nonetheless, the relative contribution of maternal diet and weight gain to the complications associated with maternal obesity is still uncertain. The IOM acknowledged the imprecision of the estimates on which the recommended weight gain ranges are based and the many additional factors that might need to be considered for an individual woman. Therefore, in 2009 new IOM guidelines were published based on the WHO cut-off point for the BMI categories [61]. Additionally, a specific narrow range of recommended weight gain for obese women was included. In this Chapter, I focus on the two critical points tightly linked to high BMI, which are maternal diet and GWG, investigating their association with birth outcome.

Therefore, the aims of this Chapter are to investigate whether:

1. high BMI and diabetes in pregnant women are associated with complications in pregnancy, such as Caesarean section, preterm delivery [46] and incidence of macrosomic offspring [33, 34], who are at increased risk of gaining more weight later in life [226]. I hypothesised that these outcomes would also be enhanced with the combined effect of both obesity and GDM
2. lower educational achievement is associated with the consumption of high energy diets and exceeding the IOM recommended gestational weight gain resulting in overweight and obesity in pregnancy [41, 46, 51, 64]
3. insulin resistance in pregnancies complicated by obesity and GDM reflects altered plasma concentrations of maternal metabolites, such as glucose and insulin, which is associated with fetal hyperinsulinaemia and consequent increased fetal growth in obese [216, 227-229] and diabetic [169, 186] pregnancies.

3.2. Methods

A description of the study design and all materials and methods used is provided in Chapter 2. Briefly, pregnant women participating in the Preobe study (P06-CTS-02341) were recruited in the 20th week of pregnancy and classified according to their BMI. At 28th weeks of gestation, they were further classified on the basis of BMI and glucose tolerance, making six groups (Chapter 2, Section 2.2, Figure 2.2). Maternal and fetal anthropometries were evaluated at 24 and 34 weeks of pregnancy for women in the BMI group whereas those of participants with gestational diabetes were measured only at the 34th gestational week (Chapter 2, Section 2.4, Figure 2.3). A similar procedure was carried on for collection of maternal venous blood for women in the BMI groups (at 24, 34 and 40 weeks of gestation) and for women with gestational diabetes at pregnancy weeks 34 and 40. After birth, umbilical venous blood samples were collected and placentas were snapped frozen and stored at -80°C for further analysis in Nottingham. Information about maternal nutrient intake before delivery (between 34-40 gestational weeks) was collected using 7 day estimated food intake records. Nutritional data were then analysed for nutrient intake by using a nutritional software program (CESNID 1.0, Barcelona University, Spain) based on validated [412] Spanish food tables ("Tablas de composición de alimentos del CESNID").

Maternal pre-pregnancy BMI was calculated as weight in kilograms divided by height in metres² by using self-reported pre-pregnancy weight, as recorded from maternal questionnaires, and height, measured at the first antenatal clinic visit. Weight gain during pregnancy was calculated using clinical data recorded at gestational week 24 and 34 as the maternal weight at 40 weeks of gestation was not measured directly but reported by the mother either at the time of delivery or by telephone. Therefore, the measurement of total weight gain in pregnancy and post-gestational weight loss were calculated referring to the last recorded weight (34 gestational weeks).

Maternal weight gain was calculated as the difference between pre-gestational weight and maternal weight at the 34th week. Maternal weight loss was defined as the difference between maternal weight at 34 gestational weeks and 6 months after pregnancy. Weight change was compared to the 2009 IOM guidelines calculated for the 34th week of pregnancy [61] and categorised as inadequate, adequate, or excess weight gain. As described in Chapter 2, Section 2.2.1, adequate GWG range until the first 34 gestational weeks was defined as 9.8-13.6 kg for women of normal BMI, 5.9-9.8 kg for overweight women and 4.2-7.6 kg for obese women. Inadequate and excessive GWG were considered as less, and more, than adequate GWG, respectively. In the infants, a birth weight of less than 2.5 kg was considered low birth weight, whereas high birth weight was considered higher than 4.0 kg. Large (LGA), average (AGA) and small for gestational age (SGA), defined as >90th, 10-90th, <10th birthweight population centile respectively [25], were calculated using the Lubchenco growth curves [405] adjusted for newborn weight and gestational age. Infant anthropometry was evaluated by a paediatrician at 3, 6 and 12 months of age respectively and maternal anthropometry at 6 months post-delivery. To determine whether changes in placental weight and gene expression were reflected in modification of placental phenotype, histological analysis and immunohistochemistry for fibronectin, an extracellular matrix molecule, were performed. However, these analyses were hampered by degradation of a large number of frozen placental samples caused by delays in fixing the tissue samples at the time of delivery (see Chapter 2, Section 2.9.2.2 and Section 2.10.1, for details). Finally, placental triglycerides (TG) were isolated and extracted with chloroform and methanol (2:1) following the adapted Folch method [439] (Section 2.7, Chapter 2). The Randox triglycerides assay kit (Cat. No: TR212, Randox laboratories Ltd, Crumlin, UK) was used to determine triglycerides concentration by spectrophotometric absorbance. After filtration, samples were dissolved in BDH and triton X-100 and placental TG concentrations calculated by interpolation of measured values from an internal control standard curve.

3.3. Results

The relative effects of maternal BMI and GDM for each outcome has been analysed both separately and combined, as shown in the following subsections.

3.3.1. Maternal socio-economical outcomes

As illustrated in Table 3.1 high BMI in pregnancy was associated with having undertaken professional studies compared to control mothers, who were more likely to enrol higher educational studies (University).

Table 3.1: Sociological characteristics of control (C), overweight (OV) and obese (OB) pregnant women at the time of recruitment (20 gestational weeks).

Maternal highest education attainment	C (n=59)	OV (n=28)	OB (n=22)
Primary school	8	4	4
Secondary school	12	4	4
Professional level	14	6	9*
University	24	11	5*
PhD	1	1	0
Maternal employment status	C (n=59)	OV (n=28)	OB (n=21)
Unemployed	16	4	7
Not working outside the home	2	4*	1
Temporary employment	13	5	7
Permanent employment	28	15	6

Data represents number of women per group (n); Statistical differences denoted by *,[†] correspond to p<0.05 compared to control and overweight respectively (Chi-square test).

Overweight women were more likely to work within the domestic environment compared to lean mothers.

On the other hand, a significant proportion of overweight and obese mothers with GDM did not continue studying after primary school (Table 3.2). The highest educational achievement of lean control women was to university degree level. This was significantly lower in obese mothers with GDM, who were also associated with the highest level of not working outside the home.

Table 3.2: Sociological characteristics of control (C), gestational diabetic lean (GDL), gestational diabetic overweight (GDOV) and gestational diabetic obese (GDOB) pregnant women at the time of recruitment (20 gestational weeks).

Maternal highest education attainment	C (n=59)	GDL (n=14)	GDOV (n=13)	GDOB (n=10)
Primary school	8	2	5*	4*
Secondary school	12	5	1	1
Diploma	14	1	1	4
University	24	5	6	1*
PhD	1	1	0	0
Maternal employment status (%)	C (n=59)	GDL (n=14)	GDOV (n=13)	GDOB (n=9)
Unemployed	16	2	1	4
Not working outside the home	2	0	0	2*
Temporary employment	13	1	4	2
Permanent employment	28	11	8	1*

Data represents number of women; n: women/group; Statistical differences denoted by * correspond to $p < 0.05$ compared to lean control (Chi-square test).

3.3.2. Maternal dietary intake

Only 86 mothers out of the 149 participants completed, and returned, the 7 day food record questionnaire. Nutritional analysis of those food records available indicated that the only difference in reported major macronutrient intake between BMI groups was in total carbohydrate intake as illustrated in Table 3.3.

Table 3.3: Maternal energy and macronutrient intake during late gestation (34 gestational weeks) in control (C), overweight (OV) and obese (OB) pregnant women.

Energy and macronutrients	C (n=37)	OV (n=16)	OB (n=8)
Energy (kcal/day)	2086.8±52.8	1933.1±150.1	1927.2±200.6
Total carbohydrates (g/day)	228.4±7.0	207.9±15.9	183.8±20.8
Monosaccharides	116.6±5.6	103.7±11.1	86.2±13.6
Polysaccharides	110.9±4.7	104.6±5.7	97.6±10.9
Total lipids (g/day)	90.3±3.3	84.3±8.6	93.6±10.4
SFA	30.2±1.7	30.0±3.6	31.6±3.4
MUFA	33.9±2.2	34.4±3.4	39.2±5.3
PUFA	12.6±0.8	12.4±1.4	15.0±2.1
Cholesterol (g/day)	0.33±0.01	0.36±0.04	0.37±0.03
Total proteins (g/day)	88.5±3.5	83.7±5.4	82.9±7.7
Animal protein	63.1±3.3	59.7±4.6	60.5±5.3
Vegetal protein	25.4±0.8	23.9±1.6	23.5±3.2
Total fibre (g/day)	19.9±0.7	17.5±1.0	18.3±3.0

Data represents means ± S.E.M. n: women/group; SFA: saturated FA; MUFA: monounsaturated FA; PUFA: polyunsaturated FA.

Although there was a trend at decreasing energy (p=0.1) and total carbohydrate (p=0.08) intake in obese mothers compared to lean women, the difference did not reach statistical difference.

Another trend in decreased total proteins, mainly of vegetable origin ($p=0.08$), was observed among obese women. Total lipids, saturated FA (SFA), monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) intake as well as cholesterol and total fibre consumption were not different between BMI groups. Obese women with GDM had a significantly lower reported total energy intake compared to lean healthy mothers without GDM (Table 3.4).

Table 3.4: Maternal energy and macronutrient intake during late gestation (34 gestational weeks) in control (C), gestational diabetic lean (GDL), gestational diabetic overweight (GDOV) and gestational diabetic obese (GDOB) pregnant women.

Energy and macronutrients intake	C (n=37)	GDL (n=11)	GDOV (n=8)	GDOB (n=6)
Energy (kcal/day)	2086.8±52.8	1900.3±120.1	1762.9±140.7	1565.1±117.5**
Total carbohydrates (g/day)	228.4±7.0	174.0±11.9**	189.4±20.6	155.2±13.4**
Monosaccharides	116.6±5.6	85.9±6.8*	89.9±10.8	73.6±9.2*
Polysaccharides	110.9±4.7	88.3±7.3	99.4±11.0	81.7±8.2
Total lipids (g/day)	90.3±3.3	90.2±7.3	70.8±6.7	66.1±7.9*
SFA	30.2±1.7	29.0±5.1	22.0±2.4*	19.6±2.1**
MUFA	33.9±2.2	38.5±3.5	30.7±3.7	29.2±4.9
PUFA	12.6±0.8	14.4±2.5	11.7±1.2	11.0±2.3
Cholesterol (g/day)	0.33±0.01	0.36±0.04	0.28±0.03	0.29±0.03
Total proteins (g/day)	88.5±3.5	96.8±7.1	90.1±7.7	85.5±4.6
Animal protein	63.1±3.3	74.5±6.9	62.1±6.1	61.9±4.6
Vegetal protein	25.4±0.8	22.4±1.1	28.1±2.8	22.4±2.3
Total fibre (g/day)	19.9±0.7	20.8±1.2	26.3±3.4*	19.6±3.0

Data represents means ± S.E.M. Statistical differences denoted by *, ** correspond to $p<0.05$ and $p<0.01$ respectively compared to control group (1-way ANOVA; Bonferroni post-hoc test); n: women/group; SFA: saturated FA; MUFA: monounsaturated FA; PUFA: polyunsaturated FA.

This was reflected by diminished total intake of carbohydrates, particularly monosaccharides, total lipid and saturated fatty acid (SFA), in those women. Similarly, total carbohydrate and monosaccharide consumption was lower in lean mothers with GDM compared to lean controls. However, their lipid and SFA intake was not different than that in the control group, reflecting similar energy intake. Total protein, both of animal and vegetable origin, were consumed in the same amount in all groups. Fibre intake was also similar among participants, although overweight mothers with GDM consumed the highest proportion compared to their healthy counterparts. When micronutrient intake was considered, 130 participants out of 149 reported daily intake of folate and iodine supplements at 24th week of pregnancy, whereas only 62 out of 130 mothers consumed iron supplements (Table 3.5).

Table 3.5: Maternal micronutrient intake during late gestation (34 gestational weeks) in control (C), overweight (OV) and obese (OB) pregnant women.

Ion intake (mg/day)	C (n=37)	OV (n=16)	OB (n=8)
Sodium	2760.6±129.8	2792.6±224.7	2451.9±258.3
Potassium	3441.0±94.5	3013.1±181.4	2784.4±314.2 [*]
Calcium	1006.3±34.2	888.6±64.3	747.3±64.9 ^{**}
Magnesium	328.4±7.7	296.6±16.5	271.9±30.0 [*]
Phosphorus	1413.7±44.7	1322.3±89.2	1251.5±98.6
Iron	12.5±0.4	11.03±0.8	11.8±1.6
Vitamin intake (mg/day)	C (n=37)	OV (n=16)	OB (n=8)
Vitamin A	0.95±0.06	0.83±0.08	0.84±0.15
Vitamin D (x 10 ⁻³)	2.20±0.21	1.70±0.21	2.70±0.59
Vitamin B1	1.57±0.07	1.45±0.10	1.36±0.13
Vitamin B2	1.88±0.06	1.71±0.13	1.54±0.12
Vitamin B6	2.02±0.07	1.83±0.12	1.74±0.23
Vitamin C	162.2±12.8	133.7±16.0	115.8±31.0

Data represents means ± S.E.M. Statistical differences denoted by ^{*}, ^{**} correspond to p<0.05 and p<0.01 respectively compared to control group (1-way ANOVA; Bonferroni post-hoc test); n: women/group.

As expected [41, 45, 46], higher BMI was associated with lower reported intake of ions compared to healthy controls. Obese mothers reported significantly lower daily intake of calcium, potassium and magnesium compared to women with lean BMI.

Table 3.6 illustrates that vitamin D consumption in lean mothers with GDM was significantly higher compared to their healthy counterparts, although no other major differences in other vitamins or ion intake were found between GDM groups.

Table 3.6: Maternal micronutrient intake during late gestation (34 gestational weeks) in control (C), gestational diabetic lean (GDL), gestational diabetic overweight (GDOV) and gestational diabetic obese (GDOB) pregnant women.

Micronutrients intake (mg/day)	C (n=37)	GDL (n=11)	GDOV (n=8)	GDOB (n=6)
Sodium	2760.6±129.8	2791.6±276.8	2606.6±217.0	2348.2±217.1
Potassium	3441.0±94.5	3539.3±163.5	3804.2±401.3	3195.6±377.6
Calcium	1006.3±34.2	931.2±75.1	1102.2±131.0	931.7±102.6
Magnesium	328.4±7.7	309.4±16.2	370.9±36.8	328.9±33.2
Phosphorus	1413.7±44.7	1403.3±86.2	1482.1±129.7	1322.5±93.2
Iron	12.5±0.4	13.0±0.9	12.7±0.9	12.5±1.5
Vitamin intake (mg/day)	C (n=37)	GDL (n=11)	GDOV (n=8)	GDOB (n=6)
Vitamin A	0.95±0.06	1.35±0.17	1.13±0.14	0.91±0.15
Vitamin D (x 10 ⁻³)	2.20±0.21	3.81±0.66*	2.01±0.65	3.01±0.67
Vitamin B1	1.57±0.07	1.52±0.10	1.57±0.13	1.44±0.14
Vitamin B2	1.88±0.06	2.04±0.18	2.05±0.17	1.80±0.17
Vitamin B6	2.02±0.07	2.22±0.14	2.28±0.23	2.10±0.20
Vitamin C	162.2±12.8	178.5±10.6	211.1±31.5	139.5±16.2

Data represents means ± S.E.M. Statistical differences denoted by * correspond to p<0.05 compared to control group (1-way ANOVA; Bonferroni post-hoc test); n: women/group.

3.3.3. Maternal clinical characteristics and anthropometry

The majority of women in this study were experiencing their first pregnancy (Table 3.7, Table 3.8). Maternal age and gestational age at delivery did not differ between BMI groups (Table 3.7). Overweight was associated with increased smoking during pregnancy (24%) compared to healthy controls (12%) and obese mothers (9%).

Table 3.7: Clinical characteristics of control (C), overweight (OV) and obese (OB) pregnant women.

Maternal clinical characteristics	C (n=59 ^Ψ)	OV (n=29 ^Ψ)	OB (n=22 ^Ψ)
Maternal age (yr)	30.4±0.6	31.9±0.8	29.0±1.0
Gestational age (wk)	39.2±0.1	39.4±0.3	39.3±0.4
Smoking status (yes/no)	7/51	7/22 ^{*F}	2/20
Parity (PP/MP)	33/24	13/14	12/7
Type of delivery (S/C)	50/7	20/7	13/7 [*]
Delivery (P/T/O)	0/52/6	1/21/7	2/13/7 [*]

Data represents means ± S.E.M.; n: women/group; ^Ψ some data are missing due to lack of recorded information; yr: years; wk: weeks; PP: primiparous; MP: multiparous; S: spontaneous; C: Caesarean P: preterm; T: term; O: overdue; gw: gestational weeks; m: months. Statistical differences denoted by ^{*},^F correspond to p<0.05 compared to control and obese respectively (Chi-square test).

All mothers gave birth to healthy offspring although obese women were 1.3 and 2.9 times more likely to experience Caesarean sections than overweight and healthy control mothers, respectively. Women in the highest BMI group had a trend (p=0.09) to increased risk of giving birth before 37 weeks of gestation (32%) followed by overweight (24.1%) and lean (10.3%) mothers.

Similarly, women who were obese had 3.1 and 1.3 times higher risk of experiencing an overdue pregnancy than overweight mothers and lean counterparts respectively.

Clinical characteristics of pregnant women with GDM, the majority of whom did not smoke during pregnancy, are shown in Table 3.8. Obese mothers with GDM were older than their lean counterparts without GDM.

Almost all mothers with GDM delivered at term (37-40 gestational weeks), whereas one preterm birth was observed among women with both overweight and GDM (<37 gestational weeks), who also gave birth post-term once (>40 gestational weeks). The highest proportion of women experiencing Caesarean section was observed among obese mothers with GDM (0.5 times) compared to lean controls.

Table 3.8: Clinical characteristics of control (C), gestational diabetic lean (GDL), gestational diabetic overweight (GDOV) and gestational diabetic obese (GDOB) pregnant women.

Maternal clinical characteristics	C (n=59^ψ)	GDL (n=14^ψ)	GDOV (n=14^ψ)	GDOB (n=11^ψ)
Maternal age (yr)	30.4±0.6	33.1±1.1	33.5±0.9	34.7±1.3**
Gestational age (wk)	39.2±0.1	39.2±0.4	38.5±0.7	38.9±0.4
Smoking status (yes/no)	7/51	0/13	1/10	0/7
Parity (PP/MP)	33/24	5/5	5/5	6/3
Type of delivery (S/C)	50/7	9/3	8/2	5/5**
Pregnancy (P/T/O)	0/52/6	0/10/2	1/9/1	0/9/1

Data represents means ± S.E.M.; n: women/group; ^ψ some data are missing due to lack of recorded information; yr: years; wk: weeks; PP: primiparous; MP: multiparous; S: spontaneous; C: caesarean; P: preterm; T: term; O: overdue; gw: gestational weeks; m: months. Statistical differences denoted by ** correspond to p<0.01 compared to control group (1-way ANOVA; Bonferroni post-hoc test for continuous variables; Chi-square test for categorical data).

Women with higher BMI in pregnancy, independently of GDM, showed reduced weight gain compared to lean control group without GDM (Figure 3.1 A). The combined effects of higher BMI and GDM were associated with considerably less weight gain during the first 34 weeks of pregnancy (Figure 3.1 B).

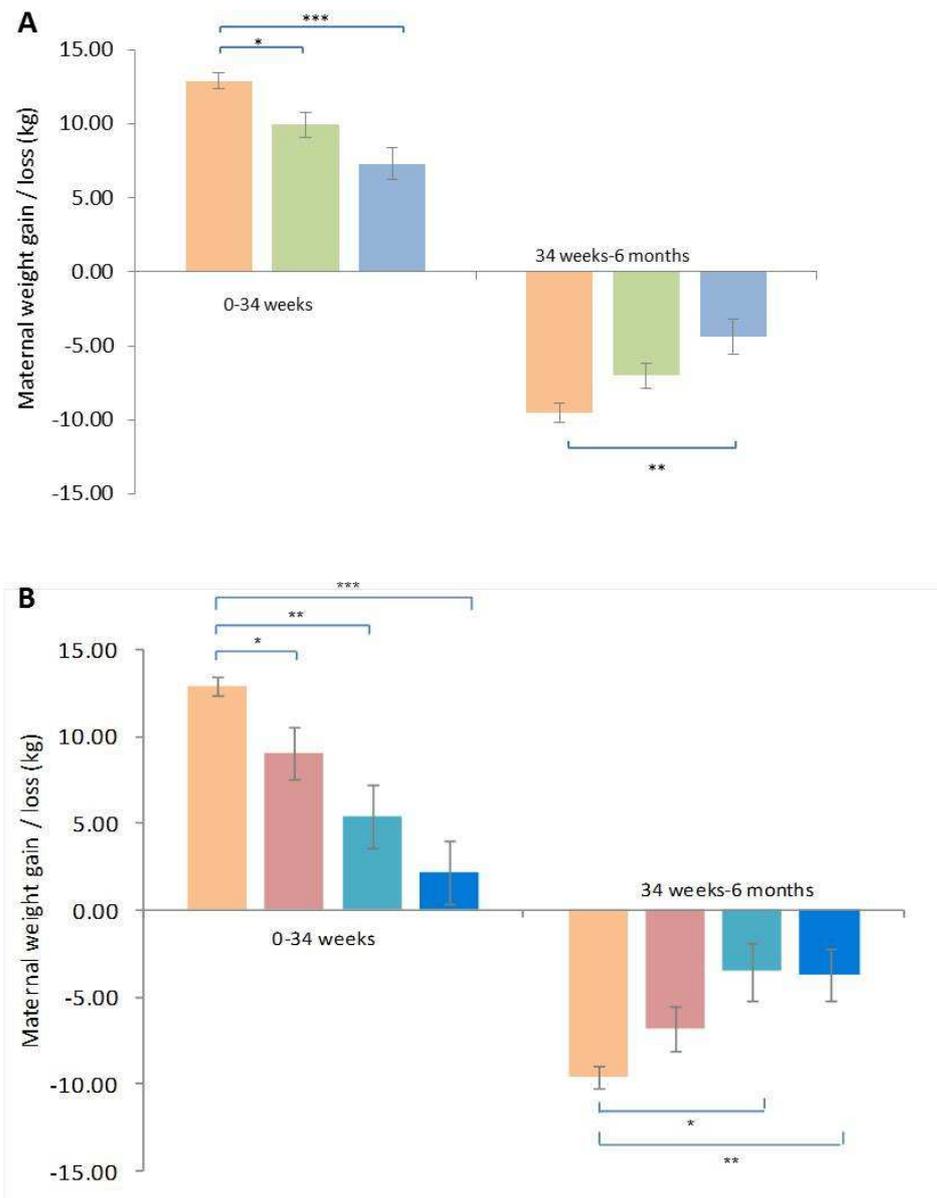


Figure 3.1: Maternal weight gain during first 34 weeks of gestation and maternal weight loss from 34 gestational weeks to 6 months post-delivery.

A) control (orange bars; n=59), overweight (green bars; n=29) and obese (light blue bars; n=22) women and in (B) control (orange; n=59), gestational diabetic lean (red; n=14), overweight (aqua; n=14) and obese (blue; n=11) mothers. Data represents means \pm S.E.M. Statistical differences denoted by *, **, *** correspond to $p < 0.05$, $p < 0.01$, $p < 0.001$ respectively (1-way ANOVA; Bonferroni post-hoc test).

Interestingly, maternal weight loss from 34 gestational weeks to 6 months after pregnancy decreased with BMI, and the highest weight retention was observed in obese women with or without GDM.

Similarly, for each IOM category (Low, Adequate, High), women with increased BMI gained less weight during gestation compared to healthy and lean mothers (Table 3.9 and 3.10). By definition, BMI was significantly higher in overweight and obese groups with (Table 3.9) or without (Table 3.10) GDM before, throughout and 6 months after gestation.

Table 3.9: Anthropometrical characteristics of pregnant women in the control (C), overweight (OV) and obese (OB) BMI groups.

Maternal BMI (kg/m²)	C (n=59)	OV (n=29)	OB (n=22)
Pre gestational BMI	21.8±0.2	27.4±0.3 ^{***}	32.5±0.5 ^{***}
Gestational BMI 24 weeks	24.9±0.3	29.7±0.4 ^{***}	33.6±0.4 ^{***}
Gestational BMI 34 weeks	26.6±0.3	31.3±0.4 ^{***}	35.4±0.5 ^{***}
BMI 6 months post-delivery	23.5±0.4	28.5±0.6 ^{***}	32.5±0.4 ^{***}
Maternal GWG 0-34 weeks (kg)^ψ	C (n=59)	OV (n=29)	OB (n=22)
Low	7.7±0.4	2.7±0.9	2.5±0.6
Adequate	12.1±0.2	8.1±0.4	5.9±0.4
High	16.1±0.6	13.4±0.6	12.3±1.1

Data represents means ± S.E.M.; n: women/group; BMI: body mass index; GWG: gestational weight gain during the first 34 gestational weeks (^ψ) based on 2009 IOM guidelines for each category^[61]:

Low: <9.8 kg for control, <5.9 kg for overweight and <4.2 kg for obese women; Adequate: 9.8-13.6 kg for control, 5.9-9.8 kg for overweight and 4.2-7.6 kg for obese women; High: >13.6 kg for control, >9.8 kg for overweight and >7.6 kg for obese women. Statistical differences denoted by ^{***} correspond to p<0.001 compared to control group (1-way ANOVA; Bonferroni post-hoc test).

Table 3.10: Clinical characteristics of control (C), gestational diabetic lean (GDL), gestational diabetic overweight (GDOV) and gestational diabetic obese (GDOB) pregnant women.

Maternal BMI (kg/m²)	C (n=59)	GDL (n=14)	GDOV (n=14)	GDOB (n=11)
Pre gestational BMI	21.8±0.23	22.4±0.5	26.98±0.4 ^{***}	35.52±1.5 ^{***}
Gestational BMI 34 weeks	26.6±0.3	25.9±0.7	29.67±0.7 ^{**}	36.4±1.2 ^{***}
BMI 6 months post-delivery	23.5±0.4	23.2±0.8	27.1±0.5 ^{***}	35.2±1.4 ^{***}
Maternal GWG 0-34 week(kg)^ψ	C (n=59)	GDL (n=14)	GDOV (n=14)	GDOB (n=11)
Low	7.7±0.4	5.2±1.2 [*]	- 0.81±2.1 ^{***}	- 3.4±2.1 ^{***}
Adequate	12.1±0.2	11.4±0.6	7.2±0.3 ^{**}	5.2±0.4 ^{**}
High	16.1±0.6	17.0±0.6	15.6±1.0 [*]	11.3±1.9 [*]

Data represents means ± S.E.M.; n: women/group; BMI: body mass index; GWG: gestational weight gain during the first 34 gestational weeks (^ψ) based on 2009 IOM guidelines^[61]:

Low: <9.8 kg for control, <5.9 kg for overweight and <4.2 kg for obese women; Adequate: 9.8-13.6 kg for control, 5.9-9.8 kg for overweight and 4.2-7.6 kg for obese women; High: >13.6 kg for control, >9.8 kg for overweight and >7.6 kg for obese women. Statistical differences denoted by ^{*},^{**},^{***} correspond to p<0.05, p<0.01 and p<0.001 respectively compared to control group (1-way ANOVA; Bonferroni post-hoc test).

Despite overweight and obese mothers without GDM gaining the least weight compared to their healthy counterparts, a great proportion of them exceeded the 2009 IOM recommendations (Figure 3.2 A).

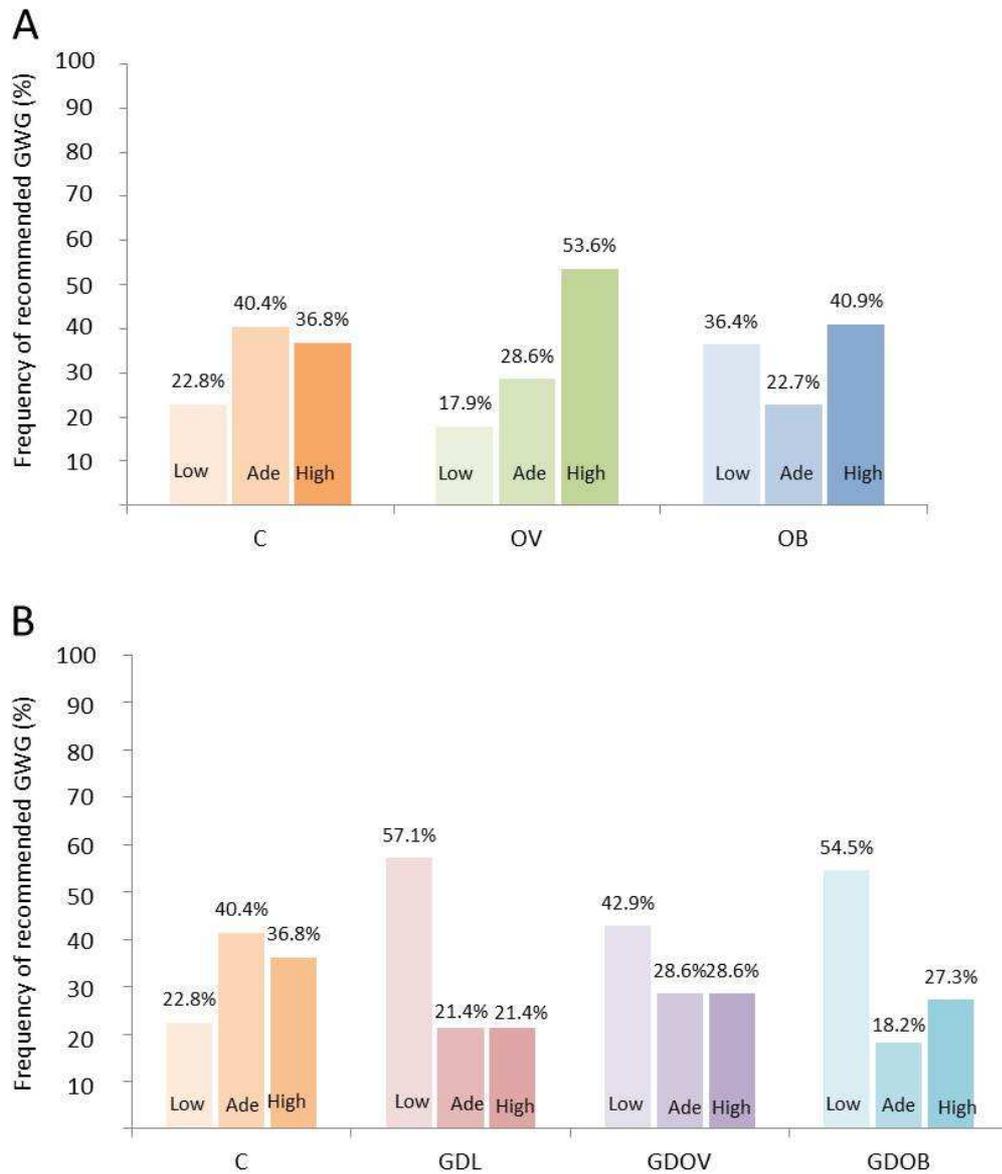


Figure 3.2: Frequency of low (Low), adequate (Ade) and excessive (High) gestational weight gain according to the 2009 IOM recommendations during the first 34 weeks of gestation ^[61].

