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Effects of Maternal Stress and Obesity on Human Feto-Placental Glucocorticoid Exposure

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Thesis presented at the University of Edinburgh for the degree of Doctor of Philosophy

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Declaration

I declare that this Thesis and the work presented in it are entirely the result of my own independent investigation, except where stated in the text. This work has not been and is not currently submitted for any other degree or diploma and to the best of my knowledge contains no material published or written by any other person, except where stated in the text.

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Abstract

Fetal exposure to excess glucocorticoids has been proposed as a key determinant of pregnancy outcome, as well as a predictor of long term health of the offspring through a phenomenon known as 'developmental programming'. Obesity and 'stress' during pregnancy are two potential sources of altered fetal exposure to glucocorticoids. One in five pregnant women is obese at antenatal booking, and maternal obesity increases risk of offspring complications including higher birth weight, potentially leading to long-term programming effects on the offspring. Likewise, maternal anxiety during pregnancy has been identified as a programming factor, increasing the risk of psychopathology in the offspring. This thesis tests the hypothesis that in humans this association is mediated by altered action of glucocorticoids, by examining circulating levels of maternal glucocorticoids during pregnancy and through measurement of key genes in the placenta regulating fetal glucocorticoid exposure.

Serum cortisol levels were measured at 16, 28 and 36 weeks gestation in n=173 class III obese (BMI 44.0±4.5kg/m^2) and n=107 lean (BMI 22.8±1.6kg/m^2) pregnant women. Serial corticosteroid binding globulin (CBG) concentrations were measured in a subset (n=39 lean, 26 obese) and free cortisol levels calculated using Coolen's equation. CRH concentrations were measured at the same time points in obese (n=20) and lean (n=22) pregnant women Salivary cortisol was measured in samples collected at bed-time, waking and 30 minutes after waking. mRNA levels of candidate genes regulating glucocorticoids and

fetal/placental growth including 11-beta hydroxysteroid dehydrogenase type 2 (*11βHSD2*), which inactivates cortisol, insulin-like growth factor 2 (*IGF2*) and glucocorticoid receptor (*GR*) were measured in first trimester (n=32), second trimester (n=15) and term (n=60) placental samples. DNA methylation of key regions controlling the expression of the *IGF2*, *GR* and *11βHSD2* genes was measured by pyrosequencing in first trimester and term samples.

Levels of mRNAs encoding *11βHSD1*, *11βHSD2*, *GR* and *MR* were measured in term placentas collected from women from Helsinki, Finland in whom anxiety during pregnancy had been prospectively assessed using validated questionnaires. Term placental samples from a subset of the obese and lean women who had also completed stress questionnaires during pregnancy were used to examine replication of findings.

Cortisol levels rose similarly during pregnancy in obese and lean but were significantly lower throughout pregnancy in obese women ($p<0.05$). The diurnal rhythm of cortisol was maintained. CBG levels also increased, though this change was lower in obese (1.21-fold (±0.9) vs 1.56-fold (±0.07), $p<0.01$). In obese women, lower calculated free cortisol at 16 weeks gestation was associated with higher birth weight after adjustment for other factors ($r=-0.46$, $p<0.05$).

Placental mRNA encoding *11βHSD2* increased in association with increasing obesity in early pregnancy ($r=0.44$, $p<0.01$) and was highest in term placenta in obese women with macrosomic (>4000g) offspring ($p<0.05$). Placental transcript

abundance of *GR* also increased in association with increasing obesity in early pregnancy ($r=0.38$, $p<0.05$), but was lowest in term placenta from obese with macrosomic offspring ($p<0.05$). *IGF2* mRNA abundance was lower in the placentas of obese women with macrosomic offspring at term compared to both lean women and obese women with normal weight offspring ($p<0.01$). Methylation results are reported.

Placental mRNA levels encoding *11βHSD1* (which converts inactive cortisone to active cortisol) at term was found to positively associate with maternal anxiety measured in the first trimester of pregnancy in a group of pregnant Finnish women ($\beta=0.3$, $p<0.05$). Findings were similar in the replication sample in lean women only ($\beta=4.6$, $p<0.05$).

Lower circulating and bioavailable cortisol levels in early pregnancy, together with a greater placental 'barrier' to maternal glucocorticoids represent key mechanisms contributing to higher birth weight in offspring of obese women. Regeneration of active glucocorticoids in placenta and increasing placental sensitivity to glucocorticoids increases fetal glucocorticoid exposure and offers insight into the biological mechanisms underlying adverse offspring effects of maternal prenatal anxiety.

Abstracts from this thesis

O'Reilly J, Drake, AJ, Jones, RL, Norman JE, Seckl JR, Reynolds RM (2011) Maternal Obesity in Human Pregnancy is Associated with Altered Placental Expression of Key Genes in Fetal Growth During the First Trimester, but not at Term, *Journal Developmental Origins of Health and Disease*, **2** (S1): S96, *Presented at DOHaD 2011, Portland, OR (Poster abstract)*

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Reynolds, RM, **O'Reilly, J**, Forbes, S, Denison, FC, Norman, JE (2012) Anxiety and depression in severely obese pregnancy: associations with gestational weight gain and birthweight, *Presented at the International Society for Psychoneuroendocrinology, New York, NY (Oral abstract)*

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Abbreviations

11βHSD1	11 *beta* hydroxysteroid dehydrogenase type 1
11βHSD2	11 *beta* hydroxysteroid dehydrogenase type 2
5mC	5 methyl cytosine
ACTH	adrenocorticotropic hormone
AMV	avian myeloblastosis virus
ANOVA	analysis of variance
APS	adenosine 5’ phosphosulfate
ATP	adenosine triphosphate
AVP	arginine vasopressin
β-ME	*beta* mercaptoethanol
B2M	beta-2-microglobulin
BSA	bovine serum albumin
CBG	corticosteroid binding globulin
cDNA	complementary DNA
Cp	crossing point
CpG	cytosine-phosphate-guanine
CRH	corticotropin releasing hormone
DAB	3,3'-diaminobenzidine
DEPC	diethylpyrocarbonate
DMR	differentially methylated region
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dNTP	deoxyribonucleotide triphosphate
DLK1	delta-like 1 homolog
EDTA	ethylenediaminetetraacetic acid
FAM	fluorescein amidite
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GILZ	glucocorticoid induced leucine zipper
GLUT	glucose transporter
GR	glucocorticoid receptor
HPA axis	hypothalamus pituitary adrenal axis
ICR	mprinting control region
kb	kilobase
IGF2	insulin like growth factor 2
IGF2R	insulin like growth factor 2 receptor
IL	interleukin
IRS1	insulin receptor substrate 1
LPL	lipoprotein lipase
MR	mineralocorticoid receptor

mRNA	messenger RNA
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NBF	neutral buffered formalin
NF-kB	nuclear factor *kappa* b
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHLDA2	pleckstrin homology-like domain, family A, member 2
PPAR	peroxisome proliferator-activated receptor
PPi	pyrophosphate
PPIA	peptidylprolyl isomerase A (cyclophilin A)
PREDO	Prediction and Prevention of Pre-eclampsia
qPCR	quantitative PCR
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse transcriptase PCR
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein variant
SEM	standard error of the mean
STAI	Spielberger State and Trait Anxiety Index
TBE	tris boric acid EDTA
TBP	TATA binding protein
UBC	ubiquitin C
UPL	Universal Probe Library
UV	ultraviolet
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

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Chapter 1:

Introduction

1.1 Fetal Programming

Fetal programming is the phenomenon by which *in utero* events can influence fetal development and therefore later offspring health. It has been proposed that the developing fetus responds to *in utero* cues that indicate the conditions of the external environment (Wells 2007). The fetus responds to conditions experienced *in utero*, such as limited nutrient supply, by making permanent changes in physiology, metabolism and structure, and these changes are thought to affect the long term health of the offspring (Barker 1998). The physiological, cellular and molecular mechanisms by which these programming effects occur have yet to be clearly elucidated, although a number of systems have been identified as possible candidates.

For many years, the orthodoxy regarding lifestyle diseases such as metabolic syndrome and coronary heart disease has been that such illnesses result from a combination of unhealthy lifestyle and genetics. In the UK however, rates of heart disease following the Second World War were found to follow a paradoxical geographical profile: rates were lowest in the most prosperous areas and amongst the highest income groups, a group traditionally thought to be at higher risk (Gardner *et al.* 1969). A study by David Barker, involving over a million deaths from coronary heart disease, found that differences in rates of death from coronary heart disease in different parts of England and Wales paralleled past differences in neonatal mortality rates, providing the first suggestion that coronary heart disease may be linked to early life experiences (Barker and Osmond 1986; Barker 2007). Rates of neonatal death, which in the early 20th Century was a phenomenon most commonly associated with low birth

weight, were found to be particularly associated with later risk of coronary heart disease (Barker 2007). The association between infant mortality and later heart disease was not unique to England and Wales: in Norway, a positive association was found between county heart disease mortality rates in people aged between 40 and 69 years and the county infant mortality rates in the early years of the same cohorts (Forsdahl 1977); in the USA, a similar association was observed between rates of infant mortality from diarrhoea in the 1917-1921 cohort and later heart disease rates in middle age (Buck and Simpson 1982). In a study of 26,000 employees of the US-based Bell System of telecommunication companies, rates of coronary heart disease were found to be higher amongst workers from so-called 'blue collar' families when compared to those whose parents were viewed as 'white collar' workers, again suggesting a link between early life conditions and illness in later life (Hinkle 1973). A similar study in the UK, the highly influential Whitehall study, found that rates of death were higher in London civil servants of lower social class and physical stature, again suggesting that those with a worse environment early in life were at highest risk of poor health later in life (Rose and Marmot 1981; Marmot *et al.* 1984).

The unexpected relationship between geography of birth and later disease risk led to the fetal origins of health and disease, or 'Barker', hypothesis: that inappropriate fetal nutrition *in utero* leads to permanent and life-long changes in physiology, anatomy and metabolism and thus leads to an increased risk of coronary heart disease and metabolic disorders such as diabetes later in life (Hales and Barker 1992; Barker 1995; Barker 1998). Low birth weight, often used as a marker of poor intra-uterine conditions, has been associated with increased incidence of a number of diseases of later life, including

cardiometabolic disorders and mental health disorders (Barker *et al.* 1993a; Barker 1995; Fall *et al.* 1995; Moore *et al.* 1996; Forsen *et al.* 1997; Rich-Edwards *et al.* 1997; Thompson *et al.* 2001; Räikkönen K and et al. 2008). This phenomenon has been observed in both the developed and developing worlds, particularly where low birth weight offspring of nutritionally-restricted mothers in poor or rural areas are met with urban diets of relative energy excess in adolescence and adulthood (Prentice *et al.* 2005; Prentice 2009).

This phenomenon of *in utero* conditions influencing later disease risk has come to be termed 'fetal programming', and is a consequence of developmental plasticity (Barker *et al.* 1993b; Seckl 1998; Barker 2007). During critical periods of development, the fetus is sensitive and plastic to substances and conditions in the *in utero* environment. Following these periods, plasticity is reduced, and a fixed functional capacity is established (Bateson *et al.* 2004). A number of factors, including maternal malnutrition, obesity and stress have been associated with alterations in fetal growth and development during these windows of developmental sensitivity (Seckl 2001; Jones 2005; Cottrell and Seckl 2009; Jones *et al.* 2009b). Fetal programming has been linked to an increased risk of a number of diseases of adulthood, including cardiovascular disease, metabolic syndrome, type 2 diabetes, cancers and obesity; taken together, these diseases represent a large proportion of mortality in the developed world, as well as a high burden on the resources of healthcare systems.

A number of authors have suggested that fetal programming may be due in part to adaptive responses made by the fetus in response to the *in utero* environment (Bateson *et al.* 2004; Gluckman and Hanson 2004b). When confronted with a

nutritionally poor environment, the mother may signal to the fetus, causing its development to be modified in such a way as to prepare it for survival in a harsh postnatal environment (a 'predictive adaptive response'); her baby may thus be born with certain physical characteristics (e.g. modified metabolism, a small body) which will help it survive in an environment where food is scarce (Bateson *et al.* 2004; Gluckman and Hanson 2004a). This response has been dubbed the 'thrifty phenotype', and may rely upon epigenetic changes (Hales and Barker 1992; Weaver *et al.* 2004). However, it is likely that many programming effects are non-adaptive, and are rather due to toxins or other insults either disrupting fetal development or forcing the fetus to adapt in such a way as to secure its immediate survival, with potential long term tradeoffs (Bateson *et al.* 2004; Gluckman *et al.* 2005). Such early-life metabolic adaptations to a difficult environment, whether predictive adaptations or not, may result in deleterious health consequences later in life, particularly if there is a mismatch between expected and actual environmental conditions.

1.1.1 Programming and Glucocorticoids

Glucocorticoids have come under particularly close scrutiny as possible candidates for mediating programming effects for a number of reasons. Exposure to excess glucocorticoids has been shown to reduce birth weight in both animal models and humans, particularly if exposure occurs during the later stages of pregnancy (Reinisch *et al.* 1978; Nyirenda *et al.* 1998; French *et al.* 1999; Bloom *et al.* 2001). Birth weight itself is a potent predictor of future offspring health, with low birth weight associated with the later development of

a number of common cardiovascular and metabolic diseases (Barker *et al.* 1993a; Barker *et al.* 1993b; Curhan *et al.* 1996; Forsen *et al.* 1997; Rich-Edwards *et al.* 1997). Glucocorticoid production and tissue sensitivity are altered in pregnancy, with dramatic rises in the production of both the glucocorticoid cortisol and the regulatory hormone corticotrophin-releasing hormone (CRH) observed during human pregnancy (Okamoto *et al.* 1989; Lindsay and Nieman 2005). Glucocorticoids also appear an attractive target when considering the potential programming effects of diet and stress: glucocorticoids are important regulators of metabolism; glucocorticoid production and clearance is altered in obesity; and glucocorticoids form a major component of the stress response (Jung 1984; Dallman *et al.* 1993; Pasquali *et al.* 1993; Torres and Nowson 2007).

1.2 Pregnancy

Human pregnancy involves a number of modifications to the maternal physiology, including hormonal and immunological changes, in order to adequately support the mother and developing fetus. Disturbances to this process, for example through poor nutrition, exposure to toxins, or disruption in hormonal or immune function, can have major consequences on birth outcome. A key component of pregnancy is the placenta, which forms the main interface between the mother and her developing offspring. The placenta is a unique and transient organ responsible for the exchange of nutrients between mother and fetus, as well as diverse endocrine and metabolic functions, all of which play key roles in pregnancy.

1.3 The Hypothalamic-Pituitary-Adrenal Axis

1.3.1 Overview

A number of important bodily processes, including digestion, immunity, energy storage and the stress response are influenced by the action of glucocorticoids. Glucocorticoid production is regulated by the hypothalamic-pituitary-adrenal (HPA) axis, a complex set of feedback interactions between the hypothalamus (specifically the paraventricular nucleus), the anterior pituitary and the cortex of the adrenal glands. These interactions are mediated by the action of a small group of hormones: corticotropin releasing hormone (CRH), arginine vasopresssin (AVP), adrenocorticotropic hormone (ACTH) and cortisol (see Figure 1-1). Cortisol production is regulated by CRH, AVP and ACTH produced

in the hypothalamus and pituitary, and is self-inhibitory through a negative feedback loop. During episodes of acute stress, CRH production by the hypothalamus is greatly increased while AVP is only moderately increased, resulting in an increase in ACTH and cortisol production. Chronic stress however elicits a greater increase in AVP secretion while CRH production is paradoxically lowered.

CRH and AVP are released from the hypothalamus and transported to the anterior pituitary, where they act to stimulate secretion of stored ACTH. ACTH is transported in the blood to the adrenal cortex of the adrenal glands, where it stimulates synthesis of corticosteroids including cortisol. Importantly, cortisol negatively feeds back to inhibit production of both CRH and ACTH in the hypothalamus and pituitary gland respectively. In healthy individuals a distinctive diurnal rhythm of cortisol production can be observed: levels generally rise rapidly shortly after wakening, reaching a peak within 30 minutes, before gradually falling throughout the rest of the day. Levels of cortisol rise once again in the late afternoon, before falling in the late evening to a trough during the middle of the night.

While the secretion of glucocorticoids is widely recognised as a classic endocrine response to stress, the physiological purpose of glucocorticoids in the stress response has been controversial (Sapolsky *et al.* 2000). The classical view, as espoused in the 1930s by the pioneering Hungarian endocrinologist Hans Seyle, is that glucocorticoids mediate the stress response either through actively stimulating the stress response, or through permissive effects (Sapolsky *et al.* 2000). A subsequent revisionist interpretation suggested that glucocorticoids in

fact suppress the stress response, in effect protecting against the defence reactions activated during the stress response (Munck *et al.* 1984). This change in view was in part a consequence of the unexpected discovery in 1949 that glucocorticoids had anti-inflammatory effects (Hench *et al.* 1949). More recently, the permissive and suppressive effects of glucocorticoids have been suggested to be complementary, with the former priming and enhancing the stress response and the latter limiting its actions (Munck and Náray-Fejes-Tóth 1992).

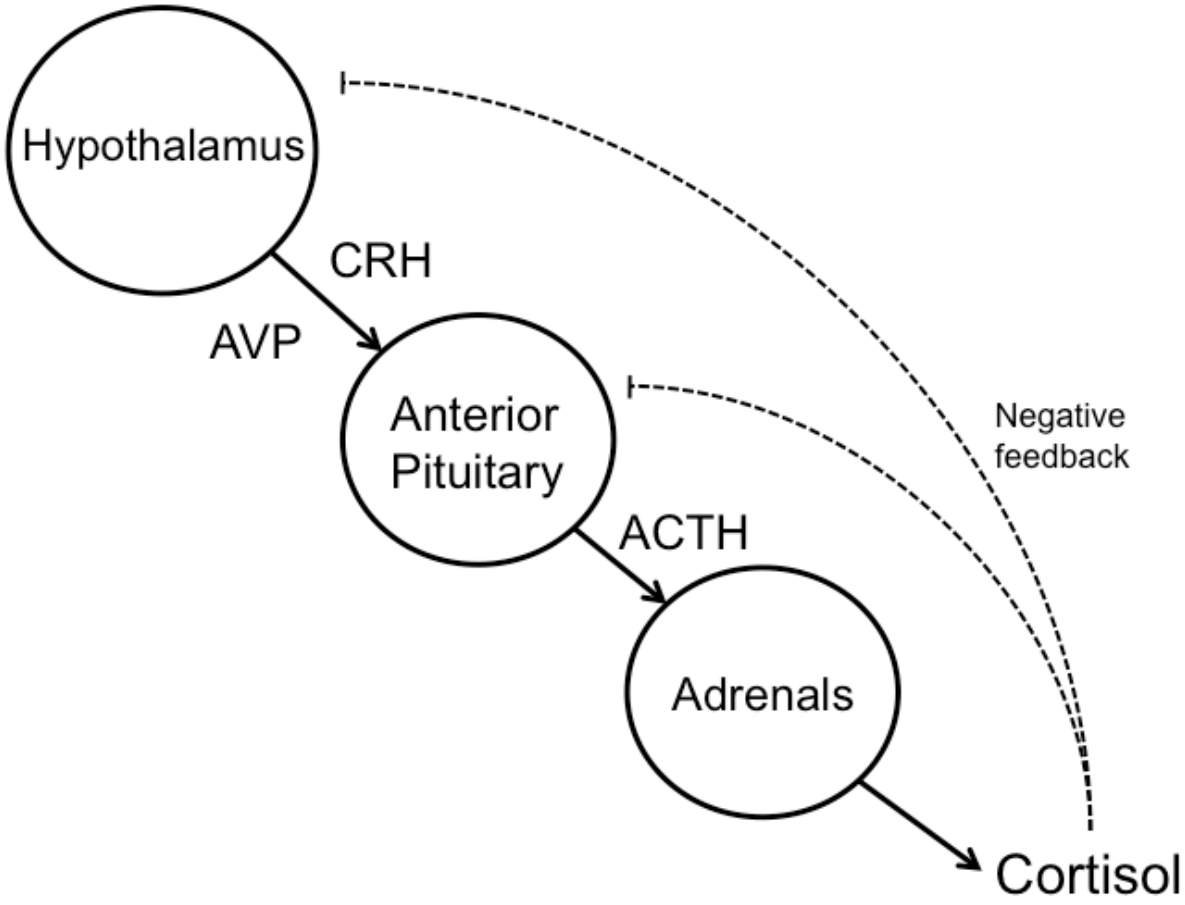


Figure 1-1. The hypothalamic-pituitary-adrenal axis in humans.

1.3.2 Cortisol

Glucocorticoids have multiple functions on a vast number of cell and tissue types. These include suppression of inflammation and immune reactions, inhibition of glucose uptake by peripheral tissues, stimulation of hepatic gluconeogenesis and effects on lipolysis (Munck *et al.* 1984; Djurhuus *et al.* 2002).

The primary glucocorticoid in humans is cortisol. Cortisol is synthesised from cholesterol in a process known as steroidogenesis. Synthesis occurs within the zona fasciculata of the adrenal cortex. The synthesis of cortisol is stimulated by ACTH through regulation of a number of key factors involved in steroidogenesis: ACTH increases the uptake of cholesterol by the adrenals through an upregulation of LDL receptors, stimulates the conversion of cholesterol to pregnenolone by upregulation of the side chain cleavage enzyme cytochrome P450SCC encoded by the *CYP11A1* gene (Hu *et al.* 2001), and hydroxylates pregnenolone to produce 17-OH-pregnenolone, a precursor to 11-deoxycortisol.

Most organs and physiological systems in the body are sensitive to glucocorticoids, with glucocorticoids having major effects on cardiovascular tone, fluid volume, immunity and inflammation, metabolism, neural function and reproduction (Sapolsky *et al.* 2000). Glucocorticoids also exert powerful effects upon metabolism. In response to stress, blood glucose levels rise rapidly as a consequence of both mobilisation from existing stores and inhibition of storage as a consequence of insulin resistance (Black *et al.* 1982). These

responses are largely the result of the influence of catecholamines, glucagon and growth hormone (Sapolsky *et al.* 2000). Glucocorticoids act to increase the levels of circulating glucose via a number of mechanisms, including stimulation of appetite (Santana *et al.* 1995), as well as to mobilise lipids through lipolysis in fat cells (Xu *et al.* 2009). Interestingly, appetite stimulation by glucocorticoids occurs at basal rather than stress levels (Dallman *et al.* 1993). At stress levels, glucocorticoids suppress appetite; this occurs by stimulating a burst of insulin secretion, which essentially negates the appetite-stimulating effects of glucocorticoids (Dallman *et al.* 1993; Sapolsky *et al.* 2000). Cortisol also increases the sensitivity of tissues to catecholamines, suggesting an integrative effect on the stress response; cortisol has been shown to regulate the sensitivity of vascular tissue to catecholamines, for example (Ullian, 1999; Magiakou et al., 2006).

1.3.3 Intracellular Action of Glucocorticoids

Glucocorticoids mainly act within the cell through the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). The GR protein is encoded by the gene *NR3C1* (Nuclear receptor subfamily 3, group C, member 1; *GR*), and is ubiquitously expressed (Yudt and Cidlowski 2002). The receptor can act as both a transcription factor and as a regulator of other transcription factors (Yudt and Cidlowski 2002).

Unliganded GR proteins form large heterocomplexes in the cytoplasm with heat shock protein 90 (hsp90) and other heat shock proteins (Pratt and Toft 1997). On hormone binding, the hormone-receptor complex is transformed, leading to an

activated hormone-receptor complex monomer that becomes hyperphosphorylated and binds to structures in the nucleus (Ortí *et al.* 1993). The activated hormone-receptor complex then binds to target genes via short palindromic nucleotide sequences in the target promoter region known as glucocorticoid response elements (GREs) in the case of transactivation, or negative GREs, disrupting the binding of positively acting factors at adjacent sites in the process, in the case of transrepression (Lucas and Granner 1992; Sapolsky *et al.* 2000). GR may also affect transcription through binding or 'tethering' to other transcription factors, rather than directly to DNA, a process that generally interferes with transcription (Sapolsky *et al.* 2000). For example, glucocorticoid suppression of the reproductive hormone GnRH is thought to occur via GR tethering to Oct-1, which binds directly to the GNRH gene (Chandran *et al.* 1999). Activated GR complexes are also thought to interact directly with general transcription factors that comprise the RNA polymerase II transcription complex.

GR is highly expressed in the placenta, and is thought to play an important role in the placental development and function (Jones *et al.* 1989a; Ringler *et al.* 1989; Hahn *et al.* 1999; Chan 2003; Lee *et al.* 2005). However, information concerning the possible gene targets of GR in the placenta is limited.

Peroxisome proliferator-activated receptor gamma (PPARγ), a gene important for placental growth and function, appears to be regulated by GR (García-Bueno *et al.* 2008; Giaginis *et al.* 2008). PPARγ mRNA and protein levels in abdominal subcutaneous adipose tissue are also reduced in obese pregnancy complicated by gestational diabetes (Catalano *et al.* 2002).

1.3.3.1 CBG

Corticosteroid-binding globulin (CBG, also known as transcortin) is thought to be a major modulator of cortisol availability in humans. This 50-60kDa glycoprotein binds to cortisol and transports it in the plasma. CBG is primarily secreted from hepatocytes with a slight diurnal variation, fluctuating in opposition to the diurnal variation in plasma cortisol levels (Hsu and Kuhn 1988; Lewis *et al.* 2006). Although there is evidence for some secretion of CBG in the kidney and testis, this is thought to regulate local glucocorticoid availability in those tissues only (Henley and Lightman 2011).

While CBG is generally defined as a serine proteinase inhibitor (or serpin), it does not act as a proteinase inhibitor, but rather as a substrate for specific proteinases such as neutrophil elastase (Hammond *et al.* 1990). Neutrophil elastase is found in high concentration at sites of inflammation, and through proteolytic cleavage causes the destruction of the steroid-binding site of CBG, thus releasing the steroid ligand (Hammond *et al.* 1990; Klieber *et al.* 2007; Lin *et al.* 2010).

Only about 5% of secreted cortisol is unbound in the plasma and thus free to bind to GR and MR. The remaining 95% of secreted cortisol is bound to carrier proteins in plasma: between 80 and 90% is bound to CBG in the plasma, with a further 10 to 15% bound to albumin (Lewis *et al.* 2005). The ‘free hormone hypothesis’ proposes that steroid hormones bound to carrier proteins such as CBG can be considered biologically inactive (Mendel 1989). The bound substance provides a reservoir of inactive hormone that is used to finely regulate

the levels of free hormone available for diffusion into cells (Henley and Lightman 2011). Following stress, CBG production can be downregulated in order to increase free cortisol levels (Neufeld *et al.* 1994; Fleshner *et al.* 1995; Spencer *et al.* 1996).

However, there is also evidence to suggest that CBG may function as a cortisol transporter, and that it may release cortisol at clinically relevant temperatures, i.e. between 34 and 37°C, and that it may have an active role in the local delivery and signaling of glucocorticoids (Petersen *et al.* 2006; Cameron *et al.* 2010). A number of Other roles have also been suggested for CBG, including regulation of intestinal sodium absorption, and direct effects in the brain (Henley and Lightman 2011).

Circulating levels of CBG are sensitive to hormonal changes; concentrations are increased by estrogens and decreased by glucocorticoids (Henley and Lightman 2011). CBG has also been shown to negatively correlate with insulin secretion (Crave *et al.* 1995; Fernandez-Real *et al.* 1999).

1.3.3.2 Free cortisol versus total cortisol

Traditionally, serum total cortisol has been used as a measure of HPA-axis activity (le Roux *et al.* 2003). This measurement does not, however, differentiate between unbound and bound levels of cortisol. Furthermore, variation in CBG concentration has been shown to significantly affect total serum cortisol levels (Dhillo *et al.* 2002). As approximately 80% of serum cortisol is bound to CBG, measurements of the unbound, or free, cortisol are thought to be a more useful

measure of available glucocorticoids (Arafah 2006). Free cortisol can be calculated from total cortisol and CBG measurements using the Coolen's equation (Coolens *et al.* 1987).