A: control (C; n=59), overweight (OV; n=29) and obese (OB; n=22) pregnant women; B: control (C; n=59), gestational diabetic lean (GDL; n=14), overweight (GDOV; n=14) and obese (GDOB; n=11) mothers.

Overall, those who entered pregnancy with a normal BMI were 1.4 and 1.7 times more likely to show adequate GWG, based on 2009 IOM guidelines, compared to overweight and obese mothers respectively (C: 40.4%; OV: 28.6%; OB: 22.7%; $X^2 = 5.24$, $p = 0.026$; Std. Residual=2.1).

Interestingly, overweight women were 1.4 and 1.3 times more likely to exceed the IOM recommended GWG compared to their lean counterparts and obese mothers respectively (C: 36.8%; OV: 53.6%; OB: 40.9%; $X^2= 5.24$, $p=0.046$; Std. Residual=1.7). Nonetheless, among obese women a similar proportion gained less than adequate weight throughout the first 34 weeks of gestation (OB low GWG: 36.4%; OB high GWG: 40.9%). On the other hand, the effect of GDM was associated with lower than recommended GWG compared on IOM guidelines (Figure 3.2 B). The proportion of lean women with GDM who gained less GWG than recommended by IOM was 2.5 higher compared to lean healthy control (C: 22.8%; GDL: 57.1%; $X^2=9.6$; $p=0.034$; Std. Residual=1.5). This was accompanied by lower weight gain throughout pregnancy (Figure 3.1 B). The effect of BMI combined with GDM was similar to that of GDM alone (GDOV: 42.9%; GDOB: 54.5%), although not statistically significant between GDM groups.

3.3.4. Maternal plasma metabolites

Physiological and clinical characteristics of mothers without GDM at different times of gestation are summarised in Table 3.11. Increased glucose concentrations in maternal plasma were detected at 40 gestational weeks in obese mothers compared to lean controls. However, these values were measured post-prandially, whilst fasting plasma glucose from an OGTT had revealed that these women were not having glucose intolerance or gestational diabetes.

Table 3.11: Physiological characteristics of maternal plasma at 24, 34 and 40 gestational weeks (gw) in control (C), overweight (OV) and obese (OB) pregnant women.

Maternal metabolites	C (n=59)	OV (n=29)	OB (n=22)
Glucose (mg/dl)			
24 gw	78.2±1.6	85.0±3.1	80.6±3.9
34 gw	84.6±1.9	86.7±4.4	86.7±3.3
40 gw	77.4±3.1	82.9±4.6	97.5±10.2*
Triglycerides (mg/dl)			
24 gw	166.3±9.2	190.4±13.8	171.9±17.8
34 gw	231.6±10.6	248.4±13.4	245.2±14.3
40 gw	210.5±9.9	237.2±15.3	223.8±18.1
Cholesterol (mg/dl)			
24 gw	243.1±5.2	246.4±8.4	242.4±13.98
34 gw	256.1±5.8	261.3±9.7	246.6±10.72
40 gw	246.4±7.3	254.0±9.8	247.2±15.67

Data represents means ± S.E.M.; n: women/group; Statistical differences denoted by * correspond to $p < 0.05$ compared to control group (1-way ANOVA; Bonferroni post-hoc test).

Similarly, women with GDM had enhanced plasma glucose concentrations at both 34 and 40 weeks of gestation. Overweight women with GDM showed no difference in glucose concentrations at 34th gestational weeks (Table 3.12). Surprisingly, no differences in maternal serum TG and cholesterol were observed in women in the highest BMI group in either presence, or absence, of GDM.

Table 3.12: Physiological characteristics of maternal plasma at 34 and 40 gestational weeks in control (C), gestational diabetic lean (GDL), gestational diabetic overweight (GDOV) and gestational diabetic obese (GDOB) pregnant women.

Maternal metabolites	C (n=59)	GDL (n=14)	GDOV (n=14)	GDOB (n=11)
Glucose (mg/dl)				
34 gw	84.6±1.9	98.7±4.4 ^{**}	87.4±4.4	98.5±8.9 [*]
40 gw	77.3±3.1	109.0±11.3 ^{**}	112.1±10.3 ^{**}	110.9±10.9 ^{**}
Triglycerides (mg/dl)				
34 gw	231.6±10.6	255.4±32.5	233.3±21.2	247.7±26.0
40 gw	210.5±9.9	237.1±15.3	215.8±16.4	221.4±18.9
Cholesterol (mg/dl)				
34 gw	256.1±5.8	253.4±14.1	257.5±10.0	254.5±15.6
40 gw	246.4±7.3	229.5±16.2	241.1±8.5	228.8±19.1

Data represents means ± S.E.M.; n: women/group. Statistical differences denoted by ^{*},^{**} correspond to p<0.05 and p<0.01 respectively compared to control group (1-way ANOVA; Bonferroni post-hoc test).

Insulin concentrations in maternal plasma were greater in the obese (BMI) group at 40 gestational weeks than in the lean comparison group regardless of GDM (Figure 3.3). By definition, insulin plasma levels at term were higher in women with GDM (Figure 3.4), although they were diet-treated.

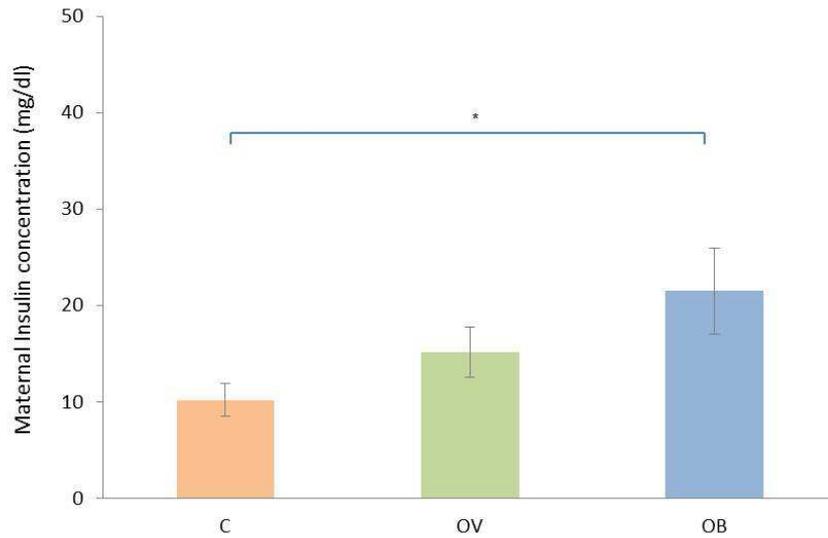


Figure 3.3: Plasma insulin concentration at 34th gestational week in control (C; n=59), overweight (OV; n=29) and obese (OB; n=22) pregnant women. Data represents means \pm S.E.M. Statistical differences denoted by * correspond to $p < 0.05$ (1-way ANOVA; Bonferroni post-hoc test).

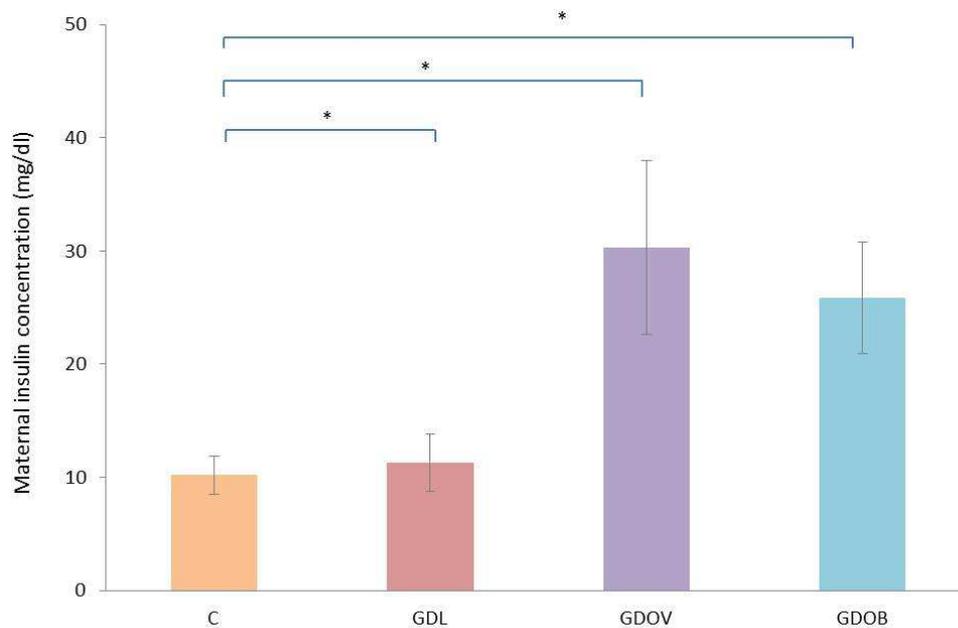


Figure 3.4: Plasma insulin concentration at 34th gestational week in control (C; n=59), gestational diabetic lean (GDL; n=14), gestational diabetic overweight (GDOV; n=14) and gestational diabetic obese (GDOB; n=11) pregnant women. Data represents means \pm S.E.M. Statistical differences denoted by * correspond to $p < 0.05$ (1-way ANOVA; Bonferroni post-hoc test).

3.3.5. Fetal and placental outcomes

Physiological characteristics of metabolites measured in cord blood in the three BMI groups and in the gestational diabetic groups are illustrated in Table 3.13 and 3.14 respectively.

Table 3.13: Cord blood and placental characteristics in control (C), overweight (OV) and obese (OB) women.

Cord blood metabolites	C (n=33)	OV (n=15)	OB (n=12)
Cord blood glucose (mg/dl)	71.2±3.1	66.1±6.2	59.3±6.6
Cord blood triglycerides (mg/dl)	47.5±2.9	46.4±4.0	45.6±6.8
Cord blood cholesterol (mg/dl)	32.3±2.5	26.1±2.3	29.9±4.1
Placental TG	C (n=50)	OV (n=26)	OB (n=14)
Total placental TG*placental weight (mg)	19.9±1.4	19.7±3.1	21.4±4.8

Data represents means ± S.E.M; n: women/group; TG: triglycerides.

Although glucose concentrations in cord blood had a trend to decrease as a consequence of obesity ($p=0.1$), it did not reach statistical significance (Table 3.13). Similarly, triglycerides and cholesterol concentrations in cord blood did not differ between BMI groups as did total placental TG per placental weight. On the other hand, overweight women with GDM showed a trend ($p=0.17$) to increased glucose concentrations in cord blood compared to lean controls without GDM (Table 3.14). Nonetheless, after running an independent t-test between cord blood glucose concentrations in GDOV group compare to control a statistical significance ($p=0.035$) was reached. Overall, a sample size calculation indicated that 40 participants from each group were required to detect a significant difference in cord blood glucose at an 80% power. Unchanged cord blood TG and cholesterol concentrations were accompanied by similar concentrations of total placental TG per placental weight in GDM groups compared to the lean control without GDM.

A sample size calculation showed that 63 samples from each group were required to detect the significance warranted at a power of 80%.

Table 3.14: Cord blood and placental characteristics in control (C), gestational diabetic lean (GDL), gestational diabetic overweight (GDOV) and gestational diabetic obese (GDOB) pregnant women.

Cord blood and placental outcomes	C (n=33)	GDL (n=7)	GDOV (n=12)	GDOB (n=7)
Cord blood glucose (mg/dl)	71.2±3.1	71.1±4.7	88.8±7.9	71.9±8.5
Cord blood triglycerides (mg/dl)	47.5±2.9	39.1±5.2	42.3±3.4	44.3±10.7
Cord blood cholesterol (mg/dl)	32.3±2.5	35.4±4.5	34.6±4.3	30.0±6.2
Placental TG	C (n=50)	GDL (n=12)	GDOV (n=4)	GDOB (n=5)
Total placental TG*placental weight (mg)	19.9±1.4	19.7±3.1	21.4±4.8	30.8±9.7

Data represents means ± S.E.M.; n: women/group; TG: triglycerides.

Anthropometric characteristics of the fetus and placenta of pregnant women without GDM indicated similar fetal weight at 24 and 34 gestational weeks (Table 3.15). Interestingly, increased placental weight, but not diameter (measured at the widest point), was observed in obese women compared to lean controls.

Table 3.15: Anthropometric characteristics of the fetus, based on ultrasound scan (USS) at 34 gestational weeks (gw), and placenta in control (C), overweight (OV) and obese (OB) women.

Placental and fetal anthropometry	C (n=59)	OV (n=28)	OB (n=22)
Estimated fetal weight at 34 gw (g)	2357±23	2360±56	2433±87
Placental diameter (cm)	19.1±0.3	19.6±0.4	20.2±0.6
Placental weight (g)	469±16	495±25	520±29 [*]

Data represents means ± S.E.M. Statistical differences denoted by * corresponds to p<0.05 (1-way ANOVA; Bonferroni post-hoc test); n: women/group.

The effect of GDM alone and combined with BMI did not influence fetal weight at 34 gestational weeks nor placental weight or diameter (Table 3.16).

Table 3.16: Anthropometric characteristics of the fetus, based on ultrasound scan (USS) at 34 gestational weeks (gw), and placenta in control (C), gestational diabetic lean (GDL), gestational diabetic overweight (GDOV) and gestational diabetic obese (GDOB) pregnant women.

Placental and fetal anthropometry	C (n=59)	GDL (n=14)	GDOV (n=14)	GDOB (n=11)
Estimated fetal weight at 34 gw (g)	2357±23	2593±116	2641±92	2612±213
Placental diameter (cm)	19.1±0.3	19.7±0.4	18.4±0.5	18.8±0.4
Placental weight (g)	469±16	497±38	491±31	462±36

Data represents means ± S.E.M.; n: women/group; m: months.

Attempt to look at placental morphology through microscopic analysis in 60 placental samples (10 C, 10 OV, 10 OB, 10 GDL, 10 GDOV, 10 GDOB) was executed. However, the analysis showed that a large number (n=30) of these frozen placental samples was degraded, probably due to delay in fixing the tissue samples at the time of delivery (Figure 3.5).

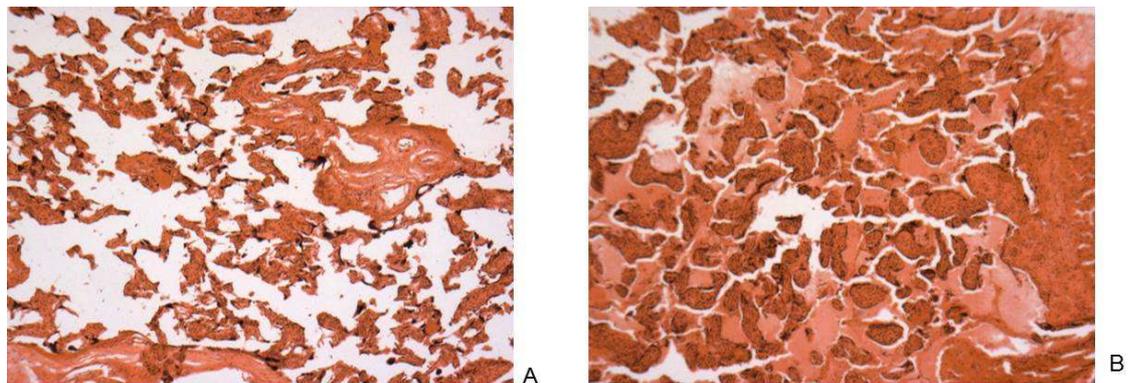


Figure 3.5: Representative haematoxylin & eosin (H&E) 5µm stained microscopic sections at 4x magnification.

A) On the left: section corresponding to placental sample from control (C).

B) On the right: section of placental sample from obese with gestational diabetes group (GDOB). The pink coloured stain represents connective tissue, red blood cells and non-nucleated cellular material. Villi surface were either deteriorated (3.9-A) or surrounded by maternal blood (3.9-B).

Few placental sections (10 out of 60) were better preserved, but the amount of frozen tissues available for quantification was limited and did not allow meaningful comparison between observational groups. Therefore, the analysis could not be completed.

3.3.6. Newborn outcomes and postnatal growth

All women without GDM gave birth at term and to healthy neonates, whose anthropometric characteristics are reported in Table 3.17.

Table 3.17: Newborn anthropometric characteristics in control (C), overweight (OV) and obese (OB) women.

Newborn anthropometry	C (n= 59)	OV (n=29)	OB (n=22)
Newborn gender (M/F)	31/28	11/17	13/8
Newborn weight (kg)	3.29±0.53	3.27 ± 0.95	3.42 ± 0.12
Newborn length (cm)	50.2±0.2	50.5±0.3	50.5±0.7
Ponderal index (g/cm³ x 100)	2.62±0.04	2.63±0.05	2.58±0.07
Placental weight/Birthweight	0.14±0.0	0.15±0.01	0.16±0.01
Infant weight gain 0-3m (kg)	2.76±0.10	2.76±0.20	2.87±0.15
Infant weight gain 3-6m (kg)	1.58±0.08	1.64±0.14	1.56±0.12
Total infant weight gain 0-12m (kg)	6.32±0.16	6.25±0.17	6.15±0.22

Data represents means ± S.E.M. n: women/group; m: months.

Neonatal ponderal index, a measure of their leanness calculated by normalising the mass with the third power of body height, did not differ between BMI groups. Despite their bigger placentas, obese mothers gave birth to neonates of similar weight to that of their lean counterparts. This may have result from the sample size being lower than expected.

In fact, the study's actual power was only 18% and, in order to detect a statistically significant difference, it would have needed 287 subjects in each group to have a target power of 80%.

After birth, infant postnatal growth at three, six and twelve months was similar between groups showing analogous weight gain in the first year of age (Table 3.17). Although women in each BMI group gave birth to newborns in the normal range of recommended weight ($2.5 < \text{birth weight} < 4.0 \text{ kg}$), obese women had the highest percentage of large for gestational age (LGA) infants (defined as $> 90\%$ birthweight population centile) compared to the other BMI groups. This was, in fact, more than twice the rate of LGA infants born to the lean group (Figure 3.6).

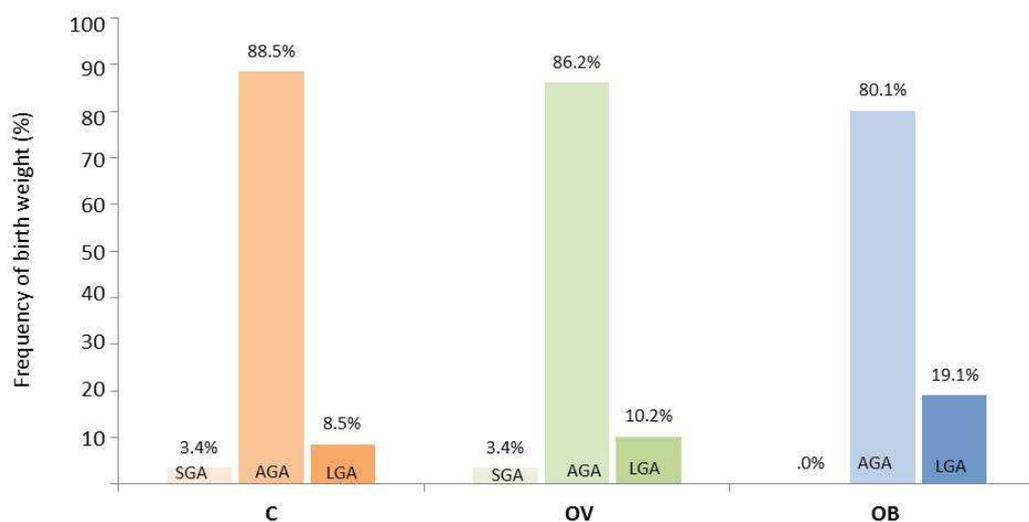


Figure 3.6: Frequency of delivering small (SGA $< 10^{\text{th}}$ birthweight population centile), adequate (AGA $10\text{-}90^{\text{th}}$ birthweight population centile) or large (LGA $> 90^{\text{th}}$ birthweight population centile) for gestational age newborns in control (C; $n=59$), overweight (OV; $n=29$) and obese (OB; $n=22$) women.

Furthermore, when obese pregnant women gained more weight (10.1-14 kg) than the 2009 IOM recommendation (4.2-7.6 kg) during the first 34 gestational weeks, they delivered bigger infants compared to lean controls mothers (Figure 3.7).

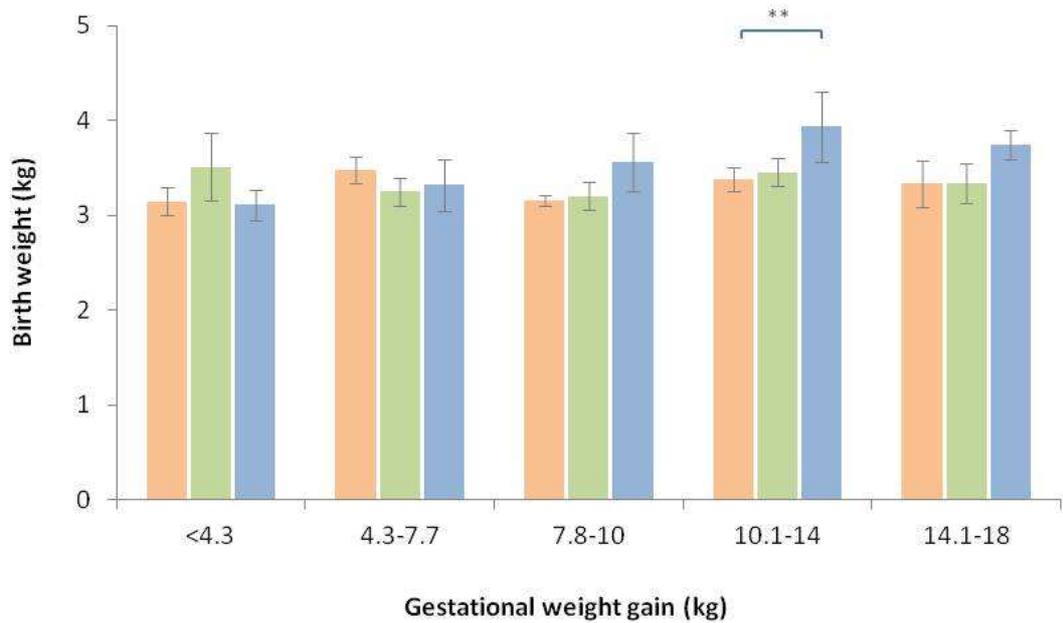


Figure 3.7: Birth weight trend based on maternal weight gain during the first 34 weeks of gestation in offspring from control (C), overweight (OV) and obese (OB) pregnant women.

Data represents means \pm S.E.M. Statistical differences denoted by ** corresponds to $p < 0.01$ (1-way ANOVA; Bonferroni post-hoc test). Orange, green and blue bars represent infants from C (n=59), OV (n=29) and OB (n=22) mothers respectively.

Unexpectedly, the effect of GDM did not influence newborn weight, length or ponderal index as reported in Table 3.18. Infant postnatal growth at three, six and twelve months was similar with all groups showing no significant influence of GDM alone, or combined with BMI, on postnatal weight gain over the first year of age (Table 3.18).

Table 3.18: Newborn anthropometric characteristics in control (C), gestational diabetic lean (GDL), gestational diabetic overweight (GDOV) and gestational diabetic obese (GDOB) pregnant women.

Newborn anthropometry	C (n= 59)	GDL (n=14)	GDOV (n=14)	GDOB (n=11)
Newborn gender (M/F)	31/28	8/7	3/4	8/3
Newborn weight (kg)	3.29±0.05	3.37±0.11	3.23±0.15	3.42±0.17
Newborn length (cm)	50.2±0.2	50.6±0.5	50.6±0.9	50.9±1.0
Ponderal index (g/cm³ x100)	2.62±0.04	2.61±0.09	2.58±0.08	2.60±0.13
Placental weight/Birthweight	0.14±0.0	0.15±0.01	0.16±0.01	0.14±0.01
Infant weight gain 0-3m (kg)	2.76±0.10	2.75±0.23	2.83±0.31	2.76±0.31
Infant weight gain 3-6m (kg)	1.58±0.08	1.61±0.12	1.83±0.18	2.04±0.47
Total infant weight gain 0-12m (kg)	6.32±0.16	6.40±0.20	6.30±0.25	6.60±0.45

Data represents means ± S.E.M.; n: women/group; M: male; F: female; m: months.

A linear regression model was undertaken in order to investigate the change in birth weight associated with BMI, GDM, GWG and other potential confounding factors (Table 3.15). In the first model, maternal pre-gestational BMI was used as the main predictor and this only resulted in an increased newborn weight of 22 grams. The second model took into account the combined effect of BMI and GWG and revealed a stronger influence of BMI on birth weight. The effect of both BMI and GWG were then combined with that of GDM in the third model, which indicated a similar influence of BMI and GDM with GWG having the weakest influence on neonatal weight. Lastly, additional confounders including newborn gender, maternal age, smoking status and parity, were added to the third model. This resulted in a negative contribution on the prediction of birth weight (Model 4), due to the gender effect as, surprisingly, males from these women weighed less than females.

Table 3.19: Association between changes in birth weight and different predicting factors in control, overweight and obese mothers with and without GDM.

Linear regression model (n=149)	Increase in birth weight (kg)	p value
MODEL 1 ^Ψ		
Maternal BMI	0.022 (0 to 0.044)	0.071
MODEL 2 ^{ΨΨ}		
Maternal BMI	0.024 (0.006 to 0.043)	0.011
Maternal GWG	0.016 (0.001 to 0.032)	0.033
MODEL 3 ^{ΨΨΨ}		
Maternal BMI	0.024 (0.006 to 0.043)	0.011
Maternal GWG	0.017 (0.001 to 0.033)	0.038
Maternal GDM	0.021 (-0.190 to 0.232)	0.845
MODEL 4 ^{ΨΨΨΨ}		
Maternal BMI	0.022 (0.002 to 0.041)	0.041
Maternal GWG	0.016 (0.00 to 0.032)	0.032
Maternal GDM	0.020 (- 0.196 to 0.236)	0.236
Other confounders	- 0.175 (-0.173 to 0.103)	0.287

^Ψ Adjusted for the a priori confounder BMI; ^{ΨΨ} adjusted for the a priori confounders BMI and GWG; ^{ΨΨΨ} adjusted for the a priori confounders BMI, GWG and GDM; ^{ΨΨΨΨ} Adjusted for the a priori confounders BMI, GWG, GDM and additional factors (newborn gender, maternal age, maternal smoking status, maternal parity, type of delivery). Numbers in brackets are 95% confidence intervals.

3.4. Discussion

3.4.1. Maternal BMI and diabetes influences maternal socio-economic status and dietary choices

There is increased awareness that high BMI and GDM have effects on pregnancy outcomes with the potential to compromise newborn health in the long-term [482, 483]. Therefore, appropriate and sustainable intervention strategies for pregnant women need to be optimised. To my knowledge, only a few investigations have attempted to discern the relative influences of overweight, obesity and GDM [474, 484, 485] on pregnancy outcomes. The novel contribution of my study was to consider the relative effects of different BMI categories in pregnancy in the presence and absence of GDM. In addition, the large amount of data available allowed me to widely analyse and interpret the adverse consequences related to these conditions. Therefore, the aim of this chapter was to focus on different critical aspects related to these diseases and their contribution on maternal and newborn outcomes.

Current evidence from epidemiological and animal investigations has highlighted the crucial contribution of maternal diet and weight gain on the development of obesity and hyperglycaemia in pregnancy [39, 486]. Unquestionably, ethnic differences play a significant role in the prevalence of obesity and GDM [61]. For instance, in Europe, GDM has been found to be more common among Asian women than among European women [487] and, in the USA, Native Americans, Asians, Hispanics, and African-American women are at higher risk for GDM than non-Hispanic white women [488-490]. In my study, the majority of the subjects were of Hispanic European white origin (95-98%) with a minor prevalence of other ethnicities as American-Indian (0-1.7%), Arabic (0-1.7%) and Romani (0-1.7%). Hispanic and Southern European culture is associated with a lifestyle and social behaviour that diverges from those of Northern European and American [491-493].

For instance, consuming an excess of saturated fat and processed food coupled with mental stress and inequality, a common feature in countries like the UK and USA [490, 493, 494], might represent factors contributing to increased obesity and diabetes in those countries [38, 495, 496]. On the contrary, Scandinavian diet is more rich in polyunsaturated fatty acids, fruit and vegetables and physical activity is a predominant part in their daily life [493, 497]. Mediterranean diet follows similar principles and it has been characterised by a less stressful life along with more homemade food [498, 499]. Furthermore, low socioeconomic status along with high levels of stress has been recognised as a frequent cause predisposing the mother to imbalanced and inadequate diets [51, 490]. More healthy women with a BMI in the normal range participating in the study had a university degree and a permanent job whereas obese mothers with or without diabetes were less likely to have continued their education to University level. In addition, obesity in pregnancy was associated with a high percentage of unemployment and not having paid work outside the home. It is possible that those women have invested less in fitness and high quality food and they could have been more stressed about their economic situation [51, 490, 493].

After analysis of the nutritional questionnaires returned by participants, however, the outcomes did not reflect what was expected, that is, a high energetic and caloric diet in women with high BMI [52]. In fact, overweight and obese mothers reported similar high caloric food consumption resulting in similar energy and total carbohydrate intake than lean women (Table 3.3). Mothers with both enhanced BMI and GDM consumed a lower energy rich diet with less total carbohydrate, lipid and SFA intake (Table 3.4). In addition, these women reported higher intake of MUFA and PUFA reflecting healthier nutritional choices [500]. A large body of evidence in both animals and human cohorts supports the beneficial effects of dietary long chain PUFA supplementation on placental and fetal development as well as birthweight [501, 502].

However, the benefits for the mother [503], as for the offspring in the long term, remain to be confirmed [504]. It is plausible to assume that the Mediterranean diet consumed by these Spanish participants played a key role on protecting them from consuming excess of saturated fat and energy rich food [479, 505]. Above all, the Mediterranean tradition of consuming homemade food [503, 506], in contrast to the increased international prevalence of fast and processed foods [490, 493, 494], may play a significant role in lowering the incidence of high caloric diets.

Evidence from epidemiological studies [79] supports the beneficial effects of higher intake of fruits and vegetables, whole grains, fish and moderate daily consumption of alcohol on a healthy lifestyle and body weight. Also, the use of olive oil as the major source of fat instead of butter and margarine that are rich in SFA, has been associated with longer life expectancy [54] and this has recently been substantiated in a larger study [491]. In this work, overweight and obese women consumed lower iron intake (Table 3.5), supporting data from epidemiological studies [38, 495, 496, 507]. Nevertheless, it is intriguing to notice that there was an overall higher intake of major micronutrients (Table 3.4) in the GDM group independent of BMI reflecting healthier food choices [508, 509]. The fact that women with GDM were routinely attending medical revisions, which included dietary recommendations for their hyperglycaemia, might have contributed to this better dietetic outcome. Researchers found that Mediterranean dietary principles are effective in clinical practice, being associated with low rates of chronic diseases and high adult life expectancy [478, 506].

Therefore, my findings substantiate that a good medical supervision in guiding patients towards more conscious nutritional choices is important and might be useful to counter-balance the adverse outcomes associated with obesity and diabetes in pregnancy.

3.4.2. Obesity, diabetes and maternal weight gain in pregnancy

Increased total energy intake as well as a higher proportion of lipids of animal origin are associated with enhanced GWG [66, 497, 510], an effect that becomes even greater if the woman is obese [38, 478]. Fraser et al. [62] studied the association between GWG, BMI and waist circumference on the maternal outcomes 16 years after pregnancy, although they did not differentiate between overweight and obesity. Not surprisingly, they observed that women with higher GWG had increased odds of overweight and central adiposity 16 years after pregnancy whereas reduced body weight and BMI was associated with previous low GWG [62]. After the IOM reevaluated the GWG guidelines in 2009, more data are available for obese women based on the severity of obesity [61], and a total GWG between 5-9 kg is currently recommended. In this study, I considered GWG in the first 34 gestational weeks, rather than up to delivery, as 34 weeks corresponds to the last weight measured by professionals and not estimated. This method is supported by current evidence [62, 482, 511] indicating that GWG is more strongly associated with early and mid-pregnancy (0-28 gestational weeks), although it remains difficult to compare across studies because of the various populations, outcomes and definition of GWG.

In contrast to Mamun et al. [512] and Fraser et al. [62], but in agreement with Bodnar et al. [513], my results revealed a lower GWG in all women in higher BMI groups during the first 34 weeks of pregnancy. Nonetheless, it is significant that overweight and obese mothers without GDM gained considerably more weight up to the 34th gestational week than those with GDM, indicating a less closer adherence to the IOM recommendations [61].

In the observational study by Bodnar et al. [513], an increase in the severity of obesity was associated with a reduction in the levels of excessive weight gain and higher levels of weight loss during pregnancy.

However, the authors were not able to address the causes as their database lacked essential information such as maternal diet, socioeconomic status and physical activity. The strength of my research reported here is that data on these important variables was available, allowing wider interpretation of the results. For instance, the lower GWG in overweight and obese women with GDM in my study could reflect their lower macronutrient and energy intake throughout late pregnancy. I am not aware of whether these women modified their diet intentionally, before or during pregnancy, with the aim of limiting weight gain or losing weight. If they did, it could have potentially adverse consequences for their infant's health in the long-term [46, 50]. However, future research should explore the metabolic mechanisms behind weight loss and/or low GWG in mothers with high BMI. A follow-up of the study includes analysis of newborn body composition data and it may assist in our understanding of the relationship between BMI, GWG and newborn adiposity. Although overweight and obese women gained, on average, less weight during pregnancy than their normal weight counterparts, overweight mothers did exceed the recommended weight gain for their BMI [3]. It must be pointed out that adequate GWG ranges until the 34th gestational week recommended to overweight (5.9-9.8 kg) and obese (4.2-7.6 kg) women are lower than those recommended to lean mothers (9.8-13.6 kg) [61]. As such, exceeding the IOM weight gain by overweight women might reflect a lack of awareness or less adherence to the recommended weight gain [3, 66]. Overweight and obese participants seemed willing to improve their diet and health condition and to be conscious of the negative consequences of gaining too much weight [514, 515]. A major problem in self-reporting questionnaires is that women with increased BMI are not very reliable when reporting their food intake [406-408]. However, energy and macronutrient intake reported by overweight and obese mothers in my study did not differ from that of lean control women (Table 3.3), indicating that the lower weight gain of these women may account for their metabolic adaptation during pregnancy.

The group of Schebendach et al. observed that, 24 h after a measured laboratory test meal, obese adults under-reported their food intake by 19% [407]. Likewise, Poppitt et al. found that obese adult females under-reported a 1 day observed and measured energy intake by 12.4% [408]. Conversely, Conway et al. found that obese individuals accurately self-reported their energy intake [516, 517], implying that diverse populations and study designs could contribute to this discrepancy.

Reduced food consumption would explain the lower GWG and the significantly minor proportion of women with GDM exceeding the IOM weight gain limits compared with their healthy lean counterparts. The majority of overweight (42.9%) and obese (54.5%) women with GDM gained less than adequate weight recommended by IOM for their BMI categories and these proportions were significantly lower than that of lean healthy women (22.4%). As mentioned in the previous section, upon diagnosis of GDM, diabetic participants had to undertake several routine medical examinations to control their glycaemia and were constantly supervised throughout pregnancy. Additionally, they were referred to a dietician for nutritional counselling in order to develop appropriate meal plans. These findings support recent studies about the importance for diabetic women of improving their lifestyle including dietary pattern [508, 518] and moderate physical activity [483]. When abnormal glucose tolerance is first detected women tend to modify their dietary habits towards healthier food choices [508, 509]. As the first line of treatment of GDM was through nutrition and lifestyle advice in maternity welfare clinics, my results illustrate that the dietary advice received by GDM women can be effective. Nonetheless, few studies with the aim of improving dietary intake and increasing physical activity in overweight and obese pregnant women have been undertaken [486, 515]. Guelinckx et al. [486] studied 195 obese pregnant women and observed that a lifestyle intervention of group sessions combined with individual advice improves nutritional habits without improving their physical activity or GWG.

As such, a personal nutritional plan consisting of a balanced low caloric diet, a diary of physical activity and monitoring energy expenditure might help improve maternal diet thus avoiding excess GWG and GDM development.

3.4.3. Placental and neonatal anthropometry related to maternal BMI, GDM and GWG

Women with increased BMI and GDM experience a higher incidence of maternal and neonatal complications [31] as well as LGA (> 90th birthweight population centile) offspring [33, 34]. This is of increasing public concern as LGA neonates have a high risk of obesity, insulin resistance and hypertension in childhood and early adulthood [70, 81]. The incidence of Caesarean deliveries in my study increased with raised BMI and GDM reaching the greatest ratio in primiparous obese women with (50%) or without (35%) diabetes. My findings substantiate earlier reports, which showed a strong association between BMI and GDM with the increased risk of Caesarean delivery [31, 35, 519]. Additionally, preterm birth was more common in participants with GDM with the greatest rate in women who were obese (25%). Conversely, the effect of obesity per se on prematurity was lower (9.5%) indicating that GDM combined with high BMI has stronger negative influence on birth outcome. This is a common complication in women who enter pregnancy with a high BMI [24, 31] and can be associated with the development of preeclampsia [37], a condition that increases with the severity of obesity [31].

Despite similar birth weight, obese patients without hyperglycaemia had bigger placentas in agreement with previous investigations [14, 47, 93]. At birth, newborn weight did not significantly differ between groups, although in the literature obesity and GDM have been associated with enhanced birth weight [14, 24, 169, 520]. GDM in my participants did not contribute to greater placental or infant birth weight although current evidence from larger studies established the additional contribution of maternal hyperglycaemia in the development of macrosomia and heavier offspring [75, 478].

In terms of LGA deliveries, the results of this study showed a higher prevalence of these newborns with increasing pre-pregnancy BMI among women with (9.1%) or without (19.1%) GDM. Once more, the effect of hyperglycaemia did not seem to increase the odds of having a LGA infant (>90th population percentile) as the incidence was higher for obese women without GDM. These findings are in agreement with epidemiological studies indicating obesity as a stronger risk factor for the increase risk of having a LGA infant than GDM [474, 485, 521, 522]. In a large cohort of 12,950 deliveries, Ehrenberg et al. [475] reported the independent association of high BMI and hyperglycaemia in pregnancy with a raised risk of LGA. In their population, overweight and obesity in pregnancy exhibit the strongest influence on the prevalence of LGA infant compared to diabetes, and increasing BMI directly enhanced the risk of delivering a LGA infant. Similarly, Ricart et al. [485] examined the relative contribution of pre-pregnancy BMI and GDM in the development of adverse outcomes in a cohort of 9,270 Spanish women. Although their subjects were overweight, rather than obese, they reported 13.4% of LGA infants in overweight women without hyperglycaemia, whereas the contribution of GDM only counted for less than 1% of LGA infants [485]. Recently, Black et al. [1] observed that 21.6% of LGA infants could be attributed to overweight and obesity without GDM, whilst 2.9% to GDM only. The contributions of both increased BMI and GDM in their population were additive accounting for the highest proportion of LGA (23.3%) [1]. Although women with GDM in Ricard's study were treated for hyperglycaemia during pregnancy, which could have reduced their probability of a LGA birth, the trends of both investigations were similar.

In contrast, I observed that the combined effects of high BMI and GDM did not increase the risk of having a LGA newborn, which was instead higher in overweight and obese mothers without GDM. This may be explained by the small sample size reported here, which significantly reduced the statistical power.

Similarly, the dietary treatment that those women with GDM monitored during pregnancy could have been protective against substantial maternal hyperglycaemia driving fetal weight gain [226].

Overall, these findings suggest that overweight and obesity in the absence of GDM may have a more substantial impact on the development of adverse maternal and perinatal outcomes than GDM itself. A healthy lifestyle along with a balanced diet and reduced caloric intake might contribute to decrease the risk of these consequences related to the combined effects of both BMI and GDM.

Because overweight and obese mothers exceeded the IOM recommendation of weight gain, I was interested in investigating the effect of GWG on birth weight and eventually the contribution of each factor (BMI, GDM and GWG) on this outcome. Weight gain in late pregnancy was positively correlated with neonatal weight that increased when women were exceeding their recommended GWG. Birth weight was significantly higher in obese mothers without GDM when their weight gain at 34 gestational weeks was in the range of 10.1-14 kg compared to the IOM recommended GWG to delivery (5-9 kg). These results are consistent with other studies reporting a strong association between excess weight gain and higher infant weight as well as pregnancy complications in obese women [1, 62, 65, 474]. Mamun et al. [512] also observed that the risk for a Caesarean section was more than doubled in their population when obese women showed excess GWG. Similarly, in this study, overweight (28.6%) and obese (25%) women without GDM, who gained excess GWG, had an increased risk of Caesarean section compared to mothers with healthy BMI. The increase in emergency caesarean sections in these obese women may be related to the increased number of LGA infants, or increased fat disposition in the soft tissues of the pelvis, which leads to dystocia during labour [523]. This risk was further increased in women with GDM, with the highest proportion of Caesarean section deliveries occurring in obese mothers when their weight gain was more than recommended by IOM.

Unfortunately, the low sample size of mothers with GDM did not permit analysis of the trend of birth weight at different ranges of maternal GWG.

In a multi-linear regression analysis to investigate the odds of increased birth weight depending on the separate effect of BMI, GDM and GWG, BMI was the greatest contributor to a raise in birth weight of 22 g, followed by GDM (21 g) and GWG (17 g). After adjusting for significant confounding variables [62, 486], the effect of each component on newborn weight was similar, whereas the contribution of other predictors such as maternal age, maternal parity and newborn gender, corresponded to a decreased in birth weight.

These results corroborate findings from larger human cohorts, which reveal that high BMI in pregnancy is associated with increased GWG to a greater extent than hyperglycaemia, resulting in a negative influence on birth weight [485, 524]. In a retrospective study of 9,835 pregnant women [1], Black and colleagues reported that the proportion of LGA infants born to overweight mothers without GDM who gained < 40 pounds (18.14 kg) was similar to that of women with a healthy BMI who gained the same gestational weight. Nonetheless, the effect of pre-pregnancy BMI and GDM are additive as the proportion of LGA infants among overweight mothers with GDM raised substantially and significantly. Whether this is due to the development of GDM or high BMI before pregnancy is not understood. Because maternal overweight and obesity is more common than GDM, this condition could be identified before pregnancy. As such, targeted interventions at overweight and obese women to lose weight before getting pregnant and to avoid excess GWG, regardless of GDM status, has the potential to decrease their high incidence of LGA offspring and negative outcomes. Finally, the GWG of the participants was accompanied by a similar trend of weight loss after delivery (Figure 3.1-A), being significantly lower in overweight and obese mothers compared to their healthy counterparts. Post-pregnancy weight loss was halved in overweight and obese women with GDM (Figure 3.1-B), reflecting their greater weight retention after pregnancy in agreement with previous findings [48].

GWG and post-pregnancy weight retention can increase the risk of long term weight gain with important implications for maternal and offspring obesity [59, 525]. Higher weight retention in obese women has been associated with enhanced infant weight gain [59, 525]. Nevertheless, this study reported no difference between infant weight gains during the first 12 months of life among any of the observational groups. This is possibly due to the fact that the study was under powered and did not allow substantiating epidemiological data found in the literature. The aim of a follow-up study is to increase the number of participants and investigate infant's health in the long term through measurement of body fat accumulation.

3.4.4. Insulin resistance in pregnancy influences maternal but not neonatal glycaemia: protective role of the placenta?

The development of maternal insulin resistance is associated with additional maternal adipose tissue deposition in early pregnancy and increased fetoplacental nutrient availability in late gestation [121, 148]. Obese [130, 482] and diabetic [33] gravidae are characterised by greater level of insulin resistance exposing the fetus to enhanced nutrient availability and insulin concentrations, which potentially result in fetal overgrowth [75]. My findings support current literature since plasma glucose and insulin in the participants increased with enhanced BMI and was significantly higher in obese mothers at 40 gestational weeks. Similarly, GDM mothers with high BMI reported enhanced circulatory glucose and insulin at 40th gestational weeks. However, in contrast to what found in the literature, I did not observe any significant changes in birth weight with either increased maternal BMI or GDM despite obese mothers having the highest frequency of LGA infants amongst all the maternal groups. A reasonable explanation is, once more, the power of the study, which was not strong enough to allow significant comparison between groups, especially among mothers with GDM.

Owing to limits on the blood volumes obtained from blood cord sampling, and the number of other factors studied, there was insufficient blood remaining for measurement of cord blood insulin. Therefore, it was not possible to investigate whether maternal hyperglycaemia was associated with increased levels of insulin in cord blood with potential negative effects in the newborn [226].

In agreement with Catalano et al. [130], the combined effect of high BMI and GDM further raised maternal blood glucose and insulin. Nonetheless, cord blood glucose was comparable between all maternal groups indicating that glucose transport across the placenta was modified with maternal hyperglycaemia. Similarly, Desoye et al. found that placental glucose transport in diet treated GDM is lower than that in normal pregnancies, whereas insulin treated diabetes led to glucose uptake in the normal range [295]. In addition, Ericsson et al. reported that insulin did not affect glucose uptake at term [117] but was associated with enhanced glucose uptake in primary villous fragments obtained at 6–8 weeks of gestation [203].

Jansson and colleagues have been investigating placental glucose transport demonstrating increased glucose transport activity in pregnancies complicated by type 1 diabetes (T1D) delivering LGA [198] but not GDM associated with this outcome [200]. They have suggested that hyperglycaemia limits the sensitivity of glucose transporters in early rather than late gestation leading to macrosomia and LGA.

Placental weight in my study rose with obesity despite similar neonatal weight, indicating lower placental efficiency [526] that could compromise fetal nutrient supply [501, 527]. Placental hypertrophy across obese mothers [526] could explain this finding, suggesting a difference in placental morphology and function with obesity. Nevertheless, the lack of knowledge on the effect of high BMI on placental growth and development triggers the need of further investigation. Additionally, the HAPO study [528] related GDM with a higher incidence of macrosomia despite an optimal glycaemic control and suggests that different nutrients other than glucose can contribute to fetal overgrowth.

Jansson also indicated that fetal macrosomia observed in GDM pregnancies might depend on greater uptake of neutral amino acids rather than glucose [179, 183]. However, opposite findings were reported by Kuruvilla [210], illustrating, once more, that further studies are warranted.

Elevated lipid concentrations in obese pregnant women have also been related to increased fetal growth and adiposity [529]. In contrast, my study showed no differences in TG concentrations in either maternal or cord blood. These results were confirmed by analysis of placental TG content, which were not altered by either BMI or GDM. Di Cianni et al. [530] reported a significant correlation between maternal TG concentrations in late pregnancy and fetal growth/adiposity. These clinical observations were supported by Radaelli et al. [215] who demonstrated that enzymes related to lipid metabolism, in contrast to glucose, are increased in placenta of obese women with GDM.

Overall, lipid and protein metabolism in women with high BMI and GDM participating in this study does not appear to be substantially modified and does not contribute to negative pregnancy outcomes. On the other hand, a positive correlation was found between maternal glucose at delivery and fetal growth at late gestation, and between placental weight and cord blood glucose in obese women with GDM. Glucose concentrations of either the mother or the fetus, in obese mothers, might be, therefore, responsible to their bigger placentas. However, it is not clear whether it is the placenta driving fetal size or the fetus causing placental overgrowth.

A growing body of evidence on the effects of GDM on placental development reports that GDM, even with optimal glycaemic control [168], is associated with greater placental weights [186] and placental-birth weight ratios [169]. On the contrary, this study showed similar placental and placental-birth weight ratios within GDM participants. Although placentas of obese mothers with GDM had similar weight to that of their obese counterparts, no statistically significant differences were observed in placental weight as a consequence of obesity combined with GDM.

This is probably due to the small sample size and their bigger variability, which reduced the power of the analysis. Whether placental function is influenced by maternal hyperglycaemia, through changes in its surface area, vascularity and cell composition is unclear. Abnormal maternal glycaemia has been associated with morphological abnormalities [174], including changes in the villous surface area [171] and deposition of perivillous fibrinoid [172, 175]. As very few studies relating maternal obesity to placental morphology can be found in the literature [153, 526, 531], an interesting comparison could be the single and combined effect of both high BMI and GDM. Maternal diet could potentially explain the lack of difference in placental or birth weights, despite maternal hyperglycaemia, in women with GDM, who reported to consume a low glycaemic index and healthy diet [486, 503]. To date, the primary means to prevent the development of insulin resistance are lifestyle measures of diet, activity and prevention of pre-pregnancy obesity [3, 532, 533]. Therefore, it appears sensible to stress the importance of lifestyle measures in order to prevent the potential long-term implications of altered fetal growth. Implementation of a low-GI diet may thus be a useful guideline in the prevention of obesity among women of childbearing age [55, 486, 497, 534].

In conclusion, my findings illustrate critical points, which are of importance for improving health and long term care in the population. Firstly, the adverse outcomes of obesity in pregnancy may be ameliorated through good regular clinical control where patients are educated towards healthy lifestyle choices as highlighted participants with GDM. Secondly, as the introduction of physical activity programmes and nutritional interventions in previous longitudinal studies have shown beneficial effects, it might be advisable to include these programmes in the health care of overweight and obese women. These targeted interventions could avoid excess weight gain throughout pregnancy and high post-delivery weight retention, thus reducing the high risk of deliver of a LGA infant often observed in those women.

Chapter 4 - Effect of BMI and gestational diabetes on placental metabolism

4.1. Introduction and hypotheses

Altered placental function is a primary mechanism, which links maternal nutritional status and the predisposition of metabolic disease as the placenta represents the main nutritional interface between the mother and her fetus [204, 205, 535]. This Chapter will further investigate the effect of entering pregnancy with a high BMI and/or gestational diabetes on key mechanisms regulating placental energy metabolism and potential implication for the newborn.

As discussed in the Introduction (Chapter 1), maternal physiology in pregnancy is further modified by obesity [263] and diabetes [252], resulting in a greater degree of insulin resistance. This is associated with an imbalanced store of nutrients and excessive release of inflammatory markers, such as leptin and cytokines [95-97]. Consequently, fetal hyperinsulinaemia and/or hyperleptinemia can stimulate fetal growth and has been associated with insulin and leptin resistance during adulthood [255, 256]. The aim of this Chapter is to examine links between maternal and fetal outcomes through modifications in placental gene expression profile.

Changes in cellular energy levels not only promote internal pathways involved in the regulation of energy metabolism and transport, they also stimulate additional mechanisms, which, in turn, govern pro-inflammatory pathways and cellular functions [535].

1. My previous results showed that insulin resistance of obese and diabetic pregnant women resulted in differences in glucose concentration gradient between mother and fetus associated with changes in placental weight but no effects on newborn growth. As post-receptor defects in the insulin signalling cascade have been proposed to link obesity, insulin resistance and inflammation [73, 106, 107], my hypothesis was that components of this signalling, i.e. insulin-like growth factor-1 receptor (IGFR1), insulin receptor substrate-1 (IRS1) and v-akt murine thymoma viral oncogene homolog 1 (Akt) within the placenta would be modified with high BMI and gestational diabetes.
2. Placental and fetal growths are regulated by maternal concentrations of leptin [101] and glucocorticoids (GC) [185], through an interaction with insulin signalling cascade. The placenta also secretes leptin, thus contributing to maternal hyperleptinaemia in pregnancy [242], and protects the fetus from an excess of GC exposure by producing the enzyme 11-beta hydroxysteroid dehydrogenase type-2 (11 β HSD2). Therefore, I tested the hypothesis that obesity and diabetes in pregnancy would increase leptin concentrations in the mother in addition to altering the expression of this hormone and its receptor (LEPR) in the placenta. Moreover, I hypothesised that placental 11 β HSD2 and GC receptor (GR α) would be reduced as a consequence of high BMI and glucose tolerance in pregnancy.
3. Insulin, IGF1 and leptin are upstream regulators of the mammalian target of rapamycin (mTOR) [302], which is a positive regulator of trophoblast transporters and enzymes involved in energy intake and metabolism [304]. Along with AMP-activated protein kinase (AMPK), mTOR regulates mitochondrial energy metabolism via changes in uncoupling protein-2 (UCP2) and nuclear factor kappa B (NF κ B) action, thus promoting cellular pro-inflammatory and pro-oxidative pathways

[536]. The activity of mTOR is negatively regulated by sirtuin-1 (SIRT1) [333], an antioxidant protein contributing to cell survival [335]. Excess fat mass and/or altered glucose metabolism can, therefore, modify the expression of these key metabolic regulators and potentially alter fetal homeostasis. Analysis of placental thiobarbituric reactive oxygen species (TBARS) concentrations, as a marker of oxidative stress [332], would help to elucidate the degree of oxidative stress in placenta of women with high BMI and gestational diabetes.

4. Finally, the peroxisome-proliferator activated receptor-gamma (PPAR-gamma) acts as an anti-inflammatory factor and regulates pathways influencing cellular fatty acid uptake and transport [308, 313]. The toll-like receptor-4 (TLR4) is also activated by free fatty acids (FFA) and is the main placental receptor that stimulates the pro-inflammatory response to bacterial infection [537]. Alterations of those proteins may play a major role in the low grade inflammatory response frequently observed with obesity [95, 538] and diabetes [94, 96, 97]. Additionally, markers of macrophage infiltration, such as CD14 and CD68, may confirm higher degree of inflammation in these complicated pregnancies [451].

4.2. Materials and methods

A more detailed description of the study can be found in Materials and Methods (Chapter 2). Pregnant women participating in the Preobe study (P06-CTS-02341) were recruited in the 20th week of pregnancy and classified according to their BMI. They were further classified at 28 weeks of gestation on the basis of BMI and glucose tolerance, making six groups (Chapter 2, Section 2.2, Figure 2.2).

Collection of maternal venous blood was performed at 24, 34 and 40 weeks of gestation for women in the BMI group whereas blood samples from participants with gestational diabetes were measured only at weeks 34 and 40 (Figure 4.1).

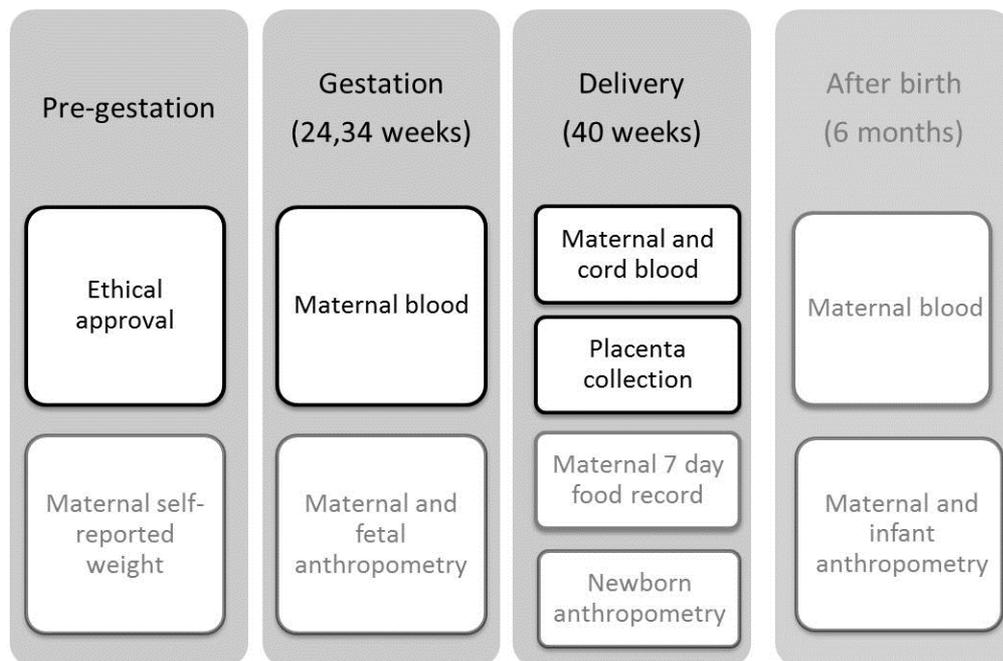


Figure 4.1: Programme of evaluations in the Preobe study.

Maternal examinations during gestation and after delivery. Collection of samples and measurements are reported in white boxes whereas greyed out boxes represent time before, during and after pregnancy. Aspects studied in this Chapter are highlighted in white boxes marked with solid black line.

At delivery, umbilical venous blood samples were collected and placentas were snap frozen and stored at -80°C for further analysis in Nottingham.

After birth, umbilical venous blood samples were collected and placentas were snapped frozen and stored at -80°C for further analysis in Nottingham.

Total RNA was extracted from 100 mg placental samples with TRI Reagent® and chloroform and cDNA obtained using the High Capacity RNA-to-cDNA kit (Applied Biosystems, CA 94404, USA). Key molecular proteins involved in insulin signalling (IGFR1, ISR1), glucocorticoid metabolism (GR α , 11 β HSD2), nutrient transport and energy sensing (mTOR, Akt, p70S6K, AMPK, leptin, LEPR), mitochondrial bioactivity (SIRT1, UCP2) and inflammation (PPAR γ , TLR4) were analysed within the placenta using RT-QPCR.

The thiobarbituric acid reactive substances (TBARS) method, based on the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA), was used as an index of lipid peroxidation to measure global oxidative stress, a well-established mechanism of cellular injury often linked to obesity and diabetes [441, 442]. As such, for quantitative measurement of TBARS concentrations, the Oxiselect™ TBARS Assay kit (Cell Biolabs Inc, CA, USA) was used [539] and the bicinchoninic acid total protein (BCA) method (Sigma-Aldrich kit) was applied to estimate the MDA content of the samples.

An attempt to look at placental inflammation through macrophages infiltration [451] by using antibodies for CD14 and CD68 was executed; however, a lack of antibody specificity did not permit successful completion of this planned analysis (see Chapter 2, Section 2.10.1, Figure 2.11 for details).

4.3. Results

4.3.1. Maternal and cord blood physiological outcomes

As discussed in Chapter 3, maternal plasma glucose concentrations at term increased with both obesity and GDM independently, and with the combined effect of the two. This was accompanied by enhanced maternal insulin at late gestation as a consequence of maternal obesity alone and when it is accompanied by GDM (Chapter 3, Figure 3.3-3.4). Leptin concentrations in maternal plasma increased throughout, and after, pregnancy with maternal obesity (Figure 4.2).

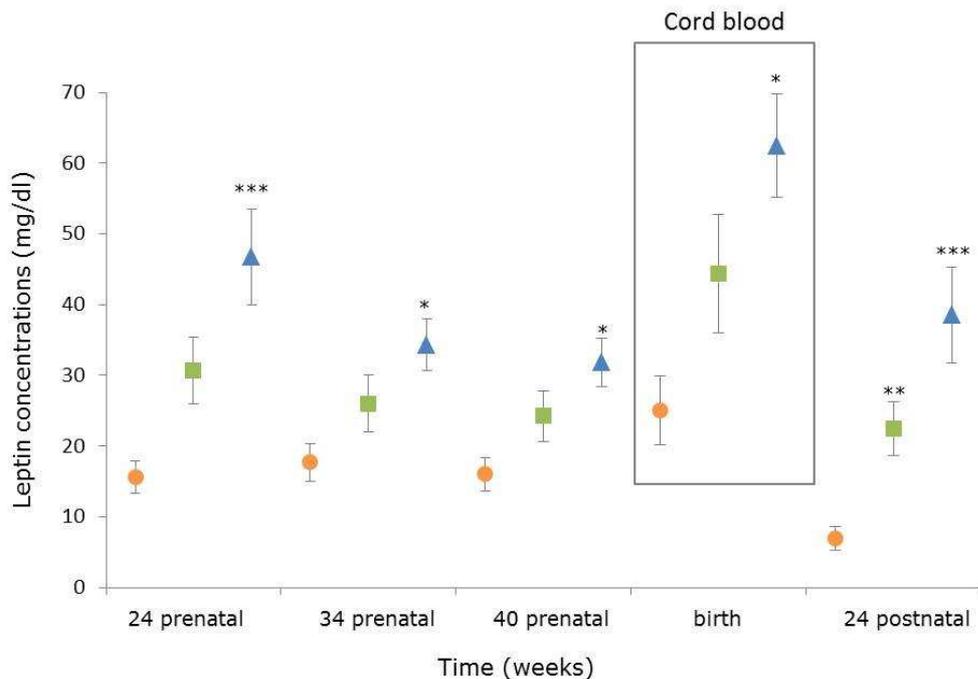


Figure 4.2: Effect of maternal BMI on maternal plasma leptin concentrations at 24, 34, 40 gestational weeks (24, 34, 40 prenatal), 24 weeks after delivery (24 postnatal), and on fetal plasma leptin concentrations in cord blood (birth).

Orange markers represent control (maternal blood, n=59; cordblood, n=33), green markers overweight (maternal blood, n=29; cordblood, n=15) and blue markers obese (maternal blood, n=22; cordblood, n=12) pregnant women. Data represent means \pm S.E.M. Statistical differences between groups denoted at each time point by *, **, *** corresponds to $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively compared to control group (1-way ANOVA; Bonferroni post-hoc test).

Similarly, cord blood leptin concentrations were significantly higher in neonates of obese mothers compared with their lean counterparts. Interestingly, leptin concentrations in maternal plasma showed very weak correlation with maternal BMI, and no correlation was found between maternal leptin concentrations and placental or neonatal weight. Conversely, leptin concentrations in cord blood were positively associated with placental ($R^2=0.43$, $p=0.049$, $n=110$) and newborn weight ($R^2=0.52$, $p=0.019$, $n=110$).

GDM alone did not affect plasma leptin in the mother but when GDM was accompanied with high BMI, leptin concentrations at term and 24 postnatal weeks were significantly raised (Figure 4.3).

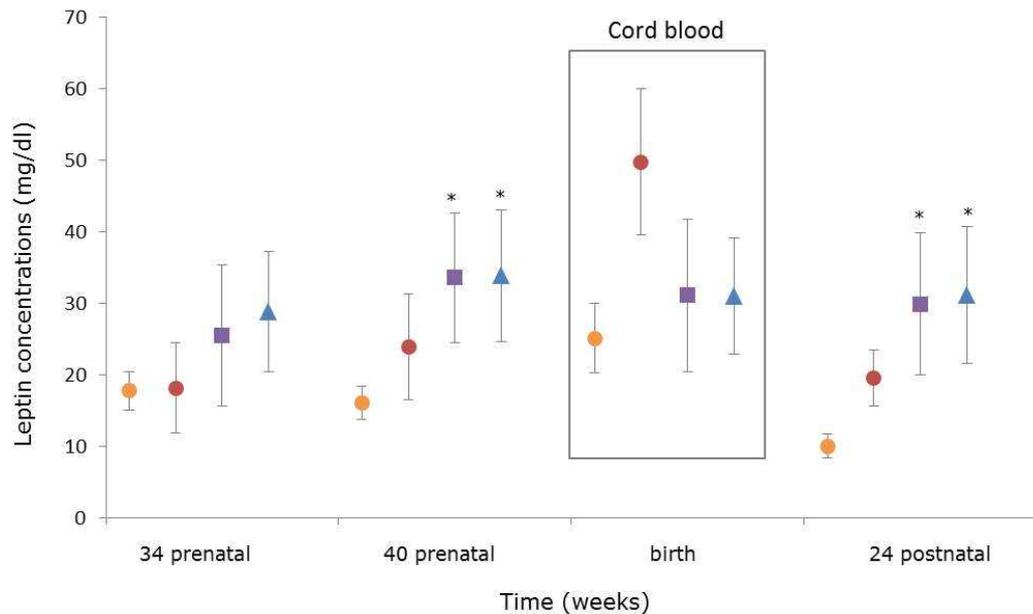


Figure 4.3: Effect of maternal BMI on maternal plasma leptin concentrations at 24, 34, 40 gestational weeks (24, 34, 40 prenatal), 24 weeks after delivery (24 postnatal), and on fetal plasma leptin concentrations in cord blood (birth).