1.3.4 The HPA-Axis During Pregnancy

Pregnancy has been described as 'a state of mild but sustained hypercortisolism', and human gestation has been shown to dramatically affect the HPA axis (see Figure 1-2)(McLean and Smith 1999). Maternal plasma concentrations of cortisol, CRH and ACTH all rise during pregnancy (Okamoto *et al.* 1989; Lindsay and Nieman 2005). The diurnal rhythm of ACTH and cortisol, as well as the cortisol response to stress, are maintained. Placental estrogen production stimulates the hepatic production of CBG, thus stimulating cortisol production and increasing levels of total and bound cortisol in the circulation (Lindsay and Nieman 2005). However, HPA axis reactivity decreases throughout pregnancy as measured by ACTH response to CRH, and stress response appears to be similarly diminished (Schulte *et al.* 1990; Lindsay and Nieman 2005).

While the rise in total plasma cortisol may be partly explained by an estrogen-related rise in CBG levels during pregnancy, free cortisol levels also increase (Scott *et al.* 1990). It is thought that increased placental CRH production is the primary factor in the increased HPA activity observed during pregnancy (McLean and Smith 1999). However, it is also important to note that the retention of the stress response and diurnal rhythm of cortisol production, even in the third trimester of pregnancy, suggests that the placenta does not entirely usurp the role of the hypothalamus in HPA axis function during pregnancy

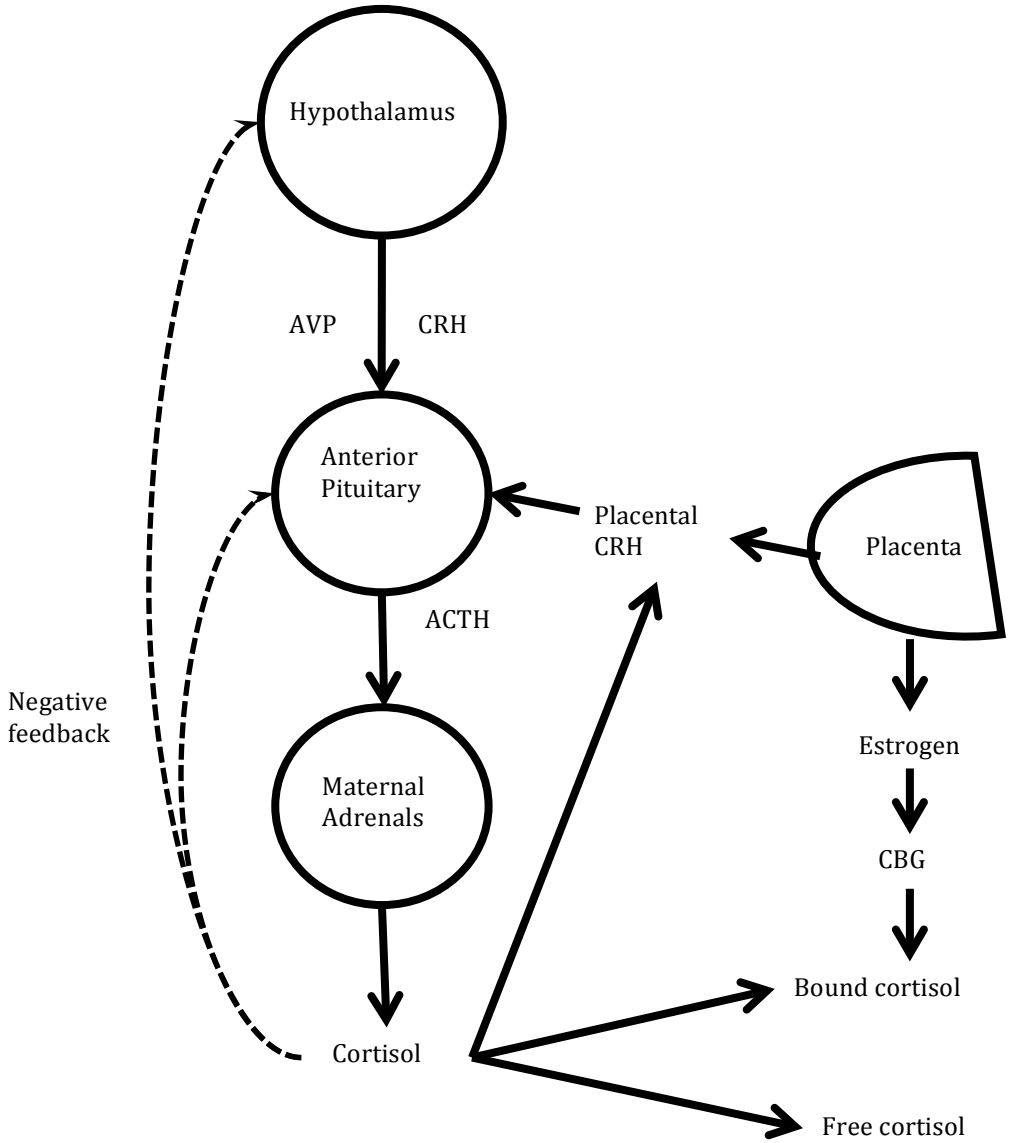


Figure 1-2. The HPA axis during human pregnancy. During pregnancy, the placenta influences maternal HPA-axis activity (ACTH, cortisol and CBG production) through the production of CRH and estrogen.

(Magiakou *et al.* 1996). It is also thought that AVP (produced by the parvocellular neurons of the paraventricular nucleus), rather than CRH, becomes the main regulator of pituitary ACTH secretion; this is similar to HPA axis regulation in chronic stress (Chowdrey et al., 1995; Engelmann et al., 2004).

1.3.4.1 CRH

Corticotrophin releasing hormone (CRH) is a peptide hormone produced in the hypothalamus responsible for stimulation of ACTH production by the pituitary, which in turn stimulates glucocorticoid secretion from the adrenals (Vale *et al.* 1981). The synthesis of CRH by the hypothalamus is regulated by negative feedback from cortisol, ACTH, as well as CRH itself.

During pregnancy, CRH is found at high concentrations in the maternal plasma and increases exponentially throughout gestation, reaching a peak concentration at term of between 1 and 10 nmol/l (Campbell *et al.* 1987; Mastorakos and Ilias 2003). This rapid rise in CRH levels is a result of production by the placenta, particularly the syncytiotrophoblast and intermediate trophoblast (Shibasaki *et al.* 1982; Riley *et al.* 1991). Unlike hypothalamic CRH, placental CRH production is stimulated by glucocorticoids; this stimulation is indicative of a positive feedback loop driving increasing production of CRH throughout pregnancy (Robinson *et al.* 1988; Jones *et al.* 1989b). CRH is an important regulator of implantation, and is thought to be involved in the production of estrogen by the fetal adrenal (Smith *et al.* 1998; Mastorakos and Ilias 2003).

The effects of CRH during pregnancy are regulated by CRH binding protein (CRH-BP), which is produced by the placenta and may be involved in reducing

stress reactivity during pregnancy by preventing inappropriate stimulation of the HPA axis (Petraglia *et al.* 1993).

1.3.4.1.1 CRH and timing of parturition

It has been suggested that CRH plays a crucial role in the timing of parturition; CRH levels at 31 weeks have been shown to predict incidence of preterm birth, indicating a role for this hormone as a 'placental clock' (Sandman *et al.* 2006). The high levels of CRH in both the maternal and fetal compartments near term, coupled with the presence of CRH receptors in the myometrium, placenta and fetal membranes, as well as the fetal pituitary and adrenal cortex, further underline the importance of this hormone during pregnancy (Ackland *et al.* 1986; Petraglia *et al.* 1990; Hillhouse *et al.* 1993; Smith *et al.* 1998). Furthermore, plasma levels of CRH-BP have been shown to fall in the third trimester of pregnancy, increasing the availability of free, unbound CRH (Linton *et al.* 1993). CRH is thought to stimulate cortisol production by the fetal adrenals (Lockwood *et al.* 1996; Sirianni *et al.* 2005). Cortisol, whether of maternal or fetal origin, may be the main driver of organ maturation, an important precursor to parturition (Challis *et al.* 2001). CRH is also thought to stimulate the release of prostaglandins from the amnion, chorion and decidua, aiding in cervical ripening, as well as regulating fetal estrogen production (Jones and Challis 1989; Smith *et al.* 1998).

1.3.5 The Placental Glucocorticoid Barrier: 11βHSD2

During pregnancy, the placenta acts as a barrier to potentially harmful substances in the maternal circulation. Of particular importance is the role of the placenta as a barrier to maternal glucocorticoids. This barrier is largely dependent upon the enzyme 11-beta hydroxysteroid dehydrogenase type 2 (11βHSD2).

Postnatally, 11βHSD2 is expressed in mineralocorticoid-target tissues throughout the body, and allows for selective access of aldosterone to the mineralocorticoid receptor, MR, by conversion of active glucocorticoids (e.g. cortisol) to an inactive form (e.g. cortisone). This allows aldosterone, generally present in much lower concentrations than cortisol, to bind to the non-specific MR (Edwards *et al.* 1988; Funder *et al.* 1988).

In the placenta, 11βHSD2 is thought to have an additional, crucial, function. Placental 11βHSD2 inactivates cortisol by converting it into cortisone, thereby protecting the fetus from the deleterious effects of excess exposure to maternal glucocorticoids as well as enabling an intricate regulation of glucocorticoid transit and subsequent fetal exposure (Brown *et al.* 1996; Wyrwoll *et al.* 2011). While 11βHSD2 expression in mice appears to be switched off in the mid-gestation placenta (from E16.5), in humans 11βHSD2 expression increases approximately 50-fold throughout gestation (with the greatest increase during the third trimester), peaking at term, and expression is localised to the syncytiotrophoblast (Krozowski *et al.* 1995; Brown *et al.* 1996; McTernan *et al.* 2001). The role of mid-term silencing of 11βHSD2 expression in the mouse placenta is unknown; while it has been suggested that 11βHSD2 protein may

persist until later in gestation, placental 11βHSD2 activity has been found to dramatically decrease by E17.5 in rodent placentas (Brown *et al*., 1996; Diaz *et al.,* 1998). Intriguingly, placental silencing of 11βHSD2 in the rodent placenta is paralleled by a reduction in 11βHSD2 expression in the mid-gestation rodent brain; similarly, 11βHSD2 expression is silenced between gestational weeks 19 and 26 in the developing human brain (Stewart *et al*., 1994; Brown *et al*., 1996). This suggests that elevated glucocorticoid exposure may be necessary for late-gestational brain development in both rodents and humans; the different patterns of placental 11βHSD2 expression may reflect differences in the level of exposure necessary and the different developmental trajectories of rodents and humans. Low levels of expression and null mutations of 11βHSD2 have been associated with intra-uterine growth restriction (IUGR), underlining the importance of this enzyme for proper growth and development prenatally (Kitanaka *et al.* 1996; Dave-Sharma *et al.* 1998).

In humans, the 11βHSD enzyme exists in two isoforms: 11βHSD1 and 11βHSD2 (Seckl 1997). While 11βHSD2 converts cortisol to its inactive form, cortisone, the isoform 11βHSD1 is involved in the reduction of cortisone to the active hormone cortisol. 11βHSD1 thus acts to regenerate active glucocorticoids, and amplify their action. 11βHSD1 is expressed throughout the adult central nervous system, and plays a key role in HPA axis function; 11βHSD1 is also thought to be an important regulator of brain development, particularly during late gestation (Wyrwoll *et al*., 2011).

Both 11βHSD1 and 11βHSD2 are expressed in the placenta (McMullen et al., 2004). In rodents, 11βHSD1 is expressed in the placenta in the latter half of

pregnancy, and has been suggested to contribute to the late-gestation rise in glucocorticoids that ensure fetal maturation (Burton *et al.*, 1996). Furthermore, both 11βHSD1 and 11βHSD2 have been found to co-localise with GR in the rodent placenta, suggesting that these enzymes may act as regulators of glucocorticoid activity in placental tissues (Waddell *et al*., 1998). It has been suggested that placental 11βHSD1 and 11βHSD2 may limit or enhance the bioactivity of maternal cortisol in different regions of the placenta; 11βHSD1 and 11βHSD2 may interact physiologically in relation to substrate availability (Waddell *et al*., 1998). However, the different patterns of localization of the two enzymes appear to preclude direct interaction to finely regulate glucocorticoid activity within individual cells or tissues.

Inside the cell, the 11β-HSD2 enzyme is localised to the endoplasmic reticulum with a cytosol-facing active site and co-factor binding domain (Odermatt *et al.* 2006). 11βHSD2 binds to cortisol with approximately 100 times the affinity that 11βHSD1 binds to cortisone; this finding implies that the glucocorticoid oxidising activity of 11βHSD2 may play a greater role in glucocorticoid regulation in tissues where the two enzymes are expressed (Wyrwoll *et al.* 2011). However, 11βHSD1 and 11βHSD2 rarely co-localise to the same cells, complicating the interplay between the two isoforms (Waddell *et al*. 1998). However, the opposing effects of the two isoforms make them of great interest when considering the effects of glucocorticoids on a macroscopic level.

Changes in the placental expression of 11βHSD2 and 11βHSD1 may lead to altered fetal development: levels of 11βHSD1 mRNA are increased in chorion of placentas from pregnancies complicated by IUGR, while 11βHSD2 activity and

gene expression is downregulated in the decidua of the same (Wachter *et al.* 2009). Furthermore, 11βHSD2 is highly expressed in fetal tissues, including the brain until mid-term, as well as in a number of adult brain structures, potentially leading to developmental effects if expression is altered (Yu *et al.* 2002; Wyrwoll *et al.* 2011).

1.4 Stress and Obesity as Programming Factors

While maternal under-nutrition has been well documented as a potential programming factor, there is increasing evidence to link maternal obesity and nutrient intake during pregnancy, as well as maternal exposure to stressors, to increased risk of a number of programmed outcomes in the offspring, such as cardiovascular disease risk later in life. The focus of this thesis is on the effects of maternal obesity (i.e. over-nutrition) and psychological stress, conditions that are increasingly relevant in the context of modern lifestyles.

Maternal stress is associated with an increased risk of offspring behavioural problems, attention deficit hyperactivity disorder (ADHD) and anxiety (Van den Bergh and Marcoen 2004; Gutteling *et al.* 2005; Rodriguez and Bohlin 2005b; Gutteling *et al.* 2006). Prenatal stress is also associated with altered HPA axis activity in the offspring (Gutteling *et al.* 2004; Gutteling *et al.* 2005; O'Connor *et al.* 2005; Yehuda *et al.* 2005; Brennan *et al.* 2008; Entringer *et al.* 2009; Grant *et al.* 2009). In humans, maternal experience of stressful events during the second trimester is associated with increased incidence of offspring schizophrenia in humans (Welberg *et al.* 2001; Koenig *et al.* 2002). Traumatic events have been

shown to alter HPA-axis response in adult humans, and schizophrenia in particular has been associated with reduced expression of glucocorticoid receptor in the brain as well as attenuated HPA-axis feedback regulation (Goldman *et al*. 1993; Goenjian *et al*. 1996; Koenig *et al*. 2002). Animal studies have suggested that prenatal maternal restraint enhances the HPA-axis responsiveness of the offspring in adulthood (Henry *et al*. 1994), and restraint during the final week of gestation in particular is associated with decreased hippocampal neurogenesis (Lemaire *et al*. 2000); intriguingly, maternal adrenalectomy has been shown to abolish the effects of maternal restraint, suggesting that maternal glucocorticoids are responsible for the increased risk of schizophrenia otherwise observed (Barbazanges *et al*. 1996). Furthermore, studies in rodents have established that overexposure to glucocorticoids prenatally is associated with altered adult behaviour and HPA-axis reprogramming (Welberg *et al*., 2001).

Maternal obesity has also been implicated as a programming factor in a number of studies, and a number of prenatal mechanisms have been proposed to explain this finding (Figure 1-3) (Whitaker 2004; Catalano and Ehrenberg 2006; Catalano *et al.* 2009; Drake and Reynolds 2010; Reynolds *et al.* 2010; Harmon *et al.* 2011; Hochner *et al.* 2012). Fetuses of obese mothers have been shown to have increased body fat, increased risk of insulin resistance, and higher levels of the inflammatory factor IL-6; furthermore, fetal adiposity has been shown to correlate with insulin resistance in these individuals (Catalano *et al*., 2009). Another study has shown that 24 hour glucose area under the curve is higher in obese pregnant women compared with lean women in both early and late pregnancy; infants born to obese women were also found to have increased

adiposity (Harmon *et al*. 2011). Maternal obesity during early pregnancy is associated with a doubling in risk of obesity at 2 and 4 years of age (Whitaker 2004). The Jerusalem Perinatal Family Follow-up Study found that higher maternal BMI before and during pregnancy is positively associated with offspring adiposity in young adulthood (Hochner *et al*. 2012). It has been suggested that these effects may simply be a result of the increased birthweight often seen among offspring of obese women, or environmental factors; however, studies such as the Jerusalem Perinatal Family Follow-up Study show strong associations even when controlling for these factors in the analysis, suggesting that the effect is independent of birthweight (Hochner *et al*. 2012). The mechanisms by which these programming effects occur remain unclear, although a number of processes have been proposed, including direct effects of nutrient availability and altered placental structure and function (Jones 2005; Mcmillen and Robinson 2005; Fowden *et al.* 2006b; Fowden *et al.* 2009). Whether changes in fetal glucocorticoid exposure are important in obese pregnancy has not been explored.

1.5 Obesity

Obesity has been classically defined as a state of excess energy or fat storage (Haslam and James 2005; Peeke and Chrousos 1995). In humans, obesity is associated with increased morbidity and mortality due to an increased risk of diabetes, cardiovascular disease and cancer (Haslam and James ; James *et al.* 2004).

Obesity has an increasing prevalence throughout the world, and represents an increasing burden on healthcare (Alberti and Zimmet 1998; Heslehurst *et al.* 2008). WHO estimates suggest that in 2008 more than 1.4 billion adults aged 20 and over were overweight, with 200 million men and 300 million women classed as obese; furthermore, 40 million children under the age of five were estimated to be overweight in 2010 (WHO 2013). In the UK, rates of obesity rose from 7.6% to 15.6% over the period between 1989 and 2007, and obesity has major effects on healthcare services and resources (Heslehurst *et al.* 2007a; Heslehurst *et al.* 2007b; Heslehurst *et al.* 2010).

The effects of obesity are dependent in part on the distribution of fat in the body. Central or abdominal obesity, for instance, is strongly associated with cardiovascular disease, as well as other metabolic and vascular conditions (Yusuf *et al*. 2004). Importantly, abdominal obesity and waist circumference are also strongly associated with dysregulation of insulin homeostasis increased incidence of metabolic syndrome via increased levels of the hormone resistin (Pouliot *et al*. 1992; Despres 2001). Other fat depots, such as subcutaneous fat, are not associated with increased risk of heart disease, cancer, or other pathologies, and has in fact been suggested to be potentially protective (Porter *et al*. 2009).

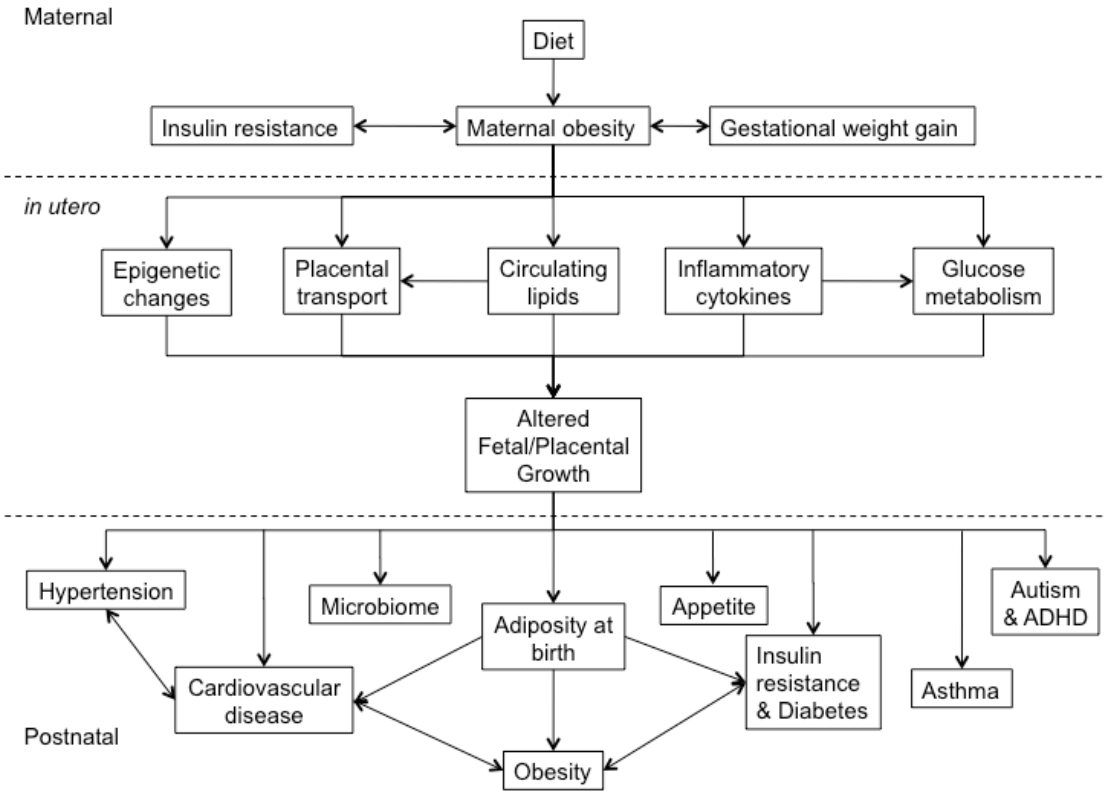


Figure 1-3. Selected factors hypothesised to influence the long-term risk of offspring obesity, including maternal influences, *in utero* mechanisms, and postnatal effects. Figure adapted from O'Reilly and Reynolds, 2013.

Body mass index, or BMI, is often used as a simple index of weight-for-height and is defined as a person's weight in kilograms divided by the square of their height in metres (kg/m^2). This index is commonly used to define overweight (a BMI greater than or equal to 25 kg/m^2) and obesity (a BMI greater than or equal to 30 kg/m^2) (Alberti and Zimmet 1998).

1.6 Obesity during Pregnancy

Just as obesity rates have risen amongst the general population, the rates of obesity among pregnant women have also increased over the last few decades (Heslehurst *et al.* 2007a; Heslehurst *et al.* 2007b; Heslehurst *et al.* 2010). Obesity has effects upon female reproductive health, including increased risk of polycystic ovarian syndrome and infertility (Heslehurst *et al.* 2008). During pregnancy, there is an increased risk of obese mothers developing gestational diabetes, hypertension and pre-eclampsia (Sibai *et al.* 1995a; Sibai *et al.* 1995b; Solomon *et al.* 1997; Sebire *et al.* 2001). The incidence of late fetal loss and stillbirth is also increased (Lashen *et al.* 2004). Obesity has also been suggested as a risk factor for a number of structural birth defects, including spina bifida, heart defects and hypospadias (Waller *et al.* 2007).

Interestingly, increasing severity of obesity has been shown to inversely correlate with gestational weight gain during pregnancy (Bodnar *et al*. 2010). In previous studies, low gestational weight gain has been associated with preterm birth and low birth weight, although low gestational weight gain may be beneficial in obese women (Hickey *et al.* 1990; Siega-Riz *et al.* 1996; Nohr *et al*. 2008), while high gestational weight gain is associated with macrosomia and an increased offspring

BMI throughout life (Hedderson *et al.* 2006; Oken *et al.* 2008; Schack-Nielsen *et al.* 2010; Hinkle *et al.* 2012). It is therefore important to account for the influence of gestational weight gain when investigating the effects of maternal obesity on pregnancy outcome and long-term offspring health effects.

1.7 Obesity and the HPA Axis

The link between stress, the HPA axis and obesity has been examined in a number of human and animal studies. Glucocorticoids are an important regulator of metabolic processes such as lipogenesis and gluconeogenesis (Dallman *et al.* 1993). Stress has been demonstrated to alter food consumption; human studies have reported both increases and decreases in eating in response to stress (Michaud *et al.* 1990; Stone and Brownell 1994; Oliver and Wardle 1999). Stress has also been associated with higher fat diet and less frequent exercise in humans, and there are suggestions that there may a gender-dependent relationship with women more likely to engage in stress-related eating (Laitinen *et al.* 2002; Ng and Jeffery 2003). While acute stress is thought to lead to a decrease in food intake through a catecholamine-mediated inhibition of appetite and digestion, chronic stress may in fact lead to an increase in food intake and weight gain through the actions of the HPA axis, and glucocorticoids in particular (Santana *et al.* 1995; Torres and Nowson 2007). Elevation of cortisol in chronic stress may thus stimulate appetite, and potentially lead to obesity (see figure 1-4). Increased secretion of glucocorticoids, as seen in Cushing's syndrome, is associated with obesity (Peeke and Chrousos 1995). Cortisol may also increase the accumulation of abdominal fat depots (Strack et al 1995; Dallman et al. 2003).

Defects or changes in the activity of the HPA axis would thus be expected to alter energy metabolism, potentially increasing the risk of obesity (Jessop et al. 2001).

Obesity is associated with alterations in some HPA axis parameters, but not others (Pasquali *et al.* 1993). Plasma cortisol concentrations, including the circadian rhythm of plasma cortisol, have been shown to be normal in obese individuals, although some data suggests that plasma cortisol levels may be lower in uncomplicated obesity (Glass *et al.* 1981; Jung 1984; Praveen *et al.* 2011). However, cortisol production and metabolism are thought to increase in obesity (Dunkelman *et al.* 1964; Jung 1984; Pasquali *et al.* 1993). This suggests that while HPA axis activity is increased in obesity, metabolic clearance of cortisol acts to maintain glucocorticoids at a normal physiological level. Interestingly, HPA axis hyperactivity is thought to be most pronounced in obese women with abdominal fat distribution (Pasquali *et al.* 1993; Jessop *et al.* 2001).

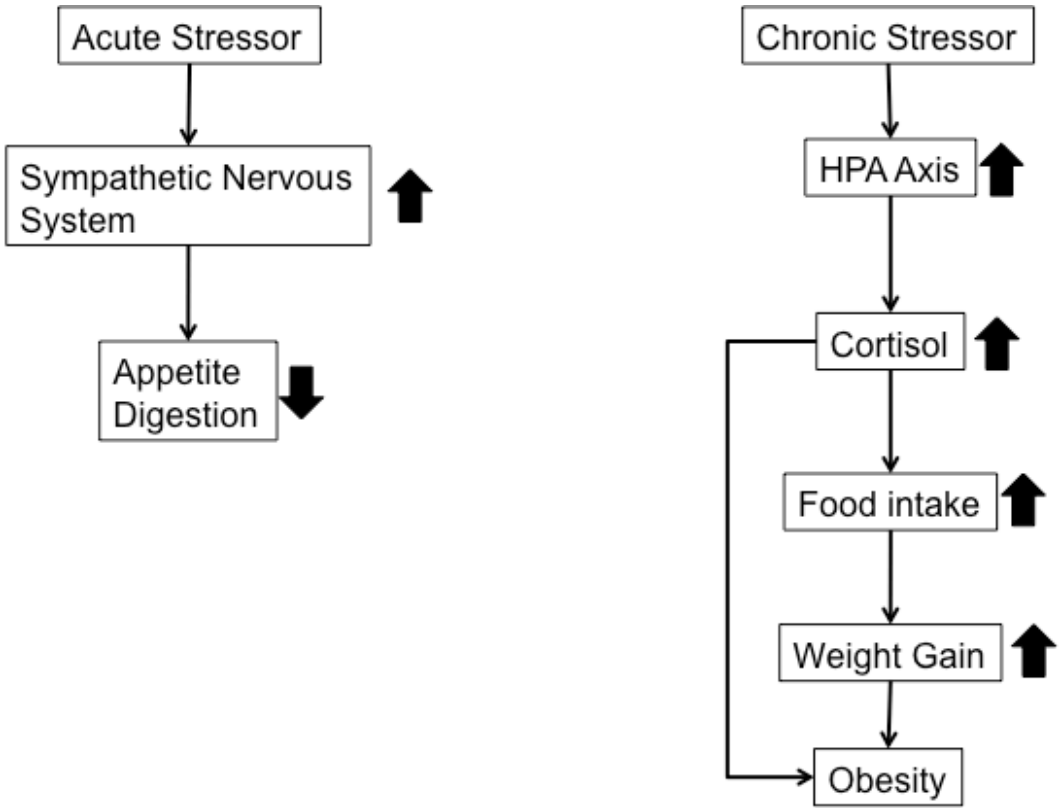


Figure 1-4. Differential effects of acute and chronic stressors on digestion and appetite.

1.7.1 Maternal Obesity and Programming

The increasing prevalence of maternal obesity has prompted research into its potential long-term health consequences on the offspring. A number of studies have linked maternal obesity during pregnancy with an increased risk of childhood and adulthood obesity and metabolic disorders among offspring (Catalano and Ehrenberg 2006; Catalano *et al.* 2009; Drake and Reynolds 2010). Maternal obesity has been associated with increased birth weight and fat mass as well as increased adiposity in childhood and adulthood (Parsons *et al.* 2001; Sebire *et al.* 2001; Whitaker 2004; Sewell *et al.* 2006; Reynolds *et al.* 2010; Harmon *et al.* 2011; Gaudet *et al.* 2012; Hochner *et al.* 2012). The increased birth weight that occurs in the children of obese women has itself been linked to an increased risk of obesity later in life, with macrosomia and maternal gestational weight gain being strong predictors of high BMI at 1 year of age, itself a predictor of weight status aged 5 to 8 years (Lindberg *et al.* 2012). While diabetes is common among obese women, the effects of maternal obesity on offspring weight status are thought to be independent of maternal diabetes, with increased adiposity also seen in the offspring of non-diabetic obese women (Sewell *et al.* 2006).

Maternal obesity has also been linked to insulin resistance in offspring. Offspring of obese mothers are more insulin resistant at delivery, and more likely to exhibit neonatal hyperinsulinaemia (Catalano *et al.* 2009; Group 2010). Offspring of overweight or obese women are also more likely to develop insulin resistance by age 11, as well as in their early 20s (Boney *et al.* 2005; Mingrone *et al.* 2008). A study conducted in Finland also suggested that higher maternal BMI during pregnancy also

contributes to an increased risk of death from coronary heart disease in the offspring (Forsen *et al.* 1997).

1.7.2 Programming versus Postnatal Obesogenic Lifestyle

In human studies of the programming effects of obesity, it has proven difficult to separate the pre- and post-natal influences on later offspring health beyond including lifestyle factors such as behaviour, level of sedentary activity and diet as confounding factors in statistical analyses. Indeed, as lifestyle factors such as diet and exercise habits do tend to cluster within families, the separation of prenatal programming effects from postnatal influences becomes an important consideration (Rogers 2003; Reilly *et al.* 2005; Branum *et al.* 2011). A number of investigators have attempted to separate pre- and post-natal influences through sibling/sibling studies and adoption studies, with conflicting findings (Branum *et al.* 2011; Lawlor *et al.* 2011). Sibling and twin studies allow the genetic contribution to traits such as obesity to be measured. Such studies allow for comparisons of individuals with similar familial environments and similar (or in the case of monozygotic twins, identical) genotypes. Classical twin studies compare monozygotic and dizogtic twins to determine the genetic contribution to traits of interest. If a specific trait is found to be more similar between monozygotic twins than in dizygotic twins (or non-twin siblings), it can be implied that the trait has a genetic basis. In the case of obesity, genetic factors have been suggested to explain between 20% and 90% of variance in offspring BMI, suggesting an important role for environmental factors in at least some instances (Maes *et al.* 1997). Studies involving twins report a genetic influence on obesity of between 50% and 90%, with the heritability of obesity increasing with

age from childhood to adolescence; genetic factors also appear to have a greater influence on levels of trunk fat compared with limb fat (Brook *et al*. 1975; Maes *et al*. 1997). In adults, however, hereditability of obesity appears to decrease with age, and is generally higher for males versus females (Korkeila *et al*. 1995; Maes *et al*. 1997). Adoption studies have shown that obesity among adopted adult offspring correlates less strongly with adoptive parents than with the biological parents (Price *et al*. 1987). However, twin studies have been criticized on the grounds that they may not be generalizable to the larger population, that there are often difficulties in determining whether twins are monozygotic or dizygotic, and that the assumption that environmental factors are identical for twins (Maes *et al*. 1997).

A particularly interesting approach to the problem of separating prenatal and postnatal influences on offspring obesity can be seen in a study by Barisione *et al*, in which women who underwent biliopancreatic diversion as a weight loss measure between pregnancies were observed (Barisione *et al.* 2011). Offspring born before the surgery were found to have significantly higher body weights by age 12 and in early adulthood compared to offspring born after the surgery, suggesting that maternal obesity may indeed have influences on offspring weight status which are independent of genetics, postnatal lifestyle and environmental factors.

1.7.3 Stress and the HPA Axis

Stress may be loosely defined as a state in which the internal homeostasis of an organism is disrupted by external factors (Peeke and Chrousos 1995). In the 1930s, Seyle proposed that the stress response was intended to accommodate such brief challenges to the body's homeostasis, with the ultimate result being a return to normal homeostatic equilibrium. The HPA axis forms a major component of the

stress response, along with the sympathetic nervous system, which is mediated largely through the action of the catecholamines: epinephrine and norepinephrine (Chrousos and Gold 1992; Peeke and Chrousos 1995).

Seyle also highlighted the potential for prolonged or chronic stress to lead to the manifestion of pathological disease states, which he termed 'diseases of adaptation' (Peeke and Chrousos 1995). Subsequently, a number of chronic conditions, including depression, anorexia nervosa, alcoholism, and seasonal affective disorder have been linked to disruption of normal HPA axis activity (Gold *et al.* 1986; Gold *et al.* 1988; Joseph-Vanderpool *et al.* 1991; Wand and Dobs 1991)

1.7.4 Acute and Chronic Stress

The HPA axis is involved in the response to both acute and chronic stress. In response to acute stress, catecholamines are rapidly secreted from the sympathetic nervous system. This is followed shortly by secretion of CRH by the hypothalamus, in turn leading to ACTH and cortisol production by the pituitary and adrenal glands (Lightman and Young 1988). Decreased hypothalamic release of GnRH and gonadotrophins is also observed, as are increased pituitary secretion of prolactin and GH and pancreatic secretion of glucagon. These changes allow for the diversion of energy resources to the musculature, increased cardiovascular tone, inhibition of energy storage, stimulation of immune function, inhibition of reproductive physiology, decreased appetite and feeding behaviour and increased cerebral glucose utilisation. These effects are characteristic of a 'fight or flight' response, whereby the physiology of the organism is shifted into a state where energy supply to the musculature is maximised for escape or combat, and cognition is enhanced, at the expense of digestion, reproduction and other processes.

In cases of chronic stress however, the response of the HPA axis is different. While levels of the ACTH-precursor pro-opiomelanocortin (POMC) are chronically raised in the anterior pituitary, mRNA levels of CRH in the paraventricular nucleus and levels of CRH in the blood are lower (Harbuz *et al.* 1992). This decline is paralleled by a rise in AVP mRNA levels and portal blood concentrations of AVP, indicating that the activation of the HPA axis in chronic stress is regulated predominantly by AVP rather than CRH (Chowdrey *et al.* 1995).

1.7.5 Maternal Stress and Programming

While glucocorticoids are of clear physiological importance during pregnancy, there are data to suggest that excess exposure to glucocorticoids is associated with reduced birth weight and adverse programmed health effects in the offspring, particularly if the exposure is in late pregnancy (Reinisch *et al.* 1978; French *et al.* 1999; Bloom *et al.* 2001). Animal studies have demonstrated a link between prenatal exposure to excess glucocorticoids late in gestation and altered adult behaviour, particularly coping and learning behaviours in aversive situations; treatment of pregnant rats with dexamethasone leads to reduced birth weight, permanent adult hypertension as well as hyperglycemia and hyperinsulinemia (Benediktsson *et al.* 1993; Nyirenda *et al.* 1998; Nyirenda *et al.* 2001; Sugden *et al.* 2001; Welberg *et al.* 2001; Seckl 2004).

There is evidence that stress and anxiety may also reduce birth weights and affect emotional behaviour (Khashan *et al.* 2008b). Offspring of women who report high levels of stress during pregnancy may have behavioural problems as toddlers, as well as impaired attention at 6 years of age (Gutteling *et al.* 2005; Gutteling *et al.* 2006). ADHD and increased anxiety are associated with prenatal maternal stress (Van den

Bergh and Marcoen 2004) (Rodriguez and Bohlin 2005b). MRI studies have also demonstrated that high anxiety during mid-gestation is associated with decreased grey matter density between 6 and 9 years of age, particularly in the prefrontal cortex, an area involved in cognition and regulation of the stress response (Diorio *et al.* 1993; Buss *et al.* 2010). Taken together, these studies suggest that prenatal stress can affect brain development and behaviour in the later life of the offspring. The importance of the timing of exposure also suggests that maternal stress may affect fetal development during critical windows of sensitivity: maternal stress during the first trimester, but not later in pregnancy, is associated with an increased risk of schizophrenia for example (Khashan *et al.* 2008a); in contrast, the risk of autism is elevated with exposure to maternal stress in mid to late gestation (Kinney *et al.* 2008).

Prenatal stress has also been associated with modifications to HPA axis function in the offspring. However, the results of studies have proven variable, possibly reflecting to the nature of the stressor. A study by Entringer *et al* found that severe prenatal life events were associated with lower cortisol but higher ACTH response to the Trier Social Stress Test, while a study by Yehuda *et al* found that cortisol levels were lower in infants born to mothers who suffered PTSD after exposure to the events of 9/11 (Yehuda *et al.* 2005; Entringer *et al.* 2009). Other studies however have suggested that prenatal stress and depressed mood may be associated with raised cortisol levels and increased reactivity in offspring (Gutteling *et al.* 2004; Gutteling *et al.* 2005; O'Connor *et al.* 2005; Brennan *et al.* 2008; Grant *et al.* 2009). Different forms of anxiety or stress may involve different psychological processes, possibly explaining the varied responses to prenatal mood (Glover *et al.* 2010).

A number of studies have attempted to address whether maternal stress associates with altered maternal glucocorticoid levels. Evans *et al*. reported elevated cortisol in pregnant women with co-morbid anxiety or depression, but not in women with anxiety or depression alone (Evans *et al.* 2008). Other studies have demonstrated only modest associations between maternal cortisol and stress and state anxiety (Diego *et al.* 2006; Sarkar *et al.* 2006). Mothers exposed to traumatic life events or high anxiety may display alterations to their circadian production of cortisol however, with one study suggesting that evening cortisol levels may rise in response to such conditions, and a second suggesting that trait anxiety is associated with a flattened afternoon decline in cortisol (Obel *et al.* 2005; Kivlighan *et al.* 2008).

HPA axis reactivity appears to be diminished during pregnancy: women administered CRH tests during late pregnancy were found to lack an ACTH or CRH response, and pregnant women exposed to simple physical stressors in late pregnancy were also found to lack a cortisol response (Schulte *et al.* 1990; Kammerer *et al.* 2002). This may help explain the low correlation between maternal stress and circulating glucocorticoids seen in other studies. As maternal cortisol production increases near term, under the influence of placental CRH, the maternal adrenal cortex may have limited capacity to respond to stress. Nevertheless, even in studies where the association between maternal anxiety and child outcome is weak, maternal cortisol levels have been found to predict infant outcomes, suggesting that the mother's HPA axis may be able to influence fetal development (Gutteling *et al.* 2006).

1.8 Programming and the Placenta

The placenta is likely to be an important site of mediation for programming effects. This organ develops early in pregnancy, and plays crucial roles in regulating proper fetal development, endocrine function, metabolism, nutrient exchange and also works as a barrier and filter against a number of potential toxins in the maternal bloodstream. Placental function is an important factor in fetal growth and development. The balance between placental and fetal growth, and any developmental compromises of each relative to one another, is also likely to be an important component of programming effects.

1.8.1 Placental Structure and Development

The placenta essentially comprises an inner network of vasculature surrounded by an outer epithelium, or trophoblast (Cross 2000). The trophoblast layer is highly differentiated, with numerous cell subtypes that have specialized endocrine, immunological and transport functions. Among these subtypes are cells that fuse into a syncytium termed the syncytiotrophoblast. In order to maximize the surface area over which nutrient exchange can take place, the conceptus must initiate intimate contact with the maternal bloodstream through extensive invasion of the lining of uterus. This involves a number of complex processes occurring throughout much of pregnancy.

The first stage of placental development involves decidualisation of the uterus, a process involving the conversion of uterine stromal cells to large, secretory decidual cells, as well as the recruitment of macrophages and lymphocytes (Malassine *et al.*

2003). In humans, rapid trophoblast proliferation takes place shortly after blastocyst adhesion (Malassine *et al.* 2003). The trophoblast cell lineage first appears at the blastocyst stage, just prior to implantation, as a simple epithelium termed the trophectoderm. Differentiation at this stage is determined in two ways: location and gene expression. Only cells in the outer layer of the blastocyst will form the trophoblast lineage, while cells on the interior differentiate into the inner cell mass. Furthermore, trophoblast lineage cells discontinue expression of the transcription factor *OCT4* at the time of differentiation, while ICM cells continue to express it (Cross 2000). By day 21 of human pregnancy, the definitive structure of the placenta is apparent as the chorionic villus becomes established; and the trophoblast further differentiates into villous and extravillous trophoblast (Malassine *et al.* 2003).

The villous phenotype involves the cytotrophoblastic cells of the floating villi remaining attached to the basement membrane to form an epithelial cell monolayer (Malassine *et al.* 2003). It is these cells that will, through proliferation and fusion, form the syncytiotrophoblast, a multinucleated cell layer that covers the entire surface of the villus. While the primary function of the syncytiotrophoblast is absorption and exchange, it also has important hormonal functions.

The extravillous trophoblast is formed as cytotrophoblasts of the anchoring villi in contact with the uterine wall begin to proliferate, detaching from the basement membrane and forming columns of cells that invade the uterine wall. Invasion takes place through two routes: the first involves invasion via the uterine stroma as trophoblastic cells migrate towards the decidual arterial wall, while the second involves infiltration of the lumens and walls of the spiral arteries by extravillous trophoblastic cells, leading to complete remodeling of the spiral artery wall and the

destruction of the muscle layer and replacement of the endothelial layer with trophoblastic cells (Pijnenborg *et al.* 1981; Kaufmann *et al.* 2003). In order to protect the developing fetus from excess oxygen early in gestation, endovascular trophoblasts form intra-arterial plugs, which prevent maternal blood from entering the intervillous space until the 12th week of gestation. Defects in arterial remodelling are thought to be involved in the development of pre-eclampsia.

The placenta is a glucocorticoid responsive organ: glucocorticoids are key regulators of the production of a number of substances in the placenta, including human chorionic gonadotrophin (hCG), CRH as well as glucose transporters (Jones *et al.* 1989a; Ringler *et al.* 1989; Hahn *et al.* 1999; Lee *et al.* 2005). Glucocorticoids are also crucial for the expression of extracellular matrix proteins by placental fibroblasts, and are thus important for placental growth and integrity (Lee *et al.* 2004).

1.8.2 Placental function

The primary function of the placenta is nutrient exchange, and the major determinant of intrauterine growth is placental nutrient supply (Fowden *et al.* 2006a). Placental weight, used as a surrogate measure of the surface area of the maternal-fetal interface, has been found to correlate with fetal weight in a number of species (Fowden *et al.* 2009). The nutrient transfer capacity of the placenta is dependent upon a number of factors, including size, morphology, transporter abundance and blood supply (Fowden *et al.* 2006a; Fowden *et al.* 2006b; Fowden *et al.* 2009). If any of these factors are altered, intrauterine growth may be affected (Fowden *et al.* 2006a; Jones *et al.* 2007). Expression of a number of key placental nutrient

transporters, including System A Amino Acid Transporters 1 and 2 (*SNAT1* and *SNAT2*), are upregulated in the placentas of rats fed high fat diets; furthermore, expression of these nutrient transporters is increased in human placental trophoblast cells when exposed to inflammatory cytokines such as IL-6 and TNF-α, suggesting that inflammation may increase placental transport (Jones *et al.* 2009a; Jones *et al.* 2009b). Cortisol has also been shown to stimulate SNAT2 expression in human placental cells, suggesting that alterations in maternal HPA activity may influence nutrient availability to the offspring (Jones *et al.* 2006).

The placenta also has important endocrine functions, many of which influence fetal growth and pregnancy outcome (Fowden *et al.* 2009). In humans, the placenta produces the steroid hormones estrogen and progesterone, as well as CRH and hCG (Shibasaki *et al.* 1982; Pepe and Albrecht 1995).

1.8.3 The Programming Effects of Placental 11βHSD2

A number of studies have highlighted the potential role that 11βHSD2 may play in mediating programming effects. Downregulation of placental 11βHSD2 may lead to an increased fetal exposure to maternal cortisol, with potential effects on development. Pharmacological blockage of 11βHSD2 by carbenoxolone during pregnancy has been associated with an increase in amygdalar glucocorticoid receptor mRNA expression and increased basal corticosterone in mice (Welberg *et al.* 2000). Genetic knockout of the gene encoding the enzyme in pregnant mice resulted in increased anxiety in the adult offspring, although corticosterone levels did not differ from controls (Holmes *et al.* 2006).

In humans, reduction in placental 11βHSD2 has been found in a number of complications of pregnancy, including pre-eclampsia, preterm birth and IUGR (McCalla *et al.* 1998; Murphy *et al.* 2002b; Kajantie *et al.* 2006; Dy *et al.* 2008). Birth weight and placental size have been shown to correlate with reduced placental *11βHSD2* (Stewart *et al.* 1995; Murphy *et al.* 2002a). Congenital 11βHSD2 deficiency has also been associated with low birth weight and increased risk of hypertension (Edwards *et al.* 1993; Kotelevtsev *et al.* 1999).

Maternal prenatal stress has been shown to affect placental 11βHSD2 activity and mRNA transcript levels. Pregnant rats exposed to stressors near the end of pregnancy have been found to have reduced levels of placental 11βHSD2 mRNA and activity; offspring were smaller and had smaller adrenals (Mairesse *et al.* 2007). Chronic stress may have particular effects: while acute stress in rats has been associated with increased 11βHSD2 activity, chronic stress did not alter 11βHSD2 activity (Welberg *et al.* 2005). Crucially, rats previously exposed to chronic stress did not show increased placental 11βHSD2 activity in response to an acute stressor, suggesting that chronic stress may prevent the potentially protective upregulation in 11βHSD2 (Welberg *et al.* 2005). In humans, maternal stress may increase the permeability, or 'leakiness', of the 11βHSD2 barrier as indicated by a strong positive correlation between maternal and fetal amniotic cortisol concentrations in women with high anxiety (Glover *et al.* 2009). A stress-induced downregulation of 11βHSD2 may allow more cortisol to cross the placenta into the fetal compartment, resulting in decreased fetal ACTH, impaired fetal adrenal growth and lower birth weight and immaturity.

Maternal diet may also affect the activity of placental 11βHSD2. Reducing protein intake by 50% in pregnant rats has been shown to reduce activity of 11βHSD2 by 33%, along with reduced birth weight and increased blood pressure at 7 weeks in the offspring (Langley-Evans *et al.* 1996a; Langley-Evans *et al.* 1996b). Similar effects have been observed in sheep (Lesage *et al.* 2001a; Whorwood *et al.* 2001). The effects on blood pressure were reversed when maternal glucocorticoid production was blocked with metyrapone (Langley-Evans 1997). These findings suggest that maternal diet may influence fetal growth and development via alterations to placental 11βHSD2 and the fetal HPA axis, potentially leading to programming effects.

A recent study by Cottrell *et al* found that pregnant mice fed a low protein diet through pregnancy showed elevated fetal glucocorticoids and reduced placental growth in mid-gestation, with reduced fetal growth near term; taken together, these results suggest that maternal diet can impact fetal glucocorticoid exposure and HPA axis function (Cottrell *et al.* 2012). However, the study also found that despite a fall in 11βHSD2 activity near term, activity was higher earlier in gestation, suggesting that the observed increase in fetal glucocorticoids may be independent of changes in placental 11βHSD2 (Cottrell *et al.* 2012). The study also raises the possibility that the fetal HPA axis takes the primary role in regulating fetal exposure to glucocorticoids and maturation and growth rates in situations of limited nutritional availability.

In light of the lack of evidence for a correlation between maternal stress and increased cortisol levels during pregnancy, an alternative mechanism has been proposed which will still result in fetal glucocorticoid overexposure: maternal stress

has been shown to downregulate expression of the placental glucocorticoid barrier enzyme 11βHSD2, leading to increased fetal exposure to maternal glucocorticoids independent of alterations in maternal HPA axis activation (O'Donnell *et al.* 2012).

1.9 Epigenetics

The term 'epigenetics' was coined in 1942 by Conrad Waddington to describe how genotype regulates development, with the epigenetic 'landscape' determining the eventual fate of genetically homogenous cells through differentiation (Holliday 2006). However, the term is now more commonly used to describe changes in gene function without alterations to the DNA sequence (Jaenisch and Bird 2003). A number of epigenetic marks are thought to mediate this effect, including DNA methylation, histone modifications and non-coding RNAs (Reik and Walter 2001; Jaenisch and Bird 2003; Holliday 2006; Bird 2007).

DNA methylation, an important regulator of gene transcription, involves the addition of a methyl group to a cytosine, and most commonly occurs at cytosines in cytosine-guanine (CpG) dinucleotides (Jaenisch and Bird 2003). In humans, 60 to 90% of all CpGs are methylated, depending on cell type (Ehrlich *et al.* 1982). DNA methylation generally inhibits the expression of genes in two ways: by physically blocking the binding of transcription factors and/or through the binding of methyl-CpG-binding domain proteins (MBDs) (Wade 2001; Jaenisch and Bird 2003; Fatemi and Wade 2006; Choy *et al.* 2010). MBDs recruit histone deacetylases and other proteins involved in chromatin remodelling, leading to the formation of inactive heterochromatin (Wade 2001; Jaenisch and Bird 2003).

Environmental cues may influence DNA methylation, particularly during critical periods of development (Jaenisch and Bird 2003). In a recent study of the Dutch Hunger Winter, a severe wartime famine in the Netherlands at the end of the Second World War, adulthood DNA methylation of the gene *IGF2* was found to be lower among individuals who were prenatally exposed to the famine compared to their unexposed siblings (Heijmans *et al.* 2008). Exposure to the famine was also associated with increased risk of impaired glucose tolerance, obesity, coronary heart disease and hypertension (Kyle and Pichard 2006). Alteration to DNA methylation at birth has also been suggested to predict later health: increased methylation of *GR* in neonatal cord blood has been associated with increased stress responsiveness at three months (measured using salivary cortisol obtained before and after a non-noxious stressor) itself a marker of later life disease (Oberlander *et al.* 2008).

DNA methylation is especially important in the regulation of transcription of imprinted genes. These are genes that are expressed monoallelically in a parent-of-origin specific manner. A number of disorders of human pre- and postnatal growth are associated with aberrant imprinting, including Beckwith-Wiedemann syndrome (which is associated with prenatal overgrowth) and Silver-Russell syndrome (associated with IUGR). *IGF2* is an imprinted gene of crucial importance in mediating normal prenatal growth, and is expressed solely from the paternally inherited allele (Constancia *et al.* 2002) (Giannoukakis *et al.* 1993). Other key imprinted genes include the maternally-expressed gene *PHLDA2*, increased placental expression of which has been associated with low birth weight and IUGR, and

maternally-expressed *CDKN1C*, also associated with IUGR (McMinn *et al.* 2006; Apostolidou *et al.* 2007).

1.9.1 IGF2

IGF2 is a paternally expressed gene in both the fetus and all zygote-derived cell types in the placenta, and acts as a growth factor during development (Reik *et al.* 2003). In the placenta, *IGF2* is a major regulator of placental growth, acting as a potent growth factor; knockout of *IGF2* in murine placentas leads to reduced growth of the placenta as well as fetal growth restriction (DeChiara *et al.* 1990; Constancia *et al.* 2002; Reik *et al.* 2003). In small for gestational age children, placental expression of *IGF2* is lower compared to controls at term (Guo *et al.* 2008).

The epigenetic control of *IGF2* expression is unusual, as silencing of *IGF2* is not a direct result of methylation of the maternal allele. In humans, imprinting of *IGF2* is controlled by CG-rich differentially methylated regions (DMRs), including the *IGF2* DMR0 located between exons 2 and 3, *IGF2* DMR2, located between exons 8 and 9, and the *H19* DMR, located 4kb upstream of the non-protein coding gene *H19* (Dejeux *et al.* 2009). Methylation of *IGF2* in placental samples, particularly at DMR 0 and DMR 2, correlates with neonatal birth weight in humans (St-Pierre *et al.* 2012). Studies involving the children of the Dutch Hunger Winter found that prenatal exposure to famine conditions was associated with lower DNA methylation of *IGF2*, suggesting that methylation of the *IGF2* DMRs may modulate birth weight in a diet-dependent manner (Heijmans *et al.* 2008).

The *H19* DMR is responsible for establishing the imprinting of both the paternally-

expressed *IGF2* as well as that of the maternally-expressed *H19* (Leighton *et al.* 1995; Ripoche *et al.* 1997; Thorvaldsen *et al.* 1998; Szabo *et al.* 2004). The activity of this DMR is dependent upon its methylation status (see Figure 1-5). The DMR is hypomethylated on the maternal chromosome, and is able to bind the protein CTCF (Bell and Felsenfeld 2000). This binding blocks the interaction of the *IGF2* promoter with downstream enchancers, leading to silencing of *IGF2* (Schoenherr *et al.* 2003; Szabo *et al.* 2004). Hypomethylation of the DMR, and CTCF binding, is necessary for the initiation of maternal *H19* expression (Thorvaldsen *et al.* 2006). On the paternal allele, the DMR is hypermethylated, a condition that is maintained throughout development (Tremblay *et al.* 1997). This not only silences *H19* expression, but also prevents CTCF binding, in turn allowing the paternal *IGF2* to access downstream enhancers and be expressed (Bell and Felsenfeld 2000; Engel *et al.* 2006).

IGF2 is further regulated by a gene expressed from the maternal allele, the insulin-like growth factor 2 receptor (*IGF2R*). IGF2R targets IGF2 proteins for degradation, thereby attenuating the signalling effects of IGF2 (Wang *et al.* 1994; Coan *et al.* 2005). This interaction is characteristic of the 'conflict theory', whereby the paternal genome drives feto-placental growth (to ensure healthy offspring) while the maternal genome constrains it (thus protecting the health of the mother).

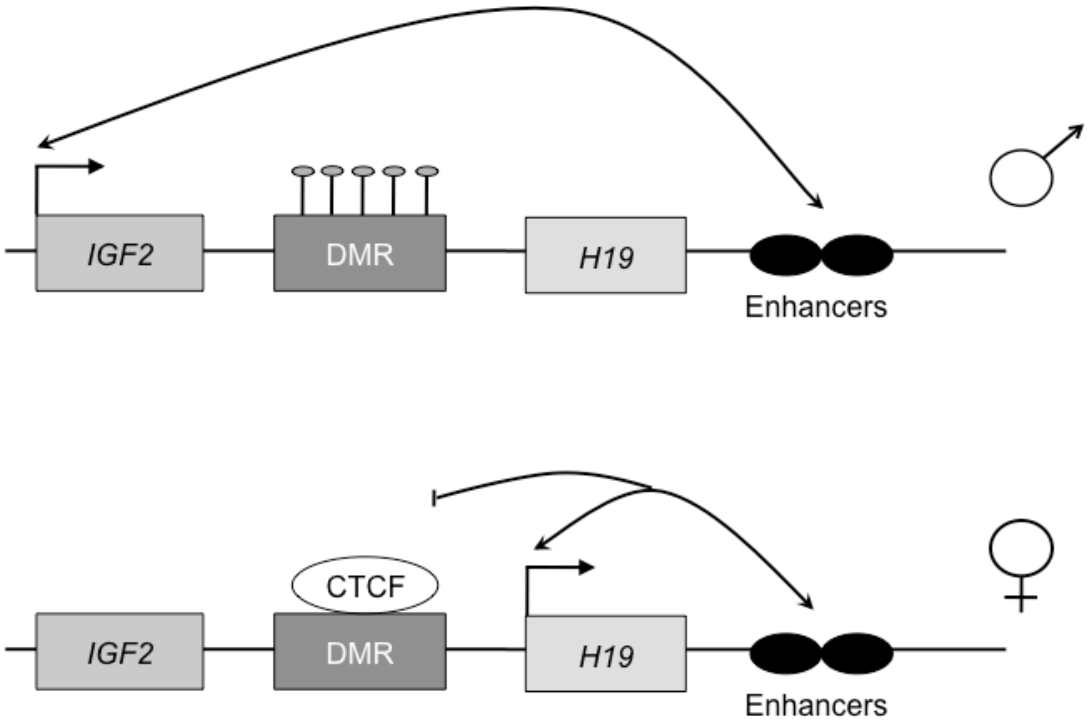


Figure 1-5. Imprinting regulation of *H19* and *IGF2* on paternal and maternal chromosomes by the *H19* DMR. Filled lollipops indicate CpG methylation, black arrows indicate interaction between enhancers and genes. *IGF2* DMR0, located upstream of *IGF2*, is not shown. Adapted from Engel, Thorvaldsen and Bartolomei, 2006.