Orange markers represent control (maternal blood, $n=59$; cordblood, $n=33$), red markers lean gestational diabetic (maternal blood, $n=14$; cordblood, $n=7$), purple markers overweight gestational diabetic (maternal blood, $n=14$; cordblood, $n=12$), and blue markers obese gestational diabetic (maternal blood, $n=11$; cordblood, $n=7$) pregnant women. Data represent means \pm S.E.M. Statistical differences between groups denoted by * at each time point corresponds to $p < 0.05$ (1-way ANOVA; Bonferroni post-hoc test).

Furthermore, there was a trend ($p=0.08$) to raised leptin concentrations in cord blood of lean women with GDM but not when GDM was associated with high BMI.

Surprisingly, inflammatory markers in maternal plasma were not influenced by high maternal BMI throughout pregnancy (Table 4.1).

Table 4.1: Maternal counts of white blood cells at 24, 34 and 40 gestational weeks (gw) in control (C), overweight (OV) and obese (OB) pregnant women.

Maternal white blood cells ($10^6 \mu\text{l}$)	C (n=59)	OV (n=29)	OB (n=22)
Monocytes			
24 gw	0.50±0.02	0.50±0.03	0.52±0.02
34 gw	0.53±0.02	0.56±0.03	0.58±0.03
40 gw	0.56±0.03	0.57±0.04	0.60±0.04
Neutrophils			
24 gw	7.57±0.26	7.68±0.36	7.69±0.57
34 gw	7.53±0.27	7.92±0.39	7.89±0.50
40 gw	11.50±0.57	11.72±1.02	12.19±0.99
Lymphocytes			
24 gw	1.84±0.07	1.80±0.09	1.74±0.10
34 gw	1.78±0.07	1.83±0.09	1.70±0.09
40 gw	1.41±0.09	1.53±0.11	1.27±0.17

Data are expressed as relative to control group and represents mean \pm S.E.M.; n = women/group.

However, monocyte levels in cord blood were higher in newborns from obese mothers compared to their lean counterparts and cord neutrophils had a trend ($p=0.09$) to increase with obesity (Table 4.2).

Table 4.2: Cord blood counts of white blood cells in infants of control (C), overweight (OV) and obese (OB) pregnant women.

Cord blood white blood cells ($10^6 \mu\text{l}$)	C (n= 33)	OV (n=15)	OB (n=12)
Monocytes	0.97±0.07	1.07±0.11	1.53±0.18**
Neutrophils	7.55±0.51	8.98±1.44	11.32±1.44
Lymphocytes	4.43±0.29	4.19±0.35	4.77±0.86

Data are expressed as relative to control group and represents mean \pm S.E.M.; n = women/group. Data represent means \pm S.E.M. Statistical differences denoted by ** correspond to $p < 0.01$ compared to infant of control women (1-way ANOVA; Bonferroni post-hoc test).

Similarly, GDM did not influence the concentrations of inflammatory markers in either maternal or cord blood as illustrated in Tables 4.3 and 4.4.

Table 4.3: Maternal counts of white blood cells at 24, 34 and 40 gestational weeks (gw) in control (C), gestational diabetic lean (GDL), gestational diabetic overweight (GDOV) and gestational diabetic obese (GDOB) pregnant women.

Maternal white blood cells ($10^6 \mu\text{l}$)	C (n=59)	GDL (n=14)	GDOV (n=14)	GDOB (n=11)
Monocytes				
34 gw	0.53±0.02	0.50±0.05	0.48±0.03	0.46±0.04
40 gw	0.56±0.03	0.64±0.09	0.42±0.07	0.39±0.08
Neutrophils				
34 gw	7.53±0.27	6.58±0.53	7.53±0.47	7.25±1.01
40 gw	11.50±0.57	11.33±1.19	9.69±2.01	9.16±1.12
Lymphocytes				
34 gw	1.78±0.07	1.76±0.19	1.52±0.12	1.61±0.12
40 gw	1.41±0.09	1.47±0.24	0.88±0.19	1.34±0.27

Data are expressed as relative to control group and represents mean \pm S.E.M.; n = women/group.

Table 4.4: Cord blood counts of white blood cells in infants of control (C), gestational diabetic lean (GDL), gestational diabetic overweight (GDOV) and gestational diabetic obese (GDOB) pregnant women.

Cord blood white blood cells (10⁶ µl)	C (n=33)	GDL (n=7)	GDOV (n=12)	GDOB (n=7)
Monocytes	0.97±0.07	1.26±0.25	1.16±0.20	1.24±0.15
Neutrophils	7.55±0.51	8.20±1.43	6.16±0.87	6.47±1.32
Lymphocytes	4.43±0.29	4.60±0.58	4.61±1.01	4.63±0.81

Data are expressed as relative to control group and represents mean ± S.E.M.; n = women/group.

4.3.2. Placental glucose metabolism and gene expression

Being either overweight or obese was not associated with changes in the expression of genes regulating cellular growth within the placenta as described in Table 4.5.

Table 4.5: Placental gene expression of target genes involved in cellular growth relative to housekeeping gene (ribosomal 18S) in control (C), overweight (OV) and obese (OB) pregnant women.

Placental gene expression (a.u.)	C (n=59)	OV (n=29)	OB (n=22)
IGFR1	1.00±0.07	1.17±0.15	1.20±0.18
IRS1	1.00±0.09	1.11±0.14	1.45±0.24
GHRL	1.00±0.15	1.24±0.20	1.24±0.23

Data are non parametric and expressed as normalised ratio relative to the mean of control group. Data represent mean ± S.E.M; a.u.: arbitrary units; n = women/group.

Gene expression of mTOR decreased with obesity (Figure 4.4) and was positively correlated with fetal, but not placental, weight at 34 gestational weeks ($R^2=0.52$, $p=0.019$, $n=110$). However, no changes in either the upstream (Akt) or downstream (p70S6K) signalling molecules were observed (Table 4.6).

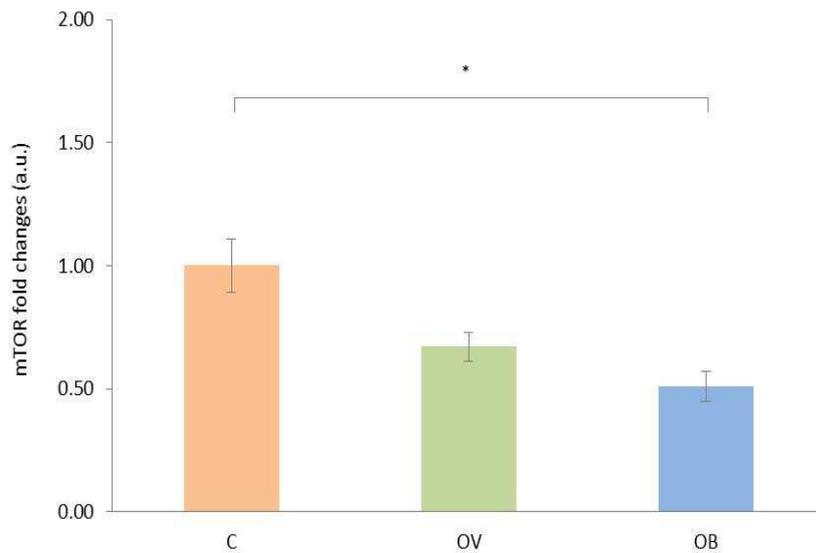


Figure 4.4: Effect of maternal BMI on the mRNA expression of placental mammalian target of rapamycin (mTOR) as determined by real-time PCR.

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give fold change. C: control (n=59); OV: overweight (n=29); OB: obese (n=22). Data are non parametric and represent mean \pm S.E.M; a.u.: arbitrary units; n = women/group. Statistical differences denoted by * corresponds to $p < 0.05$ (Kruskal-Wallis; Mann Whitney post-hoc test).

Table 4.6: Placental gene expression of target genes involved in mTOR signalling relative to housekeeping gene (ribosomal 18S) in control (C), overweight (OV) and obese (OB) pregnant women.

Placental gene expression (a.u.)	C (n=59)	OV (n=29)	OB (n=22)
Akt1	1.00 \pm 0.08	1.01 \pm 0.16	1.17 \pm 0.20
p70S6KB1	1.00 \pm 0.12	1.08 \pm 0.20	1.57 \pm 0.37

Data are non parametric and expressed as normalised ratio relative to the mean of control group. Data represent mean \pm S.E.M; a.u.: arbitrary units; n = women/group.

Despite a marked increase in plasma leptin at term and cord blood leptin in infants of obese mothers, no changes in the mRNA abundance of either placental leptin or LEPR with increased BMI were observed (Table 4.7).

Table 4.7: Placental gene expression of target genes involved in energy sensing signalling relative to housekeeping gene (ribosomal 18S) in control (C), overweight (OV) and obese (OB) pregnant women.

Placental gene expression (a.u.)	C (n=59)	OV (n=29)	OB (n=22)
AMPK	1.00±0.07	0.94±0.10	0.97±0.19
LEP	1.00±0.21	1.51±0.49	0.90±0.38
LEPR	1.00±0.20	0.83±0.14	1.06±0.29

Data are non parametric and expressed as normalised ratio relative to the mean of control group. Data represent mean ± S.E.M; a.u.: arbitrary units; n = women/group.

Neither AMPK expression (Table 4.7) nor the expression of genes implicated in corticosteroid metabolism (11HSD2, GR α , Table 4.8) were influenced by either being overweight or obesity in pregnancy.

Table 4.8: Placental gene expression of target genes involved in glucocorticoids metabolism relative to housekeeping gene (ribosomal 18S) in control (C), overweight (OV) and obese (OB) pregnant women.

Placental gene expression (a.u.)	C (n=59)	OV (n=29)	OB (n=22)
GRα	1.00±0.07	1.17±0.09	0.99±0.10
HSD11B2	1.00±0.18	1.16±0.26	0.74±0.17

Data are non parametric and expressed as normalised ratio relative to the mean of control group. Data represent mean ± S.E.M; a.u.: arbitrary units; n = women/group.

Similarly, neither GDM alone nor GDM combined with increased BMI affected the expression of genes involved in cellular growth (Table 4.9).

Table 4.9: Placental gene expression of target genes involved in cellular growth relative to housekeeping gene (ribosomal 18S) in control (C), lean with GDM (GDL), overweight with GDM (GDOV) and obese with GDM (GDOB) pregnant women.

Placental gene expression (a.u.)	C (n=59)	GDL (n=14)	GDOV (n=14)	GDOB (n=11)
IGFR1	1.00±0.07	0.98±0.12	0.91±0.06	0.83±0.10
IRS1	1.00±0.09	0.91±0.15	0.93±0.10	0.80±0.09
GHRL	1.00±0.15	1.17±0.23	0.98±0.19	1.05±0.21

Data are non parametric and expressed as normalised ratio relative to the mean of control group. Data represent mean ± S.E.M; a.u.: arbitrary units; n = women/group.

The downstream insulin signalling in placentas from GDM women was characterised by a trend in mTOR (p=0.08, Figure 4.5) to decrease when GDM was combined with obesity.

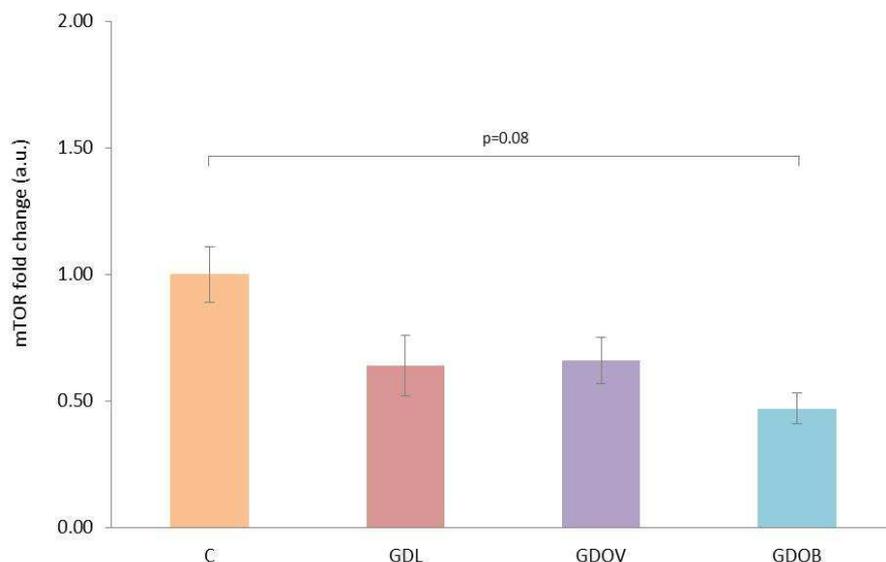


Figure 4.5: Effect of GDM on the mRNA expression of placental mammalian target of rapamycin (mTOR) as determined by real-time PCR.

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give the fold change. C: control (n=59); GDL: lean women with GDM (n=14); GDOV: overweight women with GDM (n=14); GDOB: obese women with GDM (n=11). Data are non parametric and represent mean ± S.E.M; a.u.: arbitrary units; n = women/group.

This was not accompanied by any changes in the expression of its upstream and downstream signalling molecules (Akt and p70S6K) (Table 4.10).

Table 4.10: Placental gene expression of target genes involved in mTOR signalling relative to housekeeping gene (ribosomal 18S) in control (C), lean with GDM (GDL), overweight with GDM (GDOV) and obese with GDM (GDOB) pregnant women.

Placental gene expression (a.u.)	C (n=59)	GDL (n=14)	GDOV (n=14)	GDOB (n=11)
Akt1	1.00 ± 0.08	0.82 ± 0.10	0.80 ± 0.07	0.88 ± 0.12
p70S6KB1	1.00 ± 0.12	0.73 ± 0.18	1.09 ± 0.24	1.38 ± 0.21

Data are non parametric and expressed as normalised ratio relative to the mean of control group. Data represent mean ± S.E.M; a.u.: arbitrary units; n = women/group.

The expression of genes related to energy sensing regulation was influenced by diabetes in pregnancy. Decreased AMPK expression was observed with GDM alone and when GDM was combined with high BMI (Figure 4.6).

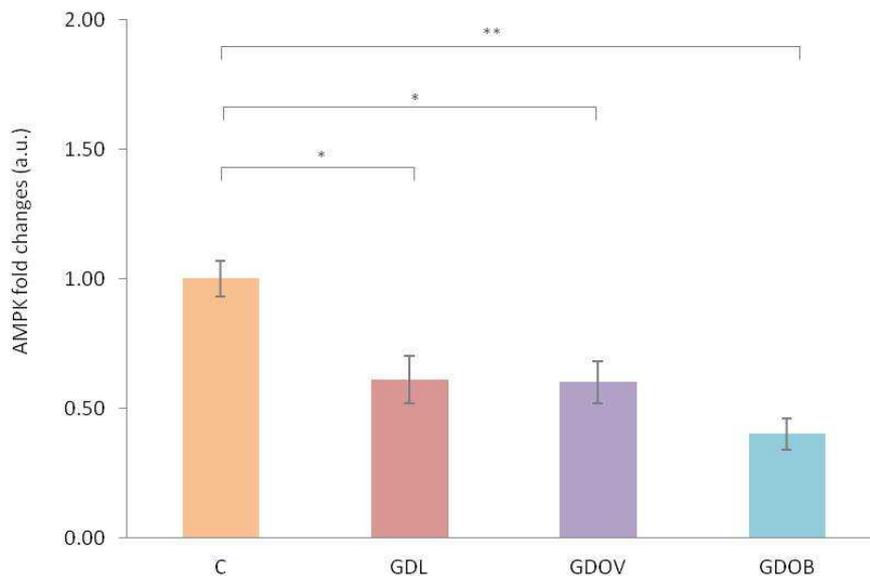


Figure 4.6: Effect of GDM and BMI on the mRNA abundance of placental AMP-activated protein kinase (AMPK) as determined by real-time PCR.

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give the fold change. C: control (n=59); GDL: lean women with GDM (n=14); GDOV: overweight women with GDM (n=14); GDOB: obese women with GDM (n=11). Data are non parametric and represent mean ± S.E.M; a.u.: arbitrary units; n = women/group. Statistical differences denoted by *, ** correspond to p<0.05 and p<0.01 (Kruskal-Wallis; Mann Whitney post-hoc test).

The increased plasma leptin at term in lean mothers with GDM (Figure 4.3) was not accompanied by any changes in LEPR expression between GDM groups, although a trend ($p=0.07$) was observed between lean mothers with GDM and lean controls (Table 4.11).

Table 4.11: Placental gene expression of target genes involved in energy metabolism relative to housekeeping gene (ribosomal 18S) in control (C), lean with GDM (GDL), overweight with GDM (GDOV) and obese with GDM (GDOB) pregnant women.

Placental gene expression (a.u.)	C (n=59)	GDL (n=14)	GDOV (n=14)	GDOB (n=11)
LEPR	1.00 ± 0.20	0.48 ± 0.05	0.49 ± 0.09	0.51 ± 0.09
11βHSD2	1.00 ± 0.18	0.67 ± 0.20	0.97 ± 0.23	0.88 ± 0.35

Data are non parametric and expressed as normalised ratio relative to the mean of control group. Data represent mean ± S.E.M; a.u.: arbitrary units; n = women/group.

However, placental LEP was upregulated in lean women with GDM (Figure 4.7), reflecting the trend of leptin concentrations to increase in cord blood of these women (Figure 4.3).

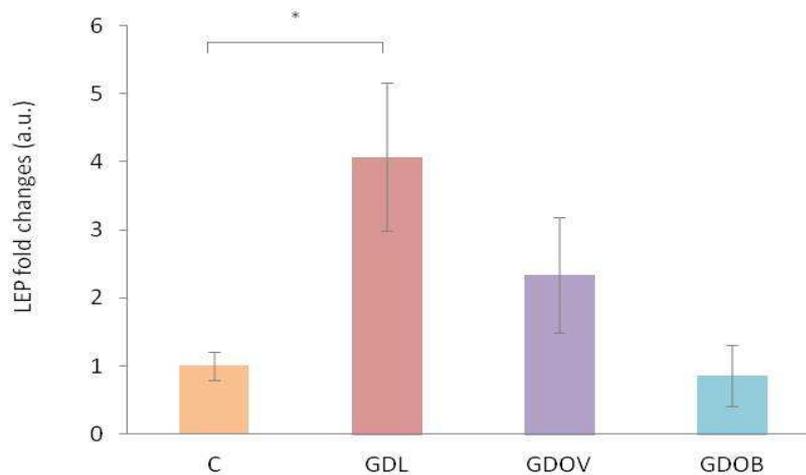


Figure 4.7: Effect of GDM and BMI on the mRNA abundance of placental leptin (LEP) as determined by real-time PCR.

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give the fold change. C: control (n=59); GDL: lean women with GDM (n=14); GDOV: overweight women with GDM (n=14); GDOB: obese women with GDM (n=11). Data are non parametric and represent mean ± S.E.M; a.u.: arbitrary units; n = women/group. Statistical differences denoted by * corresponds to $p<0.05$ (Kruskal-Wallis; Mann Whitney post-hoc test).

Genes related to local cortisol production showed a clear effect of GDM. Expression of placental GR α increased in pregnancies complicated by GDM compared to controls and the combined effect of GDM with high BMI led to overexpression of this corticoid receptor (Figure 4.8).

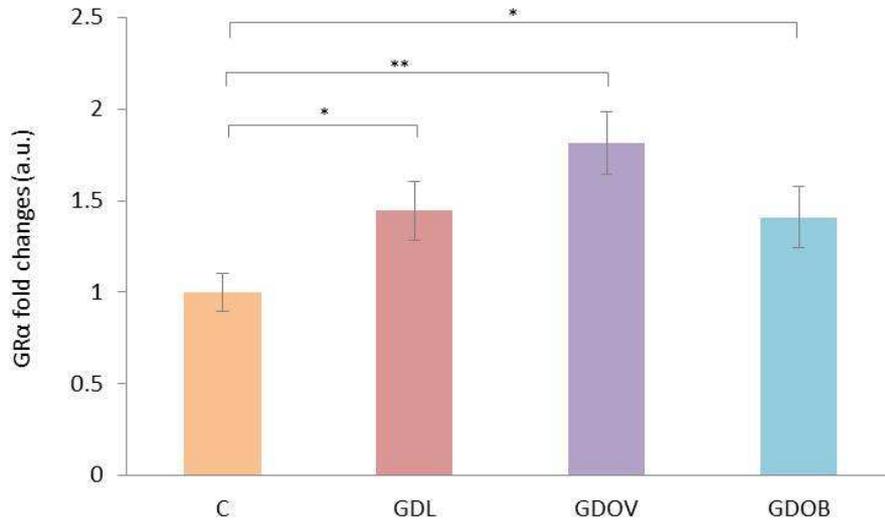


Figure 4.8: Effect of gestational diabetes on the mRNA abundance of placental glucocorticoid receptor alpha (GR α) as determined by real-time PCR.

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give the fold change. C: control (n=59); GDL: lean women with GDM (n=14); GDOV: overweight women with GDM (n=14); GDOB: obese women with GDM (n=11). Data are non parametric and represent mean \pm S.E.M; a.u.: arbitrary units; n = women/group. Statistical differences annotated by *, ** correspond to $p < 0.05$ and $p < 0.01$ respectively (Kruskal-Wallis; Mann-Whitney post-hoc test).

Interestingly, no difference in the mRNA abundance of the enzyme 11 β HSD2 was observed (Table 4.11).

4.3.3. Placental antioxidant and inflammatory response

Although both maternal overweight and obesity during pregnancy were associated with changes in placental anti-oxidative response, such differences were not detected as a consequence of GDM.

The two fold downregulation of placental mTOR with obesity (Figure 4.4) was accompanied by a 1.5 fold upregulation of SIRT1 (Figure 4.9) and UCP2 (Figure 4.10) within the placenta. Similarly, maternal overweight led to increased placental SIRT1 and UCP2 expression compared to lean control mothers.

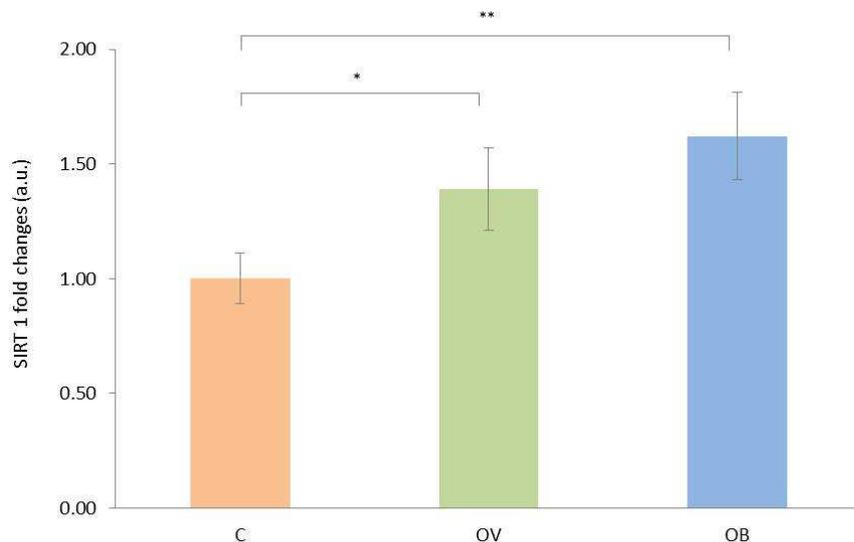


Figure 4.9: Effect of maternal BMI on the mRNA expression of sirtuin 1 (SIRT1) as determined by real-time PCR.

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give fold change. C: control (n=59); OV: overweight (n=29); OB: obese (n=22). Data are non parametric and represent mean \pm S.E.M; a.u.: arbitrary units; n = women/group. Statistical differences denoted by *,** corresponds to $p < 0.05$ and $p < 0.01$ respectively (Kruskal-Wallis; Mann Whitney post-hoc test).

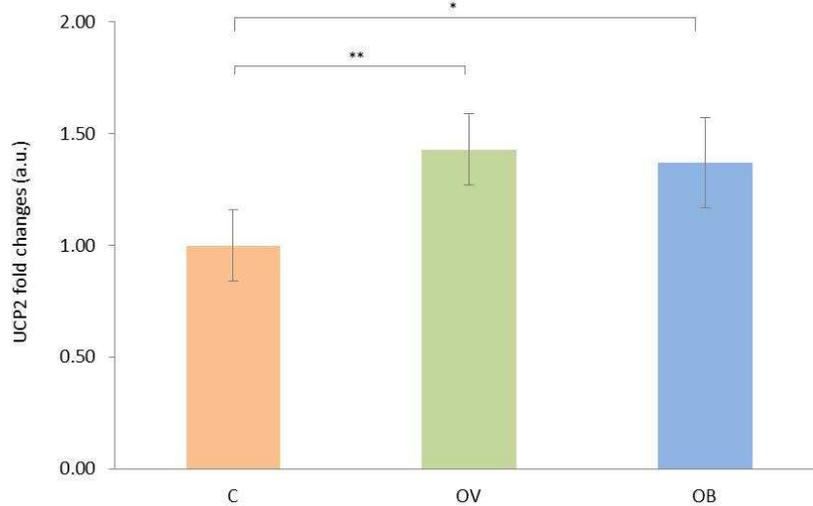


Figure 4.10: Effect of maternal BMI on the mRNA expression of placental uncoupling protein 2 (UCP2) as determined by real-time PCR.

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give fold change. C: control (n=59); OV: overweight (n=29); OB: obese (n=22). Data are non parametric and represent mean \pm S.E.M; a.u.: arbitrary units; n = women/group. Statistical differences denoted by *,** corresponds to $p < 0.05$ and $p < 0.01$ respectively (Kruskal-Wallis; Mann Whitney post-hoc test).

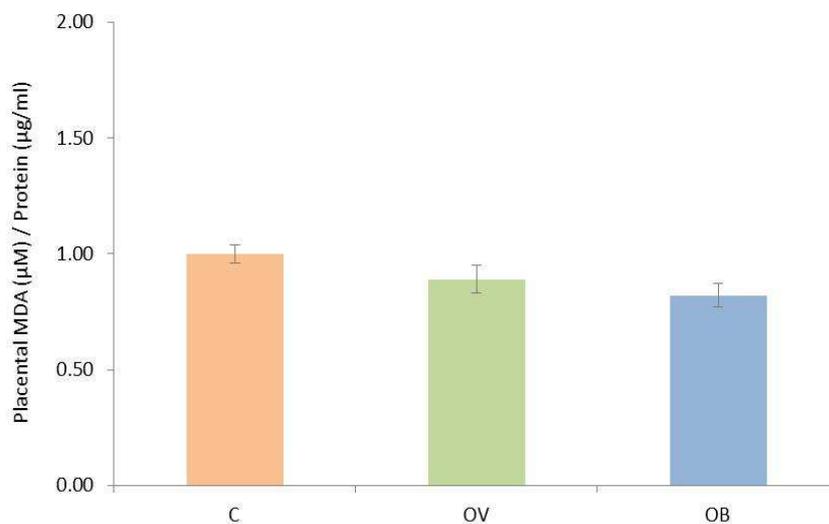


Figure 4.11: Effect of maternal BMI on placental thiobarbituric acid reactive substances (TBARS) determined as the ratio of malondialdehyde (MDA) and protein concentrations.

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give fold change. C: control (n=59); OV: overweight (n=29); OB: obese (n=22). Data are non parametric and represent mean \pm S.E.M; a.u.: arbitrary units; n = women/group.

Placental TBARS concentrations were not statistically different between any of the groups (Figure 4.11) confirming placental gene expression analysis, which indicated inhibition of placental pro-oxidative response (Figure 4.3, 4.9 and 4.10).

On the other hand, maternal GDM did not influence placental antioxidant response as reflected by an absence of an effect on UCP2 expression and TBARS concentration (Table 4.12). The mRNA abundance of SIRT1 within the placenta of GDM women had a trend ($p=0.08$) to increase when the effect of GDM was combined with high BMI.

Table 4.12: Placental gene expression of sirtuin-1 (SIRT1) and uncoupling protein-2 (UCP2) relative to housekeeping gene (ribosomal 18S), and placental thiobarbituric acid reactive substances (TBARS) in control (C), lean with GDM (GDL), overweight with GDM (GDOV) and obese with GDM (GDOB) pregnant women.

Placental gene expression (a.u.)	C (n=59)	GDL (n=14)	GDOV (n=14)	GDOB (n=11)
SIRT1	1.00±0.11	0.79±0.21	1.57±0.31	1.52±0.35
UCP2	1.00±0.16	1.31±0.38	0.93±0.23	0.76±0.30
Placental TBARS (MDA (µM) / Protein (µg/ml))	1.00±0.04	0.95±0.09	0.73±0.13	1.02±0.10

Data are non parametric and expressed as normalised ratio relative to the mean of control group. Data represent mean ± S.E.M; a.u.: arbitrary units; n = women/group; MDA: malondialdehyde.

Placental expression of genes involved in the inflammatory response (TLR4, PPAR γ) did not change with maternal BMI (Table 4.13) or gestational diabetes (Table 4.14), reflecting the concentrations of inflammatory markers in maternal plasma (Table 4.1 and Table 4.3 respectively).

Table 4.13: Placental gene expression of toll-like receptor-4 (TLR4) and peroxisome proliferator receptor gamma (PPAR γ) relative to housekeeping gene (ribosomal 18S) in control (C), overweight (OV) and obese (OB) pregnant women.

Placental gene expression (a.u.)	C (n=59)	OV (n=29)	OB (n=22)
TLR4	1.00 \pm 0.10	0.90 \pm 0.08	0.93 \pm 0.11
PPARγ	1.00 \pm 0.09	0.93 \pm 0.07	0.95 \pm 0.09

Data are non parametric and expressed as normalised ratio relative to the mean of control group. Data represent mean \pm S.E.M; a.u.: arbitrary units; n = women/group.

Table 4.14: Placental gene expression of toll-like receptor-4 (TLR4) and peroxisome proliferator receptor gamma (PPAR γ) relative to housekeeping gene (ribosomal 18S) in control (C), lean with GDM (GDL), overweight with GDM (GDOV) and obese with GDM (GDOB) pregnant women.

Placental gene expression (a.u.)	C (n=59)	GDL (n=14)	GDOV (n=14)	GDOB (n=11)
TLR4	1.00 \pm 0.10	0.80 \pm 0.13	0.84 \pm 0.09	0.81 \pm 0.16
PPARγ	1.00 \pm 0.09	1.03 \pm 0.16	1.08 \pm 0.10	0.87 \pm 0.07

Data are non parametric and expressed as normalised ratio relative to the mean of control group. Data represent mean \pm S.E.M; a.u.: arbitrary units; n = women/group.

4.4. Discussion

This study is the first to systematically compare the effects of both high maternal BMI and GDM on the regulation of placental energy homeostasis focusing on the main factors regulating placental nutrient transport capacity. As such, critical cellular mechanisms, which can contribute to long term adverse outcomes associated with both maternal obesity and gestational diabetes, have been highlighted.

4.4.1. Influence of high maternal BMI and diabetes in pregnancy on placental insulin signalling

As discussed in Chapter 3, one of the major interventions aimed at decreasing the risk of becoming obese in pregnancy is to limit maternal weight gain by reducing food intake [24, 523, 540]. The outcomes presented in this Chapter indicate that such an intervention should be carefully considered as in the obese groups, characterised by the smallest increase in gestational weight gain, upregulation of hormonal regulators involved in insulin signalling within the placenta did not occur.

The pathophysiology of insulin resistance involves abnormalities of insulin-sensitive tissues and insulin signalling [73, 106, 107], which lead to an inadequate insulin response [96]. My previous results showed that increased maternal insulin and glucose concentrations at term in obese and diabetic women occurred without altered fetal growth, newborn weight or changed glucose concentrations in cord blood (Chapter 3). As such, I hypothesised that components of insulin signalling within the placenta would be modified by altered maternal BMI and glucose tolerance to compensate for enhanced maternal substrate supply. Insulin and IGFs are growth factors implicated in fetal growth, which act through the activation of their receptors (IR and IGF1R) leading to phosphorylation of IRS1 and activation of insulin downstream signalling [240, 541].

In this study, no changes in the expression of placental IGFR1 and IRS1 were observed in either women with high BMI or GDM. Previous studies on skeletal muscle and adipose tissue [109, 131], showed decreased expression of IRS1 protein and upregulation of IRS2 associated with impaired insulin action in lean and obese pregnant women with GDM (Figure 4.13). Similarly, Colomiere et al. [195] found a significant reduction of IRS1 protein expression in human term placenta of lean and obese mothers with insulin-dependent GDM, whilst obesity or GDM diet-treated alone did not influence the expression of this receptor. The same authors reported enhanced IRS2 with insulin- and diet-treated GDM and with obesity alone, suggesting a compensatory relationship between these two insulin substrate receptors [195].

The lack of difference in the expression of placental IRS1 and IGFR1 observed in my study can be explained as a placental compensatory mechanism against maternal hyperinsulinaemia and hyperglycaemia (see details in Chapter 3). In fact, placental adaptive regulation involves a change in the expression or activity of specific transporters, like insulin/IGFs receptors and glucose transporters, in response to altered substrate concentrations [183, 270]. However, this placental response is not seen within the range of glucose concentrations normally observed in maternal diabetes [542]. Therefore, the severity of diabetes and/or the method of glycaemic control after GDM diagnosis might account for the disparity between my study and those of others. This would additionally accord with similar concentrations of cord blood glucose reported here.

Table 4.15 summarises some of the findings reporting the effects of obesity and GDM on the expression of insulin signalling components, leptin and leptin receptor in different maternal tissues, placenta and fetus.

Table 4.15: Effect of maternal obesity (A) and gestational diabetes (B) on the concentrations and/or expression (mRNA or protein) of different hormones and receptors in the mother, placenta and fetus.

A) Factor	Mother	Placenta	Fetus
Insulin [83, 93, 216, 236, 253, 543]	↔ ¹ ↑ ¹	-	↔ ¹ ↑ ¹
IGF1 [236, 241]	↑ ¹	n.m.	↑ ¹
IGF2 [236, 544]	↑ ¹	n.m.	↑ ¹
IRS1 [2, 109, 195]	↓ ²	↑ ¹ ↓ ² ↔ ²	n.m.
IRS2 [195]	↑	↑ ¹ ↔ ²	n.m.
Leptin [83, 93, 216, 236, 253, 543]	↑ ¹	↔ ¹	↔ ¹ ↑ ¹
LEPR [253]	n.m.	↓ ¹	n.m.
B) Factor	Mother	Placenta	Fetus
Insulin [11, 130, 237, 295]	↑ ^{3,4}	-	↑ ^{3,4}
IGF1 [237, 295]	↑ ⁴ ↔ ^{3,4} ↓ ³	↔ ³ ↓ ⁴	↑ ^{3,4}
IGF2 [237, 295]	↑ ^{3,4} ↔ ^{3,4}	↑ ^{3,4} ↓ ⁴	↑ ^{3,4}
IRS1 [109, 131, 195, 545]	↓ ³	↓ ³ ↔ ⁴ ↑ ³	n.m.
IRS2 [131, 195]	↑ ³	↑ ^{3,4}	n.m.
Leptin [214, 259, 545-548]	↑ ³	↑ ^{3,4} ↔ ^{3,4}	↑ ^{3,4}
LEPR [214, 259, 545]	↑ ³ ↔ ³	↑ ^{3,4} ↔ ³	n.m.

Higher (↑), lower (↓) or similar (↔) levels in obese (¹), obese gestational diabetes insulin (²), T1D (³), GDM (⁴) compared to normal pregnancies; n.m.: not measured. In the legend IGF1: insulin growth factor-1; IGF2: insulin growth factor-2; IRS1: insulin substrate receptor-1; IRS2: insulin substrate receptor-2; LEPR: leptin receptor.

Similar to insulin, the adipokine leptin stimulates cell proliferation by inducing the IRS1/MAPK pathway in a glucose-dependent manner [549]. Circulating leptin dramatically increases during late pregnancy [550], suggesting a contribution of placental leptin [550, 551] to maternal hyperleptinaemia.

Leptin produced within the placenta has been reported to have a different role from that in the hypothalamus, where it acts as a sensor for appetite regulation [242, 246]. In agreement with previous studies [252, 253], my results reported higher maternal leptin concentrations with obesity alone and when obesity was accompanied by GDM. Increased adipocyte numbers, as the main source of leptin [263], in pregnant obese women might account for their higher leptin concentrations compared to lean mothers. Nonetheless, the contribution of locally produced leptin by trophoblast cells is also expected to underlie the hyperleptinaemia of pregnancy [244, 550]. Placental leptin mRNA abundance was not different in pregnancies complicated by high BMI than those in lean control women. This suggests that increased adiposity, rather than placental leptin, accounts more for enhanced leptin at term in these mothers. Similar conclusions can be drawn for obese mothers with GDM, who had higher maternal leptin concentrations but no differences in placental leptin expression.

Conversely, placentas of lean women with GDM had higher leptin expression despite similar maternal blood concentrations at term. These results are in agreement with the findings of other authors [214, 259], who have shown increased placental leptin in GDM pregnancies, although these studies did not look at maternal or fetal outcomes.

The mRNA abundance of the placental leptin receptor, mainly located on the maternal side of the syncytiotrophoblast [550], was unaltered with either obesity or GDM, substantiating the results of Meller et al. [259] but not Radaelli et al. [214], who reported, instead, a 4.3 fold upregulation of this receptor with GDM. In the study of Gavrilova et al., the soluble leptin receptor (ObRe) was reported to increase with insulin-dependent diabetes [258]. My findings reported total leptin receptor expression without differentiating all its splice variants. As such, it might be that different receptors account for maintaining similar leptin concentrations in both mother and fetus because of placental hyperleptinaemia in lean women with GDM.

Obesity in pregnancy, which was associated with maternal hyperleptinaemia at term, did not result in changes of leptin receptor gene expression within the placenta. Leptin is co-localised to both syncytiotrophoblast and villous endothelial cells, and it is likely to be released into both the maternal and fetal blood circulation [244, 252]. The expression of its receptor on the MVM of the syncytiotrophoblast suggests a possible autocrine role of leptin at the maternal side [244, 252]. Although total leptin receptor expression was not modified at a gene expression level, alteration in its activity or changes in the expression of different isoforms might have occurred with obesity, thus contributing to the higher placental weight observed. Furthermore, the peak in maternal leptin found in their circulation can reflect leptin resistance at the end of pregnancy suggesting an endocrine activity of leptin [258]. On the other hand, unchanged placental leptin receptor expression was accompanied by increased cord blood leptin concentrations in infants of obese mothers. This might indicate that, on the fetal side, leptin release by vascular endothelial cells may act directly on the fetus [244, 252]. Umbilical cord leptin concentrations can be viewed as a marker of adiposity in the fetus and a marker of obesity after birth and in adulthood [552]. This is further supported by the ability of fetal adipose tissue to synthesise leptin in proportion to neonatal ponderal index [546]. Alternatively, fetal hyperleptinemia can contribute to induce leptin resistance by chronic activation of leptin receptors in several fetal tissues [553]. Animal studies have indicated that adult offspring of obese mothers show impairment of hypothalamic metabolic regulation associated with leptin resistance [554, 555]. However, it is not known whether the hypothalamic targets of leptin are already responsive to its metabolic signals in the fetus. The main effect of placental leptin is thought to be on placental and fetal growth through autocrine and paracrine regulation [550, 551] and its influence on maternal physiology is less clear [120]. These observations are consistent with the positive correlations described here between cord blood leptin and both placental and birth weight, whereas no correlation with maternal leptin was found.

A potential role of leptin on placental and fetal growth, independent of maternal leptin metabolism [254], is hereby highlighted. The pleiotropic role of this adipokine could, therefore, explain the increased placental weight observed in obese subjects, who are more likely to experience placental hypertrophy [526]. As discussed in Chapter 3, an alternative role of insulin or glucose, which was also positively related to placental weight in obese subjects, could also explain enhanced placental weight with obesity.

Corticosteroids can also be involved in the regulation of insulin and leptin signalling. Excess GC have been associated with reduced leptin transport across the placenta [249] with a potential role in post-receptor inhibition of insulin signalling [265, 266, 269]. Giorgino et al. found that total tyrosine phosphorylation of insulin receptors was decreased in glucocorticoid-treated skeletal muscle, and this was accompanied by decreased IRS1 content and reduced serine and/or threonine phosphorylation of IRS1 [266]. Although the expression of key metabolic markers involved in placental insulin signalling and energy homeostasis was not influenced at a gene expression level in this study, post-transcriptional alteration might have occurred, accounting for the observed placental outcome. Interestingly, placental mRNA abundance of the GC receptor increased with GDM alone and when GDM was associated with high BMI, whilst overweight or obesity alone did not influence its expression. As such, GDM, rather than high BMI, seems to be the driving force upregulating this receptor. The activation of GC receptor initiates transcription of genes involved in metabolic homeostasis [292] and, additionally, it is associated with the transcription of inflammatory genes [293]. Thus, increased placental GC receptor observed in GDM pregnancies might contribute to a placental immunosuppressive response [292, 293] and this will be addressed in the following section. The 11β HSD2 enzyme expressed by the trophoblast represents a functional placental barrier to maternal GC by converting cortisol to inactive cortisone [288]. Equivalent placental 11β HSD2 gene expression in both obese and diabetic participants may indicate good placental protection of the fetus against high maternal cortisol [282, 288].

This was confirmed by the delivery of normal weight neonates from these pregnancies [284].

Insulin, IGF1 and leptin, as well as glucose and amino acids, are upstream regulators of mTOR, which regulates protein translation, thereby controlling cellular growth and metabolism [556-559]. In the placenta, mTOR has a major role in the regulation of trophoblast cell proliferation by stimulating amino acids placental transporters [300, 306]. I found that increased maternal BMI and GDM, both resulting in higher insulin and glucose plasma concentrations, were not associated with upregulation of mTOR signalling in the placenta as judged by gene expression. In contrast with previous studies [2, 300, 306], these results show inhibition of placental mTOR gene expression with obesity. This was accompanied by unchanged mRNA abundance of Akt, reflecting no differences in the expression of either IGFR1, ISR1 or p70S6K, a downstream regulator of mTOR signalling [298, 299]. These findings do not support those from Jansson et al. [2], who found activation of mTOR and insulin/IGF1 signalling in association with high BMI. Although placental and birth weight were not statistically different between groups, the authors found a positive association between these outcomes and activation of mTOR signalling. This correlation was not observed in my study, suggesting that mTOR signalling within the placenta did not significantly contribute to changes in placental or fetal growth. The BMIs considered here were pregestational whereas in Jansson's work BMI was measured between 8 and 12 weeks of gestation. Therefore, my study cannot exclude the possibility that a few subjects, classified as lean and overweight before pregnancy, might have moved to the obese group by gaining excess gestational weight at the beginning of pregnancy, potentially affecting the results. Furthermore, the average BMI of obese participants in my study was significantly lower than the mean BMI in Jansson's population. More importantly, the gestational weight gain of their participants was 2-3 fold higher than the weight gained by obese mothers in my study, and this was reflected by higher placental weight.

Although the authors did not measure food intake or maternal physiological outcomes, it is likely that their participants considerably increased their nutrient intake during pregnancy, and this was not observed in my study (Chapter 3). Raised nutrient intake in the obese mothers participating in Jansson' study might be explanatory of their enhanced placental weight [512] and raised mTOR expression [300, 558, 559]. The type of diet and reduction in food intake observed here may account for downregulation of placental mTOR expression [299, 482]. It is established that individual nutrients, such as glucose, regulate mTOR signalling by AMPk leading to inhibition of mTOR gene expression [305, 560], although how changes in diet through pregnancy can reset this mechanism is currently unknown.

Obese women showed a reduction in their food intake and lower rate of weight gain, but their plasma glucose concentrations at term were higher. Additionally, no alterations in AMPK expression with obesity were observed, indicating that some other mechanisms are responsible for mTOR downregulation. As another function of mTOR is to act as a homeostatic ATP sensor with autophosphorylation activity [307], it is possible that high BMI acts as a signal to silence this protein.

Despite the adaptations described above, these pleiotropic genes in the placenta can be also involved in cellular metabolism and pro-inflammatory pathways as discussed in the following section. An overview of the effect of both obesity and gestational diabetes on maternal, placental and fetal metabolic outcomes is illustrated in Figure 4.12 and 4.13.

Obesity

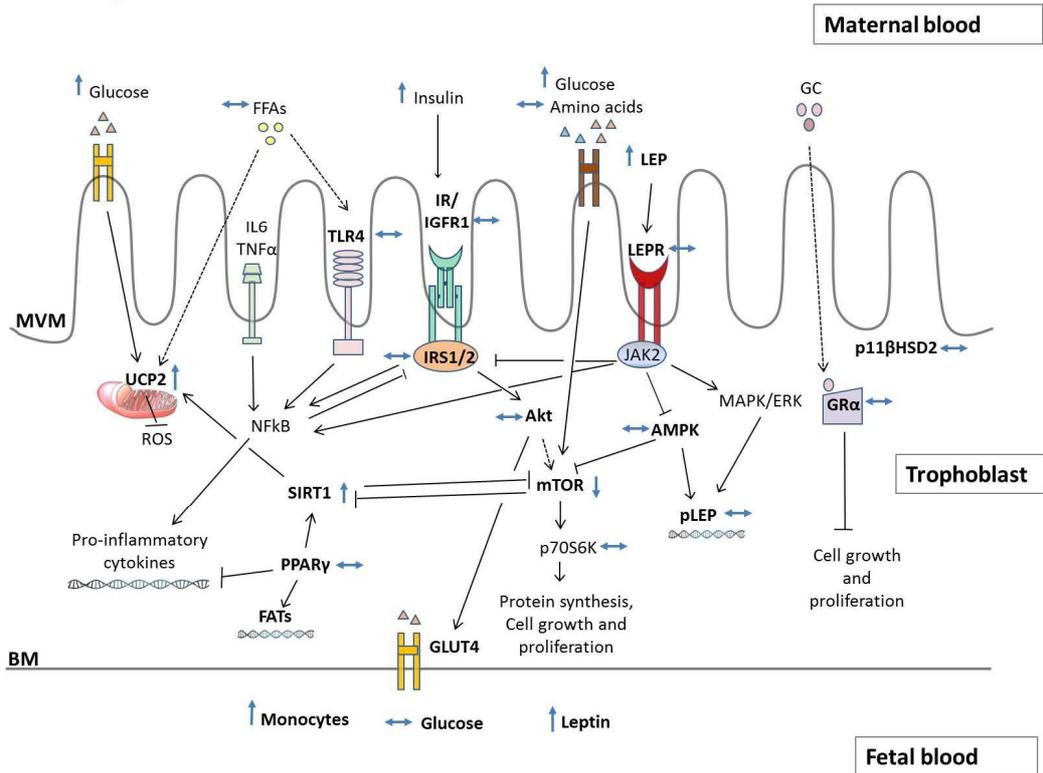


Figure 4.12: Effect of obesity on maternal, placental and fetal metabolism.

MVM: microvillous plasma membrane; BM: basal plasma membrane; FFAs: free fatty acids; UCP2: uncoupling protein-2; ROS: reactive oxygen species; IL6: interleukin-6; TNF α : tumour necrosis factor- α ; NF κ B: nuclear factor kappa B; TLR4: toll like receptor-4; IR: insulin receptor; IGFR1: insulin-like growth factor-1 receptor; IRS1/2: insulin substrate receptor-1/2; Akt: v-akt murine thymoma viral oncogene homolog; mTOR: mammalian target of rapamycin; p70S6K: ribosomal protein S6 kinase 70kDa polypeptide; LEP: leptin; LEPR: leptin receptor; GC: glucocorticoids; 11 β HSD2: 11-beta hydroxysteroid dehydrogenase-2; GR α : glucocorticoid receptor-alpha; MAPK/ERK: mitogen-activated protein kinase/extracellular regulated kinase; JAK2: janus kinase-2; AMPK: AMP-activated protein kinase; PPAR γ : peroxisome proliferator-activated receptor gamma; SIRT1: sirtuin-1. Arrows and bars represent activation and inhibition respectively. Blue arrows represent the effect driven by obesity: \uparrow upregulation, \leftrightarrow unchanged, \downarrow downregulation.

Gestational Diabetes

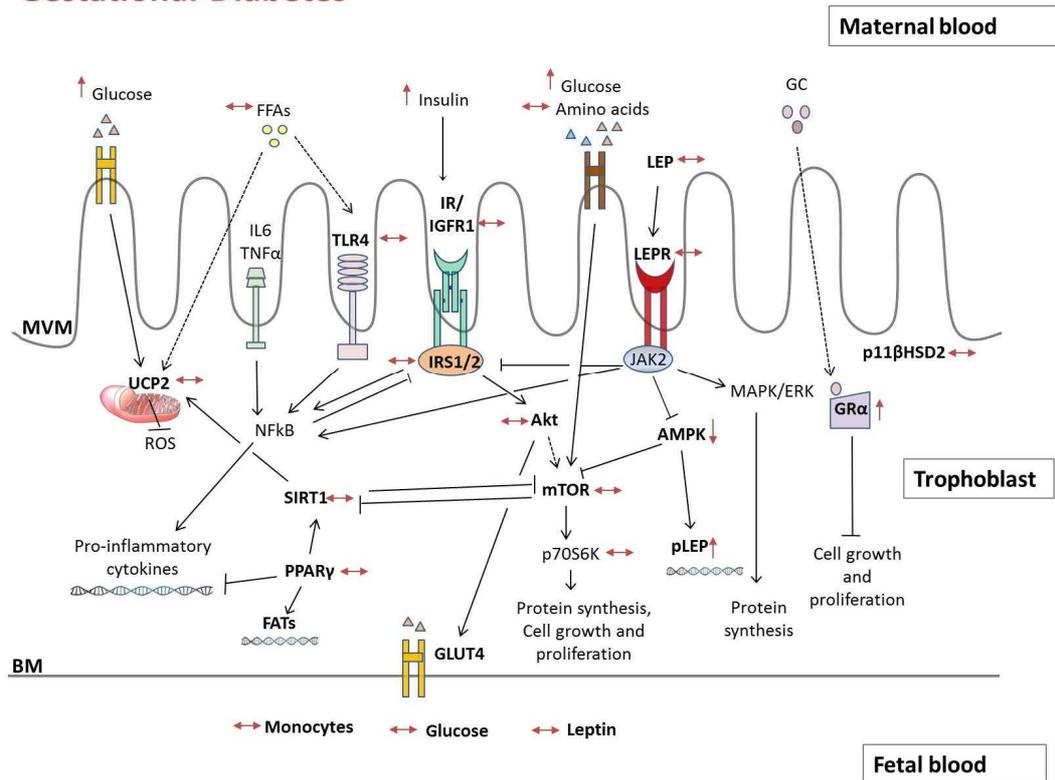


Figure 4.13: Effect of gestational diabetes on maternal, placental and fetal metabolism.

MVM: microvillous plasma membrane; BM: basal plasma membrane; FFAs: free fatty acids; UCP2: uncoupling protein-2; ROS: reactive oxygen species; IL6: interleukin-6; TNF α : tumour necrosis factor- α ; Nf κ B: nuclear factor kappa B; TLR4: toll like receptor-4; IR: insulin receptor; IGFR1: insulin-like growth factor-1 receptor; IRS1/2: insulin substrate receptor-1/2; Akt: v-akt murine thymoma viral oncogene homolog; mTOR: mammalian target of rapamycin; p70S6K: ribosomal protein S6 kinase 70kDa polipeptide; LEP: leptin; LEPR: leptin receptor; GC: glucocorticoids; 11 β HSD2: 11-beta hydroxysteroid dehydrogenase-2; GR α : glucocorticoid receptor-alpha; MAPK/ERK: mitogen-activated protein kinase/extracellular regulated kinase; JAK2: janus kinase-2; AMPK: AMP-activated protein kinase; PPAR γ : peroxisome proliferator-activated receptor gamma; SIRT1: sirtuin-1. Arrows and bars represent activation and inhibition respectively. Red arrows represent the effect driven by gestational diabetes: \uparrow upregulation, \leftrightarrow unchanged, \downarrow downregulation.

4.4.2. Placental anti-oxidant and anti-inflammatory response

It is well established that obesity induces chronic low-grade inflammation and increases circulating concentrations of inflammatory molecules, which can further induce insulin resistance [83, 561]. Diabetes in pregnancy also elicits major changes in the expression profile of placental genes with a prominent increase in markers and mediators of inflammation such as interleukins, leptin, and TNF α , linking inflammatory pathways and gestational diabetes-associated insulin resistance [214]. The resulting inflammatory environment can cause an antioxidant response by increasing antioxidant gene expression [562] and represents a protective or adaptive mechanism to prevent damage from further oxidative insults in utero.

Both maternal body fatness and the prevailing maternal glucose environment influence the magnitude of hormonal adaptations to pregnancy which, in turn, modulate adjustments in maternal and fetal energy metabolism [315]. This study appears to demonstrate that a diverse, but protective, response of the placenta is generated within the altered intra-uterine environments caused by obesity and gestational diabetes.

The impaired insulin resistance seen in these pregnancies [88] increased the circulatory availability of glucose, which could have developed an oxidative reaction within the placenta [82]. As placental metabolism is dependent on the availability of oxygen and glucose [7], any reduction in utero-placental oxygen uptake results in the increased production of placental ROS seen in both obesity and diabetes during pregnancy [14, 175, 519]. Changes in ROS production might act on pro- or anti-apoptotic pathways that interact with DNA stability, with potential re-programming of fetal energy homeostasis [536]. My findings showed that reduced placental mTOR observed with obesity was associated with overexpression of SIRT1 and UCP2, suggesting an enhanced placental antioxidant capacity [332].

Conversely, placentas of gestational diabetic women, with lean or increased BMI, were characterised by similar levels of antioxidant markers, possibly because of the small sample size or the mild severity of diabetes in these participants.

In normal physiologic situations, mitochondria directly regulate cellular ATP and AMP concentrations [563] through the activity of AMPK, Akt, and mTOR with the former sensing energy depletion [321], whereas the latter are stimulated by raised energy [300]. Mitochondria also regulate ROS production and oxidative stress by uncoupling energy supply. Both AMPK and mTOR regulate oxidative stress through changes in UCP2 [538] and NF κ B action [326-328], thereby promoting pro-inflammatory and pro-oxidative pathways within the trophoblast cells. On the other hand, mitochondrial replication is dependent on SIRT1 activity, which has been implicated in cell survival and senescence through inhibition of mTOR activity [333, 334]. Consequently, because SIRT1 and mTOR have been linked to age-associated diseases with SIRT1 activation and mTOR inhibition having beneficial effects [333, 335, 564], these findings might indicate an increased placental protective response against oxidative stress [333, 334]. In addition, higher placental weight in obese women was associated with similar placental TBARS concentration, which is considered a marker of oxidative stress [332]. Unchanged TBARS concentrations may indicate that placenta of mothers with high BMI participating in this study is not altered by exaggerated ROS production. This would therefore support the gene expression outcome reflecting the generation of a compensatory response to protect the fetus from excess maternal or placental ROS.

Excess availability of oxidised lipids has potential implications for placental development, lipid transport and metabolism by inhibiting trophoblast invasion leading to effects on fetal development [84]. Maternal concentrations of TG, which are the primary source of FFA, were similar between BMI groups and this was reflected by similar expression of placental genes involved in inflammatory response [216, 330].

PPAR γ has been proposed as an anti-inflammatory factor in both human and animal studies [308, 313] with a potential role in the initiation of labour [310]. High concentrations of circulating FFA and oxidised lipids have been associated with decreased PPAR γ expression and subsequent cytokine activation [309, 329]. This nuclear transcription factor is primarily involved in lipid metabolism [337], and is directly regulated by FFA [343, 344].

Similarly, the receptor TLR4, which plays an important role in inducing inflammation by activating NF κ B pathway, is also activated by FFA [345]. The lack of changes in the expression of these two pro-inflammatory genes with increased BMI are in keeping with the similar TG content in placenta and cord blood (Chapter 3), thus suggesting that no major signs of inflammation were present in these participants [329]. As measurement of placental markers of macrophage infiltration (CD14, CD68) [91-93] was not possible due to the poor specificity of available antibodies, these findings could not be further substantiated.

Despite apparently good metabolic homeostasis within the placenta and normal fetal and neonatal growth, some inflammatory markers in the cord blood of the infants of the obese participants were altered with potential adverse outcomes for the offspring [87, 88]. Enhanced circulating leptin in obese women was associated with higher leptin and monocyte concentrations in the cord blood of their infants. In addition to its potential role in newborn adiposity [546, 552], growing evidence has linked leptin with the maturation of fetal and neonatal immune system [244, 565]. In normal physiological situations, this adipokine can have a protective role in setting an acute systemic immune response and protect the infant from allergic conditions [259, 260]. However, in newborns with abnormally raised leptin concentrations impaired immune response is observed and this may result in the perpetuation of adaptive immunity through local modulation of pro-inflammatory cytokines [263] such as TNF α and IL10 [565].

Alternatively, increased proinflammatory cytokines expression, including TNF α and IL6, and/or enhanced circulating monocyte chemoattractant protein 1 (MCP1) concentrations in the maternal circulation of obese women may account for the monocytes rise observed in cord blood of their infants [566]. MCP1 is well established to regulate macrophage recruitment to sites of inflammation [567]. Hence, higher plasma levels of this protein may be involved in the monocyte recruitment into adipose tissue of newborns from obese individuals [566]. Here, they differentiate into macrophages and produce proinflammatory cytokines, contributing to a state of insulin resistance and low grade inflammation.

The effect of gestational diabetes on the placental inflammatory milieu resulted in upregulation of placental leptin and GR α associated with reduced AMPK expression. Leptin can regulate its own expression within the placenta through a mechanism involving the mitogen-activated protein kinase (MAPK) pathway and, alternatively, the suppression of AMPK [551]. A direct transcriptional action of GC on placental leptin expression has also been highlighted [568], suggesting that a local inflammatory response within the placenta of lean and obese diabetic women may have been generated [250, 261, 262, 293]. Nonetheless, cord blood inflammatory markers were similar in these participants, indicating that placental inflammation, if any, was not transferred to the fetus.

In conclusion, these findings suggest that changes in cellular energy status within the placenta can be very different between women that are obese compared to those that exhibit gestational diabetes. Overexpression of pleiotropic genes involved in key metabolic pathways within the placenta of diabetic mothers could regulate nutrient uptake, thereby contributing to a localised chronic pro-inflammatory state, which, eventually, does not seem to negatively affect the fetus. Conversely, high BMI in pregnancy might contribute to the development of an impaired immune response in the fetus despite a good antioxidant response within the placenta.

Chapter 5 - Effects of
maternal BMI and diabetes on
folate metabolism and placental
methylation

5.1. Introduction and hypotheses

Folate is essential for DNA synthesis and cell proliferation and, together with vitamin B12 and methylenetetrahydrofolate reductase (MTHFR), is involved in the homocysteine-methionine cycle through complex biochemical pathways (Figure 1.17, Chapter 1). This micronutrient serves as single carbon donor in the vitamin B12-dependent remethylation of homocysteine into methionine [43] whilst normal MTHFR activity maintains the pool of circulating folate by preventing a rise of cellular homocysteine [569, 570].

Folate deficiency in pregnancy increases the risks of premature delivery, with NTDs and lower birthweight, all of which can be reduced by adequate maternal folate intake [350, 518, 571]. The incidence of these complications in diabetic pregnancies is lowered by good glycaemic control and folate administration [508]. However, despite being recommended in the management of obesity in pregnancy, folate supplementation in pregnant women with high BMI [38, 45] does not reduce the risk of delivering a child with NTD [352]. In addition, large epidemiological studies have reported a strong association between obesity and lower folate concentrations in women of childbearing age [572, 573]. The present study investigated the association between maternal dietary intake of folate and maternal and cord blood folate in pregnancies complicated by high BMI and gestational diabetes. Its novel focus was to examine the contribution of different BMI categories as well as the distinct effects of gestational diabetes amongst lean, overweight and obese subjects on folate metabolism. Folate and its metabolites are strongly involved in DNA methylation. S-adenosyl methionine (SAM) is used by DNA methyltransferases (DNMTs) to methylate DNA [371, 379], an essential process for the developing embryo. Previous studies have shown that altered levels of DNA methyl transferases -1 and -3 (DNMT1, DNMT3) in liver, serum and the uterus during embryo implantation are associated with dietary folate deficiency [574].

Defective folate metabolism, or limited folate availability, can contribute to modifications in feto-placental function by influencing DNA methylation status in peripheral blood cells [369] or altering expression of inflammatory markers in the placenta [500]. The resulting individual is, therefore, at an increased risk of metabolic dysfunction and CVD in later life [366, 575]. As the relationship between maternal folate and DNMTs has not been fully investigated, this Chapter also contributes to current knowledge about the influence of maternal diet on gene expression during fetal development.

Oyama, Wentzel and colleagues have recently demonstrated in rodents that diabetes leads to decreased gene expression of folic acid binding protein (Folbp) in the mother resulting in intracellular folate deficiency in the embryo [518, 576]. Although the placenta is crucial for ensuring fetal development, few studies have focused on folate transport within the placenta and the resulting outcomes for the fetus. In the Introduction (Chapter 1) to this thesis, I have described how cellular uptake of folate is mediated by specific transport mechanisms in the placenta, which include the folate receptor alpha (FR α), proton coupled folate transporter (PCFT), and reduced folate carrier (RFC) [329, 330, 331]. Briefly, folate is bound to the FR α on the maternal side and transported within the placenta by endocytosis/exocytosis at a neutral to mildly acidic pH. Subsequently, PCFT mediates the co-transport of folate and protons at low pH and, finally, RFC mediates the cellular uptake of folate in exchange for specific anions such as organic phosphates. RFC has been proposed as the major route of folate delivery to the fetal circulation at a physiological pH [577]. Carter et al. suggested that placental folate transport in overweight and obese pregnancies is impaired and results in lower fetal folate concentrations [354]. Nevertheless, few studies have investigated placental transport of folate nor have focused on its effect on pregnancies complicated by epidemic diseases such as obesity or diabetes.

Dietary components such as folate have been shown to influence methylation patterns during development [578] with potential effects on the offspring's health [579, 580].

Changes in epigenetic profile in response to perturbations within the in utero environment could contribute to modifications in fetoplacental function that ultimately place the resulting individual at increased risk of metabolic disease in later life [575, 579-581]. This is because differences in the methylation pattern of genes expressed in the placenta are associated with changes in gene expression, which could impair placental function [575] resulting in gestational and early life diseases [370]. The use of genome-wide methylation arrays enables a more detailed examination of human epigenomics although this can lead to controversial interpretations [402].