1.9.2 Methylation and the regulation of glucocorticoid exposure: GR and 11βHSD2

A number of studies have attempted to address the possible role of methylation of GR and 11βHSD2 in pregnancy outcome.

Transcription of the gene encoding GR is regulated in part by a complex 5' structure, made up of a number of alternative untranslated first exons that are spliced onto a common acceptor site in exon 2 (McCormick *et al.* 2000; Presul *et al.* 2007; Turner *et al.* 2010). These alternative first exons are located in two promoter regions: a proximal promoter region approximately 5kb upstream of the translation start site, and a distal promoter region more than 30kb further upstream (Presul *et al.* 2007). These alternative first exons are thought to regulate *GR* expression in a tissue-specific manner, with the majority of placental GR derived from the GR 1C promoter (Johnson *et al.* 2008a).

Studies have suggested that methylation of the GR promoter is affected by maternal stress in the early-life environment, potentially leading to programming effects (Mueller and Bale 2008; Oberlander *et al.* 2008; Turner *et al.* 2008; Turner *et al.* 2010; Cao-Lei *et al.* 2011; Hompes *et al.* 2013). There is also evidence to suggest a role for GR methylation in predicting birth weight: increased methylation of the GR promoter in the placenta, predicted to result in lower expression of GR and thus reduced glucocorticoid signalling, has been linked to higher birth weight (Filiberto *et al.* 2011). Furthermore, a small study demonstrated a link between maternal depression/anxiety and increased methylation of the GR promoter in neonate cord blood, itself associated with increased stress response 3 months postnatally, a marker of later life disease (Oberlander *et al.* 2008).

The regulation of 11βHSD2 transcriptional activity may be an important determinant in pregnancy outcomes, including birth weight. Increased methylation of the 11βHSD2 promoter is associated with lower transcription, and increased methylation of this gene in the placenta has also been shown to associate with lower birth weight (Alikhani-Koopaei *et al.* 2004; Marsit *et al.* 2012). Increased placental 11βHSD2 methylation was associated with altered newborn behaviour with reduced quality of movement scores (Marsit *et al.* 2012).

Few studies have examined the effects of maternal obesity on methylation of either GR or 11βHSD2 during pregnancy. However, a study by Drake *et al* found that both 11βHSD2 and GR methylation positively associated with increased adiposity and blood pressure in buffy coat samples taken from adults whose mothers had consumed unbalanced diets during pregnancy; importantly, methylation of GR was increased in the offspring whose mothers had the most unbalanced diets, suggesting that nutrient intake may affect glucococorticoid exposure and sensitivity via alterations in DNA methylation (Drake *et al.* 2012).

Aims and Hypothesis

This thesis aims to test the hypothesis that in humans the programming effects of maternal obesity and maternal stress are mediated by altered action of glucocorticoids on the developing fetus.

In chapter 3, the effects of maternal obesity on the maternal HPA axis are explored through the measurement of circulating cortisol, CBG and CRH throughout pregnancy in a cohort of very severely obese and lean women. The relationships between maternal HPA axis activity, maternal weight gain and birth outcomes are investigated.

In chapter 4, whether maternal obesity is associated with altered placental expression of genes regulating fetal glucocorticoid exposure and fetal/placental development in each trimester of pregnancy is explored.

In chapter 5, pyrosequencing is used to test whether differences in gene mRNA levels associated with maternal obesity in the placenta are explained by differences in DNA methylation.

Finally, in chapter 6, the effects of maternal stress and anxiety on placental expression of genes regulating glucocorticoid exposure are explored in two prospective cohorts of women who completed stress and anxiety questionnaires during pregnancy.

Chapter 2:

Materials and Methods

2.1 Clinical Methods

2.1.1 Longitudinal study of very severely obese pregnancy

2.1.1.1 Subject Recruitment

The University of Edinburgh and NHS Lothian Hospitals Trust established an Antenatal Metabolic Clinic (AMC) in Edinburgh, with support from Tommy's the Baby Charity (www.tommys.org), to care for pregnant women who are very severely obese at antenatal booking (i.e. with a BMI >40kg/m^2). The main research focus of the clinic is to understand problems that arise during pregnancy due to maternal obesity. Women attending this clinic receive normal antenatal care and are also seen by a specialist dietician who provides them with personalised advice about healthy eating during pregnancy. A lean group (with a BMI between 20 and 25kg/m^2) was recruited in parallel. These women received their antenatal care in the community but attended the research clinic for research visits. Exclusion criteria included pre-existing type 1 or type 2 diabetes mellitus and non-singleton pregnancy. All women participating in the research study are characterised in terms of BMI, stress and anxiety levels and blood pressure throughout pregnancy. The ethical approval for this study was obtained from the NHS Lothian Research Committee (REC reference number 08/S1101/39). Written informed consent was obtained from all participants.

2.1.1.2 Assessing Maternal Mood and Anxiety

Participants were asked to complete validated questionnaires to assess mood (including stress at home, work, stress over money, stress over life events, satisfaction with life and anxiety) as well as the Spielberger State and Trait Anxiety

Inventory. Maternal anxiety was measured using the Spielberger State and Trait Anxiety Inventory (STAI), commonly used to measure acute situational anxiety ('state' anxiety) as well as anxiety as a general, long term personality trait ('trait' anxiety). The test consisted of 25 items including both positive (e.g. 'I feel calm') and negative (e.g. 'I feel exhausted') emotions, each scored using a four-point scale ranging from 'Never' (score 1) to 'Always' (score 4). Questionnaires were completed at Visit 1 (~16 weeks gestation) and at Visit 2 (~28 weeks gestation).

2.1.1.3 Sample Collection

Fasting morning blood samples were taken; height and weight were measured by nurses at three time points during pregnancy: 16 weeks gestation ('visit 1'), 28 weeks gestation ('visit 2'), and 36 weeks gestation ('visit 3'). Participants were also invited to attend a follow-up visit at 3 months postnatal. Samples were immediately centrifuged and serum and plasma stored at -80C for subsequent analysis. Three saliva samples were taken at visit 1, one at bedtime, a second at waking, and a third 30 minutes after waking. Samples were centrifuged and stored at -20C for subsequent analysis.

2.1.2 First Trimester Placenta Study

Pregnant women with a healthy singleton pregnancy who underwent surgical termination of pregnancy during the first trimester were eligible for the study.

The ethical approval for first trimester tissue study was obtained from the NHS Lothian Research Committee (LREC/04/S1103/20 (F)). Written informed consent was obtained from all participants. Patients with diabetes mellitus, patients receiving

corticosteroid-based medications, and patients who smoked were excluded from this study.

2.1.2.1 Tissue Collection

First trimester placental samples were collected from surgical terminations of pregnancy conducted between weeks 8 and 12 of gestation (mean gestation: 10 weeks). Trophoblast tissue was separated from decidua and fetal tissues immediately following termination, and placed into RNAlater solution at 4˚C for 24 hours before freezing at -80˚C. Tissues for immunohistochemistry were collected and stored in 4% neutral buffered formalin solution for 24 hours before transfer to ethanol.

2.1.3 Second Trimester Placental and Liver Study

Pregnant women with a healthy singleton pregnancy who underwent termination of pregnancy during the second trimester were eligible for the study. Written informed consent was obtained from all participants prior to tissue collection.

2.1.3.1 Tissue Collection

Second trimester placental and liver samples were collected from terminations of pregnancy conducted between weeks 12 and 16 of gestation.

Approximately 30mg of tissue from the fetal side of the placenta was collected and stored in RNAlater solution at 4˚C for 24 hours before freezing at -80˚C. Tissues to be used for immunohistochemistry were collected and stored in 4% neutral buffered formalin solution for 24 hours before transfer to ethanol.

2.1.4 Term placental samples

Term placenta samples (n = 60) were collected from healthy singleton term pregnancies after elective caesarean delivery as part of the Edinburgh Reproductive Tissue BioBank (http://www.crh.ed.ac.uk/biobank/). A single large biopsy was obtained from each placenta, equidistant between the edge of the placenta and the umbilical cord, avoiding areas of obvious infarction or damage. Biopsies were used for both gene expression and histology specimens. Placenta samples were stored in RNAlater solution for 24 hours prior to freezing at -80°C. Tissues for immunohistochemistry were collected in 4% neutral buffered formalin solution within 2 hours of birth, and stored for 24 hours before transfer to ethanol.

2.1.5 PREDO Stress In Pregnancy Study

2.1.5.1 Subject Recruitment

The placental samples for this work were collected by our collaborator Professor Katri Raikonnen of the University of Helsinki, Finland. Participants in this study were recruited as part of the multidisciplinary PREDO-Project ('Prediction and Prevention of Pre-eclampsia'). The PREDO study is composed of two arms, both of which provided placental samples for the gene expression study. The first arm comprised 947 pregnant women at high risk for pre-eclampsia and 117 pregnant control women without known pre-eclampsia risk factors. The recruitment of these women took place between September 2005 and December 2009, when the women attended their first ultrasound screening (between 12 and 14 weeks of gestation) in one of the ten hospital maternity clinics participating in the PREDO Project (the Women's Hospital, Kätilöopisto Maternity Hospital and Jorvi Hospital at Helsinki

University Central Hospital, Kanta-Häme Central Hospital, Päijät-Häme Central Hospital, Tampere University Hospital, Kuopio University Hospital, Northern Karelia Central Hospital and Iisalmi Hospital). The inclusion and exclusion criteria, and the aspirin trial embedded in the study design of the first study arm, are described in Villa et al 2013. A further 4171 mothers were recruited for the epidemiological arm of the PREDO project between April 2007 and February 2010 while attending their first ultrasound screening (between 12 and 14 weeks of gestation) in one of the five hospital maternity clinics participating in the PREDO Project (Women's Hospital, Kätilöopisto Maternity Hospital and Jorvi Hospital at Helsinki University Central Hospital, Kanta-Häme Central Hospital, or Päijät-Häme Central Hospital). Written informed consent was obtained from all participants in both study arms.

Maternal age, smoking status, parity, occupation, pre-pregnancy body mass index (BMI), and medical/obstetric history were derived from hospital birth records and the Finnish national birth register. Education and alcohol consumption during pregnancy were self-reported.

2.1.5.2 Assessing Maternal Mood and Anxiety

All participants of the PREDO study were invited to complete a set of standardized and validated questionnaires, aimed at measuring stress, depression, anxiety and anger throughout pregnancy at two-week intervals beginning between 12 and 14 weeks of gestation, and ending two weeks postpartum. Questionnaires took approximately 30 to 40 minutes to complete each time, and the mothers returned the questionnaires after the final assessment. Maternal anxiety was measured using the Spielberger State and Trait Anxiety Inventory (STAI), commonly used to measure

acute situational anxiety ('state' anxiety) as well as anxiety as a general, long term personality trait ('trait' anxiety). Anxiety was assessed using the STAI every two weeks, at 14 time points during pregnancy beginning approximately at 12+0 to 13+6 weeks+days of gestation. The mean scores for first, second and third trimesters, as well as the mean score for pregnancy overall, were calculated and used in subsequent analyses. Scores for the positive and negative emotion subscales, as well as scores for the questions related to curiosity, were also calculated, and the mean values for each trimester and over the entirety of pregnancy were used in analyses.

Maternal personality traits were measured using the NEO Personality Inventory at the first assessment, between approximately 12 and 14 weeks of gestation. Personality was assessed by 96 questions measuring Neuroticism and Extraversion. Each item was measured using a five-point scale, ranging from 'Strongly disagree' (score 0) to 'Strongly agree' (score 4). Anxiety was measured as a subcomponent of 'neuroticism' with 8 questions.

2.1.5.3 Sample Collection

Placenta samples (n = 67) were collected from healthy singleton term (37 to 42 weeks of gestation) pregnancies a maximum of 90 minutes after vaginal (n = 48) or caesarean delivery (n = 19). Given the heterogeneity of the placenta, 9-site biopsies were obtained avoiding areas of obvious infarction or damage. Placenta samples were collected in RNAlater solution and stored at -20°C.

2.2 Materials

2.2.1 General Chemicals

Unless otherweise stated, all chemicals were purchased from Sigma (see Appendix for supplier addresses).

TaqMan Gene Expression Assays AmpliTaq Gold PCR master mix	Applied Biosystems
GelRed Nucleic Acid Gel Stain	Biotium, Inc
Liquid DAB+ Chromogen System	Dako
Cortisol Radioimmunoassay, 96 tests CBG Radioimmunoassay, 96 tests	DIASource
1 kB DNA ladder NuPage LDS Sample Buffer NuPage Transfer Buffer NuPage MES SDS Running Buffer NuPage Sample reducing agent NuPage 4-12% Bis-Tris Gels Oligo Primers	Invitrogen
Streptavidin Sepharose Beads	GE Healthcare
IRDye goat anti-rabbit 800CW IgG	Li-Cor Biosciences
SeaKem LE Agarose PBS	Lonza
100bp DNA ladder	New England Biolabs
dNTPs RNAseIn Random Primers Taq DNA Polymerase 1kB DNA ladder Reverse Transcriptase	Promega
RNeasy Mini Kit RNase-free DNase set PyroMark Q24 Reagents kit PyroMark Wash Buffer Epitect Bisulfite Kit	Qiagen
LightCycler 480 Probes Master Universal Probe Library Probes	Roche Applied Science

2.2.2 Equipment

Equipment	**Manufacturer**
Agilent 2100 Bioanalyser	Agilent Technologies
Graduated microtubes 2.0ml	Anachem Ltd
Power Pack 200 agarose gel system	Bio-Rad
BioMat Class II microbiological Safety cabinet	Contained Air Solutions
Eppendorf 5415 R centrifuge Eppendorf 5810 R centrifuge	Eppendorf
GS-1 Thermal Cycler	G-Storm
XCell SureLock Mini-Cell Electrophoresis System Nitrocellulose Membrane Filter Paper Sandwich pore size 0.45 um	Invitrogen
Wetzlar 1512 rocking microtome	Leitz
Odyssey Infrared Imager	LI-COR Biosciences
Milli-Q integral water purification system	Millipore
OPTImax tunable microplate reader	Molecular Devices
Nanodrop ND-1000	Nanodrop Technologies
0.5ml, 1.5ml tubes	Sarstedt
Surgical Instruments	Sigma Aldrich
0.2 ml strip-tubes with lids	StarLab
Disposable Scalpels	Swann-Morton
Shandon Sequenza racks MB35 Premier microtome blades Microscopic slides Cover Glass Round 22x22mm Thickness no.1 Sterlilin Petri dishes	Thermo-Scientific
UviPro system	UviTec
Zeiss Axioscop Microscope	Zeiss

2.2.3 Software

Equipment/applicaton	Software
Agilent 2100 Bioanalyser	2100 Expert Software (Agilent Technologies)
LightCycler 480	LightCycler 480 release 1.5 O SP3 (Roche)
Nano-Drop ND-1000 spectrometer	ND-1000 v3.3 (Nanodrop Technologies)
Odyssey Infrared Imager	Odyssey v3.0 (LI-COR Biosciences)
Statistical analysis	Minitab 16 (Minitab) GraphPad Prism 5 (GraphPad) SPSS (IBM)
PyroMark Q24	Q-CpG (Biotage/Qiagen)
Zeiss Axioscop Microscope	KSM 300 3.0 (Zeiss)

2.2.4 Solutions and Buffers

All solutions were prepared with Milli-Q water unless stated otherwise.

Acid alcohol: 1% v/v hydrochloric acid (37% HCl) in 74OP

Blocking buffer (western blot): 5% w/v Blotting-grade non-fat dry milk in TBST

Citrate buffer (10X, immunohistochemistry): 0.1M citric acid, pH 6

DEPC-water: 1 drop of diethylpyrocarbonate (DEPC) per 100ml, leave for 1-24h, autoclave

DNA ladder: 25% v/v 1kb DNA ladder (Promega), 25% v/v TE buffer, 50% v/v Orange G loading dye

DNA/RNA loading buffer: 0.3% w/v Orange G, 40% v/v glycerol

Lysis buffer (SDS-PAGE): 25 mM HEPES, 68.5 mM NaCl, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM NaF, 1 mM EDTA, 5 mM sodium pyrophosphate, 1% NP-40, 10% glycerol, 1X PIC

Phosphate buffered saline (PBS): 1 PBS tablet per 200 ml

PIC (Protease Inhibitor Cocktail, 25X): Tablet of complete protease inhibitor cocktail dissolved in 2ml

Running buffer (SDS-PAGE): 1X NuPAGE MES SDS running buffer (Invitrogen), 0.05% v/v NuPAGE® Antioxidant

Scott's tap water: 24 mM sodium bicarbonate, 166 mM magnesium sulphate in tap water

TBE (10x): 0.9M Tris, 0.9M boric acid, 12.5 mM EDTA

TBS (10X): 0.2M Tris, 1.37M NaCl; pH 7.6

TBST: 0.1% v/v Tween-20 in 1xTBS

TE: 10 mM Tris pH 8.0, 1 mM EDTA

Transfer buffer (western blot): 1X NuPAGE Transfer buffer, 20% v/v methanol, 0.1% NuPAGE Antioxidant

Tris-Phosphate buffer: 0.1M Tris, 32 mM phosphoric acid; pH 7.8

2.3 Laboratory Materials and Methods

2.3.1 Quantitative Real Time PCR

The mRNA transcript abundance of specific genes of interest was quantified using qPCR. This involves the extraction of RNA from tissues, followed by reverse transcription to generate complimentary DNA (cDNA). The cDNA undergoes qPCR using specific primers (generally intron-spanning) and *Taq* DNA polymerase in order to amplify specific genes of interest. Quantification is achieved through the use of fluorescent probes. These probes incorporate a fluorescent reporter and a quencher. The close proximity between the two prevents detection of the reporter's fluorescence. As polymerisation of the cDNA takes place, the 5' to 3' exonuclease activity of *Taq* polymerase leads to the degradation of the probe, separating the reporter and the quencher. The separation of the reporter from the quencher results in an increase in fluorescence. By measuring the rate of change in fluorescence, the abundance of mRNA transcript can be determined. The mRNA transcript concentrations of genes of interest are assessed relative to the mRNA levels of reference, or control, genes, in order to control for reverse transcription efficiency.

2.3.1.1 RNA Extraction From Placenta and Liver

RNA extraction was performed using RNeasy mini kits from Qiagen, West Sussex, UK.

Approximately 30mg of tissue was excised from each sample on dry ice, and placed in a flat-bottomed 2ml microtube with 600µl of buffer RLT with 10µl/ml of b-mercaptoethanol, as described in the Qiagen RNeasy Mini Kit Handbook Animal

Tissues protocol. All samples were homogenised until no visible particles of tissue remained in the lysate. Samples were then stored on dry ice prior to RNA extraction.

Lysates were centrifuged for 3 minutes at 13,000rpm, and the supernatant transferred to a 2ml microtube, and the remainder (including pellets) discarded. 600µl of 70% ethanol was added to the homogenate and mixed thoroughly with a pipette. Up to 700µl of this solution was transferred to spin columns placed in 2ml collection tubes. The columns were centrifuged for 15 seconds at 10,000rpm. Flow-through was discarded. For samples with total volumes exceeding 700µl, the remainder (up to 700µl each time) was transferred to the spin columns and centrifuged for 15 seconds at 10,000rpm, and the flow-through was discarded.

350µl of Buffer RW1 was added to each spin column, and spin columns were then centrifuged for 15 seconds at 10,000rpm. Flow-through was discarded. 80µl of DNase I solution (Qiagen) was added to each sample, and left to incubate for 15 minutes at room temperature. After incubation, 350µl of Buffer RW1 was added to each sample, and samples were centrifuged for 15 seconds at 10,000rpm. Flow-through was discarded.

500µl of Buffer RPE (Qiagen) was added to each column, and columns were centrifuged for 15 seconds at 10,000rpm. Flow-through was discarded. A further 500µl of Buffer RPE was added to each column, and columns were centrifuged for 2 minutes at 10,000rpm. Flow-through was discarded. Each spin column was placed into a fresh 2ml collection tube, and centrifuged for 1 minute at 10,000rpm.

Spin columns were then placed in fresh 1.5ml microtubes. 35µl of RNase-free water was added to each column for elution of total RNA. Columns were centrifuged for 1 minute at 10,000rpm. The flow-through was then re-added to the spin columns, and

columns were once again centrifuged for 1 minute at 10,000rpm. Purified samples were stored at -80°C.

2.3.1.2 RNA Quantification

The RNA concentration and purity of all samples was assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, UK). The ND-1000 software calculated RNA concentrations in ng/µl using the Beer-Lambert law. 2µl of RNA was quantified by ultraviolet absorbance at wavelengths of 260nm, and purity was assessed by comparison to absorbance at 230nm (260/230 ratio) for contaminants such as salts, and 280nm (260/280 ratio) for contaminants including protein. Samples that were measured to have ratios between 1.8 and 2.1 were considered suitable for use.

2.3.1.3 RNA Quality Assessment

The integrity of RNA was confirmed by separating ribosomal RNA (rRNA) using electrophoresis in a 1% agarose/0.5xTBE (45mM Tris-borate, 1mM EDTA) gel with 0.1µl/ml Gel Red (Biotium, Hayward, CA, USA) at ~90V for 30 to 50 minutes. To prepare the gels, agarose was melted in TBE buffer using a microwave oven, and GelRed nucleic acid dye was added to the molten agarose (at 0.0005% concentration). Once cooled, the mix was poured into a tray. Samples were mixed with Orange G loading dye in a 1:5 ratio and loaded onto the gel together with a DNA ladder.

Gels were visualised under UV light (λ=260nm), and RNA integrity was determined by the presence of two clear bands corresponding to 28S and 18S RNA with no smearing. Smeared samples without clear bands were regarded as degraded RNA.

2.3.1.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was reverse transcribed using the Access RT-PCR system (Promega, Southampton, UK).

The measured concentration of total RNA in each sample was used to calculate the volume of nuclease free water needed to dilute the RNA concentration to 25ng/µl in a total volume of 12.7µl. The total RNA concentration of samples was then confirmed using a Nanodrop ND-1000 spectrophotometer as previously described, leaving a volume of 10.7µl.

Diluted samples were then incubated at 70°C for 10 minutes in a PCR machine (GS1, G-Storm, Somerset, UK). Reagents were added to each sample as follows:

4µl of $MgCl_2$ (5mM)
2µl of 10x AMV RT Buffer
2µl of 1mM dNTP mixture
0.5µl of Recombinant RNasin Ribonuclease Inhibitor (1u/µl)
0.6µl of Avian Myeloblastosis Virus (AMV) RT polymerase enzyme (15u/µg)
0.2µl of oligo dT mix

Two negative controls, one with no RNA and a second with no AMV-RT enzyme, were used to ensure that reagents were not contaminated and contained no DNA.

Samples were incubated at room temperature for 10 minutes for annealing and extension, then incubated at 42°C for 15 minutes, then at 95°C for 5 minutes. Samples were then stored at -20°C.

2.3.1.5 Real Time Quantitative PCR

The abundance of cDNA was quantified using a LightCycler 480 machine (Roche Applied Science, Burgess Hill, UK).

2.3.1.5.1 Assay design and Evaluation

Assays were designed using the Universal Probe Library system (UPL; Roche). This system utilises a fluorophore, fluorescein amidite (FAM), to enable quantification of PCR product. Oligonucleotide primers for UPL assays were ordered from Invitrogen (Invitrogen Ltd, Paisley, UK). UPL assays were designed using the UPL Assay Design Centre (Roche) to include intron-spanning boundaries where possible. Where no suitable UPL assay was available, a commercially designed assay was used, TaqMan Gene Expression Assays purchased from Applied Biosystems (ABI), CA, USA). For a list of primers and probes used, see Tables 2-1 and 2-2.

2.3.1.5.2 Real Time PCR Preparation

Equal volumes of each sample were pooled together to make a ‘stock’ solution, which was serially diluted from 1:4 to 1:2048. These serial dilutions were used as a standard curve against which the relative cDNA concentrations of samples could be calculated. Primer/probe sequences were validated against the standard curve on 384 well plates using the Roche Lightcycler 480 system (Roche).

Samples (diluted in nuclease-free water to 1:20), standards and controls (diluted as per samples) were added, 2µl, to a 384 well plate in triplicate. To this 8µl of master mix was added.

Master mix constitution varied between UPL and ABI assays. For UPL assays, 5µl of Lightcycler 480 Master Mix, 2.86µl of PCR-grade water, 0.02µl of 100µM forward primer, 0.02µl of 100µM reverse primer, and 0.1µl of probe was added per well. For ABI assays, 5µl of Lightcycler 480 Master Mix, 2.5µl of sterile water and 0.5µl of ABI primer-probe mix was added to each well.

Plates were sealed, and centrifuged at 1500rpm for 2 minutes to ensure samples and mastermix were at the bottom of the wells. Reaction conditions for real-time PCR included a pre-incubation step of 5 minutes at 95°C. Samples then underwent 50 PCR cycles consisting of: denaturation at 95°C (10 sec), annealing at 60°C (30 sec), elongation at 72°C (30 sec). This was followed by a cooling step (30s at 40°C).

The LC480 software (Roche) measured fluorescence during PCR and plotted amplification curves (fluorescence against PCR cycle number). The crossing point (Cp) was calculated as the maximum point of the second derivative of the amplification curve.

A standard curve of Cp versus log 'concentration' (i.e. serial dilution) was generated by the LC480 software. Curves were considered acceptable if reaction efficiency fell between 1.8 and 2.1. Mean mRNA 'concentration' from triplicates was interpolated from this graph for unknown samples using their Cp. The abundance of mRNA transcript for each sample was expressed relative to the abundance of a control gene. Control genes were selected for each tissue used by identifying genes for which abundance did not appear to be affected by maternal phenotype (i.e. BMI).

Gene	Assay ID
UBC	Hs00824723_m1
TBP	Hs00427629_m1
PPIA	Hs99999904_m1
YWHAZ	Hs00237047_m1
GR	Hs00230818_m1
PHLDA2	Hs00168368_m1
IGF2	Hs01005970_m1
11BHSD2	Hs00388669_m1
SDHA	Hs00188166_m1

Table 2-1. List of Applied Biosystems TaqMan gene Expression Assays used for qPCR.