Therefore, in this Chapter the hypotheses are:

1. Pregnant women with high BMI and gestational diabetes are characterised by inadequate dietary intake of folate and this will result in changes in serum folate. Due to the consumption of a poor quality diet [38, 41, 45], overweight and obese mothers will have lower folate concentrations whereas these will be increased in gestational diabetic women, who are advised to follow healthier food choices [508, 509].
2. Alterations in maternal folate concentrations will influence folate status in the newborn via changing placental folate transport and metabolism. The fetus can survive on maternal folate stores even in conditions of profound dietary deficiency [348] resulting in newborn folate concentrations within the normal range [582]. This raises the possibility that the placenta buffers maternal folate deficiency to protect the fetus. I hypothesise that placental folate transport in overweight and obese women will be upregulated in order to compensate for lower maternal folate. On the other hand, placental folate transport in diabetic women, associated with higher plasma folate concentrations, will be reduced contributing to a newborn with normal folate levels.

3. Due to the crucial role of folate in providing methyl donors [583] and its proposed influence on DNMT1 and DNMT3 [574], I hypothesise that changes in placental folate transport resulting from maternal high BMI and/or gestational diabetes, will reflect alterations in expression of these DNMTs and possible modifications in DNA methylation within the placenta [583]. Epigenetic alterations can be of critical importance in highlighting both novel pathways and regulatory molecules associated with an adverse nutritional environment [581].

5.2. Methods

The design of the study and all materials and methods used are detailed described in Chapter 2 (Materials and Methods). In summary, pregnant women participating in the Preobe study (P06-CTS-02341) were recruited in the 20th week of pregnancy and classified according to their BMI. At 28th weeks of gestation, they were further classified on the basis of BMI and glucose tolerance, making six groups (Chapter 2, Section 2.2, Figure 2.2).

Maternal and fetal anthropometries were evaluated at 24 and 34 weeks of pregnancy as illustrated in Figure 5.1. As maternal weight at 40 weeks of gestation was self-reported, and may not have equal accuracy in all BMI groups, this was not used in calculations of gestational weight gain and postnatal weight loss [62, 482]. After birth, infant anthropometry was evaluated by a paediatrician at 3, 6, 9 and 12 months of age respectively and maternal anthropometry was performed at 6 months.

Information relating to folate and vitamin B12 intake before delivery was collected using 7 day estimated food intake records at 34 weeks of gestation. Nutritional data were then analysed for nutrient intake by using a nutritional software program (CESNID 1.0, Barcelona University, Spain) based on validated [412] Spanish food tables ("Tablas de composición de alimentos del CESNID"). Maternal venous blood was collected at 34 and 40 weeks of gestation as well as at 6 months post-delivery. At birth, umbilical venous blood samples were collected and placentas were snapped frozen and stored at -80°C for further analysis in Nottingham.

Total RNA was extracted from 100 mg of placental samples with TRI Reagent[®] and chloroform and cDNA obtained using the High Capacity RNA-to-cDNA kit (Applied Biosystems, CA 94404, USA) as described in Chapter 2. Gene expression of target genes implicated in placental folate transport and metabolism (FR α , PCFT (SLC46A1), RFC (SLC19A3) and MTHFR) and mRNA abundance of DNMT1 and DNMT3 were assessed by real-time PCR.

Gene expression of PCFT and RFC was examined in the later part of the study on a representative random selection of samples (C=20; OV=20; OB=20; GDL=10; GDOB=10).

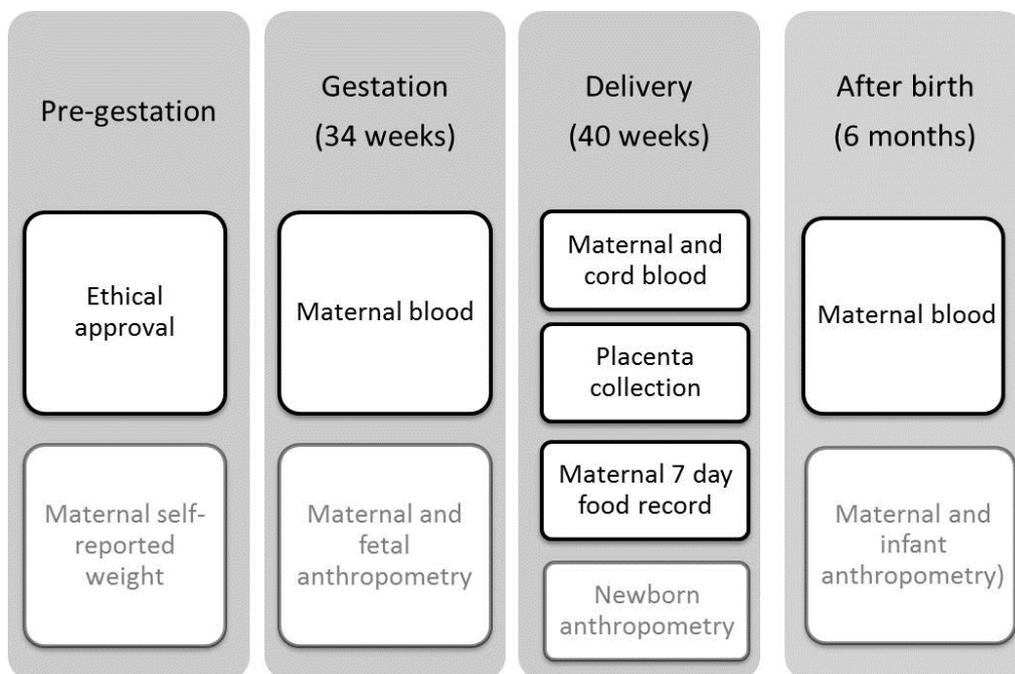


Figure 5.1: Programme of evaluations in the Preobe study.

Maternal examinations during gestation and after delivery. Collection of samples and measurements are reported in white boxes whereas greyed out boxes represent time before, during and after pregnancy. Aspects studied in this Chapter are highlighted in white boxes marked with solid black line.

Total protein was extracted from frozen tissues using CellLytic MT and protease inhibitor cocktail (Chapter 2, Section 2.11.1), whilst a further extraction using RIPA buffer was performed from a few samples stored in RNA later (Chapter 2, Section 2.11.2). Protein abundance of FR α and RFC was determined by Western Blotting; however, none of the two antibodies were found to be specific for the target products. All primer sequences and antibodies used in these analyses are listed in Tables 2.4 and 2.7 (Chapter 2, page 82 and page 110, respectively).

Lastly, a set of 24 placental samples were selected from 5 of the groups (C, n=5; OV, n=4; OB, n=5; GDL, n=5; GDOB, n=5) to guarantee homogeneity of the population, by maternal age (between 25 and 35 years), BMI (no borderline BMIs), smoking during pregnancy (only no smokers), nulliparity (only first pregnancy) and newborn weight (only AGA). Genomic DNA isolation was achieved by thorough disruption and digestion of the tissues, which were then purified using QIAamp DNA Mini Kit (50) (QIAGEN) (see Chapter 2, Section 2.12.1, page 119).

The HumanMethylation450 15017482 v.1.1 manifest from Illumina [400] was used to measure genomic DNA methylation patterns and the Matlab package NIMBL (Numerical identification of Methylation Biomarker Lists) [402] for the analysis of annotation data (including, for example, gene and gene region information). According to this, Infinium methylation profiles for all samples were provided as a text file and analysed by Emes et al. using NIMBL [470]. Several output plots were generated to visualise the sample quality including a plot of the beta value distribution of each sample, which represents the estimates of methylation levels at a given locus [400]. Additionally, gene centric plots providing genomic DNA sequence and coordinate of gene coding regions, were generated for the novel genes, which were most differentially methylated, and their expression determined by QPCR.

5.3. Results

5.3.1. Folate intake and physiological outcomes

Characteristics of the participants are given in Table 5.1 and Table 5.2. Of all the participants, 130 women out of 149 reported daily intake of folate and iodine supplements at 24th week of pregnancy. Only 86 out of 149 participants completed and returned the 7 day dietary questionnaires and these showed no influence of maternal BMI in maternal folate and vitamin B12 intake in late gestation. Whilst the reduction in folate intake as assessed by dietary questionnaire did not reach statistical significance in this subset of women, when data from more women within the Preobe study was available, overweight and obese women had a significantly lower folate intake compared to lean controls [520]. On the other hand, maternal folate intake increased with gestational diabetes as shown in Table 5.3, but this was statistically significant only in the overweight group (GDOV).

Folate concentrations in all subjects were within the normal range (5-16 ng/ml) with no evidence of folate deficiency (<5 ng/ml) [584]. However, a marked decrease in folate concentrations from mid to late pregnancy was observed in both overweight and obese subjects ($p < 0.001$) compared to lean women.

Table 5.1: Maternal nutrient intake and plasma folate during late pregnancy and after delivery in lean control (C), overweight (OV) and obese (OB) pregnant women.

Maternal nutrient intake ($\mu\text{g}/\text{day}$)	C (n=37)	OV (n=16)	OB (n=8)
7 day folate intake	298.3 \pm 12.0	258.6 \pm 18.6	260.4 \pm 46.2
7 day vitamin B12 intake	5.76 \pm 2.91	4.7 \pm 2.2	5.0 \pm 2.7
Maternal folate (ng/ml)	C (n=59)	OV (n=29)	OB (n=22)
Plasma folate at 34 gw	12.9 \pm 0.6	10.6 \pm 0.9*	11.1 \pm 0.9
Plasma folate at 40 gw	12.9 \pm 0.6	9.5 \pm 0.9**	8.4 \pm 0.9*
Plasma folate 6 m post-delivery	10.3 \pm 0.7	10.8 \pm 0.9	8.09 \pm 1.19

Data represents means \pm S.E.M.; n = women/group; gw: gestational weeks; m: months. Statistical differences denoted by *,** correspond to $p < 0.05$ and $p < 0.01$ respectively (1-way ANOVA, Bonferroni post-hoc test).

Cord blood folate concentrations in newborns from overweight and obese mothers were significantly lower compared to controls (Table 5.2). In addition, the difference between umbilical cord folate and maternal folate at 40 gestational weeks (Delta folate) increased with maternal BMI.

Table 5.2: Cord blood folate in lean control (C), overweight (OV) and obese (OB) subjects.

Cord blood folate (ng/ml)	C (n=33)	OV (n=15)	OB (n=12)
Cord blood folate	18.7 \pm 0.4	17.1 \pm 0.8*	17.4 \pm 0.7*
Delta folate	5.80 \pm 0.81	7.66 \pm 0.82*	9.01 \pm 0.90*

Data represents means \pm S.E.M.; n = women/group; gw: gestational weeks; m: months.

Delta folate: difference between maternal folate at 40 gestational weeks and umbilical cord folate. Statistical difference denoted by * corresponds to $p < 0.05$ (1-way ANOVA, Bonferroni post-hoc test).

As reported in Table 5.3, lean mothers who developed diabetes during gestation showed the highest serum folate concentrations at both 34 gestational weeks and 6 months after delivery compared to lean women without GDM. Interestingly, this difference was not observed with the additional effect of BMI, which was associated with similar folate concentrations to that of lean controls without GDM.

Table 5.3: Maternal nutrient intake and plasma folate during late pregnancy and after delivery in lean control (C), lean gestational diabetic (GDL), overweight gestational diabetic (GDOV), and obese gestational diabetic (GDOB) pregnant women.

Maternal nutrient intake ($\mu\text{g}/\text{day}$)	C (n=37)	GDL (n=11)	GDOV (n=8)	GDOB (n=6)
7 day folate intake	298.3 \pm 12.0	342.9 \pm 33.9	387.8 \pm 43.6*	299.5 \pm 53.5
7 day vitamin B12 intake	5.76 \pm 2.91	10.08 \pm 4.13	4.49 \pm 0.96	5.29 \pm 1.21
Maternal folate (ng/ml)	C (n=59)	GDL (n=14)	GDOV (n=14)	GDOB (n=11)
Plasma folate 34 gw	12.9 \pm 0.6	17.0 \pm 1.0**	13.9 \pm 1.5	15.2 \pm 1.0
Plasma folate 40 gw	12.9 \pm 0.6	15.4 \pm 1.2	10.7 \pm 1.7	13.9 \pm 1.2
Plasma folate 6 m post-delivery	10.3 \pm 0.7	15.3 \pm 1.4**	12.1 \pm 2.1	11.5 \pm 1.9

Data represents means \pm S.E.M.; n = women/group; gw: gestational weeks; m: months. Statistical difference denoted by **, ** corresponds to $p < 0.05$ and $p < 0.01$ respectively (1-way ANOVA, Bonferroni post-hoc test).

Cord blood folate concentrations and Delta folate were similar between gestational diabetic groups with or without the influence of BMI (Table 5.4).

Table 5.4: Cord blood folate in lean control (C), lean gestational diabetic (GDL), overweight gestational diabetic (GDOV), and obese gestational diabetic (GDOB) pregnant women.

Cord blood folate (ng/ml)	C (n=33)	GDL (n=7)	GDOV (n=12)	GDOB (n=7)
Cord blood folate	18.7±0.4	19.3±0.7	18.6±0.8	20.0±0.1
Delta folate	5.93±0.81	4.45±1.43	7.56±1.72	6.78±1.62

Data represents means ± S.E.M.; n = women/group; gw: gestational weeks; m: months.

Delta folate: difference between maternal folate at 40 gestational weeks and umbilical cord folate.

5.3.2. Placental gene expression of folate transporters and metabolism

The relative expression of placental genes implicated in folate transport and metabolism and in DNA methyl transfer is reported below.

Despite obese subjects showing the highest difference in folate levels between mother and fetus, their placental expression of FR α was significantly lower compared to control group (Figure 5.2).

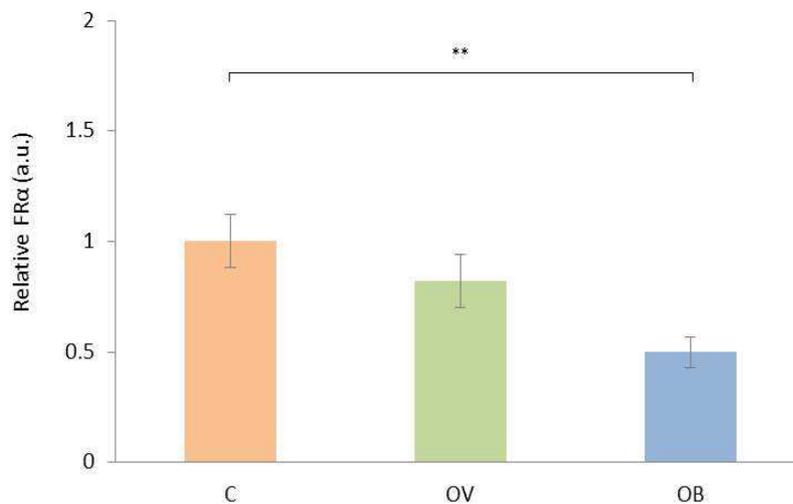


Figure 5.2: Placental folate receptor alpha (FR α) mRNA expression relative to 18S of lean control (C), overweight (OV), and obese (OB) pregnant women.

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give fold change. C: control (n=59); OV: overweight (n=29); OB: obese (n=22). Data are non parametric and represent mean \pm S.E.M; a.u.: arbitrary units; n = women/group. Statistical difference detected by ** corresponds to $p < 0.01$ (Kruskal-Wallis; Mann Whitney post-hoc test).

A multi-regression analysis investigating predictors for placental FR α highlighted pre-gestational BMI and placental weight as the factors with greatest influence on the expression of this transporter (Table 5.5). After adjusting for other cofounders, such as gestational age and maternal folate at 40 gestational weeks, no additional factors were found to be associated with pre-gestational BMI, indicating an independent contribution of these factors on placental gene expression.

Table 5.5: Association between placental gene expression of folate receptor alpha (FR α) and different predictors in control, overweight and obese pregnant women.

Linear regression model (n=110)	Changes in placental FR α gene expression	p value
Model 1^Ψ	1.66 (0.85 to 2.47)	0.047
Model 2^{ΨΨ}	2.13 (1.20 to 3.05)	0.017
Model 3^{ΨΨΨ}	5.47 (0.13 to 10.82)	0.118

^Ψ Adjusted for the *a priori* confounders BMI; ^{ΨΨ} adjusted for the *a priori* confounders BMI and placental weight; ^{ΨΨΨ} adjusted for the *a priori* confounders BMI, placental weight, gestational week and maternal folate at 40 gestational weeks. Numbers in brackets are 95% confidence intervals.

The FR α is responsible for folate uptake from the maternal circulation into the placenta and, together with PCFT and RFC, ensures its transport to the fetus [328, 329]. The mRNA abundance of placental folate transporters PCFT and RFC was measured in a randomly selected subgroup of samples but no differences were detected (Table 5.6). Furthermore, gene expression of placental MTHFR did not change significantly between groups although a trend (p=0.09) to decrease was observed with obesity (Table 5.6).

Table 5.6: Placental mRNA expression relative to housekeeping gene (ribosomal 18S) of lean control (C), overweight (OV), and obese (OB) pregnant women.

Placental gene expression (a.u.)	C (n=59)	OV (n=29)	OB (n=22)
PCFT ^ψ	1.00±0.15	0.96±0.11	1.11±0.14
RFC ^ψ	1.00±0.19	0.93±0.12	1.04±0.17
MTHFR	1.00±0.16	1.04±0.19	0.76±0.13
DNMT3	1.00±0.20	1.08±0.23	0.66±0.10

Data are non parametric and expressed as relative to housekeeping gene (ribosomal 18S). Data are normalised to the lean control group to give fold change and represent means ± S.E.M; a.u.: arbitrary units; n = women/group. The abundance of genes denoted by ^ψ has been measured in a representative selection of samples (n=20).

Although there were no significant changes in DNMT3a expression (Table 5.6), mRNA expression of DNMT1 was upregulated 2 fold with overweight (p=0.001), and 1.5 fold with obesity (p=0.023) (Figure 5.3).

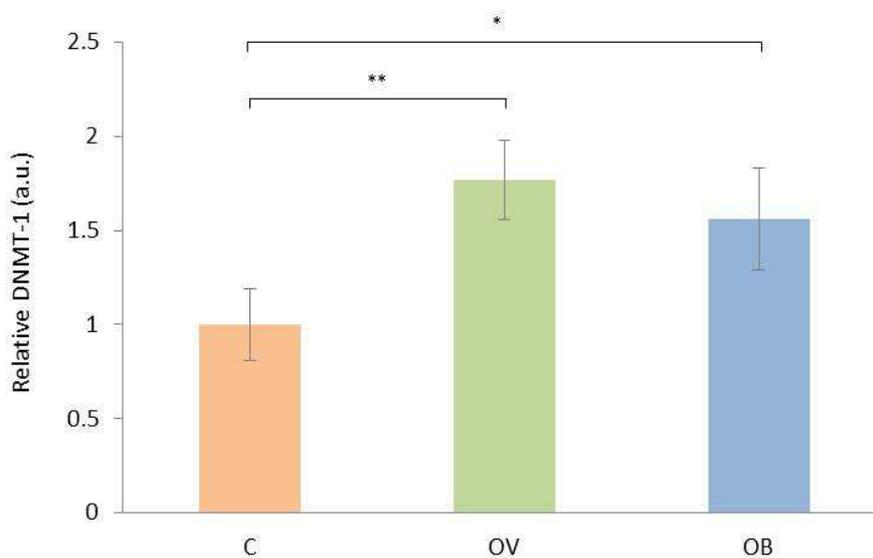


Figure 5.3: Placental DNA methyl transferase 1 (DNMT1) mRNA expression of lean control (C), overweight (OV), and obese (OB) pregnant women

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give fold change. C: control (n=59); OV: overweight (n=29); OB: obese (n=22). Data are non parametric and represent mean ± S.E.M; a.u.: arbitrary units; n = women/group. Statistical difference detected by *, ** correspond to p<0.05 and p<0.01 respectively (Kruskal-Wallis; Mann Whitney post-hoc test).

Similarly, high BMI combined with gestational diabetes was associated with a decrease in the mRNA abundance of FR α (Table 5.7), an effect that became statistically significant with maternal obesity in a similar manner to that seen in the non-diabetic groups (Figure 5.2). In addition, no differences in other folate transporters were observed in mothers with GDM, possibly due to the smaller number of samples.

Table 5.7: Placental mRNA expression relative to housekeeping gene (ribosomal 18S) of lean control (C), lean gestational diabetic (GDL), overweight gestational diabetic (GDOV), and obese gestational diabetic (GDOB) pregnant women.

Placental gene expression	C (n=59)	GDL (n=14)	GDOV (n=14)	GDOB (n=11)
FR α	1.00 \pm 0.12	0.61 \pm 0.09	0.76 \pm 0.13	0.48 \pm 0.08 [*]
PCFT ^{Ψ}	1.00 \pm 0.15	0.85 \pm 0.15	n.m.	0.84 \pm 0.11
RFC ^{Ψ}	1.00 \pm 0.19	0.73 \pm 0.14	n.m.	0.71 \pm 0.18
DNMT3 α	1.00 \pm 0.20	0.86 \pm 0.33	1.30 \pm 0.49	0.64 \pm 0.08

Data is expressed as relative to housekeeping gene (ribosomal 18S) and normalised to the lean control group to give fold change. Data are non parametric and represent mean \pm S.E.M; a.u.: arbitrary units; n = women/group; n.m.: not measured. Statistical difference annotated by ^{*} corresponds to $p < 0.05$ (Kruskal-Wallis). The abundance of genes denoted by ^{Ψ} has been measured in a representative selection of samples (n=20).

As a consequence of gestational diabetes combined with high BMI, gene expression of placental MTHFR, the enzyme that catalyses the synthesis of 5-methyl THF, was upregulated (Figure 5.4). Interestingly, enhanced expression of placental MTHFR was observed with gestational diabetes alone in overweight (GDOV vs OV) and obese (GDOB vs OB), but not lean (GDL vs C) GDM groups (Figure 5.5).

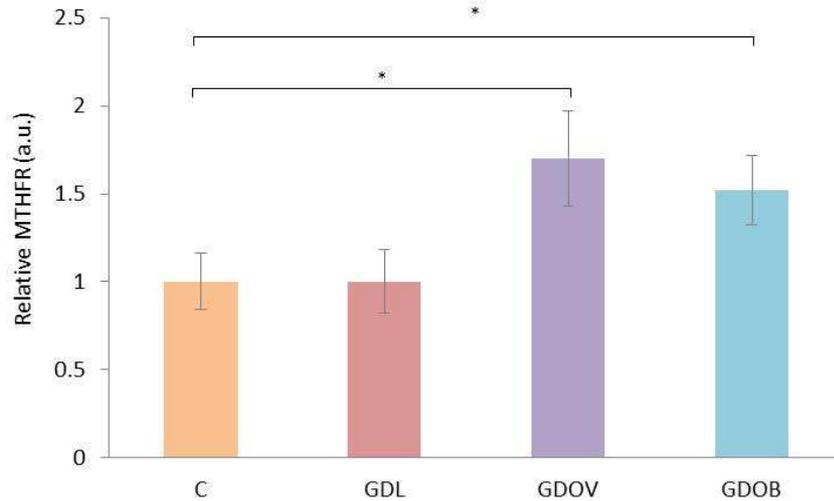


Figure 5.4: Effect of GDM and BMI on the mRNA abundance of placental methylenetetrahydrofolate reductase (MTHFR) as determined by real-time PCR.

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give the fold change. C: control (n=59); GDL: lean women with GDM (n=14); GDOV: overweight women with GDM (n=14); GDOB: obese women with GDM (n=11). Data are non parametric and represent mean \pm S.E.M; a.u.: arbitrary units; n = women/group. Statistical differences denoted by *, ** correspond to $p < 0.05$ and $p < 0.01$ respectively (Kruskal-Wallis; Mann Whitney post-hoc test).

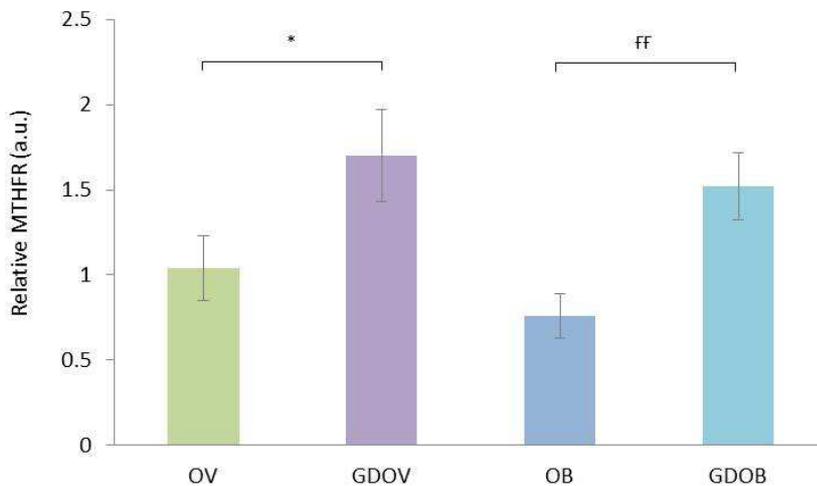


Figure 5.5: Effect of GDM on the mRNA abundance of placental methylenetetrahydrofolate reductase (MTHFR) as determined by real-time PCR.

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give the fold change. C: control (n=59); GDL: lean women with GDM (n=14); GDOV: overweight women with GDM (n=14); GDOB: obese women with GDM (n=11). Data are non parametric and represent mean \pm S.E.M; a.u.: arbitrary units; n = women/group. Statistical differences denoted by * and ** correspond to $p < 0.05$ and $p < 0.01$ respectively (Kruskal-Wallis; Mann Whitney post-hoc test).

The abundance result of folate transporters was further investigated by measuring the protein expression. In total, three different antibodies for FR α and one for RFC were used with the aim of detecting their specific targets. Protein concentrations in previously frozen placental tissues were unusually low and the antibody did not bind the protein of interest (see Chapter 2, Section 2.11.7). This suggests that these tissues might have been degraded during collection or storage and were not suitable for any further analysis. Consequently, attempt to extract proteins from placental tissues stored in RNA later solution, was performed. Although protein was better preserved from degradation, as demonstrated by running the samples in agarose gel (Figure 2.5, Section 2.6.2, Chapter 2), none of the polyclonal or monoclonal antibodies used detected specific bands for their target antigens. Any binding was non-specific to proteins of similar molecular weight as shown by incubation of the membrane with non-immune rabbit serum (NRS) (Figure 5.6). This negative control is known not to express the target protein and is used to check for non-specific binding and false positive results.

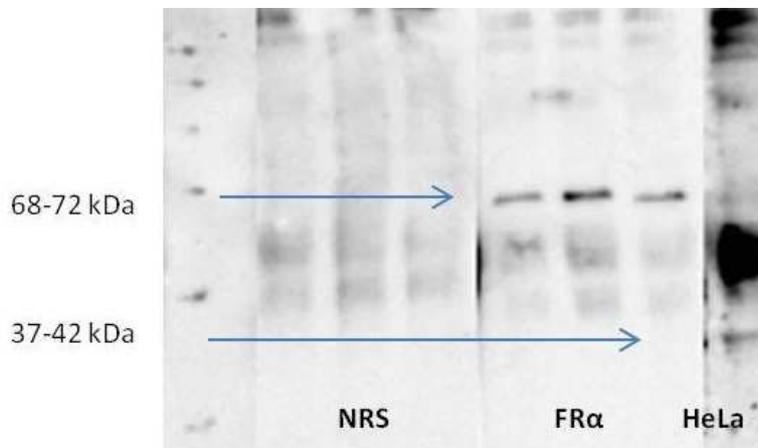


Figure 5.6: Example of immunodetection for FR α in RNA later placental samples.

The antibody binds a specific protein of approximately 70 kDa in the samples (FR α) and this can be due to protein aggregation (i.e. albumin). A protein of the correct molecular weight (37-42 kDa) is instead recognised by the antibody in the positive control (HeLa cells). FR α : folate receptor alpha; NRS: non-immune rabbit serum; HeLa: cervical cancer cells.

A positive control (HeLa cells lysate) known to express the target protein, was also run alongside the placental samples to test the specificity of the antibody. The 37 kDa band of interest for FR α was detected, showing that the antibody was specific for the target protein.

As a specific band of 68-72 kDa was recognised by the monoclonal antibody to FR α , it is likely that the protein of interest interacted with another present in the sample impeding FR α protein free migration through the polyacrylamide gel. As discussed in Chapter 2 (section 2.11.8), in the case that protein aggregation occurred, the interaction can be broken by adding a reducing agent, such as anti-ubiquitin or urea 4-8M. Therefore, a further procedure using urea 4M to separate the aggregate was performed giving a better resolution of the protein bands. Once more, the immunodetection did not show a specific binding of the antibody to the target protein as highlighted in positive controls (HeLa) and by incubation of the membrane with NRS (Figure 5.7).

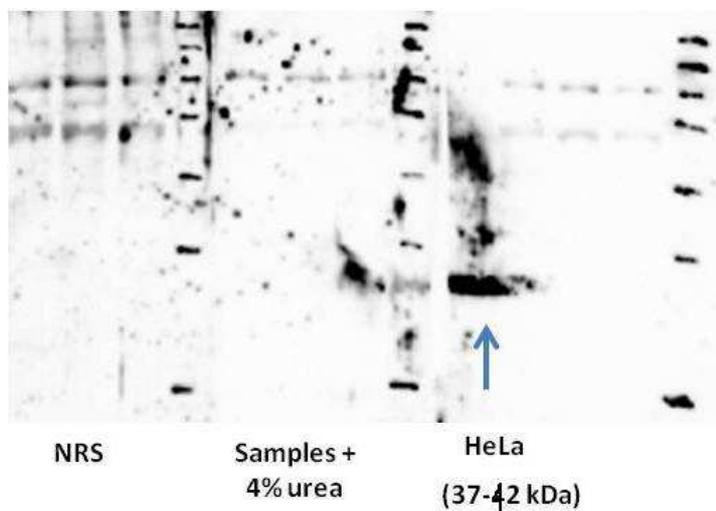


Figure 5.7: Immunodetection for FR α after treatment of samples with 4% Urea.

The 37-42 KDa band of interest was detected only in the positive control (HeLa).

5.3.3. Methylation analysis

5.3.3.1. Identification of differentially methylated sites using NIMBL

A plot of beta value distribution [400], representing the relative methylation level of each sample is illustrated in Figure 5.8. Array sites with one or more of the 24 samples having a p value of less than 0.05, and sites with missing beta values measurements, were excluded from further analysis. The figure represents twenty three of the 24 samples being of good quality with one showing measurements with low confidence of methylation accuracy (e.g. detection p-value > 0.05).