Gene	Forward Primer	Reverse Primer	Probe
PPARγ	gacaggaaagacaacagacaaatc	ggggtgatgtgtttgaacttg	7
CDKN1C	aaccgctgggattacgact	caggcgctgatctcttgc	18
Leptin	ttgtcaccaggatcaatgaca	gtccaaaccggtgactttct	25
GILZ	ccgttaagctggacaacagtg	atggcctgttcgatcttgtt	36
LPL	aaactggtgggacaggatgt	ccaaggctgtatcccaagag	25
IGF2R	agcagcaggaagataccacaa	cacctccaaaatatatcaaggtga	58
DLK1	gacggggagctctgtgatag	gggcacaggagcattcata	68
IL-6	caggagcccagctatgaact	gaaggcagcaggcaacac	45
GLUT 3	gggtgtggttaatactatcttcactg	tcatatgcagagtccttcttcct	31
H19	ttacttcctccacggagtcg	ttgagctgggtagcaccatt	46
11BHSD1	caatggaagcattgttgtcg	ggcagcaaccattggataag	71
IRS1	tgcatcttcgctccttcc	atcctccgagagccaagtct	70
IL-1β	tacctgtcctgcgtgttgaa	tctttgggtaatttttgggatct	78
MR	tgggaattctgacttacttaacca	aatacaaaaagctgatgcagacc	58

Table 2-2. List of UPL designed assays for qPCR.

2.3.2 DNA Methylation Analysis

2.3.2.1 Principle

DNA methylation status of the genes *IGF2*, *H19*, *GR* and *11βHSD2* was analysed by pyrosequencing. Pyrosequencing involves the sequencing of DNA using a 'sequencing by synthesis' principle. A single-stranded DNA sequence of interest is sequenced by enzymatic synthesis of a complementary strand one base pair at a time. Solutions of specific dNTPs are sequentially added and removed from the reaction. Pyrosequencing relies upon the detection of pyrophosphate released after nucleotide incorporation by DNA polymerase, producing light that can be detected by a camera (see Figure 2-1).

Treatment of genomic DNA with bisulfite converts cytosine residues to uracil. However, 5-methylcytosine, or methylated cytosine (5mC), residues are not affected by this process (see Figure 2-2). Bisulfite treatment thus induces specific and predictable changes in the DNA sequence in a manner that is dependent upon the methylation status of individual cytosine residues. Individual sites of interest (e.g. promoter regions) can then be amplified using PCR, whilst retaining the distinction between methylated and unmethylated sites. During PCR, uracil residues (i.e. unmethylated cytosines in the original sequence) are converted to thymine.

Bisulfite treated DNA is hybridised to a sequencing primer (and a biotinylated primer), and incubated with DNA polymerase, ATP sulfurylase, luciferase, apyrase, adenosine 5' phosphosulfate (APS) and luciferin. One of four dNTPs (dTTP, dGTP, dCTP or dATPαS) is added. If DNA polymerase is able to incorporate the dNTP (i.e. it is correct and complementary to the original sequence), pyrophosphate (PPi) is released. PPi is converted by ATP sulfurylase to ATP in the presence of APS. The

ATP generated is used in the luciferase-mediated conversion of luciferin to oxyluciferin, producing light proportional to the amount of ATP present. The light produced is detected by a camera and quantified and analysed by a software program. Unincorporated nucleotides are degraded by the enzyme apyrase, allowing the reaction to restart with another nucleotide.

As bisulfite treatment of DNA selectively modifies unmethylated cytosines to thymine while not affecting methylated cytosines, methylation state of the original DNA can be determined by comparing the original sequence and the bisulfite-treated sequence. Pyrosequencing allows the ratio of C to T at specific sites to be determined quantitatively.

2.3.2.2 DNA Extraction

DNA extraction was carried out using a phenol-chloroform extraction method. This method relies on phase separation by centrifugation of a phenol-chloroform mixture and an aqueous sample to isolate nucleic acids and protein. Nucleic acids partition into the aqueous phase, and can be precipitated using ethanol.

Approximately 30mg of tissue was incubated overnight at 55°C with 600µl of tail buffer (1% SDS, 0.1M NaCl, 0.1M EDTA, 0.05M Tris) and 35µl of proteinase K (10mg/ml, Qiagen). Once tissue was fully lysed, 20µl of 20µg/ml DNase-free RNase A was added to each sample, and incubated for 2 hours at 37°C. After incubation, 37.5µl of 2M β-mercaptoethanol (Sigma Aldrich) and 600µl of phenol was added to each sample. Samples were rotated on a spinner for 15 minutes before centrifuging at 16000g for 2 minutes. The organic lower phase was discarded, while the aqueous

supernatant and interphase layers were then transferred to a fresh tube. To this, 300µl of phenol and 300µl of chloroform were added. Samples were then rotated again on a spinner for 5 minutes, and centrifuged at 16000g for 2 minutes. The supernatant layer was then transferred to a fresh tube. DNA concentration and integrity were assessed by Nanodrop and agarose gel electrophoresis, as described in section 2.3.1.3.

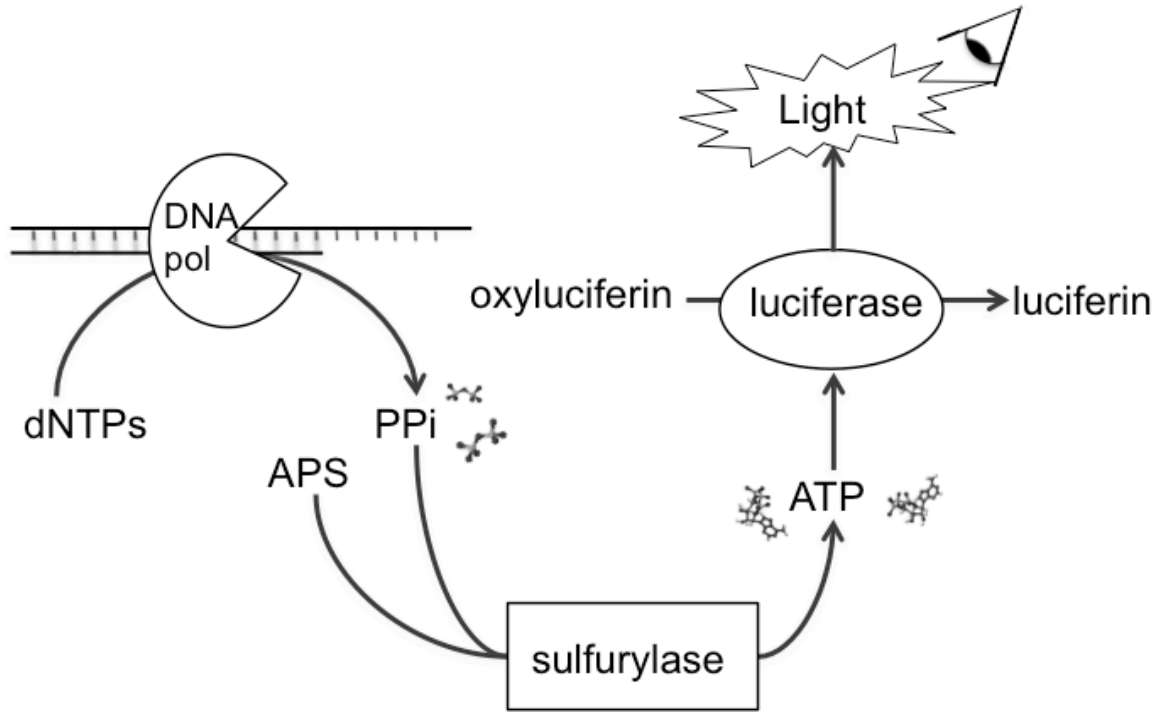


Figure 2-1. Principle of pyrosequncing.

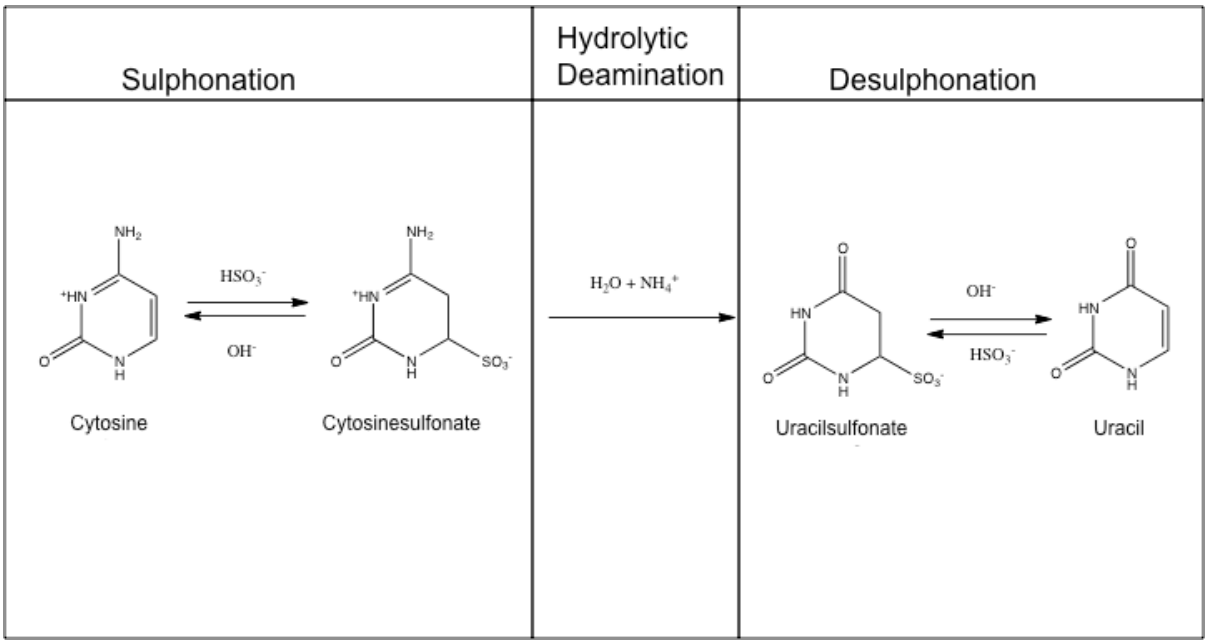


Figure 2-2. Bisulfite-dependent conversion of cytosine to uracil.

2.3.2.3 Bisulfite Treatment

Bisulfite treatment was carried out using the Epitect Bisulfite Kit (Qiagen). Using nuclease-free water, 1µg DNA was made up to 20µl in a 200µl PCR tube. To this, 85µl of bisulfite mix and 35µl of DNA Protect Buffer were added. Bisulfite DNA conversion was performed on the mixture using a thermal cycler (G-Storm, UK). The thermal cycler conditions were as follows:

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	25 min	60°C
Denaturation	5 min	95°C
Incubation	85 min	60°C
Denaturation	5 min	95°C
Incubation	175 min	60°C
Hold	Indefinite	20°C

Once the bisulfite conversion was complete, the PCR tubes were centrifuged briefly and transferred to clean 1.5ml microcentrifuge tubes. To each sample, 560µl of Buffer BL containing 10µg/ml of carrier RNA, was added and tubes were briefly centrifuged. The mixture was then transferred to a fresh Epitect spin column.

Epitect spin columns were centrifuged at 14,000 rpm for 1 minute. The flow-through was discarded, and 500µl of Buffer BW was added to each. Tubes were then centrifuged at 14,000 rpm for 1 minute. Flow-through was discarded, and 500µl of

Buffer BD was added to each spin column and incubated at room temperature for 15 minutes.

After incubation, spin columns were centrifuged at 14,000 rpm for 1 minute. Flow-through was discarded and 500µl of Buffer BW was added to each spin column. Spin columns were centrifuged at 14,000 rpm, flow-through was discarded and a further 500µl of Buffer BW was added to each spin column. Spin columns were centrifuged at 14,000 rpm for 1 minute and flow-through was discarded. Spin columns were placed into new 2ml collection tubes and centrifuged at 14,000 rpm for 1 minute to remove any residual liquid. Spin columns were then placed, with open lids, into clean 1.5ml microcentrifuge tubes and incubated for 5 minutes at 56°C for 5 minutes in a heating block. Spin columns were then placed into clean 1.5ml microcentrifuge tubes, and 20µl of Buffer EB was dispensed onto the centre of each spin column membrane. The purified DNA was eluted by centrifugation for 1 minute at 12,000 rpm. Purified DNA was stored at -20°C.

2.3.2.4 PCR

Pyrosequencing primers were designed for exon 1C of GR and for the promoter region of 11βHSD2 by Amanda Drake using the PyroMark Assay Design 2.0 software (Qiagen). Published assays were used for pyrosequencing of DMRs known to control expression of IGF2 (DMR0, DMR2 and H19 imprinting control region) (Dupont et al. 2004; Murrell et al. 2008). IGF2 DMR2 methylation was analysed using two previously published assays, DMR2.1 and DMR2.2, covering separate regions within the DMR. Primers for GR and 11βHSD2 were designed using the PyroMark Assay Design 2.0 software (Qiagen) Primer sequences and PCR conditions are listed in

Table 2-3. All primers were purchased from Eurogentec (Southampton, UK).

2.3.2.5 Agarose Gel Electrophoresis

PCR products were electrophoresed on a 1.5% agarose/0.5xTBE gel at 90V for 30 minutes to assess the success of PCR. Agarose was melted in the buffer using a microwave oven, and GelRed nucleic acid dye was added to the molten agarose (at 0.0005% concentration). Once cooled, the mix was poured into a tray. Samples were mixed with Orange G loading dye in a 1:5 ratio and loaded onto the gel together with a DNA ladder. Gels were visualised under UV light (λ=260nm) using a transilluminator. The strength of each visualised band was noted, and PCR was repeated for samples with weak bands (i.e. low PCR product copy number, see Figure 2-3).

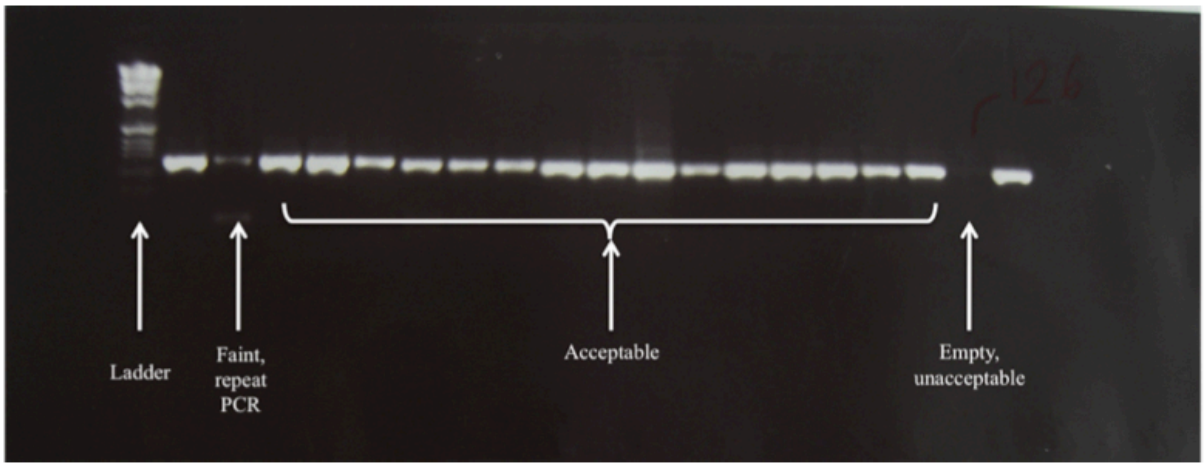


Figure 2-3. Example of bisulfite converted PCR product run out on a 1% agarose/0.5xTBE gel at 110V for 45 minutes, showing acceptable and unacceptable DNA and a 1Kb ladder.

2.3.2.6 Pyrosequencing

Pyrosequencing was carried out using a Pyromark Q24 instrument. Appropriate volumes of enzyme mix (DNA polymerase, ATP sulfurylase, luciferase and apyrase), substrate mix (adenosine 5' phosphosulfate and luceferin) and dNTPs (dATPαS, dCTP, dTTP and dGTP) were added to the Pyromark Q24 instrument as indicated by the Pyro Q-CpG assay design software. To a 24-well plate, 10µl of PCR product was added, along with 2µl of streptavidin-coated sepharose beads (GE Healthcare Life Sciences, UK), designed to bind to biotinylated compounds, and 40µl of Binding Buffer. The volume was made up to 80µl using 28µl of Milli-Q (18.2 MΩ·cm) purified water. Plates were sealed using strip caps and agitated constantly for 5 to 10 minutes at 14000rpm. A second shallow-welled 24-well plate was prepared by adding 0.3µM sequencing primer in 25µl of annealing buffer (see

Table 2-3). Samples, bound to the sepharose beads, were then captured using a Pyromark suction device and flushed with 70% ethanol for 5 seconds, denaturation solution for 5 seconds and washing buffer for 10 seconds whilst under suction. The beads were then released into the sequencing primer plate by removing suction. This plate was then incubated at 80°C for 2 minutes to facilitate annealing of the sequencing primer to samples while the suction device was flushed with Milli-Q purified water. Plates were then processed using the Pyromark Q24 instrument and analysed using Pyro Q-CpG software.

Schematic diagrams of the methylated regions for analysis are shown in Figure 2-4.

Gene	PCR Primers and hybridization temperature	Sequencing primer	Product size
GR (exon 1C)	Forward GGGGGAGAGTTTTTATTTAAGAAAGT Reverse (biotinylated) CCCCCCTCCTCCATTTTA 51°	TTTTTTTTATTTATAGAATT	235
11βHSD2 (-763 to -704)	Forward GTTTTGGAAGGAAAGGGAAAGA Reverse (biotinylated) CACATCCCCATACCCTTTACTAAT 55°	GGGGTAGAGATTTTAAGAA	304
IGF2, DMR0	Forward TGAGGATGGGTTTTTGTTTGGTAT Reverse (biotinylated) TCCTCAATCCACCCAAAAATAATAT 50°	AAAAGTTATTGGATATATAGT	205
IGF2, DMR2, product 1 (DMR2.1)	Forward GGGTTTTGGGTGGGTAGAGT Reverse (biotinylated) CCAAAACAACTTCCCCAAAT 60°	GTTTGGTTTTTTTGAA	195
IGF2, DMR2, product 2 (DMR2.2)	Forward GGGAAAGGGGTTTAGGATTTTTAT Reverse (biotinylated) ATAATTTACTCCCCCTTCAACCTC 60°	GATTTTTATYGGAAGTA	255
H19	Forward TGGGTATTTTTGGAGGTTTTTTT Reverse (biotinylated) TCCCATAAATATCCTATTCCCAAA 57°	GTAGGTTTATATATTATAG	220

Table 2-3. Primer sequences and hybridization temperatures for pyrosequencing assays.

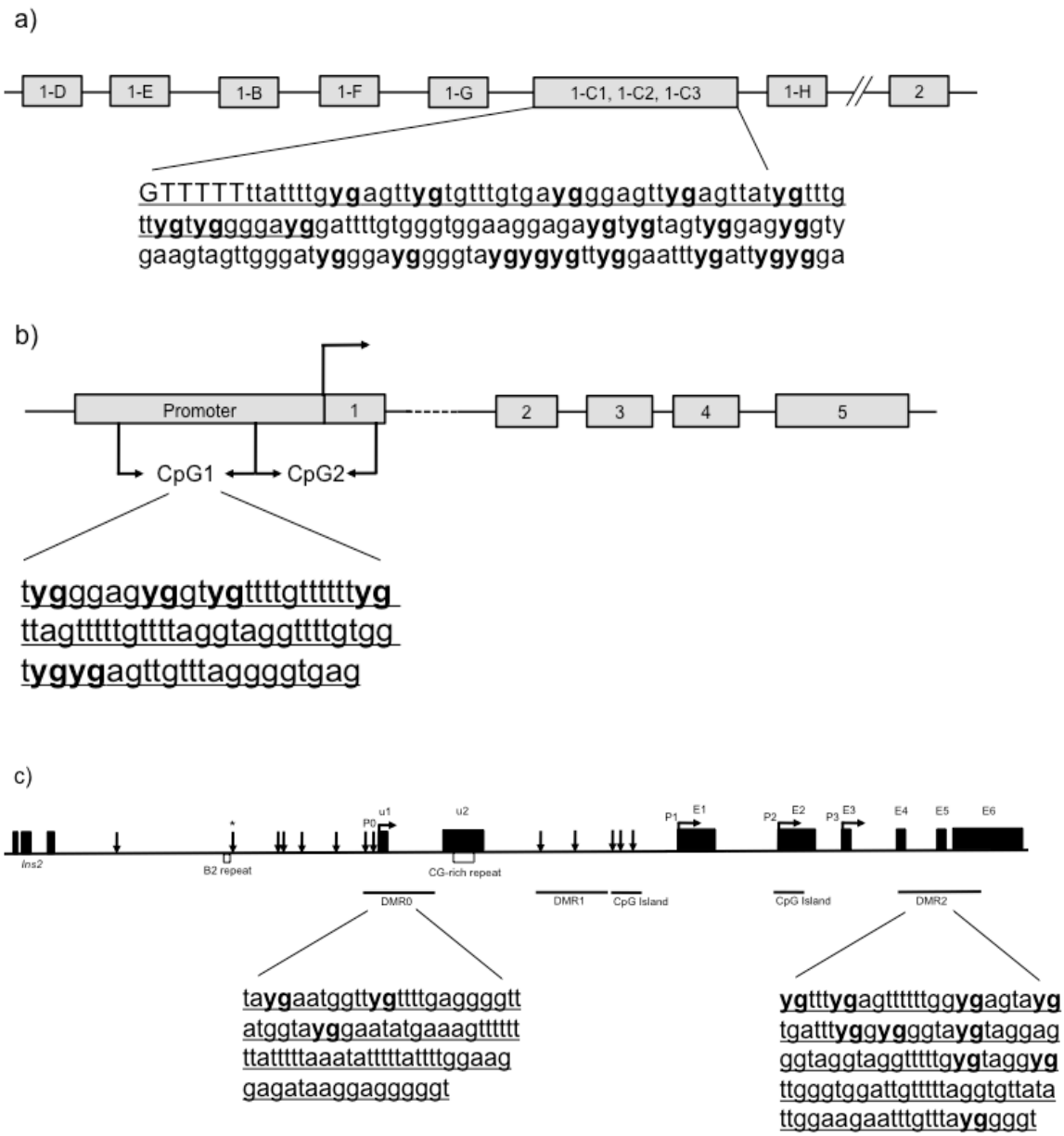


Figure 2-4. Schematic diagaram of the areas assessed for methylation at exon 1C of the GR promoter (a), across the 11βHSD2 promoter (b), and across DMR0 and DMR2 of the IGF2 gene (c) Exons are shown in lower-case letters; the area assayed is shown as underlined, with specific CpGs of interest highlighted in bold lettering. Figure adapted from Drake *et al.* 2012.

2.3.3 Protein Analysis

2.3.3.1 Protein Extraction

Total protein was extracted from frozen tissue. Placental tissues were homogenized in lysis buffer and incubated for 1 hour at 4°C with rotation before centrifugation at 10,000g for 5 minutes at 4°C. The supernatant was transferred to a fresh tube and stored at -20°C.

2.3.3.2 Protein Quantification

Total protein concentration was measured using colorimetric assay. Sample concentrations were determined using a standard curve derived from a series of bovine serum albumin (BSA, Bio-Rad Laboratories, Hertfordshire, UK) dilutions ranging from 0 to 1.4mg/ml. 5µl of samples or standards were added to individual wells in a 96-well plate, followed by 25µl of Bio-Rad reagent A and 200µl of reagent B. The 96-well plate was then incubated for 15 minutes and absorbance at 750nm at room temperature was recorded using a plate reader (OPTImax tunable microplate reader, Molecular Devices, PA, USA).

2.3.3.3 SDS-Page

Protein samples (30µg) were made up to 14µl with water, 5µl of NuPAGE LDS sample buffer and 2µl of sample reducing agent. Samples were incubated at 70°C for 10 minutes to denature the protein. A Bis-Tris Novex 4-12% pre-cast gel (Invitrogen, Paisley, UK) was locked to the buffer core in a vertical XCell SureLock electrophoresis tank. The inner chamber was filled with 1x running buffer, and

samples were loaded with 5µl of Full-Range Rainbow marker (GE Healthcare Life Sciences, Buckinghamshire, UK). The outer chamber was filled with buffer while 0.5ml of NuPAGE antioxidant was added to the inner chamber. Samples were electrophoresed at 200V for 50 to 60 minutes.

2.3.3.4 Western Blotting

Following electrophoresis, the gel was transferred to a transfer sandwich (comprising sponge, paper, gel, membrane, paper, sponge). Sponges were pre-soaked in 4°C transfer buffer for 1 hour. The sandwich cassette was placed in the tank, and the chambers were filled with transfer buffer. Proteins were transferred at 30V on ice for 80 minutes. Following transfer, the membranes were placed into blocking buffer and incubated with agitation for 1 hour at room temperature. The blocking buffer was removed, and the membrane was incubated with primary antibody at 4°C overnight at a concentration of 1:2,000 for rabbit anti-11βHSD2 antibody and 1:10,000 for rabbit anti-α-tubulin antibody. The membrane was then washed 3 times, 10 minutes each time, in TBST buffer at room temperature with agitation. The membrane was then incubated for 1 hour at room temperature with secondary antibodies (kindly provided by Kerry McInnes) diluted 1:10,000 in blocking solution. The membrane was then rinsed with TBST for ten minutes 3 times. Finally, the membrane was scanned using a Licor Odyssey infrared scanner (Licor Biotechnology Ltd, Cambridge, UK), and protein bands were quantified suing the Odyssey V3 software package.

2.3.4 Immunohistochemistry

2.3.4.1 Immunohistochemistry

Representative samples of first trimester, second trimester and term trophoblast tissue (3 samples for each time point) were collected in 10% neutral buffered formalin (NBF) and stored for 24 hours at room temperature. Samples were then dehydrated in an ethanol gradient: 70% ethanol, 95% ethanol and absolute ethanol, and soaked in xylene. Samples were then embedded in paraffin wax and blocked. Paraffin blocks were cut using a microtome into 5μm slices, and slices were mounted onto microscope slides.

Slides and samples were dewaxed in xylene for five minutes, twice, then rehydrated through an ethanol gradient, with 20 seconds in absolute ethanol (twice), 20 seconds in 95% ethanol, and 20 seconds in 70% ethanol. Slides were then washed in several changes of tap water. Slides were then washed in deionised water for approximately 30 seconds.

For antigen retrieval, slides were pressure-cooked, from cold, for 5 minutes at pressure in 2 litres of 10 mM TRIS, 1 mM EDTA and 0.05% Tween-20 (pH 9.0). Slides were once again washed in distilled water for approximately 30 seconds.

Slides were then blocked for 30 minutes in 1% Hydrogen Peroxide in TBST (pH 7.5 20 mM Tris; 150 MM NaCl; + 0.5% Tween20) and washed in several changes of tap water. Slides were then washed in TBST for 5 minutes. Slides were transferred to a Sequenza Immunostaining Center apparatus (Thermo Scientific, Waltham, MA, USA) and incubated for 15 minutes in a staining buffer of TBST + 5% donkey horse serum. Primary antibodies were diluted to 1:10000 in staining buffer, and slides were

incubated for 2hrs at room temperature with either rabbit anti-11βHSD2 serum (1:10000) for samples, or control rabbit serum (1:10000) for negative controls.

Slides were then washed in TBST. Slides were then blocked for 5 minutes with 2.5% blocking (normal horse) serum (Vector Laboratories, CA, USA). Slides were incubated with secondary antibody for 30 minutes (one drop of ImmmPress anti-rabbit horseradish peroxidase). Slides were washed twice in TBST, and flow-through was decanted from the Immunostaining apparatus. Slides were stained with DAB (3,3-diaminobenzidine) for 5 minutes, and then washed in TBST 20 and transferred to distilled water. Slides were then counterstained with haemotoxylin for 30 seconds. Excess haemotoxylin was washed off in tap water and soaked in Scott's Tap Water for 10 seconds. Slides were washed in tap water and examined for excessive haemotoxylin staining, with acid alcohol used to reduce any heavy haemotoxylin staining. Slides were dehydrated in an increasing ethanol gradient, with 20 seconds in 70% ethanol, 20 seconds in 95% ethanol, and two incubations of 20 seconds each in absolute ethanol. Slides were then incubated in xylene for 10 minutes, and coverslips were mounted with pertex.

2.3.5 Radioimmunoassays

Radioimmunoassay involves the use of radioactive antigens mixed with a known amount of antibody for the antigen. A sample of serum, containing an unknown concentration of the same antigen, is added. Radiolabelled and unlabelled antigens are thus able to compete for antibody binding sites. A higher concentration of unlabelled antigen will reduce the ratio of antibody-bound radiolabelled antigen to free radiolabelled antigen. Bound and unbound antigens are then separated, generally

through washing, and the radioactivity of the bound antigens is measured using a gamma counter. The separation of bound and unbound antigen may be aided by covalent bonding of the antibody to another substance, such as the inner surface of a polypropylene tube. A binding curve, generated by the use of known standards, can then be used to derive the concentration of antigen in the sample serum.