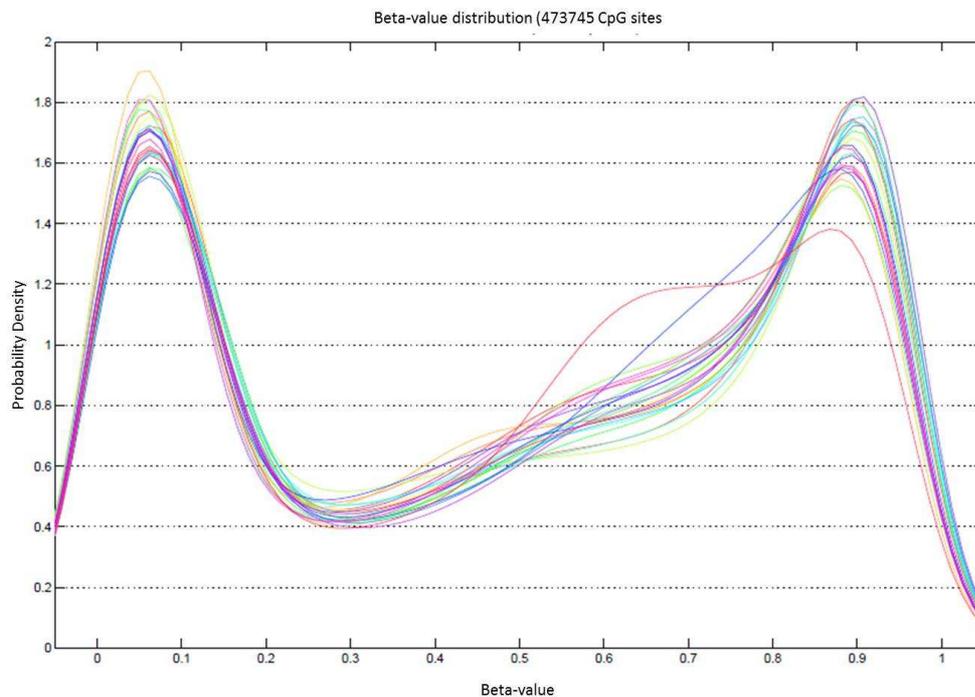


Figure 5.8: Summary output plots of the distribution of beta-values of all samples.

Estimation of the proportion of methylation at a given locus (beta value) of 24 samples (5 controls, 4 overweight, 5 obese, 5 gestational diabetic lean, 5 gestational diabetic obese). A ratio of 0 equals to non-methylation of the locus whereas a value of 1 equals to total methylation; a ratio value of 0.5 means that one copy is methylated and the other is not, in the diploid human genome. One of the samples in purple shows an aberrant distribution and was excluded from further analysis.

Each beta-value indicates the proportion of methylation at a given CpG site with values ranging from 0 (CpG site always found unmethylated in sample DNA) to 1 (CpG site always found methylated in sample DNA) [468].

The differential methylation settings used are based on a constant level of beta value inter-group distance of 0.2 corresponding to 20% minimum difference between groups. Therefore, differentially methylated sites were identified as sites with the largest difference in methylation levels between two groups with a minimum beta value distance between non-overlapping groups (Chapter 2, Figure 2.15). If this minimum beta value distance was not reached, samples were masked and sites with previously overlapping groups or low discrimination were additionally identified and flagged.

The greatest difference in methylation was found between placentas sampled from normal and overweight mothers. These showed more than 200 differentially methylated CpG sites, which was almost 10 fold greater when compared with the obese group. Examples of gene centric plots of the sites of methylation changes were then generated for the genes with most differential methylation (i.e. FAM3B and WNT2) as follows.

Methylation of the promoter regions for both FAM3B and Wnt2 was raised in placenta of overweight women as shown in their methylation profile in Figure 5.9 and 5.10 respectively.

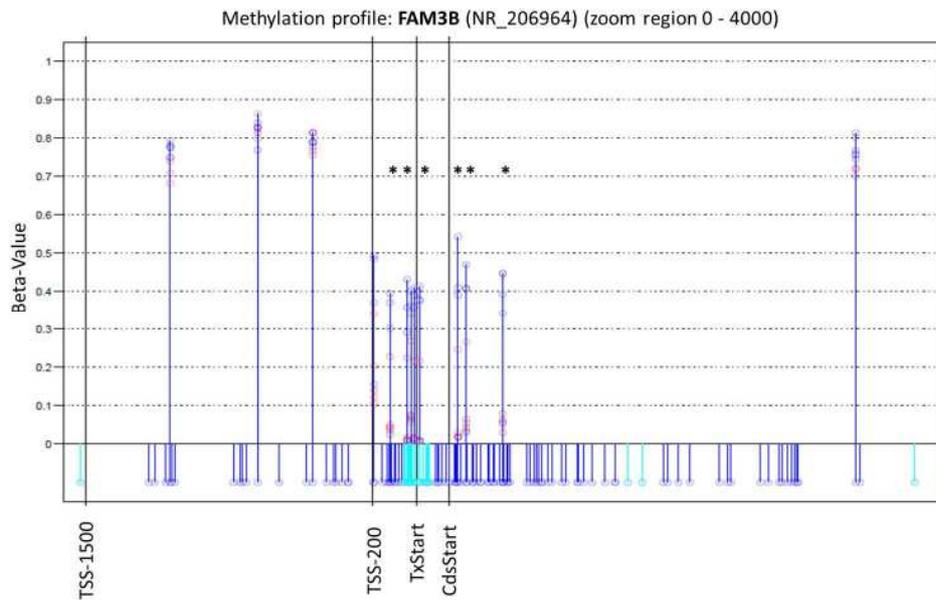


Figure 5.9: Methylation profile of gene FAM3B.

NIMBL-gene output showing a zoomed region of the genomic location of the gene FAM3B displaying 14 out of 20 CpG sites associated with this gene on the array. The stems below the x-axis correspond to all CpG sites located in this region. CpG sites identified as differentially methylated by NIMBL ($d=0.2$, $m=1$) are highlighted with asterisks. TSS-1500, TSS-200: position 1500bp and 200bp respectively upstream of transcriptional start site (TxStart).

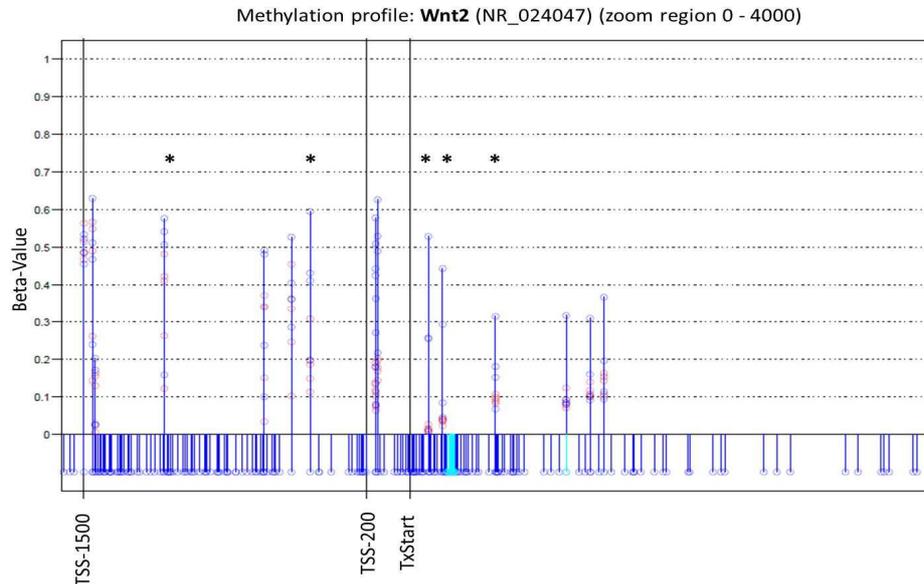


Figure 5.10: Methylation profile of gene Wnt2.

NIMBL-gene output showing a zoomed region of the genomic location of the gene Wnt2 displaying 14 out of 20 CpG sites associated with this gene on the array. The stems below the x-axis correspond to all CpG sites located in this region. CpG sites identified as differentially methylated by NIMBL ($d=0.2$, $m=1$) are highlighted with asterisks. TSS-1500, TSS-200: position 1500bp and 200bp respectively upstream of transcriptional start site (TxStart).

5.3.3.2. Gene expression of differentially methylated genes

Gene expression analysis of FAM3B and Wnt2 was further performed in all five groups to determine if the results from the methylation analysis were accompanied by alterations in gene expression. Interestingly, greater methylation in the promoter region of FAM3B and Wnt2 was not accompanied by comparable changes in gene expression.

Figure 5.11 shows downregulation of placental FAM3B in obese compared to overweight subjects, but not between overweight and lean control groups, as was expected from the methylation analysis.

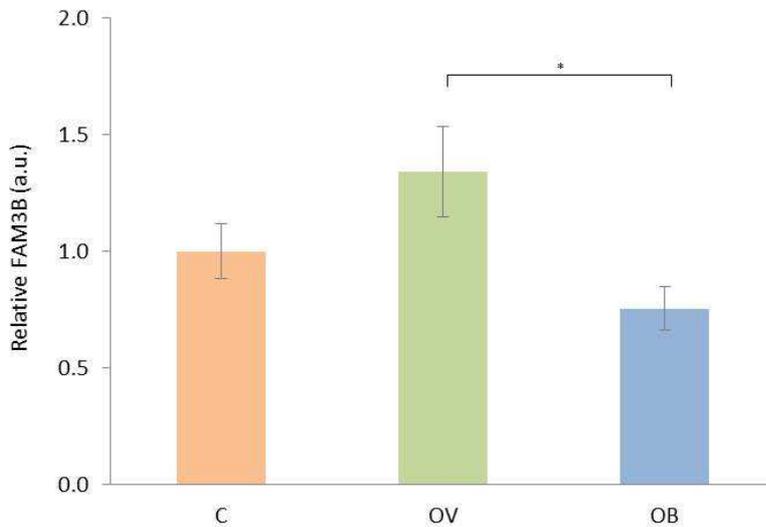


Figure 5.11: Placental FAM3B mRNA expression in lean control (C), overweight (OV) and obese (OB) pregnant women.

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give fold change. C: control (n=20); OV: overweight (n=20); OB: obese (n=20). Data are non parametric and represent mean \pm S.E.M; a.u.: arbitrary units. Statistical differences detected by * corresponds to $p < 0.05$ (Kruskal-Wallis; Mann Whitney post-hoc test).

Similarly, mRNA abundance of placental FAM3B was reduced with the combined effect of GDM and obesity (Figure 5.12), although the promoter region of this gene had not been previously found to have greater methylation.

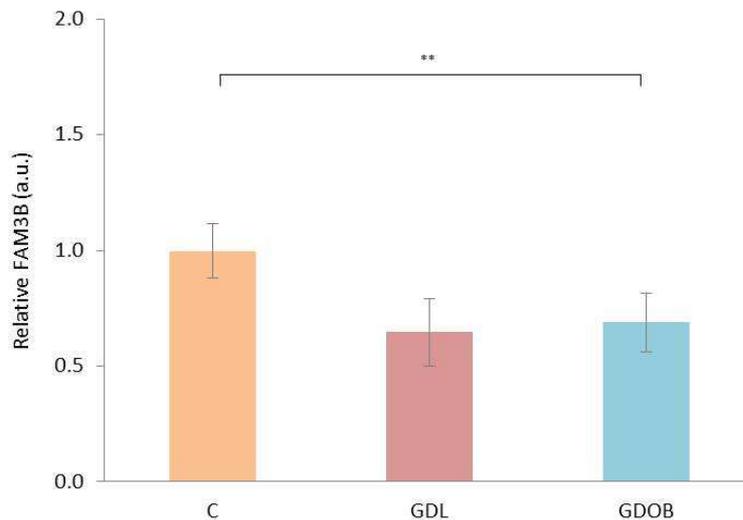


Figure 5.12: Placental FAM3B mRNA expression in control (C), gestational diabetic lean (GDL) and gestational diabetic obese (GDOB) pregnant women.

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give fold change. C: control (n=20); GDL: gestational diabetic lean (n=10); GDOB: gestational diabetic obese (n=10). Data are non parametric and represent mean \pm S.E.M; a.u.: arbitrary units. Statistical differences detected by ** corresponds to $p < 0.01$ (Kruskal-Wallis; Mann Whitney post-hoc test).

As predicted by methylation analysis, gene expression of placental Wnt2 was downregulated with overweight (Figure 5.13). However, hypomethylation of the Wnt2 promoter region detected on Illumina analysis, as a consequence of obesity and gestational diabetes, was not accompanied by higher or similar expression of this gene. On the contrary, Wnt2 expression was reduced with obesity (Figure 5.13) and gestational diabetes alone and when the two were both present (Figure 5.14).

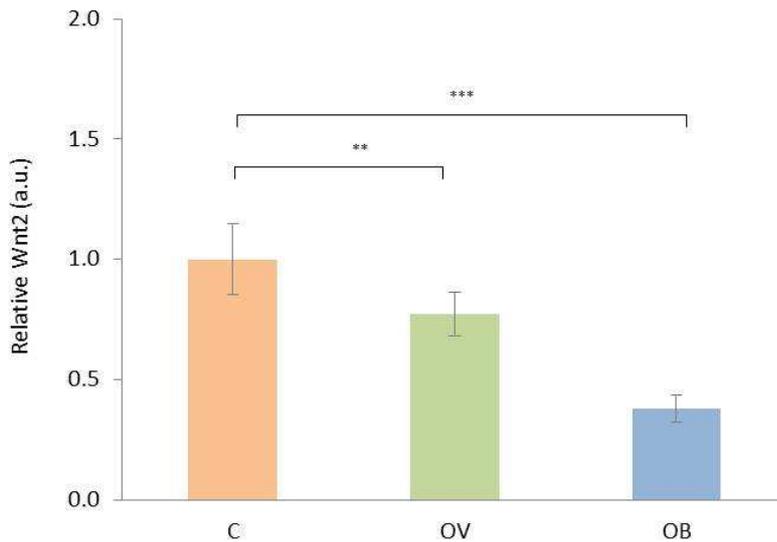


Figure 5.13: Placental Wnt2 mRNA expression in control (C), overweight (OV) and obese (OB) pregnant women.

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give fold change. C: control (n=20); OV: overweight (n=20); OB: obese (n=20). Data are non parametric and represent mean \pm S.E.M; a.u.: arbitrary units. Statistical differences annotated by **, *** correspond to $p < 0.01$ and $p < 0.001$ respectively (Kruskal-Wallis; Mann Whitney post-hoc test).

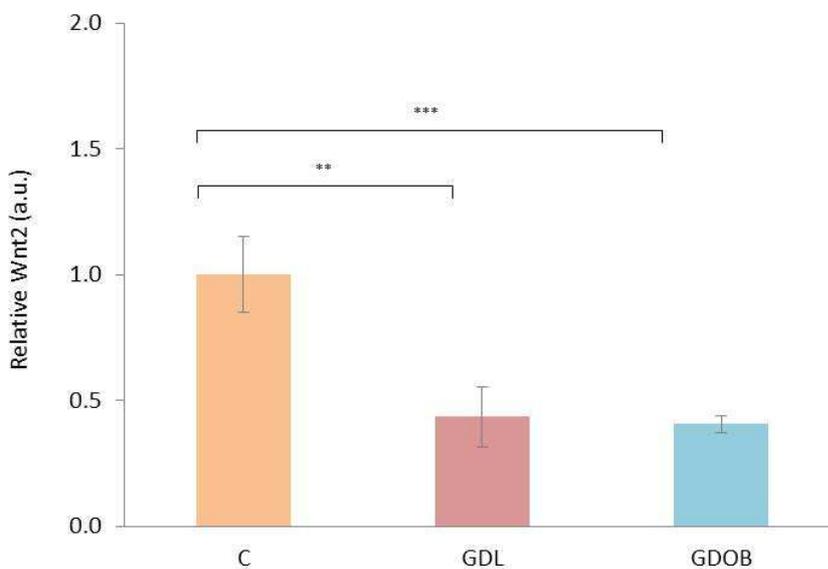


Figure 5.14: Placental Wnt2 mRNA expression in control (C), gestational diabetic lean (GDL) and gestational diabetic obese (GDOB) pregnant women.

Data were expressed relative to housekeeping gene (ribosomal 18S RNA) and normalised to the control group to give fold change. C: control (n=20); GDL: gestational diabetic lean (n=10); GDOB: gestational diabetic obese (n=10). Data are non parametric and represent mean \pm S.E.M; a.u.: arbitrary units. Statistical differences detected by **, *** correspond to $p < 0.01$ and $p < 0.001$ respectively (Kruskal-Wallis; Mann Whitney post-hoc test).

5.4. Discussion

Obesity and diabetes in pregnancy are associated with a variety of complications including increased risk of premature delivery and NTD, which have been related to maternal folate deficiency [44, 353, 585]. In this study, the effects of both high maternal BMI and gestational diabetes on folate concentrations and folate metabolism from mother to fetus have been investigated.

5.4.1. Effect of high BMI

Epidemiological studies have demonstrated that clinical complications associated with folate deficiency are reduced by adequate dietary intake [350, 518]. As folate supplements in obese women do not improve, or reduce, the risk of fetal malformations [352], I hypothesised that placental transport is impaired with raised BMI. As such, I investigated whether low maternal folate intake and reduced folate concentrations in maternal serum resulted in decreased levels of folate in the fetus.

My results suggest that folate metabolism differs according to the body weight of pregnant women as previously indicated by others [349, 572]. It is likely that women entering pregnancy with a high BMI consumed a poor diet lacking in essential micronutrients, including folate [38, 41, 45]. In this regard, overweight and obese mothers in this study reported reduced dietary folate intake, and a lower percentage (OV: 44.1%; OB: 33.3%) reported taking folate and iodine supplements at 24 weeks of pregnancy. Although folate deficiency (<5ng/ml) was not observed amongst these women, serum folate concentrations at both 34 and 40 gestational weeks were reduced in overweight and obese participants, reflecting their lower folate intake. Maternal BMI was inversely related to serum folate and this can be explained by changes in plasma volume and folate distribution amongst maternal tissues [586].

In addition, hormonal changes in pregnancy and endocrine modifications associated with high BMI might be responsible for reduced serum folate concentrations [349]. Interestingly, cord blood folate concentrations in overweight and obese women were within the normal range but lower than in lean controls. The fact that those offspring showed appropriate cord blood folate concentrations despite reduced maternal folate reflects the dependence of the fetus on maternal stores even in conditions of lower folate concentrations. This illustrates the crucial function of the placenta in buffering the low maternal folate, therefore, ensuring adequate fetal supply. One previous study on the effect of high BMI on folate transport in the placenta was conducted by Carter et al. They hypothesised that obesity in pregnancy was associated with lower fetal folate concentrations and impaired placental folate transport [354]. However, their findings indicated that maternal obesity did not alter maternal or fetal serum folate concentrations. In addition, the effect of obesity on protein expression and activity of folate transporters in placental homogenates and placental microvillus membrane (MVM) was markedly different [354]. The authors suggested that post-translational modifications are responsible of this discrepancy. Gene expression data in my study showed that mRNA abundance of FR α , which is the main folate transporter on the maternal side [351], was significantly reduced with obesity. Conversely, no differences in the expression of the other two transporters, PCFT and RFC were observed. The smaller number of samples used for analysing the expression of these two transporters might have resulted in a lack of statistical power to show any difference. A retrospective sample size calculation to estimate the number of samples needed to show a statistical difference at a power of 80%, using the mean and SD obtained in my study, revealed that the necessary sample size would be very large (n=1127). The lack of difference PCFT and RFC mRNA expression between body weight groups was unexpected as the ratio between maternal folate at 40 gestational weeks and umbilical cord folate was higher in obese mothers (Table 5.2) suggesting increased folate transfer in these pregnancies.

A multilinear regression model confirmed an independent effect of pre-gestational BMI and maternal folate at term on placental FR α . A possible explanation for the increased transfer of folate to the fetus to compensate for maternal deficiency is that the mRNA transcripts of those folate transporters undergo post-transcriptional or post-translational modifications. According to this, upregulation at a protein expression level or higher activity of placental folate transporters might have occurred resulting in normal fetal folate status. In my study, protein expression of FR α and RFC has been analysed using different antibodies, two of which were also used by Carter et al. [354]. However, due to the non-specific nature of available antibodies, it was not possible to quantify those proteins, leaving the question of how the placenta can compensate low maternal supply unanswered [577]. In agreement with Carter et al., my data does not indicate increased fetal malformations in obese pregnancies as a consequence of fetal folate deficiency and placental impairment [365]. As obese women participating in this study were not folate deficient, it is unsurprising that they gave birth to infants who did not have NTDs. However, the lack of an observed increase in the incidence of NTD may result from the sample size reported here. Furthermore, as folate concentrations at term were analysed, normal values of folate in the fetus at term do not exclude that folate availability was limited during early pregnancy. Folic acid, vitamin B12 and MTHFR are involved in the homocysteine-methionine cycle. Normal MTHFR activity maintains the pool of circulating folate thus preventing an increase in intracellular homocysteine levels [587]. The latter, together with low folate concentrations, is associated with an increased risk of adverse pregnancy outcomes [365, 369]. In the present study, the lower folate intake in women with high BMI was not accompanied by changes in dietary vitamin B12 intake. Wilson et al. studied the methionine cycle in the pregnant rat and advocated that the placenta is not contributing to homocysteine in the maternal circulation. [367]. These authors found that, even in conditions of severe methyl-deficient diets, little or no methionine cycle activity was present in the placenta.

Conversely, methionine synthesis from homocysteine in fetal tissues was maintained or increased, indicating protection to some extent of these rats within fetal tissues. In my study, neither homocysteine nor choline were measured in either maternal or cord blood, therefore it is difficult to draw conclusions regarding these metabolites and their contribution to folate metabolism. Folate deficiency has been associated with reduced hepatic SAM and increased placental SAM/SAH ratios, which are commonly used as indicators of cellular methylation potential [361, 500]. It is, therefore, possible that the lower levels of folate observed in obese mothers contribute to reduce the availability of placental SAM, which is used by DNMTs to methylate DNA, as a key epigenetic contribution associated with gene silencing [371, 379].

The hypothesis currently tested was that limited maternal folate supply would influence methyl group transfer to the fetus via alteration in DNMT expression [43]. DNMT1, an enzyme required for both the maintenance of the DNA methylation patterns and essential for cellular development, was upregulated in both high BMI groups compared to controls, reaching the highest values in the placenta of overweight women. DNMT1 exists in a complex with a sequence specific DNA-binding factor and has implications for targeting methylation, transcriptional control, DNA replication and tumourgenesis [588]. The increased mRNA abundance of DNMT1 might be a placental compensatory response to reduced maternal folate concentration in order to maintain adequate methylation pattern in the fetus [589]. This upregulation of DNMT1 with increased BMI was not accompanied by changes in DNMT3 and this may reflect the fact that the latter enzyme is only highly expressed and active during early gestation when the embryo is developing and not at term [574].

A summary of the above findings is illustrated in Figure 5.15.

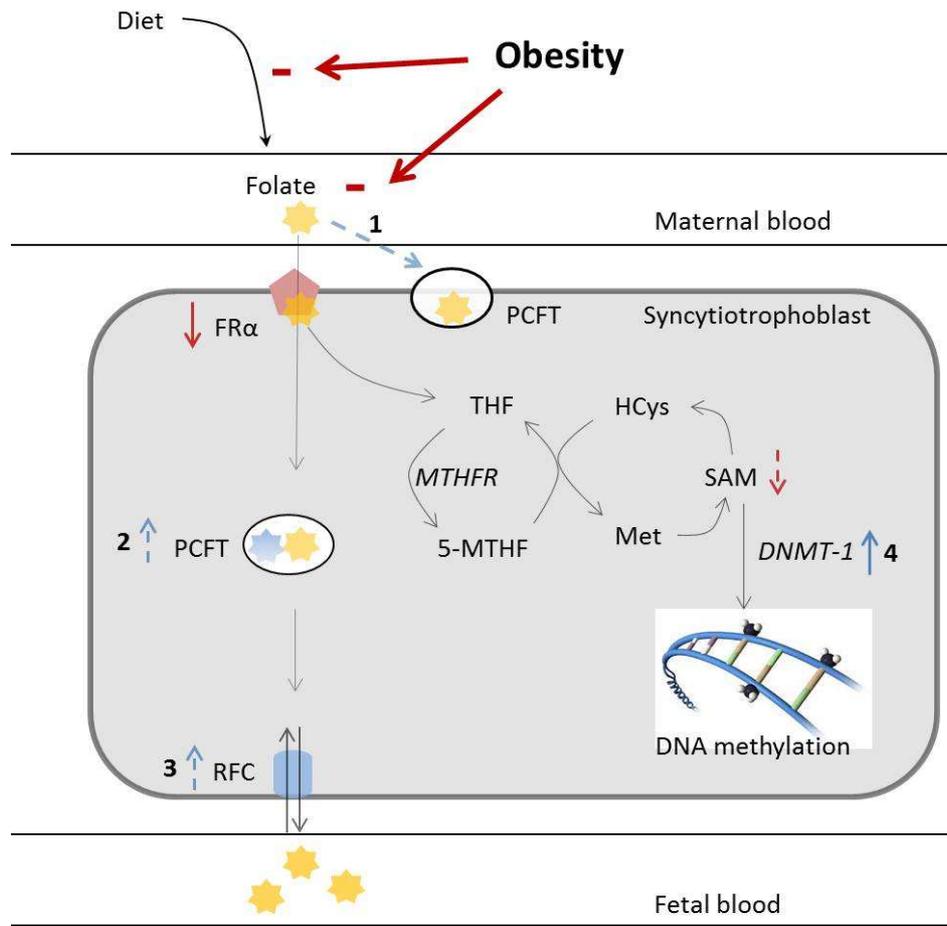


Figure 5.15: Effects of maternal obesity on folate intake and placental transport and metabolism.

A lower dietary intake of folate in obese women leads to decreased folate concentrations in maternal serum. As a consequence, gene expression of placental folate receptor alpha (FR α) decreases leading to reduced tetrahydrofolate (THF) and S-adenosylmethionine (SAM). To buffer low maternal folate concentrations and provide the fetus with adequate folate, the placenta can increase the expression of other folate transporters on the microvillous plasma membrane (1), within the syncytiotrophoblast (2) or on the basal plasma membrane (3). By increasing DNA methyltransferase-1 (DNMT1) gene expression (4) to compensate a decrease in the methyl donor (SAM), the placenta ensures the maintenance of the methylation pattern in the fetus.

PCFT: proton coupled folate transporter; RFC: reduced folate carrier; 5-MTHF: 5-methylenetetrahydrofolate; MTHFR: methylenetetrahydrofolate reductase; HCys: homocysteine; Met: methionine.

Red arrows show the effects of obesity as found in the study (solid line) and its hypothesised effects (dotted line) in folate metabolism within the syncytiotrophoblast; blue arrows represent the resultant placental response (solid line) and the hypothesised compensatory mechanisms (dotted line); yellow stars show folate concentrations as found in the mother, placenta and cord blood, whereas blue stars indicate the hypothesised placental response at a transporter level. \uparrow represents upregulation and \downarrow downregulation.

5.4.2. Effect of gestational diabetes

Diabetic pregnancies have also been associated with congenital malformations and NTD even though the incidence of these can be reduced by good glycaemic control and folate supplementation [508, 518]. Upon diagnosis of gestational diabetes mellitus (GDM), diabetic participants in my study were referred to a dietician for nutritional counselling in order to develop appropriate meal plans. Their higher folate intake compared to lean controls could reflect that they were more conscious of a healthier diet [508, 509]. Diabetic women with lean BMI showed significantly higher serum folate at 34 gestational weeks and 6 months post-delivery compared to lean controls. Interestingly, gestational diabetes and higher BMI combined did not change serum folate concentrations compared to lean women without diabetes. Nonetheless, cord blood folate in all four groups was similar, and within the normal range, highlighting a regulatory role of the placenta in controlling the passage of folate to the fetus. The effect of diabetes on placental transport of macronutrients including glucose and amino acids is being currently investigated by other authors [145, 179, 183] and has been discussed in Chapter 4.

However, the passage of micronutrients during pregnancies complicated by GDM is less well established. Folic acid deficiency is associated with compromised fetal growth resulting in reduced birth weight with high risk of developing metabolic syndrome and CVD later in life [500]. The risk of diabetes-induced congenital defects in hyperglycaemic mice has been shown to be reduced by folic acid treatment [518, 576]. As already discussed in Chapter 3, weight at birth and weight gain in infants of gestational diabetic mothers with lean as well as high BMI, did not differ from those born from healthy pregnancies. Thus, good glycaemic control with a healthy diet and/or folic acid supplements during gestation, was sufficient to ensure this positive outcome. Nonetheless, the small number of participants in the diabetic groups might have reduced the power of the study, thus skewing the results.

To my knowledge, there are currently no published studies in vivo investigating the effect of diabetes on folate transport within the placenta. Keating et al. examined, in vitro, the modulation of folic acid uptake by human syncytiotrophoblast under detrimental conditions, including hyperglycaemia and hyperinsulinaemia [590]. Despite insulin not affecting placental folate uptake in vitro, hyperglycaemia chronically inhibited placental uptake of this micronutrient [590], suggesting downregulation of FR α as principal cause. An association between diabetes and reduced mRNA expression of folic acid binding protein in the embryo of hyperglycaemic mice was also found, resulting in decreased embryonic concentrations of folate [518, 576]. In contrast, my findings did not suggest placental modifications in folate transport, since no difference was found in gene expression of any folate transporters as a consequence of gestational diabetes. The unchanged placental transport of folate might reflect the lack of difference in cord blood folate found in these pregnancies, despite enhanced maternal serum folate at late gestation and at 6 months post-delivery. This may represent a placental way of compensating any excess maternal folate to avoid a surplus in the fetus, which can also be detrimental [591]. Interestingly, upregulation of the placental MTHFR gene was observed when gestational diabetes was associated with high BMI reflecting higher folate intake. The lack of difference in folate intake between gestational diabetic obese and lean controls may also be due to the small number of women (43 out of 70) who completed the questionnaire. By enhancing folate metabolism and, therefore, the concentrations of its metabolites, the placenta avoids accumulation of homocysteine within the trophoblast, which could result in fetal complications [364, 365]. Therefore, the increased MTHFR expression may be due to higher folate within the placenta although no differences in FR α were observed at a gene expression level. Other post-translational mechanisms might be responsible to increase placental folate metabolism, which results in the observed MTHFR upregulation.

Methylenetetrahydrofolate (MTHF) uptake in the MVM can be measured on published techniques for amino acid uptake in syncytiotrophoblast plasma membrane vesicles as reported by other authors [351, 354, 358].

The enzymes DNMT1 and DNMT3, involved in DNA methylation, did not significantly change because of diabetes probably reflecting good maternal folate concentrations. The overall effect of gestational diabetes on folate metabolism and transport is summarised in Figure 5.16.

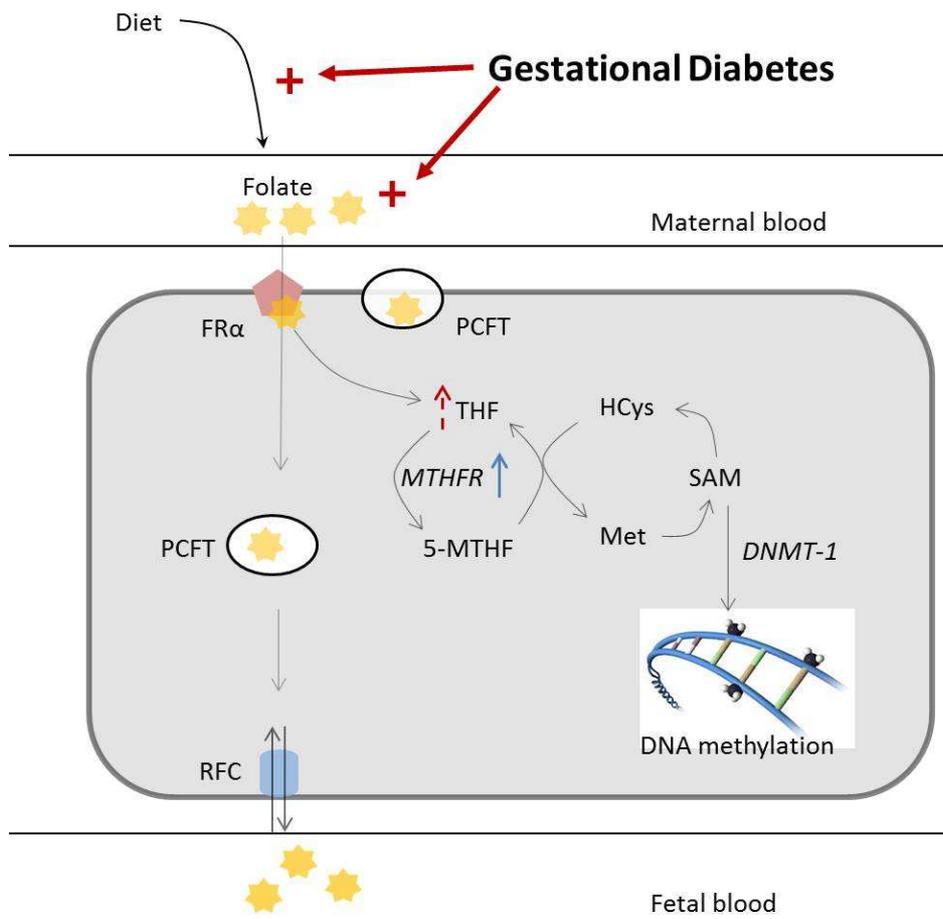


Figure 5.16: Effects of gestational diabetes on folate intake and placental transport and metabolism.

A higher dietary intake of folate in diabetic women leads to an increase in tetrahydrofolate (THF) within the placenta. As a consequence, placental methyltetrahydrofolate reductase (MTHFR) gene expression increases thus ensuring transformation of homocysteine (HCys) into methionine (Met).

FR α : folate receptor alpha; PCFT: proton coupled folate transporter; RFC: reduced folate carrier; 5-MTHF: 5-methylenetetrahydrofolate; S-adenosylmethionine (SAM); DNA methyltransferase-1 (DNMT1). Red arrows show the hypothesised effects (dotted line) of gestational diabetes in folate metabolism within the syncytiotrophoblast; blue arrows represent the resultant placental response (solid line) in folate metabolism; yellow stars show folate concentrations as found in the mother, placenta and cord blood; \uparrow represents upregulation.

Finally, a potential mechanism by which folate could influence body mass and obesity later in life is via epigenetic control of gene expression [349, 371, 592, 593]. Folate depletion in humans has been observed to reduce genomic DNA methylation [368, 594]. Additionally, previous studies have linked modifications in the methylation pattern of placental genes with changes in gene expression contributing to impaired placental function [575] and development of early life disease [370]. Variations in DNA methylation could be linked to adult obesity since, in humans, key genes, which are closely correlated with fetal growth [595] and body fat distribution [596] exhibited changes in expression. Therefore, it was reasonable to hypothesise that obesity, which was associated with lower folate concentrations, would result in the greatest modification of DNA methylation, and this would be reflected in gene expression, with potential long term implications. In this study, the placenta of overweight women showed a much greater adaptation in their global methylation profile, with about 200 genes differentially methylated, compared with obese women, for whom only 50 genes were differentially methylated. The "DAVID" database [597] was used to analyse the function of these genes and elucidate potential molecular pathways in which they were involved. The outcome of the analysis indicated that most of these markers were related to neurodevelopment and brain function and in the immune response signalling. This substantiates the hypothesis stated in Chapter 4, according to which obesity might contribute to the development of an impaired immune response in the fetus.

However, the apparently reduced methylation response with the highest maternal BMI was unexpected as the majority of changes in placental gene expression observed here were with obesity rather than overweight. In addition, a highly methylated promoter was not always directly related with changes in gene expression, which might reflect inaccuracy of array probes in capturing the levels of the target [403, 404].

The discrepancy between the degree of methylation of the promoter region and the resulting gene expression might be also explained by the distribution of large partially methylated domains (PMDs). In the human placenta, these domains are associated with distinct methylation compared with that of the global genome [598]. Genes located in PMDs are usually repressed and have tissue-specific functions unrelated to the tissue of origin. It could be hypothesised that the placenta of obese mothers, although less globally methylated than that of overweight women, is more rich in PMD loci on the promoter region. This would explain the higher variation in gene expression with obesity despite their lower global methylation. Nevertheless, the extent to which these PMDs may be related to regulation of gene expression is still under investigation.

Taken together, my results show that pregnancies complicated by high BMI and gestational diabetes are differentially associated with altered maternal serum folate at mid and late gestation. The placenta responds to both low and high maternal folate intake and serum folate by modulating its transport and metabolism, highlighting its protective role for the fetus as demonstrated by the delivery of healthy offspring. My outcomes, therefore, support the need to provide sufficient folate intake in pregnancy for adequate fetal nourishment in particular in obese pregnant women, who are encouraged to take a daily folate supplement [534]. Nonetheless, even in situations of relative folate deficiency, such as in pregnancies complicated by obesity, the placenta seems to be able to compensate and supply the newborn with appropriate amount of nutrients. Despite potential epigenetic biomarkers highlighted from analysis of methylation at specific genes, interpretation of methylation differences still require further investigation (for example, through consideration of both global and local methylation context).

Chapter 6 - Conclusions

6.1. General aims

The aims of this thesis were to investigate the independent and combined effects of a high maternal BMI and gestational diabetes on the placenta in a population of Spanish pregnant women and their potential contributions to neonatal growth and health.

These objectives were first achieved by assessing the metabolic status of the mother through analysis of her dietary intake, weight gain, and physiological characteristics during pregnancy.

Secondly, the contributions of these maternal conditions to placental size, fetal weight and postnatal growth were assessed by analysing anthropometric outcomes of the placenta, fetus and infant until 12 months of postnatal age, and the fetus' physiological characteristics at the time of delivery as reflected in cord blood.

Placental metabolism was assessed by analysis of gene expression within the placenta focusing on specific molecular pathways, including placental insulin signalling, oxidative stress and inflammation.

Finally, folate concentrations in the mother and cord blood were examined alongside placental folate metabolism and placental DNA methylation. This allowed elucidating the contribution of folate on epigenetic modifications within the placenta and highlighting potential critical pathways.

6.2. Summary of findings

6.2.1. Maternal diet and weight gain in women with high BMI and GDM

According to self-reported food records, the independent and the combined effects of obesity and diabetes in pregnancy were associated with reduced carbohydrate intake in late gestation. This was accompanied by a lower gestational weight gain (GWG) in women in all observational groups (high BMI, GDM, both high BMI and GDM) compared to lean controls mothers without GDM.

In addition, the majority of overweight and obese women with GDM gained less weight than recommended by IOM for their BMI categories [3]. Among lean women with GDM and among mothers with both high BMI and GDM, a significantly greater proportion gained less than adequate gestational weight compared to their lean healthy counterparts. Interestingly, overweight and obese mothers without GDM, although gaining less weight than their lean counterparts, did exceed the recommended weight gain for their BMI by IOM [3]. The latter finding suggests a lack of reliability in reporting food intake [406] and/or a lack of awareness or less adherence to the recommended weight gain [3, 66] by women with a higher BMI, especially overweight mothers without GDM.

Nevertheless, reduced food consumption, lower GWG and the significantly lower proportion of women with GDM exceeding the IOM weight gain limits may also reflect a better control and awareness of their condition. The first line of treatment of GDM in this study was through nutritional [508, 518] and lifestyle advice [483] delivered to pregnant women in maternity welfare clinics. Therefore, the dietary advice received by women with GDM may have been effective in eliciting these outcomes. As such, it might be possible to ameliorate the adverse outcomes of obesity in pregnancy through good regular clinical management.

According to this, patients would be educated towards healthy lifestyle choices with the introduction of physical activity programmes and nutritional interventions [486, 515]. The aim of the latter is to avoid excess weight gain throughout pregnancy, thus reducing the high risk of delivery of a LGA infant often observed in those women.

6.2.2. Influence of obesity and GDM on placental and newborn anthropometry and metabolism

Despite similar fetal weight, obese women without GDM had heavier placentas [14, 47, 93] but newborn of similar weights, the latter in contrast with the findings commonly reported in the literature [14, 24, 169, 520]. Similarly, GDM in the participants of the present study did not contribute to greater infant birth weight, even though maternal hyperglycaemia has been strongly associated with the development of macrosomia and heavier offspring [75, 478]. Enhanced maternal glucose concentrations and/or increased leptin plasma concentrations in obese mothers might be associated to their higher placental weight. On the other hand, good glycaemic control through the diet could have contributed to similar placental weight in women with GDM.

My findings support a higher prevalence of LGA neonates (>90th population percentile) with increasing pre-pregnancy BMI among women with or without GDM. Once more, the effect of GDM on increasing the risk of having a LGA infant was less significant than that of obesity independently. This indicates that, in the present study, high BMI is a stronger risk factor for the increased risk of having a LGA infant, rather than GDM [474, 485, 521, 522]. One reason contributing to the delivery of LGA infants despite lower GWG in obese women without GDM could be the metabolic adjustments [87] or placental hypertrophy [526] in these mothers.

The findings, in this study, that newborn weight and infant weight gain during the first year of age were not influenced by either maternal obesity, GDM or the combined effect of the two could reflect good metabolic control by the placenta.

In the present study, insulin and mTOR signalling, within the placenta of women with high BMI and GDM, did not significantly contribute to changes in placental or fetal growth. Glucose and insulin plasma concentrations increased in both obese and GDM mothers whilst similar glucose concentrations were observed in cord blood of their infants. As gene expression of components in the insulin and mTOR signalling pathways was unaltered, it is likely that other factors contribute to compensate for maternal hyperglycaemia. Obesity in pregnancy was associated with increased placental gene expression of antioxidant factors, including UCP2 and SIRT1, and decreased pro-inflammatory markers, such as mTOR. However, monocyte concentrations in cord blood were higher in infants of obese mothers. These findings suggest that maternal obesity might contribute to the development of an impaired immune response in the fetus despite a good antioxidant response within the placenta. Gestational diabetes was associated with different outcomes, probably reflecting improved glycaemic control through the diet. Upregulation of pleiotropic genes involved in key metabolic pathways within the placenta, including leptin and GR α , was observed in lean women with GDM. The direct transcriptional action of glucocorticoids on placental leptin expression [568] might indicate the generation of a localised pro-inflammatory state within the placenta of lean diabetic women [250, 261, 262, 293]. Nonetheless, cord blood inflammatory markers were similar in infants of these participants, indicating that placental inflammation, if this occurred, did not adversely affect the fetus. A summary of the effects of obesity and gestational diabetes on placental metabolism is illustrated in Figure 4.12 and 4.13 respectively.

6.2.3. Folate availability and DNA methylation

Pregnancies complicated by high BMI were associated with reduced plasma folate in the mother at both 34 and 40 gestational weeks compared to lean controls. This most likely reflects the lower dietary folate intake and folate supplements reported by these women during pregnancy. Infants of overweight and obese women had similar cord blood folate concentrations, although these were lower than those of lean controls. This resulted in a greater difference between maternal folate at 40 gestational weeks and umbilical cord folate in those pregnancies complicated by high BMI, suggesting a crucial function of the placenta in compensating the low maternal folate by ensuring adequate fetal supply.

Therefore, it is expected that folate transport across the placenta increased with overweight and obesity. However, placental gene expression of FRA [351] was significantly reduced with obesity, whilst no differences in the expression of the other two transporters, PCFT and RFC were observed. It is hence likely that other factors, such as modifications at a post-translational level or higher protein activity, might be responsible of enhanced folate transport, ensuring adequate folate concentrations in the fetus.

Furthermore, increased mRNA abundance of DNMT1 was observed with overweight and obesity. As folate supply can influence the availability of methyl donors to the fetus via alteration in DNMT expression [43], this finding suggests that the placenta can compensate for reduced maternal folate concentrations by maintaining adequate methylation pattern in the fetus [589]. On the other hand, gestational diabetes was associated with increased maternal folate intake compared to lean controls, probably reflecting a healthier diet [508, 509]. In lean women with GDM, serum folate at mid and late gestation was enhanced and might be a result of their higher folate intake. The effect of overweight and obesity in mothers with GDM was, once more, associated with lower serum folate concentrations compared to their lean counterparts.

This indicates that metabolic differences associated with high BMI, such as those related to adipose tissue function [263, 314, 546], could influence the availability of this micronutrient. In addition, infants of lean GDM women showed similar cord blood folate concentrations alongside unchanged placental expression of folate transporters. This finding suggests that, despite enhanced maternal serum folate in late gestation and at 6 months post-delivery, the placenta is able to buffer any excess maternal folate to avoid a surplus in the fetus, which can also be detrimental [591]. Gene expression for the enzymes DNMT1 and DNMT3, involved in DNA methylation, did not significantly change as a result of diabetes, probably reflecting good maternal folate concentrations. An overview of the effects of obesity and gestational diabetes on folate metabolism and transport are summarised in Figure 5.15 and 5.16 respectively.

These outcomes, therefore, support the need to provide sufficient folate intake in pregnancy for adequate fetal nourishment in particular in obese pregnant women, who are encouraged to take a daily folate supplement [534]. Nonetheless, it seems that the placenta is able to compensate and supply the newborn with appropriate amounts of folate, even when this is provided by the mother in lower quantities, such as was observed with obesity.

Methylation analysis performed in this study indicated a greater influence of being overweight on DNA methylation at specific promoter regions compared to obesity or gestational diabetes. This was unexpected as the major gene expression differences were detected as a consequence of obesity rather than overweight or GDM. In addition, analysis of the expression of some of the genes, which had been found to be differentially methylated, did not always confirm the methylation analysis. Several reasons, which could explain this discrepancy, include, for example, consideration of both global and local methylation. However, to achieve comprehensive interpretation of methylation differences further investigation is required.

6.3. Limitations of the study

6.3.1. Sampling and data collection

As in many investigations involving human populations, the main limitation of this study lay in the collection of an adequate number of samples to reach substantial clinical evidence. Recruitment of volunteers is not usually straight forward, and the principles of informed participant consent mean that subjects need time and think about what has been proposed to them. This is even more difficult with pregnant women, especially those who already have to routinely undertake visits for other conditions, such as diabetes. For instance, most of the women with gestational diabetes initially recruited, preferred not to take part or continue to participate to the study because of additional visits and difficulty of organising their time. Owing to the incidence of GDM in this population, a limited number of subjects from this group were available for recruitment and contributed to reduce the power of the analysis. As such, increasing the sample population in the future would be beneficial.

In addition to the above difficulties in recruitment, unexpected circumstances have sometimes occurred during sample collection. For instance, when delivery took place overnight, out of usual working hours, collection of placenta and cord blood could have been subject to delay out of our control. As described in the Materials and Methods (Chapter 2), degradation of placental samples, due to delay in collection, was responsible for the unsuccessful histological analysis, performed at a first attempt in frozen placentas. Although placental tissues stored in RNA later did not suffer such degradation, a limited number of samples stored in this way was available at the time of analysis and, therefore, this did not allow meaningful further examination. Attempts to examine protein expression of FR α and RFC were undertaken, as reported in Chapter 5. However, due to the non-specific nature of available antibodies, these proteins could not be quantified.

6.3.2. Measurements and self-reported data

There is general concern about self-reported data, especially related to BMI estimations in obese women [406-408]. Unfortunately, at the time of recruitment, it was not possible to measure maternal anthropometry. Therefore, pregestational maternal weight was self-reported. This could have contributed to erroneously classifying some subjects as lean or overweight before pregnancy. In fact, those women might have moved into the obese group by gaining excess gestational weight at the beginning of pregnancy, before the first study measurements of height and weight could be made at 24 weeks of gestation.

Furthermore, the nature of the study in a multidisciplinary clinic setting did not enable visits of the participants to be undertaken in the morning, where subjects were in a fasting state. Indeed, all the visits in the hospital took place in the afternoon, between 3-7 pm. This resulted in postprandial maternal blood collection, the analysis of which could have contributed to skew some results (i.e. maternal glucose, TG and cholesterol), which are classically performed after an overnight fast.

Finally, although detailed information regarding the completion of food records was given to all participants, it is likely that some of them did not complete them correctly, underestimating or overestimating their portions. As discussed in Chapter 2 (Section 2.3), some nutritional data was missing due to the mother not returning or completing the questionnaires.

6.4. Future work

In order to further explore the above findings, further investigations would be required as suggested in the following sub-sections.

6.4.1. Future directions for the study of offspring obesity

As previously discussed, a limitation of this study was the small number of samples, especially from mothers with gestational diabetes. During the Preobe Follow-on study, more participants have been recruited and more samples are now available. Analysis of samples from the follow-on studies, which have a similar design, would allow inclusion of a larger and, thus, more powerful sample size to more specifically investigate the effects of gestational diabetes and high BMI on the placenta and on the offspring metabolic health. A larger number of samples would also allow further investigation of the effects of overweight and obesity in women with gestational diabetes, thus elucidating the separate effects of the two conditions on infant weight gain.

Since one of the most alarming issues of today's western societies is obesity and the consequent development of offspring diabetes, these further studies should examine, in addition to weight and ponderal index, fat and lean mass in the infants at different stages of the first year of life. This would allow to further determine whether fetal hyperleptinaemia observed in the umbilical cord blood of infants of obese mothers is associated with increased adiposity in those infants. It would also contribute to investigate whether these infants have a higher incidence of obesity and metabolic diseases in adulthood. Analysis of neonatal and infant nutrition, through maternal recording at the first 1, 3, 6, 12 months of age under paediatric supervision, would be useful to corroborate infant anthropometric data.

6.4.2. Future directions for the study of offspring neurodevelopment

The role of epigenetic modifications in the placenta was assessed through analysis of DNA methylation, providing a list of several genes differentially methylated, some of which were common to overweight, obesity and/or diabetes. A first analytical procedure to analyse the function of these genes was performed using the "DAVID" database [597], which indicated that most of them were related to neurodevelopment and brain function as well as to immune response signalling. It would be of interest, therefore, to further investigate novel pathways in which these genes are implicated in order to focus clinical fields of potential future research. As discussed in Chapter 5, gene expression of these potential novel markers can be a useful tool in hypothesis-setting research, although interpretation of these results requires caution. With regards to the potential long term implications in the neurodevelopment of these infants, further observational studies could use functional measures of infant neurodevelopment, such as Bayley III neurodevelopmental scores at three years of age, and developmental assessments at school entry by formal validated testing.

6.5. Final remarks

This PhD study has illustrated relationships between key factors in pregnancy, the placenta and fetal development to term, and later neonatal growth, and how these are affected by maternal overweight, obesity and diabetes in pregnancy. Importantly, the placenta could compensate for maternal hyperglycaemia and folate deficiency of obese mothers by increasing its own use of glucose and/or placental folate transport respectively, resulting in normal glucose and normal folate concentrations in the newborns. However, increased concentrations in cord blood monocyte of infants of obese mothers and hypermethylation of genes involved in placental immune response pathways requires further investigation.

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Appendix A: Conference abstracts

European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN), London, UK, May 2013 (poster presentation by Cristina Campoy)

DNA methylation in the human placenta and its association with high maternal body mass index.

J. Martino, R. D. Emes, L. García-Valdés, M. T. Segura, I. Rusanova, M. D. C. Padilla, H. McArdle, H. Budge, M. Symonds, C. Campoy.

Objectives and Study: Changes in epigenetic profile in response to perturbations within the in utero environment could contribute to modifications in feto-placental function that ultimately place the resulting individual at increased risk of metabolic disease in later life. This is because differences in methylation pattern of genes expressed in the placenta are associated with changes in gene expression that could impair placental function. Furthermore, the use of genome-wide methylation arrays enables a more detailed examination of human epigenomics although this can lead to controversial interpretations. The present study, therefore, aimed to examine the influence of maternal body weight on the epigenomic profile in the human placenta followed by the determination of gene expression of genes identified to be differentially methylated.

Methods: Term placental samples as part of the PREOBE study* were collected from 3 groups of pregnant women with normal glucose tolerance, according to pre-pregnancy BMI classified as normal weight ($BMI < 25$ kg/m²; n=5), overweight ($25 \leq BMI < 30$ kg/m²; n=4), and obese ($BMI \geq 30$ kg/m²; n=5). Illumina Infinium HumanMethylation450 BeadChip array was used to examine genome-wide DNA methylation patterns and annotation data were analysed using NIMBL software. Gene centric plots were generated for novel genes which were most differential methylated and their expression was determined by QPCR.

Results: The greatest difference in methylation was found in placenta sampled from overweight mothers in which there were more than 200 differentially methylated CpG sites, an adaptation that was largely absent in placenta sampled from obese women. Methylation of the promoter regions for both FAM3B and Wnt2 was raised in placenta of overweight women, but this adaptation was only accompanied with comparable changes in FAM3B gene expression.

Conclusion: Placenta of overweight women show greater adaptation in their global methylation profile than those of women who are obese although this is

not consistently directly related to changes in gene expression. The global methylation response, therefore, with increasing maternal BMI suggests that enhanced maternal overweight may result in adverse fetal outcomes.

World Congress on Developmental Origins of Health and Disease (DOHaD), Portland, OR, USA, September 2011 (poster presentation by Jole Martino)

Effect of high maternal BMI on folate concentrations and placental DNA methyltransferase 1 (DNMT-1) gene expression in humans: the PREOBE Follow up study.

Martino J, Sebert S, Segura MT, Rusanova I, Martínez-Zaldívar MC, García-Valdés L, Padilla MC, McArdle HJ, Budge H, Symonds ME and Campoy C.

Background: The importance of maternal methyl donors, including folate, in DNA methylation and embryogenesis has been demonstrated in animal studies. Maternal obesity has been associated with adverse neonatal outcomes which could be linked to folate deficiency. Therefore, we investigated whether maternal BMI can alter folate status and whether that, in turn, modifies the activity of placental DNMT-1 and offspring folate concentrations.

Methods: Women were recommended to take folate supplements (0.4mg) during the first 3 months of gestation. Pregnant women were recruited at 20 weeks of gestation and classified according to their pre-pregnancy BMI into control (BMI < 25 kg/m²; n=56), overweight (25 kg/m² < BMI < 30 kg/m²; n=26) and obese (BMI > 30 kg/m²; n=21) groups. Plasma folate concentrations were analysed in maternal samples taken at 34 weeks of pregnancy and at delivery (38.7 ± 1 weeks of gestation), and in cord blood. Placental DNMT-1 gene expression was determined using real-time PCR. Data were analysed according to their parametric distribution with Kruskal-Wallis and 1-way ANOVA.

Results: Although there was no difference in maternal folate intake in early pregnancy as determined by lifestyle questionnaire, overweight mothers had lower folate concentrations at both 34 weeks of pregnancy (C=12.9±0.6; OV=10.3±0.9, (p=0.02)) and at delivery (C=12.8±0.7; OV=9.5±0.9, (p=0.005)) whilst in obese women, plasma folate was only reduced at delivery (OB=8.9±1.2ng/mL (p=0.007)). There were no differences in cord blood folate. Placental mRNA abundance of DNMT-1 was upregulated 2 fold with overweight (C=0.0115±0.0019; OV=0.021±0.002; (p=0.0004)), and 1.5 fold with obesity (OB=0.0168±0.0029; (p=0.04)) but was not directly associated with folate concentrations.

Conclusions: Although plasma folate concentrations were lower in women of increased BMI, there was no reduction in their newborns, suggesting enhanced placental folate transport. The extent to which upregulation of placental DNMT-1 may act with altered DNA methylation in the placenta to increase folate supply to the fetus is currently being investigated.

The physiological Society main summer meeting, Oxford, UK, July 2011 (Oral presentation by Jole Martino)

Placental sirtuin 1 (SIRT-1) and mammalian target of rapamycin (mTOR) in the Preobe Study*: a protective response for the fetus against the adverse outcomes of maternal obesity?

Martino J, Sebert S, Segura MT, Rusanova I, Martínez-Zaldívar MC, García-Valdés L, Padilla MC, McArdle HJ, Budge H, Symonds ME and Campoy C.

Background: The maternal metabolic environment influences both fetal development and long-term health in the offspring [24]. These responses can be modulated by the placenta due, in part, to its capacity to protect the fetus from adverse conditions.

Aim: We, therefore, investigated whether maternal body mass index (BMI) influences placental oxidative status as a result of changes in gene expression for SIRT-1 and mTOR.

Methods: Pregnant women were recruited at 20 weeks of gestation and classified according to their pre-pregnancy BMI as control (BMI<25kg/m²; n=56), overweight (BMI=25-30kg/m²; n=26) or obese (BMI>30kg/m²; n=21). At delivery, placentae were sampled and SIRT-1 and mTOR gene expression determined using real-time PCR. OxiSelect™ TBARS assay was used as a measure of lipid peroxidation. Data were analysed according to their parametric distribution by Kruskal-Wallis or 1-way ANOVA.

Results: Obesity had no effect on gestational length, birth weight, or postnatal morbidity but obese mothers did produce the largest placentae (C=468.4±15.8g; OB=520±29.1g; (p=0.022)). Placental SIRT-1 gene expression showed a 1.5 fold upregulation with obesity (C=88±10; OB=134±19 x10⁻⁵ a.u. (p=0.024)) whereas mTOR expression was halved (C=177±18; OB=87±11 x10⁻⁴ a.u. (p=0.022)). TBARS concentrations were similar between groups.

Discussion and conclusion: Overexpression of the anti-oxidant gene SIRT-1, combined with reduced mTOR in obese mothers, may indicate an enhanced placental anti-oxidative capacity. Surprisingly, higher placental weight was not associated with increased oxidative stress as measured by TBARS concentrations suggesting a placental compensatory response to raised maternal BMI. A follow up study of the offspring is currently being undertaken up to 18 months of age to establish whether obesity in pregnancy is accompanied with longer term responses.

European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN), Istanbul, Turkey, June 2010 (oral presentation by Cristina Campoy)

Influence of maternal BMI and gestational diabetes on mTOR and PPARG gene expression in the term placenta

Martino J, Sebert S, Budge H, García-Valdés L, Segura MT, Anjos T, López-Tarragona R, Marti-Romero MA, Florido J, Symonds M.E. and Campoy C.

Background: It is now well established that changes in the maternal diet at defined stages of pregnancy can affect offspring's risk of later metabolic disease [473]. Such alterations of fetal growth can be due to substantial modifications of materno-fetal energy partitioning regulated in part by the mammalian target of rapamycin (mTOR) (2) and the peroxisome-proliferator activated receptor (PPAR-gamma) (3,4). However, the influence of maternal body mass index (BMI) and/or insulin sensitivity on the regulation of placental energy metabolism has yet to be established. This is clearly important as it will improve our understanding of the potential adverse effect of maternal obesity and gestational diabetes on pregnancy outcomes.

Methods: Placental samples were collected from healthy pregnant women participating in the PREOBE Project*(P06-CTS-02341) directed by the University of Granada (Spain). The subjects were recruited at 20 weeks of gestation and divided into four groups according to their BMI and glucose tolerance. Mothers were either classified as lean (pre-pregnancy BMI < 25 kg/m²; normal glucose tolerance; n = 56), overweight (pre-pregnancy 25 kg/m² < BMI < 30 kg/m²; normal glucose tolerance; n = 23), obese (pre-pregnancy BMI > 30 kg/m²; normal glucose tolerance; n = 12) or gestational diabetic (pre-pregnancy BMI < 25 kg/m²; glucose intolerant; n = 17). At delivery, placenta from each individual was sampled in order to analyse mTOR and PPAR-gamma gene expression by real-time RT-PCR.

Results: The mRNA abundance for mTOR in placenta of lean mothers was respectively two and three times lower than in the placenta of overweight (but not obese) (p < 0.05) and gestational diabetic mothers (p < 0.001). Interestingly, diabetic mothers overexpressed mTOR when compared to overweight (p < 0.05) and obese (p < 0.005). In addition, the placenta of overweight, obese and gestational diabetic mother overexpressed the gene encoding for PPAR-gamma compared to lean women (P < 0.05).

Conclusions: We have shown that both being overweight and glucose intolerance during pregnancy alters placental energy metabolism pathways. Overexpression of mTOR and PPAR-gamma within the placenta could promote nutrient uptake thereby altering fetal growth and body composition. This may also contribute to local chronic pro-inflammatory states within the placenta or fetus and further suggests that mTOR and PPARG are key gene targets for fetal programming.

International Conference on The Early Nutrition Programming Project (EARNEST), Munich, Germany May 2010 (Oral presentation by Jole Martino)

Gene expression in the term placenta: effect of maternal BMI and glucose tolerance on uncoupling protein 2 (UCP-2), toll-like receptor 4 (TLR-4) and DNA methyltransferase-1 (DNMT-1)

Martino J., Sebert S., García-Valdés L., Segura MT., Anjos T., Marti-Romero MA., Florido J., Budge H., Symonds M.E. and Campoy C.

Background: Maternal obesity and diabetes are critical public health issues that can compromise maternal and offspring health. The regulation of genes involved in placental energy metabolism, pro-inflammation and DNA remodelling (UCP-2, TLR-4, DNMT-1) can determine the partitioning of energy between the mother and fetus¹. In addition, they may be altered by weight gain and diabetes during pregnancy and subsequently result in long-term adverse outcomes.

Methods: As part of The PREOBE project* pregnant women were recruited at 20 weeks of gestation and classified as lean (BMI<25kg/m²; n = 58), overweight (BMI = 25-30kg/m²; n = 25), obese (BMI>30kg/m²; n = 12) or gestational diabetic (BMI<25 kg/m²; glucose intolerant; n = 13). At delivery, placentas were sampled and UCP-2, TLR-4 and DNMT-1 gene expression determined using real-time PCR.

Results: In overweight and obese mothers the genes encoding for UCP-2 and TLR-4 were increased two-fold compared to lean mothers (P<0.05). Diabetes resulted in further overexpression (p<0.001) of these genes. Additionally, the mRNA abundance of DNMT-1 was enhanced two-three times in all groups compared to lean controls (P<0.001).

Conclusions: Upregulation of UCP-2 and TLR-4 in the placenta with increased maternal body weight or diabetes indicates a resetting of fatty acid oxidation and chronic pro-inflammatory status, whilst raised DNMT-1 is indicative of epigenetic adaptations. These findings suggest that UCP-2, TLR-4 and DNMT-1 are key gene targets for fetal programming and highlight critical cellular mechanisms that contribute to long term adverse health outcomes associated with maternal obesity and diabetes.

Appendix B: Suppliers

Abcam®, Cambridge, UK
Abgene Ltd, Epsom, UK
Alpha Laboratories Ltd, Eastleigh, UK
Anglia Scientific Ltd, Cambridge, UK
Applied Biosystems, Carlsbad, California (CA), USA
Biotek UK, Potton, UK
Bio-Rad Laboratories Ltd, Hemel Hempstead, UK
Bioron, Ludwigshafen, Germany
Cell Biolabs Inc., San Diego, California (CA), USA
Diagnostic Products Corporation, Caernarfon, UK
Ecolabs®, Saint Paul, Minnesota (MN), USA
Fisher Scientific UK Ltd, Loughborough, UK
Hoefer® Inc., Holliston, Massachusetts (MA), USA
IBM UK Ltd, Portsmouth, UK
Invitrogen Ltd, Paisley, UK
Leica Microsystems (UK) Ltd, Buckinghamshire, UK
Linton Instrumentation, Norfolk, UK
Manor Farm Feeds Ltd, Rutland, UK
Mercodia, Uppsala, Sweden
Menzel-Gläser Inc., Braunschweig, Germany
Microsoft Corporation, Redmond, Washington (WA), USA
Millipore, Billerica, Massachusetts (MA), USA
Minitab Ltd, Coventry, UK
PerkinElmer, Cambridge, UK
Premier Biosoft International, Palo Alto, California (CA), USA
Primer Design Ltd, Southampton, UK
Qiagen UK Ltd, Crawley, UK
Randox Laboratories Ltd, Crumlin, UK
Roche Diagnostics Ltd, West Sussex, UK
Scientific Laboratory Supplies (SLS) Ltd, East Riding of Yorkshire, UK
Severn Biotech Ltd, Worcestershire, UK
Sigma-Aldrich Company Ltd, Dorset, UK
VWR International, Lutterworth, UK