2.3.5.1 Radioimmunoassay for Cortisol

To measure serum cortisol levels, ImmuChem Cortisol ^{125}I kits were used (ICN Biomedicals, CA, USA). Kits were equilibrated to room temperature prior to assaying samples. Samples were defrosted, and 25µl of sample was added in duplicate to rabbit anti-cortisol coated tubes. Standards (at concentrations 0, 1, 3, 10, 30 and 100 µg/dL) were also added to tubes in duplicate. 1ml of I^{125} labelled cortisol was then added to each tube and the tubes were gently vortexed prior to incubation at 37˚C for 45 minutes. After incubation, all fluid was aspirated. Tubes were then washed with 1ml of 0.05% Tween-20 and re-aspirated. The tubes were then left to air-dry, inverted on paper towels. Levels of I^{125} labelled cortisol were then measured using a gamma counter calibrated for I^{125}.

To calculate cortisol levels, the average gamma counts for duplicate tubes were divided by the averaged counts of the zero standard, and then multiplied by 100. This value (the percentage bound to unbound, or %B/B0) was then plotted against the concentration for the cortisol standards (1 to 100 µg/dL) to plot the standard curve (see Figure 2-5). Sample concentrations were then calculated by interpolation from the standard curve graph.

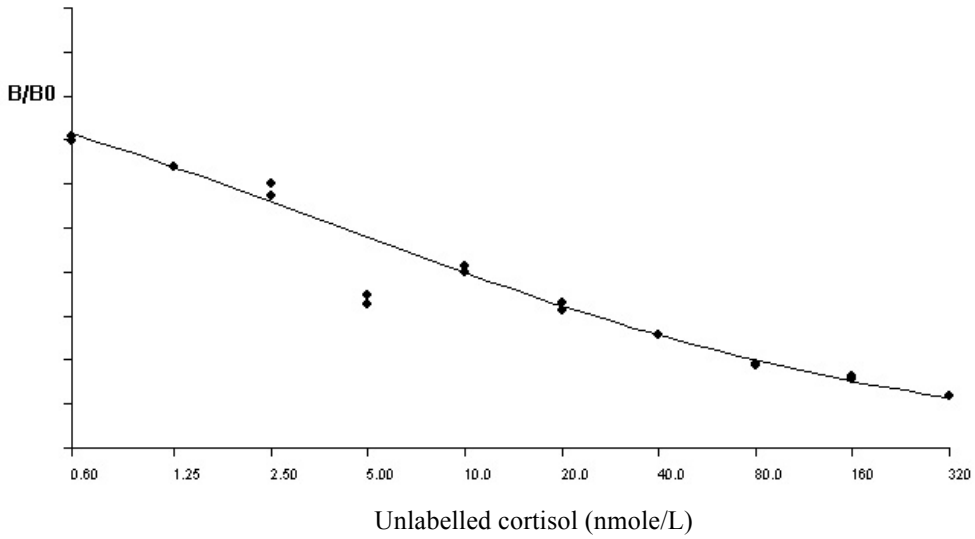


Figure 2-5. Example cortisol radioimmunoassay standard curve. The proportion of bound radiolabelled cortisol (B/B0, y-axis) decreases as it is displaced by increasing concentrations of unlabelled cortisol (x-axis). The cortisol concentration of samples was determined from their B/B0 by interpolation from the curve.

2.3.5.2 Radioimmunoassay for CBG

DIAsource CBG-RIA-CT kits (DIAsource, Belgium) were used to measure serum CBG. A fixed quantity of ^{125}Iodine labelled CBG competes with sample CBG for a known quantity of anti-CBG antibody. These antibodies then bind to goat anti-mouse antibodies bound to the walls of polystyrene tubes, allowing for immobilization and measurement of CBG.

Serum samples were diluted 25 times in dilution buffer, and 100µl of the diluted sample was added in duplicate to tubes coated with goat anti-mouse antibody (including two 'normal' or total count tubes) Two zero controls (containing phosphate buffer but no CBG), and duplicate standards (containing 0.44µg/ml, 0.81µg/ml, 1.50µg/ml, 2.20µg/ml, 4.00µg/ml, and 8.00µg/ml of CBG in phosphate buffer) were also prepared. 100µl of I^{125} labeled CBG was added to each tube (including total count tubes and standards). Finally, 100µl of CBG antiserum was added to each tube (excluding the total count tubes).

Tubes were then incubated for two hours at room temperature with shaking at 400rpm. The contents of each tube (excluding total counts) were then aspirated. Tubes were then washed with 2 ml of working wash solution (tris-HCl), and left to stand for two minutes before aspirating the last drops of liquid.

Levels of I^{125} labelled CBG were then measured using a gamma counter calibrated for I^{125} for 60 seconds. The mean of the duplicate tubes were calculated and concentrations were determined by extrapolation using a standard curve of Bound/Unbound values against CBG concentration for each standard.

2.3.5.3 Coolen's Equation for Calculating Free Cortisol

In order to estimate the levels of free (i.e. unbound) cortisol from total cortisol concentration and CBG concentration, Coolen's equation was used.

In human serum, cortisol is bound to CBG. A less significant quantity of cortisol is bound more weakly to albumin. Coolen's equation can be used to calculate the levels of unbound cortisol not bound to either CBG or albumin (i.e. cortisol which is biologically available in serum). This equation can be represented as:

$$U = \sqrt{Z^2 + \frac{C}{(1-N)\,K}} - ZM$$

where:

$$Z = \frac{1}{2K} + \frac{T - C}{2\,(1 + N)} \quad M$$

U represents the unbound cortisol (in μM), C the molar concentration of total cortisol (in μM), N the proportion of albumin-bound cortisol, and K the affinity of CBG for cortisol at 37˚C. If a value for K is assumed to be 3 x 10^{-7} M^{-1} and a value of N to be 1.74 (as precise albumin levels were unavailable), the following equation can be used:

$$U = \sqrt{Z^2 + 0.0122\,C} - Z$$

where Z = 0.0167 + 0.182 (T-C) μM.

2.3.6 CRH

Levels of CRH were measured in serum (collected in tubes coated in lithium heparin) by radioimmunoassay as described in Smith *et al* 1993, and were performed by Maria Bowman and Roger Smith at the University of Newcastle, Australia (Smith *et al.* 1990; Smith *et al.* 1993)

2.4 Statistical Analysis

Normal distribution of data was assessed visually using histograms. Data that were not normally distributed were normalised using natural log transformation. Correlations of normally distributed variables were analysed using Pearson correlation. Adjustments for confounding factors (e.g. maternal age, smoking status, parity, DEPCAT category, and length of gestation) were performed using multiple linear regression. Statistical analyses are described in further detail in experimental chapters.

All data are presented as mean (± s.e.m), and p values of less than 0.05 were considered significant. The Bonferroni correction was used to determine values for alpha where multiple testing was carried out.

Analysis was performed using Minitab (Minitab Inc, PA, USA), SPSS (IBM, NY, USA) and Statistica (StatSoft Inc, OK, USA). Graphs were prepared using GraphPad Prism (GraphPad Software Inc, CA, USA).

Chapter 3:

The Maternal HPA Axis in Obese Pregnancy and Associations with Gestational Weight Gain, Birth Weight and Gestation at Delivery

3.1 Introduction

As previously discussed (see Introduction section 1.3.4), the regulation of the HPA axis undergoes dramatic changes during pregnancy, with circulating levels of cortisol rising to around three-fold non-pregnant levels by the third trimester (Okamoto *et al.* 1989; Jung *et al.* 2011). These changes contribute to the changes in maternal, fetal, and placental physiology that occur during pregnancy, including fat deposition, tissue maturation, and mobilization of fuel to the fetus for growth (Murphy *et al.* 2006). High circulating levels of maternal cortisol have also been implicated in 'programming' of low birth weight (Goedhart *et al.* 2010; Reynolds 2013). Likewise, the placenta secretes large quantities of CRH into the maternal circulation during the second and third trimesters (McLean and Smith 1999). CRH concentrations may be important in regulating the timing of delivery, with high levels of the hormone at 31 weeks associating with subsequent preterm birth (Sandman *et al.* 2006).

The prevalence of obesity among pregnant women is rising (see Introduction section 1.6) and is associated with macrosomia and longer gestation (Denison *et al.* 2008). No studies have examined whether changes in the maternal HPA axis in obese pregnancy are associated with these adverse pregnancy outcomes. Indeed, whether the HPA axis undergoes the normal physiological changes during pregnancy in women who are obese has not been studied. In the non-pregnant state, obesity is associated with increased hepatic/urinary clearance of circulating glucocorticoids (Pasquali *et al.* 1993; Rask *et al.* 2001; Praveen *et al.* 2011). Some data suggest that plasma cortisol is lower in uncomplicated obesity, however the effects of obesity on

plasma cortisol levels during pregnancy have not been extensively studied (Praveen *et al.* 2011). During pregnancy, the activity of the placental enzyme 11βHSD2 will also impact on cortisol levels and it is not known if this differs in women who are obese. The aim of this study was to characterize the hormones of the HPA axis in a group of very severely obese women and lean controls participating in a longitudinal study of obese pregnancy and to test associations with pregnancy outcomes.

3.1.1 Hypotheses

It was hypothesized that:

a) maternal obesity would be associated with lower maternal cortisol, CRH and CBG levels during pregnancy.
b) Lower cortisol levels in obese women would be associated with lower gestational weight gain
c) lower maternal cortisol and CRH levels in obese women would be associated with higher offspring birth weight and longer gestation, respectively.

3.1.2 Aims

1.To compare circulating levels of cortisol, CBG and CRH measured at three time points in pregnancy and at 3 months post-partum in very severely obese and lean pregnant women.

2. To test the relationship between maternal cortisol and CRH levels and gestational weight gain as well as birth outcomes including birth weight and gestation at delivery.

3.2 Methods

3.2.1 Study Design

This was a case-control study comparing lean and very severely obese pregnant women who had been recruited among participants of a larger prospective cohort study.

3.2.2 Subject Recruitment

Participants for this study were recruited as part of a longitudinal study of obesity in pregnancy between April 2008 and September 2012 as described in methods section 2.1.1.1. Briefly, lean (BMI between 20 and 25 kg/m^2) and obese (BMI > 30 kg/m^2) pregnant women were recruited from the Tommy's Antenatal Metabolic Clinic in Edinburgh. All study participants attended the research clinic at three time points during pregnancy: 16 weeks gestation ('visit 1'), 28 weeks gestation ('visit 2'), and 36 weeks gestation ('visit 3'); fasting morning blood samples were taken at these visits to be used for the measurement of cortisol, CBG, and CRH; height and weight were also measured by medical staff in order to calculate gestational weight gain and BMI. Study participants were also invited to attend a fourth visit 3 months postnatally. At Visit 1, women were also asked to collect three saliva samples into Sarstedt salivette tubes: one sample was collected at bedtime, a second at waking, and a third 30 minutes after waking. Gestational weight gain was calculated as the difference in weight between visit 3 and visit 1. Birthweight, gender and gestation at delivery and mode of delivery were recorded after delivery. Women who attended for blood sampling for any of the three visits during pregnancy were included in the

study, while women with gestational diabetes (n=37) and pre-eclampsia (n=17) were excluded. Pre-term births (delivery <37 weeks gestation, n=17) were also excluded, except when analyzing associations between cortisol and length of gestation.

3.2.3 Laboratory methods

ImmuChem Cortisol ^{125}I kits (ICN Biomedicals, CA, USA) were used to measure serum cortisol concentrations in all samples as described in section 2.3.5.1. Briefly, samples were added to rabbit anti-cortisol coated tubes, to which I^{125} labelled cortisol was added. Tubes were incubated at 37°C for 45 minutes, at which point fluids were aspirated and tubes were washed to remove unbound cortisol. Bound cortisol was measured using a gamma counter calibrated for I^{125}. Similarly, CBG and CRH were measured by radioimmunoassay in samples collected from a subset of women for whom samples were available at all 3 prenatal visits (as described in methods sections 2.3.5.2 and 2.3.6). Free cortisol was calculated in the samples for which both cortisol and CBG measurements were available using Coolen's equation, as described in methods section 2.3.5.3.

3.2.4 Statistics

Normal distribution of data was assessed visually using histograms. Data that were not normally distributed (total serum cortisol concentrations, serum CBG concentration, calculated free cortisol concentration) were normalised using natural log transformation. Comparisons of variables between obese and lean groups were tested using independent t-tests. Differences in the pattern of cortisol levels during

pregnancy were tested using repeated measures analysis of variance (ANOVA) for subjects that had serial cortisol measurements. Correlations between HPA axis variables and birth weight and gestational weight gain were tested using Pearson correlation. Multiple regression was used to adjust for confounding factors (neonate gender, length of gestation, maternal smoking status, parity and ethnicity). All data are presented as mean (±s.e.m), and p values of less than 0.05 were considered statistically significant. Analysis was performed using Minitab (Minitab Inc, PA, USA) and Statistica (StatSoft Inc, OK, USA) software packages. Graphs were prepared using GraphPad Prism (GraphPad Software Inc, CA, USA).

Statistical power calculations were performed to determine the sample size required for these analyses. For correlation analyses where alpha = 0.05 and statistical power = 0.8, a sample size of 70 was determined to be necessary to detect a correlation of r = 0.3, while a sample size of 30 was calculated to be necessary to detect a correlation of r = 0.4, and a sample size of 23 was calculated for detection of a correlation r = 0.5. For multiple regression analyses using 6 predictors (e.g. correcting for neonate gender, gestational age, maternal smoking status, parity, and ethnicity) with an alpha of 0.05, a sample size of 38 was calculated as being necessary to detect an $r^2 \geq 0.3$. For group analyses, a sample size of 60 was determined to be necessary to detect a difference of 20% between groups with a power of 0.8, while a sample size of 238 was determined to be necessary to detect a difference of 10% between groups.

3.3 Results

3.3.1 Demographics

Table 3-1 shows the characteristics of the 173 obese and 107 lean pregnant women. Obese women were younger than lean, had greater social deprivation as measured by DEPCAT score and were of significantly higher parity.

3.3.2 Total Serum Cortisol Profiles Differ Between Lean and Obese Pregnant Women

Total serum cortisol concentrations increased throughout pregnancy in both obese ($p<0.001$) and lean women ($p<0.0001$, see Figure 3-1). Cortisol levels were significantly lower in obese than lean at each time-point during pregnancy but did not-differ post-partum.

To explore the pattern of cortisol measurements during pregnancy, only participants who had provided samples for all three antenatal time points were considered (see Figure 3-2). Using repeated measures ANOVA, while both groups were found to rise significantly throughout gestation ($p<0.001$), the pattern of rise did not differ between the two groups. As in the complete group, obese women had lower cortisol levels than lean at each time point in pregnancy.

3.3.3 Salivary diurnal patterns

Diurnal pattern of cortisol secretion was maintained in both obese and lean women with significantly lower levels at bed-time compared to waking and 30mins post waking. Salivary cortisol concentrations did not differ significantly between lean and obese women at any time point (see Figure 3-3).

	Lean (n=107)	Obese (n=173)	P-value
Maternal Characteristics			
BMI (kg/m^2)	22.76 (0.16)	44.04 (0.34)	<0.0001
Age (years)	33.30 (0.42)	30.80 (0.39)	<0.0001
Gestational Weight Gain (GWG)(16 weeks to 36 weeks, kg)	10.01 (0.36)	5.96 (0.43)	<0.0001
GWG (16 weeks to 28 weeks, kg)	6.46 (0.24)	2.99 (0.29)	<0.0001
GWG (28 weeks to 36 weeks, kg)	3.52 (0.23)	2.78 (0.44)	0.09
Multiparous*	62 (61%)	89 (55%)	<0.01
Nulliparous*	40 (39%)	74 (45%)	
Smokers*	2 (1.8%)	12 (6.9%)	0.75
Higher socioeconomic status (DEPCAT 1-3)*	77 (72%)	44 (26%)	<0.0001
Lower socioeconomic status (DEPCAT 4-7)*	29 (28%)	126 (74%)	<0.0001
White British*	91 (96%)	152 (97%)	0.73
Other Ethnicity*	3 (4%)	6 (3%)	
Offspring Characteristics (n=280)			
Gestation (days)	283.3 (0.78)	282.10 (0.66)	0.445
Birth weight (g)	3581 (48.19)	3638 (42.75)	0.276
Caesarean Section Delivery*	15 (16%)	63 (43%)	<0.0005
Males *	49 (46%)	79 (45%)	0.92
Females *	58 (54%)	95 (55%)	0.92

Table 3-1. Demographic data for lean and obese pregnant women. Data are mean (s.e.m), or *N(%). Socioeconomic status was measured using deprivation category (DEPCAT) scores based on postcode (McLoone 2004). DEPCAT information was unavailable for 1 lean woman and 3 obese women. Ethnicity information was unavailable for 13 lean women and 15 obese women. 'Smokers' are defined as those who considered themselves as current smokers. Parity data was unavailable for 5 lean women and 10 obese women. Caesarean Section delivery included elective and emergency caesarean sections.

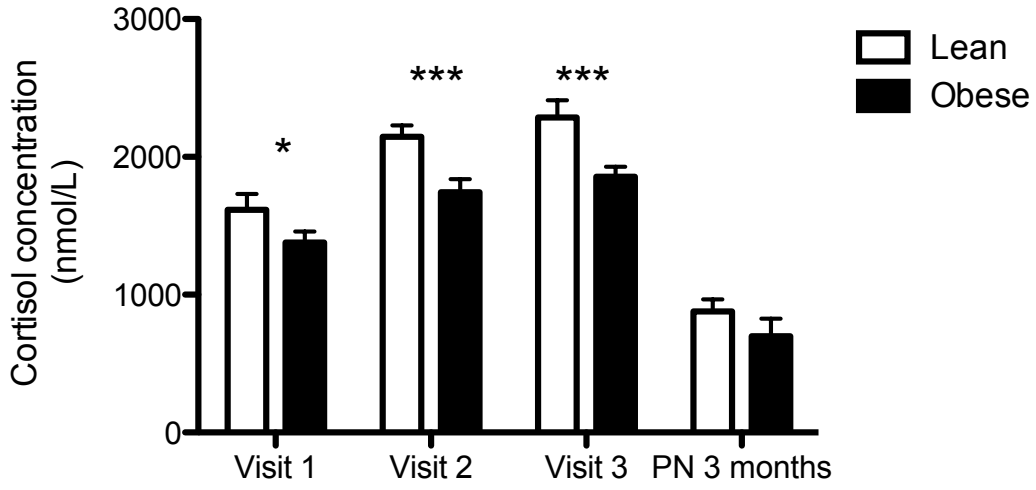


Figure 3-1 Total serum cortisol concentrations measured at three time points during pregnancy (16 weeks, 28 weeks and 36 weeks) and 3 months postnatally in lean (n=107) and obese (n=173) women (mean ±s.e.m; * difference in cortisol concentration between lean and obese p<0.05, *=p<0.01, ***=p<0.005).

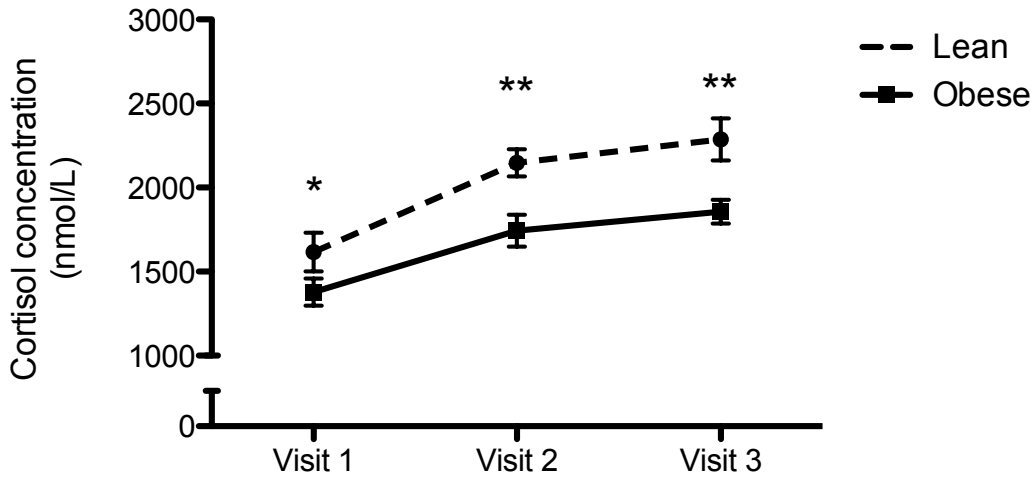


Figure 3-2. Total serum cortisol concentrations measured at three time points during pregnancy (16 weeks, 28 weeks and 36 weeks) in lean (n=48) and obese (n=62) women; only pregnant women who had cortisol measurements at all three time points are shown (mean ±s.e.m, * difference between cortisol concentration between lean and obese *=p<0.05, **=p<0.01).

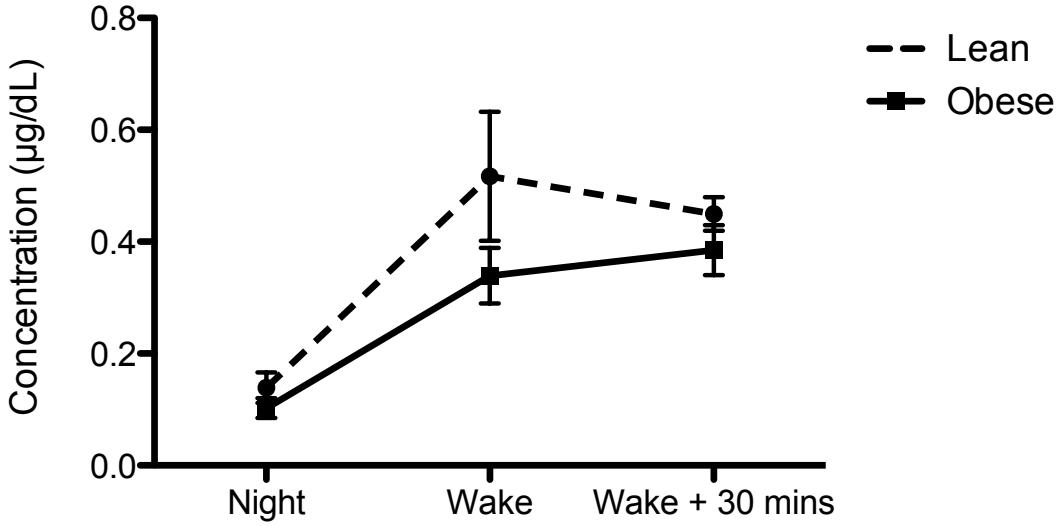


Figure 3-3. Diurnal profile of salivary cortisol in a subset of lean (n=17) and obese (n=7) pregnant women at visit 1 (mean ±s.e.m). Values did not differ between lean and obese at bedtime (p=0.43), waking (p=0.35) or 30 minutes post waking (p=0.29).

3.3.4 Serum CBG Is Lower in Obese Women than Lean

Serum CBG was measured in patients with viable cortisol measurements from all 3 prenatal research visits. At visit 1, serum CBG concentrations did not differ between obese and lean. CBG concentrations then increased throughout pregnancy in both lean ($p<0.0001$) and obese women ($p<0.05$, see Figure 3-4). CBG concentrations at both visit 2 and visit 3 were lower in obese compared to lean (both $p<0.005$) and thus the rise in CBG between visits 1 and 3 was significantly lower in obese women compared to lean ($p<0.05$, see Figure 3-5).

In lean women, serum CBG positively correlated with total serum cortisol at visits 1 ($p<0.05$) and 3 ($p<0.0001$), but not at visit 2 (see Figure 3-6). The association between CBG and total cortisol at visit 1 was no longer significant when adjusting for maternal age, parity, ethnicity, social class and smoking status ($b=0.31$, adjusted $p=0.3$), but remained significant at visit 3 ($b=0.62$, adjusted $p<0.005$). In obese women, no correlations were seen between CBG and cortisol at any time point; other than a trend at visit 2 in adjusted analyses ($b=0.21$, adjusted $p=0.051$).

3.3.5 Calculated Free Cortisol Differed Only at 36 Weeks

When unbound, or free, cortisol was calculated using Coolen's equation, calculated free cortisol concentrations did not differ between lean and obese women at visits 1 and 2 ($p=0.857$ and $p=0.322$ respectively, see Figure 3-7). At visit 3, obese women were observed to have significantly higher calculated free cortisol concentration than lean women (t-test, $p<0.05$).

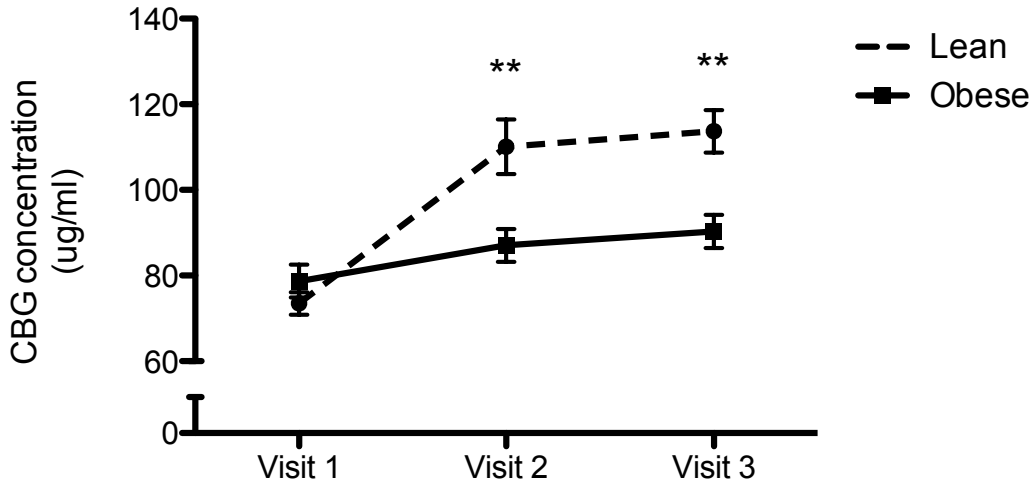


Figure 3-4. Total serum CBG concentrations measured at three time points during pregnancy (16 weeks, 28 weeks and 36 weeks) in lean (n=39) and obese (n=26) women; only pregnant women who had cortisol measurements at all three time points are included in this analysis (mean ±s.e.m; ** difference in CBG concentration between lean and obese p<0.01).

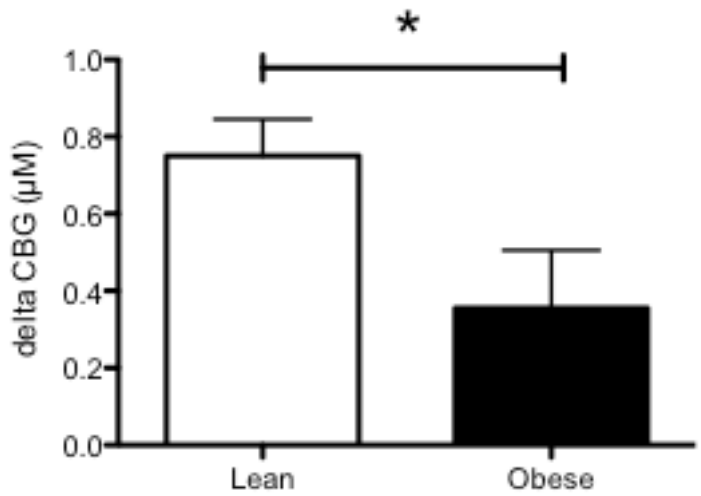


Figure 3-5. Change in CBG concentration (µM) between visits 1 (16 weeks) and visit 3 (36 weeks) in 26 lean and 15 obese women (data are mean ±s.e.m; *difference in change in CBG between lean and obese p<0.05).

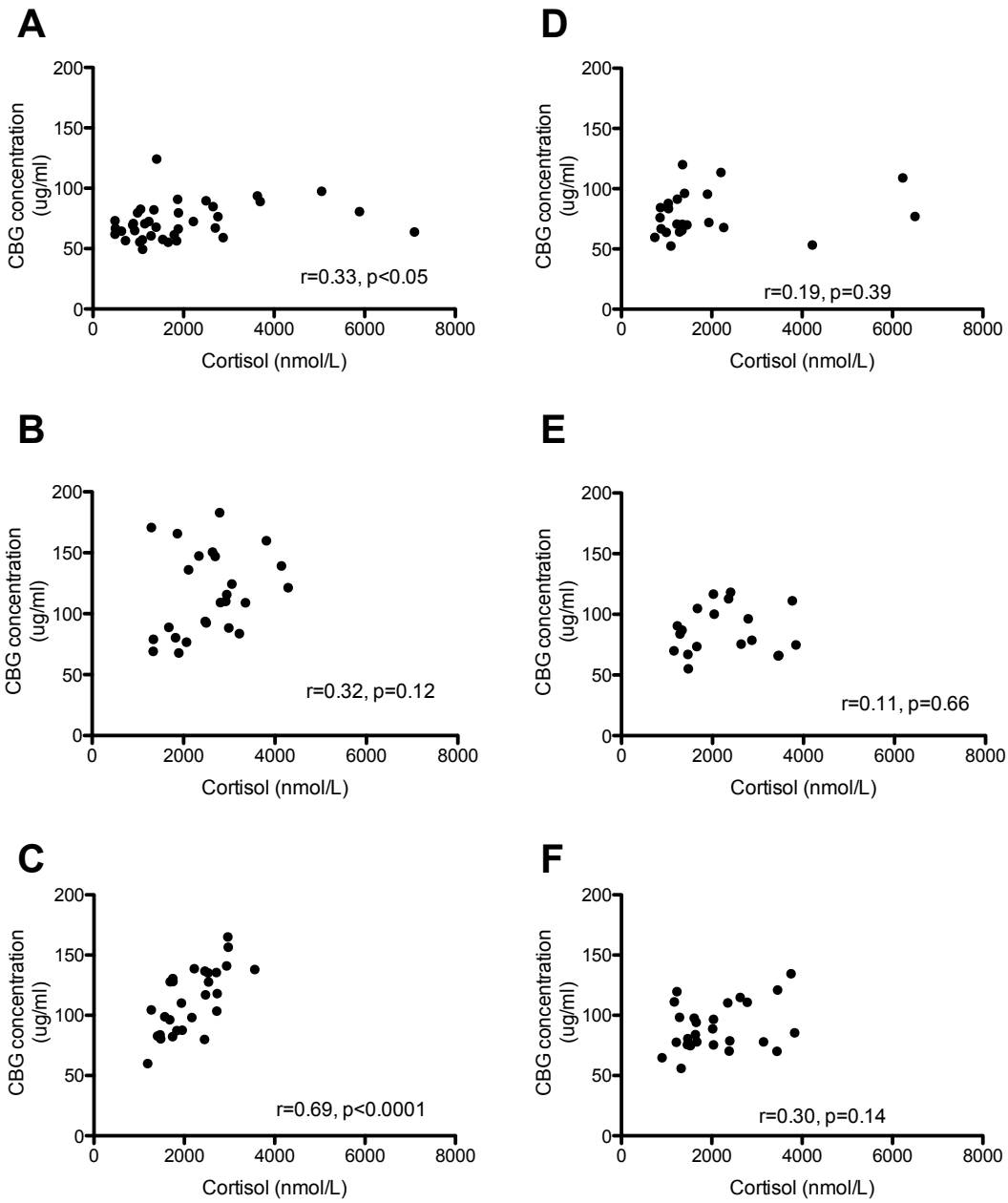


Figure 3-6. Correlations between serum cortisol and serum CBG in lean women at visit 1 (A), visit 2 (B), visit 3 (C), and in obese women at visit 1 (D), visit 2 (E), and visit 3 (F).

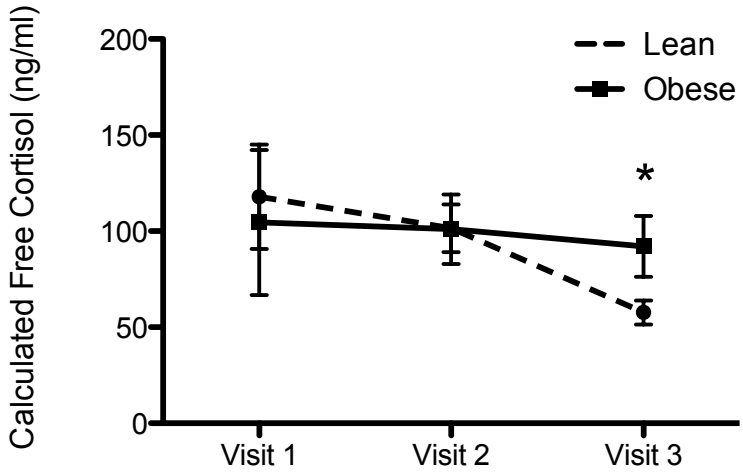


Figure 3-7. Calculated free cortisol concentrations in blood measured at three time points during pregnancy (16 weeks, 28 weeks and 36 weeks) in lean (n=37) and obese (n=26) women as calculated using the Coolens equation; only pregnant women who had cortisol measurements at all three time points are included in this analysis (mean ±s.e.m; * difference between calculated free cortisol $p<0.05$).

3.3.6 CRH

Maternal CRH concentrations rose in both lean and obese women throughout pregnancy, with the greatest rise occurring between visits 2 and 3 (see Figure 3-8). While there was no difference in CRH levels between lean and obese women at visit 1, by visit 2 obese women had significantly lower CRH measurements compared to lean women ($p<0.05$). Similarly, at visit 3, CRH concentrations in obese women tended to be lower than in lean women ($p=0.06$).

As CRH is thought to be involved in regulating the timing of parturition, and levels of CRH rise near parturition, CRH levels were also compared for those women who delivered by spontaneous vaginal delivery (SVD) only. When women delivering by SVD only were considered, obese women were found to have a significantly lower CRH concentration compared to lean at visit 2 ($p<0.01$) and visit 3 ($p<0.05$).

3.3.7 Gestational Weight Gain

Table 3-1 and Figure 3-9 show that total gestational weight gain between visits 1 and 3 was greater in lean than obese ($p<0.0001$). Lean women gained significantly more weight in the period between visits 1 and 2 than in the period between visits 2 and 3 ($p<0.0001$, see Figure 3-9) whereas obese women gained similar amounts of weight between visits 1 and 2 compared with 2 and 3 ($p=0.303$).

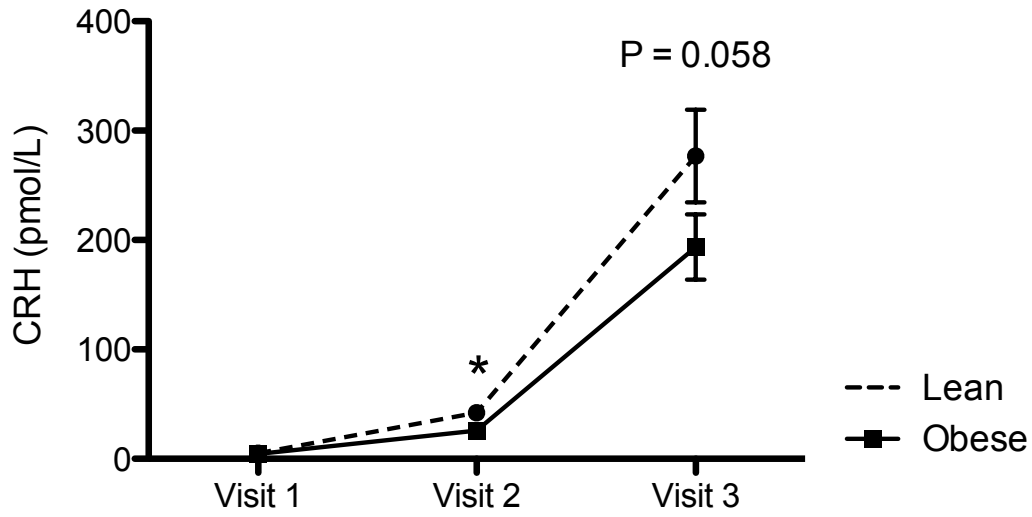


Figure 3-8. Total serum CRH concentrations measured at three time points during pregnancy (16 weeks, 28 weeks and 36 weeks) in lean (n=20) and obese (n=20) women (mean ±s.e.m; * difference in CRH concentration between lean and obese p<0.05).

3.3.8 Gestational Weight Gain and Birth Weight

Increased gestational weight gain between 16 and 36 weeks correlated with increased birth weight in lean women ($r=0.26$, $p<0.01$, see Figure 3-10A). The association was stronger after adjustment for neonate gender and length of gestation ($r = 0.31$, adjusted $p<0.005$), and further strengthened after additional adjustment for maternal smoking status, parity and ethnicity ($r = 0.41$, adjusted $p<0.005$).

In obese women there was no association between birth weight and gestational weight gain (16 to 36 weeks) in unadjusted ($r=0.09$ $p=0.199$, see Figure 3-10B) or adjusted analyses ($p=0.162$ adjusted for neonate gender and length of gestation; $p=0.380$ including maternal smoking status, parity and ethnicity in the model).

3.3.9 Associations Between Maternal Cortisol Concentrations and Gestational Weight Gain

Higher maternal serum cortisol concentrations at visit 1 were associated with lower gestational weight gain between 16 and 36 weeks in both lean and obese women (lean

$r=-0.18$, unadjusted $p=0.08$, adjusted $p<0.05$ after adjustment for maternal age, maternal weight at visit 1, parity, smoking status, social class and ethnicity; obese $r=-0.16$, $p<0.05$; adjusted $p<0.05$). No other significant associations were observed between total cortisol concentrations and gestational weight gain measurements in lean women or obese women. Calculated free cortisol did not correlate with gestational weight gain at any time point in either lean or obese women.

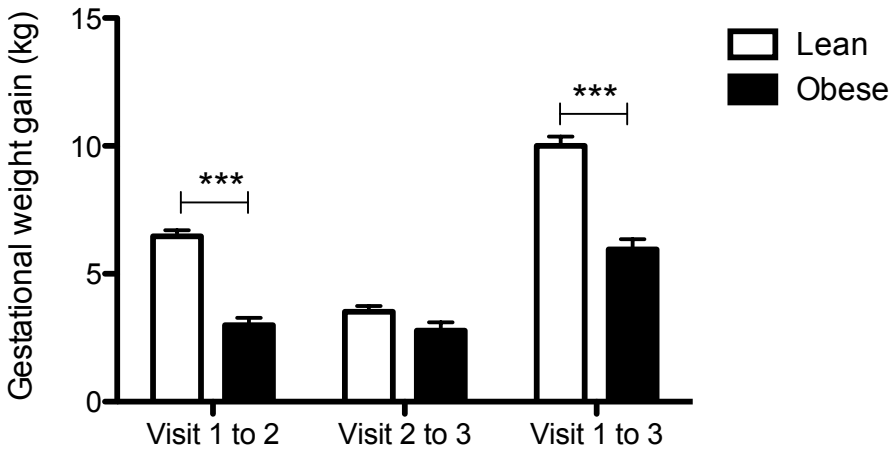


Figure 3-9. Gestational weight gain throughout pregnancy. Weight gain was greater in lean women compared to obese women between visits 1 and 2 and between visits 1 and 3 (* difference in weight gain between lean and obese $p<0.0001$, data are mean±s.e.m).**

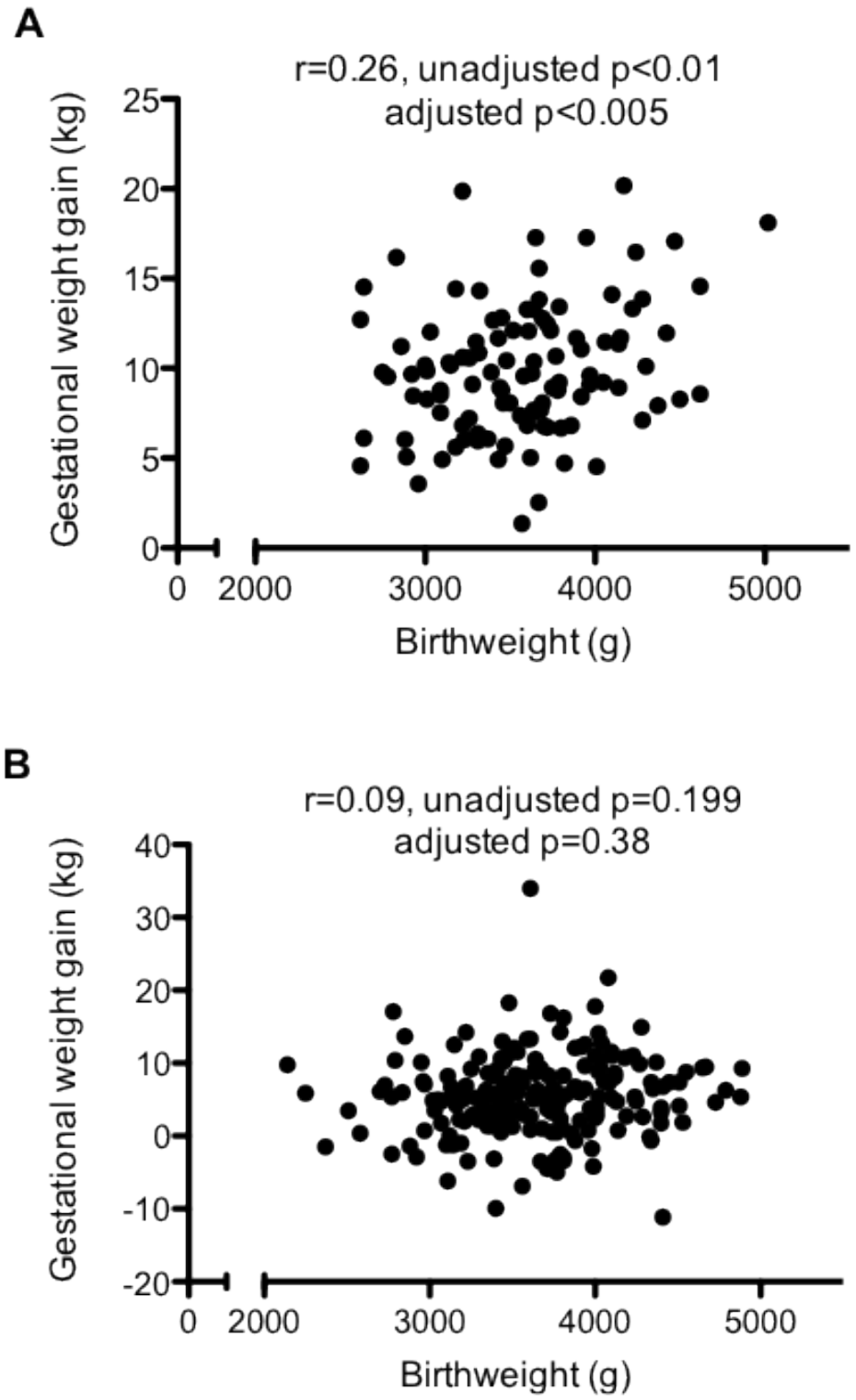


Figure 3-10. Gestational weight gain plotted against birth weight for 107 lean (A) and 173 obese (B) women, adjusted p with neonate gender, length of gestation, maternal smoking status, parity and ethnicity included.

3.3.10 Birth weight and Total Maternal Cortisol

Birth weight did not differ between lean and obese women in unadjusted analyses (p=0.276, see Figure 3-11) or after adjustment for gestation at delivery and newborn gender (adjusted p=0.18).

No correlations were found between birth weight and any cortisol measurement in obese (see Figure 3-12) or lean women (see Figure 3-13) in univariate analyses or after adjustment for gestation at delivery, gender, maternal smoking status, parity, ethnicity and social class.

3.3.11 Birth Weight and Calculated Free Maternal Cortisol

In lean women there was a positive correlation between birth weight and calculated free cortisol at visit 1 (r=0.35, p=0.05, see Figure 3-14). When length of gestation and neonate gender were adjusted for, the association was weakened (b=116, adjusted p=0.08), and was no longer significant when length of gestation, neonate gender, maternal social status (as measured by DEPCAT), smoking status, parity and ethnicity were adjusted for (b=122, adjusted p=0.112). No other correlations were observed between birth weight and calculated unbound cortisol in lean women.

In obese women, lower birth weight was associated with higher calculated free cortisol at visit 1 (r=-0.46, p=0.05, see Figure 3-15). This finding was strengthened after adjustment for neonate gender and length of gestation (b=-171, adjusted p<0.05). Furthermore the association remained significant after

further adjustment for maternal social status (as measured by DEPCAT), smoking status, parity and ethnicity (b=-267, adjusted $p<0.05$). No other correlations were observed between birth weight and calculated unbound cortisol at any time point.

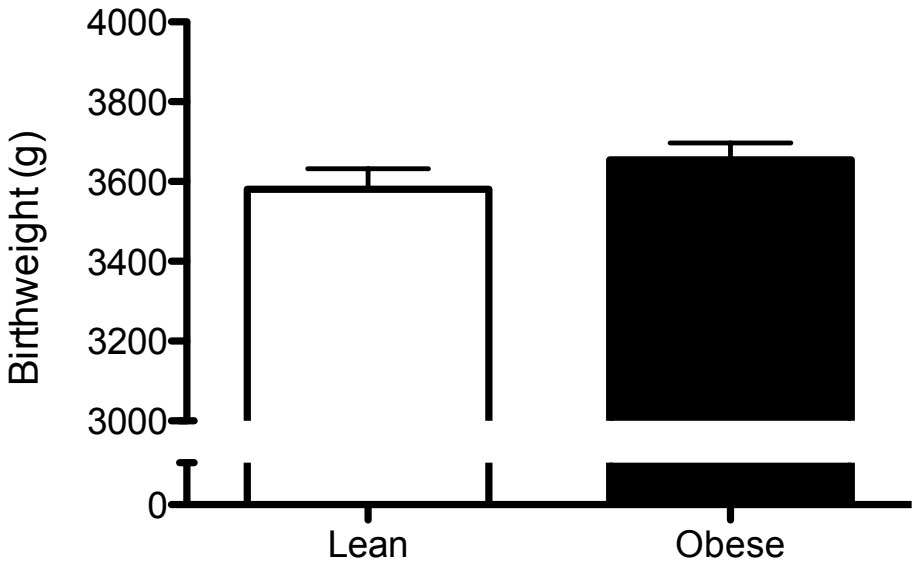


Figure 3-11. Birth weight in grams for lean (n=94) and obese (n=159) groups (p=0.276;, data are mean ±s.e.m).

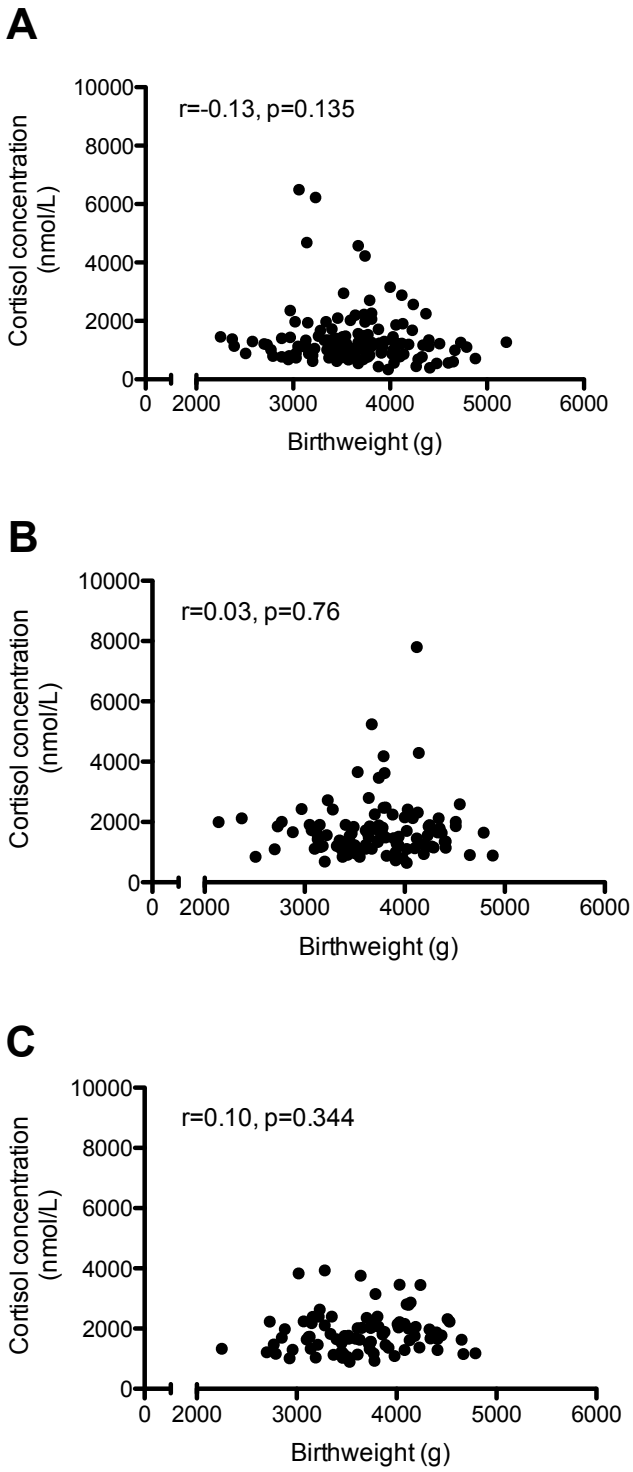


Figure 3-12. Birth weight and concentration of total blood cortisol measured at visit 1 (A), visit 2 (B) and visit 3 (C) in 173 obese women.

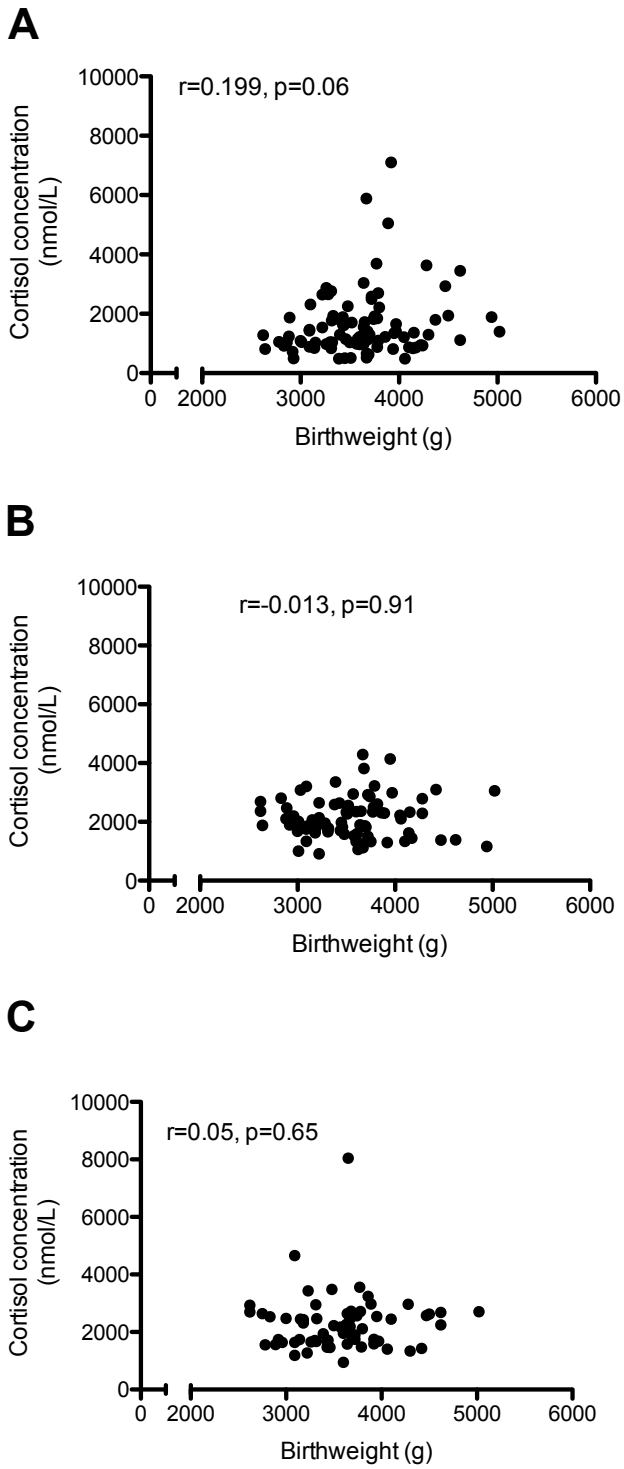


Figure 3-13. Birth weight and concentration of total blood cortisol measured at visit 1 (A), visit 2 (B) and visit 3 (C) in 107 lean women.

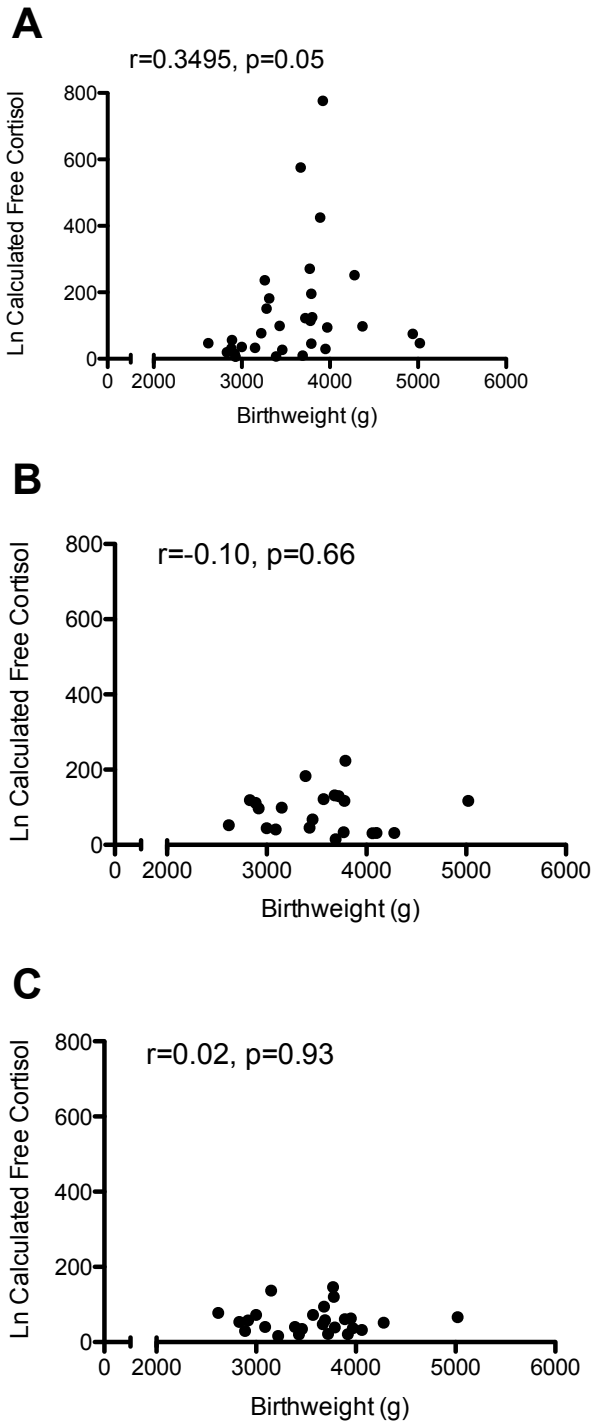


Figure 3-14. Birth weight and concentration of calculated free cortisol measured at visit 1 (A), visit 2 (B) and visit 3 (C) in serum samples from 37 lean women.

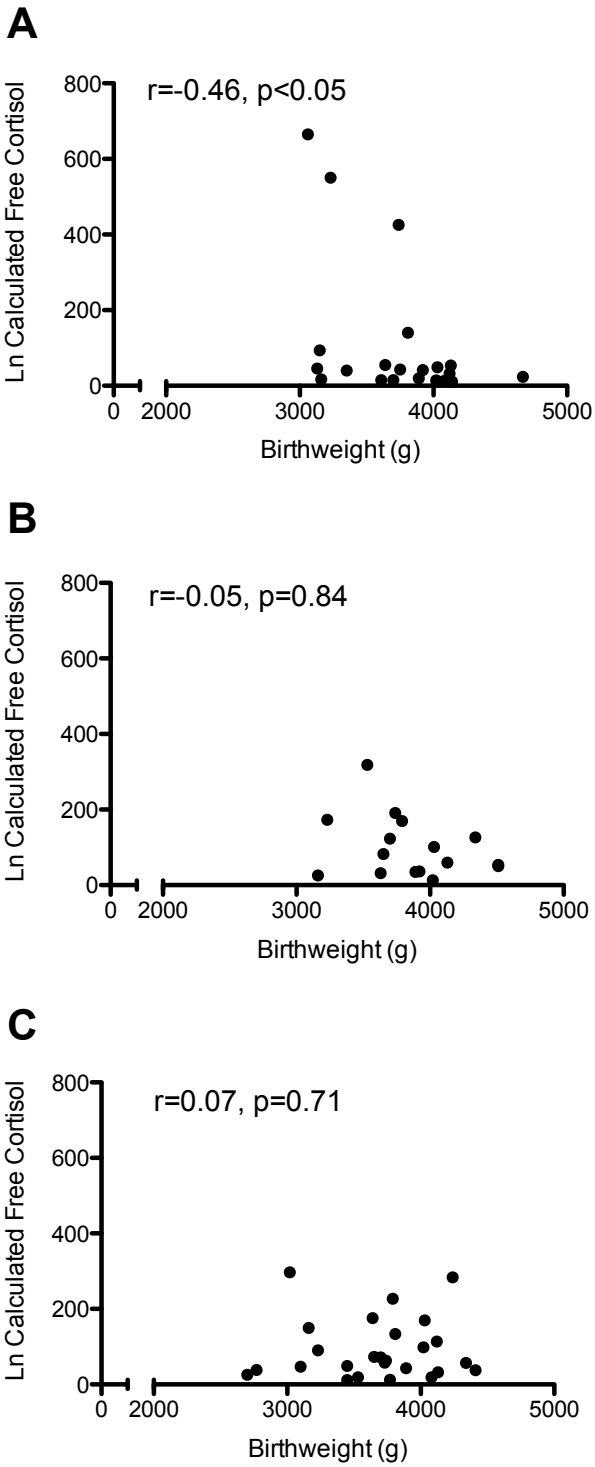


Figure 3-15. Birth weight and natural logged concentration of calculated free cortisol measured at visit 1 (A), visit 2 (B) and visit 3 (C) in serum samples from 26 obese women.

3.3.12 Cortisol and Timing of Gestation

Total cortisol concentration measured at visit 2 was found to correlate with length of gestation in lean women in unadjusted analysis ($r=0.22$, $p<0.05$; adjusted $p=0.26$), however there were no other associations between length of gestation and total cortisol at any time point in either lean (visit 1: $r=0.15$, $p=0.14$, adjusted $p=0.22$; visit 3: $r=0.003$, $p=0.98$, adjusted $p=0.19$) or obese women (visit 1: $r=-0.03$, $p=0.72$, adjusted $p=0.29$; visit 2: $r=-0.06$, $p=0.50$, adjusted $p=0.54$; visit 3: $r=0.05$, $p=0.67$, adjusted $p=0.16$) in unadjusted analysis or when mode of delivery was adjusted for. Furthermore, when only those women who gave birth by SVD were analysed, no associations were observed.

Similarly, no associations were found between length of gestation and free cortisol at any time point in either lean (visit 1: $r=0.16$, $p=0.42$; visit 2: $r=0.23$, $p=0.31$; visit 3: $r=-0.15$, $p=0.51$) or obese women (visit 1: $r=0.28$, $p=0.25$; visit 2: $r=0.23$, $p=0.42$; visit 3: $r=0.07$, $p=0.77$) in unadjusted analysis or when mode of delivery was adjusted for. Furthermore, when only those women who gave birth by spontaneous vaginal delivery were analysed, no associations were observed.

3.3.13 CRH and Timing of Gestation

CRH concentration measured at visit 2 was found to correlate with length of gestation in obese women in unadjusted analysis, but not when adjustments were

made for mode of delivery, maternal age, smoking status, DEPCAT category and parity

(r=-0.48, p<0.05, adjusted p=0.22). No associations were found between length of gestation and CRH at any other time point in either lean (n=19, visit 1: r=-0.37, p=0.12, adjusted p=0.35; visit 2: r=-0.27, p=0.99, adjusted p=0.99); visit 3: r=-0.07, p=0.75, adjusted p=0.48) or obese women (n=20, visit 1: r=0.25, p=0.29, adjusted p=0.39; visit 3: r=-0.26, p=0.28, adjusted p=0.57) in unadjusted analysis or when adjustments were made.

When only women who gave birth by SVD are included, significant correlations between length of gestation and CRH concentration at visit 2 (r=-0.73, p<0.05, adjusted p<0.05) and visit 3 (r=-0.73, p<0.05, adjusted p<0.05), but not visit 1 (r=-0.57, p=0.07, adjusted p=0.09) are observed in obese women in unadjusted analysis and when adjusting for maternal age, smoking status, parity and DEPCAT category. No associations are observed in lean women who gave birth by SVD at visit 1 (r=0.04, p=0.91, adjusted p=0.13), visit 2 (r=0.42, =0.18, adjusted p=0.09) or visit 3 (r=0.34, p=0.28, adjusted p=0.35).

3.4 Discussion

An adverse fetal intra-uterine environment has been associated with increased risks of metabolic and cardiovascular disorders later in life (Barker *et al.* 1993a; Godfrey *et al.* 1994; Barker 1995; Godfrey and Barker 2000). In animal models exposure to excess glucocorticoids during pregnancy has been associated with reduced birth weight, and affects development and maturation of organs (Seckl 2004) and thus glucocorticoid overexposure is one of the key mechanisms proposed to underlie fetal programming. Here, we demonstrated for the first time that total cortisol levels in very severely obese women were lower throughout pregnancy than with a normal weight control group. Furthermore, levels of CBG were also lower. Reflecting the lower total cortisol and CBG levels, free cortisol levels were found to be similar between lean and obese women until near term. CRH levels were also found to be lower among obese women and predicted length of gestation.

3.4.1 Cortisol Levels are Lower in Obese Women Throughout Pregnancy

Total serum cortisol measurements were found to be lower in obese women compared to lean women throughout pregnancy, while postnatal serum measurements were similar between the two groups. This suggests a difference in overall maternal HPA-axis response to pregnancy during pregnancy according to maternal BMI. The lower cortisol levels were observed from the first measurement in the early second trimester, but the pattern of change (rise) of

cortisol throughout pregnancy did not differ between lean and obese women as pregnancy progressed. This suggests that there is a change in HPA-axis activity in early pregnancy in obese women, leading to the lower observed total cortisol measurements. It is possible that the observed difference in total cortisol levels may reflect increased urinary clearance of circulating glucocorticoids in obese women compared to lean, a phenotype observed in non-pregnant obese individuals (Andrew *et al.* 1998). However total urinary glucocorticoid metabolites did not differ between lean and obese (n=4 per group) (R.Reynolds personal communication). It is also possible that differences in the activity of the placental glucocorticoid-inactivating enzyme *11βHSD2* may contribute to differences in maternal cortisol levels; this will be further explored in Chapter 4.

Diurnal production of cortisol (as measured by saliva sampling) was maintained in both lean and obese in the first half of pregnancy. Due to the small sample size no differences in cortisol measurements between lean and obese were observed, despite the lower plasma levels in obese. A larger sample size is required.

3.4.2 CBG Is Lower In Obese Women

In humans, 90 to 95% of circulating cortisol in the blood is bound to the specific steroid transport protein CBG. The concentrations and binding activity of this protein is thought to regulate the bioavailability of the steroid to target cells, with bound cortisol being considered essentially 'biologically inactive' (Fernandez-Real *et al.* 1999). However, recent evidence suggests that CBG may in fact be

directly involved in signal transduction and bioavailability of glucocorticoids (Petersen *et al.* 2006). In this study, CBG was lower in obese women compared to lean, with a significant lower rise throughout pregnancy. The lack of rise in CBG may help explain the lower cortisol concentrations observed in obese women throughout pregnancy. Furthermore, CBG concentration was found to positively correlate with total cortisol concentration in both early and late pregnancy in lean women, but not in obese, again suggesting that obese women may lack a CBG-dependent rise in plasma cortisol concentrations. The reasons for the lack of rise in CBG in obese women are unknown, but may reflect differences in estrogen levels in obese pregnant women (which is currently undergoing investigation in this cohort), alterations in liver function of obese women (Torpy and Ho 2007), insulin levels or inflammatory mediators (Fernandez-Real *et al.* 1999). No association was found between insulin levels and serum CBG production (data not shown).

3.4.3 CRH is Lower in Obese Women and Predicts Length of Gestation

Placental CRH is an important regulator of HPA axis activity during pregnancy, and plasma concentrations of CRH increase throughout pregnancy to a concentration of 1 to 10nmol/L at term (Shibasaki *et al.* 1982; McLean *et al.* 1995). CRH increased from visit 2 onwards in both groups, reflecting increasing placental production of the hormone from the second trimester. Obese women had a lower level of CRH compared to lean during the second half of pregnancy.

As CRH is a key promoter of cortisol production, this finding may explain the observed reduction in circulating cortisol, suggesting an overall suppression of HPA-axis activity in obese pregnancy. This may have the ultimate effect of reducing fetal exposure to maternal glucocorticoids, although further work on placental transit and fetal tissue effects is required. CRH concentration was found to negatively correlate with length of gestation in obese women who gave birth by SVD, reinforcing the hypothesis that this hormone is involved in the timing of parturition (McLean *et al.* 1995). This finding suggests that in obese women, increased HPA axis activation may predict length of pregnancy, presumably through the maturational effects of glucocortoicoids. Intriguingly, this association was not observed in lean women. This may reflect a higher sensitivity to the hormone in obese women in response to lower circulating levels. However, free cortisol levels are similar in obese and lean women, suggesting that sensitivity may in fact be unaffected by weight status.

The lower levels of CRH observed in obese pregnancy may explain in part the lower levels of cortisol and CBG also observed. This in turn may lead to a lower production of CBG by the maternal liver to compensate for the lower levels of circulating glucocorticoids. This may imply that the changes in CBG are primarily directed at regulating maternal glucocorticoid exposure in response to lower CRH production; i.e. to ensure sufficient levels of cortisol are present for normal physiological function (e.g. gluconeogenesis and immune functions) despite reduced production. The regulation of cortisol in pregnancy is complicated by the need for balance between regulating fetal exposure and the normal physiological role of cortisol in the mother.

3.4.4 Free Cortisol Concentrations Are Higher in Obese Women by Term

While total cortisol provides a useful measure of glucocorticoid production, it is thought that measurements of free (or unbound) cortisol are more biologically significant, reflecting the levels of glucocorticoid that are biologically active and available (Arafah 2006). In order to calculate free cortisol values, Coolen's equation was used. This equation uses constant values for variables such as the binding affinity of CBG and the albumin content of blood (Coolens *et al.* 1987). The equation has been validated in patients with sepsis and healthy controls (Ho *et al.* 2006; Dorin *et al.* 2009) but not in pregnancy where CBG binding affinity may be altered (Ho *et al.* 2007).

Nevertheless the values of calculated free cortisol were found to be very similar between lean and obese women between visits 1 and 2, but obese women were found to have significantly higher calculated values near term (at visit 3) indicating a higher fetal exposure to bioavailable (as opposed to total) cortisol (Vogeser *et al.* 1999; le Roux *et al.* 2003). Thus the increase in free cortisol at the end of pregnancy in obese women may induce late pregnancy specific effects, in particular affecting the timing of organ maturation, potentially leading to programming effects. In accord with this, in obese the higher free cortisol levels at visit 1 were associated with lower birth weight. Importantly, recent work has suggested that CBG may act as a transporter or carrier of glucocorticoids, with direct involvement in signal transduction in target tissues, with CBG thought to be a necessary component of glucocorticoid signaling (Henley and Lightman 2011). If this is the case in humans, the lower levels of

CBG seen in obese pregnancy, and by association the higher free cortisol levels, may not be indicative of higher bioavailability of cortisol to target tissues.

Regardless, lower levels of total maternal cortisol may have detrimental effects on the fetus and pregnancy outcome. Post-traumatic stress disorder has been associated with hypocortisolism (Rohleder *et al.* 2004), and psychological trauma among pregnant women associated with the World Trade Center attacks in 2001 has been linked to lower maternal salivary morning cortisol levels postnatally, longer gestation and reduced infant head circumference at birth (Engel *et al.* 2005; Brand *et al.* 2006). However, hypocortisolism has not been found in all studies of post-traumatic stress disorder, and in fact cortisol levels may be normal, or higher or lower than normal (Young et al., 2004; Young and Breslau, 2004a; Young and Breslau, 2004b; Fink 2011). Nevertheless, Addison's disease (a condition characterized by reduced production of glucocorticoids) during pregnancy, whether diagnosed or undiagnosed, increases the risk of both cesarean delivery and preterm delivery (Bjornsdottir *et al.* 2010). The underlying mechanisms are unknown, but in the case of Addison's disease have been suggested to be related to reduced fetal adrenal production of DHEAS, an important precursor for placental production of estradiol. The lower estradiol production that results may therefore lead to a rise in the estradiol/estriol ratio, giving rise to an increased risk of preterm birth (Bjornsdottir *et al.* 2010). Therefore, the lower cortisol production may be a marker rather than a direct cause of poor pregnancy outcome in hypocortisolaemic women. Cortisol is also crucial for proper fetal development, with roles in the maturation and function of

a number of fetal organs, including the liver, lungs and fetal adrenals (Smith *et al.* 1974; Avery 1975; Liggins 1994).

3.4.5 Maternal Weight and GWG

Obese women were found to gain less weight than lean women throughout pregnancy (between 16 and 36 weeks), and in particular obese women gained less weight than lean women in the period between visits 1 and 2. Mean gestational weight gain was at the lower end of the Institute of Medicine guidelines for both lean and obese women (defined as approximately 11 to 16 kg for women with a normal BMI and at least 5kg for obese women) (Rasmussen and Yaktine 2009). Gestational weight gain between visits 1 and 3 was found to correlate positively with birth weight in lean women. This may reflect the increased weight of the fetal-placental unit contributing to the mother's weight, or alternatively may suggest that women with higher nutritional intake were better able to support increased fetal growth. Gestational weight gain was not found to correlate with birth weight in obese women however, suggesting that other factors, such as compromised placental function (e.g. nutrient transport), may be more important in obese women. Higher maternal serum cortisol concentrations in early pregnancy were associated with lower gestational weight gain between 16 and 36 weeks in both lean and obese women. This is in agreement with the findings of animal studies that have suggested glucocorticoids can suppress appetite and inhibit food intake, and highlights a possible link between maternal diet and HPA axis activity (Liu *et al.* 2011).

While gestational weight gain may be useful as a surrogate of fetal growth, future studies may benefit from a more direct measure such as ultrasound in order to more effectively track fetal growth.

3.4.6 Conclusions

In this study of very severely obese women, total cortisol levels were lower throughout pregnancy than in lean women. A lower concentration of total cortisol in obese women may indicate a substantial reduction in the level of glucocorticoids that the developing fetus is exposed to during pregnancy, potentially protecting the baby from detrimental programming effects. If lower cortisol measurements are common amongst the wider obese pregnant population, this finding may partly explain the increased prevalence of higher birth weights and macrosomia amongst obese pregnant women (Ehrenberg *et al.* 2004).

The placenta acts as a barrier to glucocorticoids, but is also intimately involved in both the maternal HPA-axis and regulating fetal growth. The effects of maternal obesity on this organ, particularly on the barrier enzyme 11βHSD2, shall be explored in the next chapter.

Chapter 4:

Placental Gene Expression

4.1 Introduction

In Chapter 3, it was shown that maternal obesity was associated with lower levels of total circulating cortisol throughout pregnancy. Glucocorticoids are powerful regulators of fetal growth and development, and the lower levels of cortisol that the fetus of an obese woman is exposed to may partly explain the macrosomia associated with maternal obesity.

In order to protect the fetus from the potentially harmful effects of circulating glucocorticoids, the placenta expresses a 'barrier enzyme', 11βHSD2, which inactivates cortisol by converting it to the biologically inert cortisone (Brown *et al.* 1993). Deficiencies of this enzyme have been associated with decreased birth weight and poor placental development. Maternal malnutrition in rodents has been shown to reduce transcript levels of *11βHSD2*, suggesting a link between maternal diet and the efficacy of the placental glucocorticoid barrier (Lesage *et al.* 2001b). Furthermore, low placental *11βHSD2* has been observed in low birth weight babies (Stewart *et al.* 1995).

The growth and development of the placenta itself is also an important variable when considering birth outcomes. A larger placenta is likely to be more efficient than a smaller placenta, due to an increased surface area contacting the maternal bloodstream over which nutrient exchange can occur. Placental growth and development is dependent on a number of key regulatory genes, including the growth factor *IGF2* and the nuclear receptor *GR*. A number of other genes, including inflammatory regulators and targets of *GR* are also important in placental function and potential programming effects on the fetus. These genes,

which include *GILZ*, *PPARγ*, *IL6* and *IL1β*, have been shown to regulate several important mechanisms, including steroidogenesis, inflammation, placental invasion and growth (Jauniaux *et al.* 1996; Roth *et al.* 1996; Roberts *et al.* 2003; Makris *et al.* 2006; Zhao *et al.* 2006; Matsuda *et al.* 2013). The genes *IGF2R* and *H19*, which are thought to antagonistically interact with the growth factor *IGF2* are also important modulators of fetal and placental growth, and are thought to be particularly important in growth restriction through the phenomenon of imprinting, whereby allele-specific expression of certain genes, and their interactions with one another, tightly regulate both fetal and placental growth, with potentially significant impacts on birth weight as a result (Perkins *et al.* 2012; St-Pierre *et al.* 2012; Turan *et al.* 2012).

In order to investigate the effects of maternal obesity on placental gene and protein levels and localisation, samples were taken from three time points in pregnancy: first trimester, second trimester and at term. Furthermore, fetal liver tissues were also sampled during the second trimester in order to determine the potential effects of maternal obesity in the fetal compartment.

4.1.1 Hypothesis

It was hypothesized that maternal obesity would be associated with increased placental mRNA transcript levels of the glucocorticoid barrier enzyme *11βHSD2*, as well as other key genes involved in nutrient transport, placental growth and development, resulting in lower fetal exposure to maternal cortisol but higher exposure to nutrients for growth.

4.1.2 Aims

1. To investigate protein localization and abundance of the glucocorticoid barrier enzyme 11βHSD2 in placental trophoblast tissue obtained from the first trimester and second trimester of pregnancy, and at term.

2. To identify suitable control 'housekeeping' genes for use in real time PCR analysis for first trimester, second trimester and term tissues.

3. To investigate the effects of maternal obesity on placental mRNA transcript levels of key genes, including *11βHSD2*, *GR* and *IGF2*, and other genes involved in growth and development of the placenta in the first trimester and second trimester of pregnancy, as well as at term.

4. To investigate the effects of maternal obesity on fetal liver mRNA transcript levels of key genes, including the glucocorticoid barrier enzyme *11βHSD2*, the glucocorticoid receptor *GR* and the growth factor *IGF2* in the second trimester of pregnancy.

4.2 Methods

4.2.1 Tissues

First and second trimester tissue was collected from women at time of termination of pregnancy and term placental tissue was collected at elective caesarean section as described in methods section 2.1. For first trimester and second trimester studies, where samples were limited in number, placental biopsies were collected from women with a range of BMIs, including lean, normal, overweight, and obese women. For the term placental gene expression arm of the study, placental biopsies were collected from lean women (BMI < 25 kg/m^2) and obese women (BMI > 30 kg/m^2) at delivery; the lean and obese groups were analysed separately in order to identify differences in gene expression.

4.2.2 Immunohistochemistry

A single NBF-stored sample selected at random from placental samples from each gestation was used for imaging 11βHSD2 protein using immunohistochemistry techniques as described in Section 2.3.4.1.

4.2.3 RNA Extraction

Tissues stored in RNALater were used for RNA purification and extraction as described in Section 2.3.1.1.

4.2.4 Reverse Transcription and Real Time PCR

RNA successfully extracted from tissues was converted to cDNA using Roche Reverse Transcription kits as described in Section 2.3.1.4. cDNA was used to perform qPCR in order to estimate mRNA transcript levels of key target genes as described in detail in Section 2.3.1.5.

4.2.5 Statistical Analyses

mRNA transcript data was normalised to the control genes *PPIA* for first trimester samples, *YHWAZ* for term samples, and the mean of *TBP* and *β-ACTIN* for second trimester samples. Normal distribution of data was assessed visually using histograms, and statistically using the D'Agostino & Pearson omnibus normality test. mRNA transcript data were not normally distributed and were normalised using natural log transformation. Correlations between mRNA transcript levels and maternal BMI in first and second trimester tissues were analysed using Pearson correlation. Adjustments for confounding factors were performed using multiple linear regression. Differences between mRNA transcript levels between obese and lean groups at term were compared using independent t-tests.

All data are presented as mean (±s.e.m), and p values of less than 0.05 were considered significant.

Power calculations were performed to determine the sample sizes for analyses at each time point during pregnancy. Statistical power calculations are necessary to

determine the sample size necessary to limit the probability of commiting a type II error (i.e. incorrecty rejection of the null hypothesis)

For correlation analyses (i.e. first trimester and second trimester gene expression), a minimum sample size of 29 biopsies was determined necessary to detect a correlation $r \geq 0.4$ with an alpha of 0.05 and a statistical power of 0.8.

For grouped analyses (i.e. term placental tissue), a minimum sample size of 56 was determined necessary to detect a 20% difference in gene expression between groups, based on an alpha of 0.05, and a statistical power of 0.8.

Analysis was performed using Minitab (Minitab Inc, PA, USA) and Statistica (StatSoft Inc, OK, USA). Graphs were prepared using GraphPad Prism (GraphPad Software Inc, CA, USA).

4.3 Results

4.3.1 Immunohistochemistry

4.3.1.1 11βHSD2 Protein Positive and Negative Controls

Mouse ectopic trophoblast tissue was used as a positive control for the 11βHSD2 immunohistochemistry antibody, as shown in Figure 4-1. This antibody has been validated in both human and mouse tissues; murine ectopic tissue has also been validated and used as a positive control in prior work by Jeremy Brown (unpublished data).

Figure 4-2 shows nuclear counterstaining without use of the 11βHSD2 antibody in the same tissue as a negative control.

4.3.1.2 11βHSD2 Protein in First Trimester Placental Tissue

Figure 4-3 shows a representative slide showing the distribution of 11βHSD2 protein in first trimester tissue at low and high magnifications. In first trimester trophoblast tissue, 11βHSD2 protein appears to be limited to the syncytiotrophoblast, or the outermost layer of the placental villi, with an apparent absence of any protein in the inner cytotrophoblast tissue (see Figure 4-3B). 11βHSD2 protein is present throughout the syncytiotrophoblast, forming a complete barrier around the entirety of the placental villi. At this stage of pregnancy, the syncytiotrophoblast appears as a thick layer surrounding the entire villus. Furthermore, the villi appear relatively undifferentiated, with a low

surface area to volume ratio, typical of early placental development (see Figure 4-3A).

4.3.1.3 11βHSD2 Protein in Second Trimester Placental Tissue

In second trimester tissue, 11βHSD2 protein is again limited to the syncytiotrophoblast as seen in first trimester tissue, and again appears to be expressed throughout most of the syncytium surrounding the placental villi (see Figure 4-4). It should be noted that there appears to be greater differentiation of the villi when compared to first trimester tissues (see Figure 4-4A) and the area in which 11βHSD2 is expressed, and by extension, the syncytiotrophoblast appear reduced, with a much thinner layer of tissue stained when compared to first trimester samples (see Figure 4-4E).

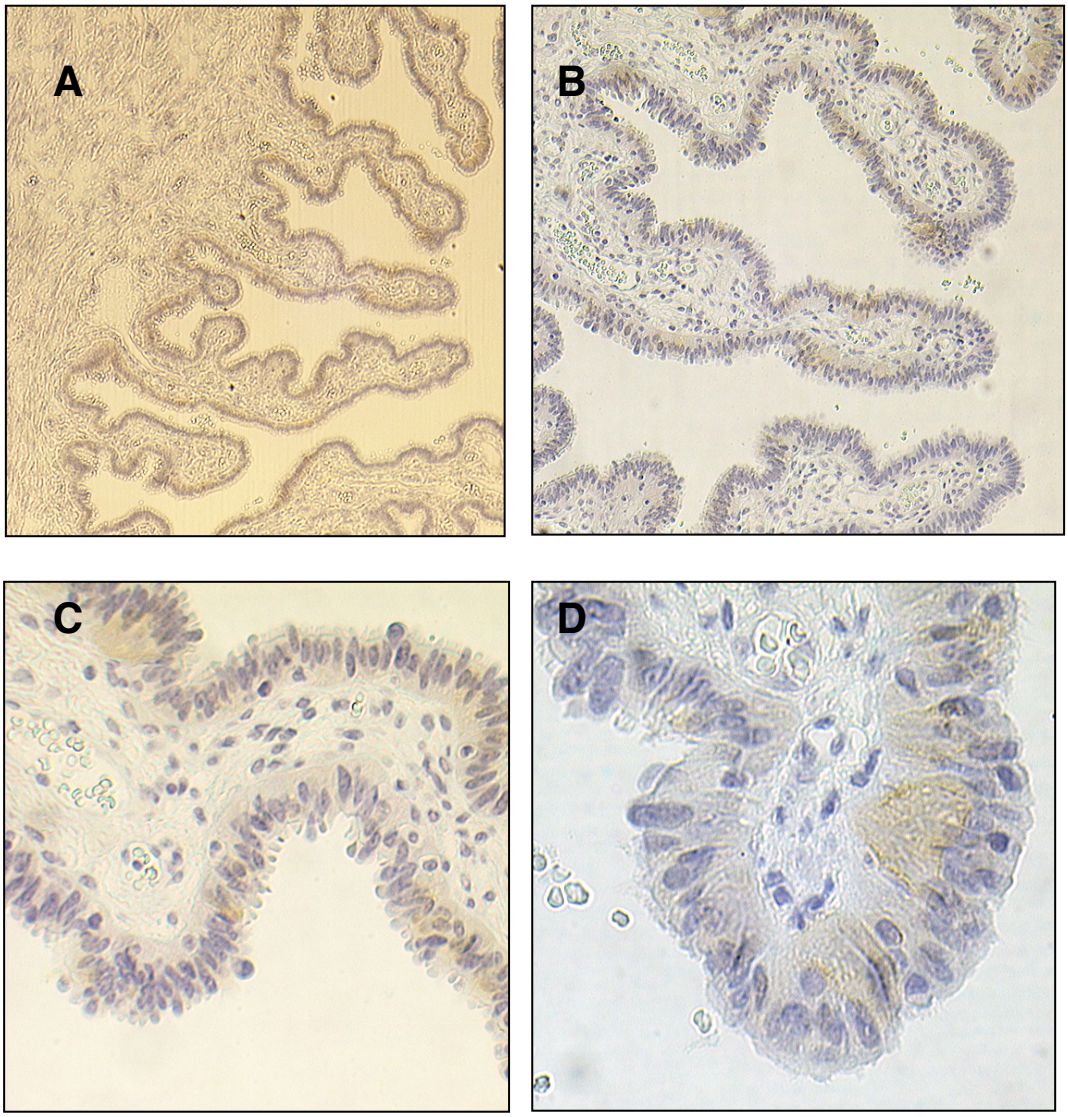


Figure 4-4. A-D: Immunohistochemistry staining (positive control) for 11βHSD2 protein with nuclear counterstaining (blue) in ectopic trophoblast tissue, A: 50x magnification, B: 100x magnification, C: 250x magnification, D: 400x magnification of trophoblast villi.

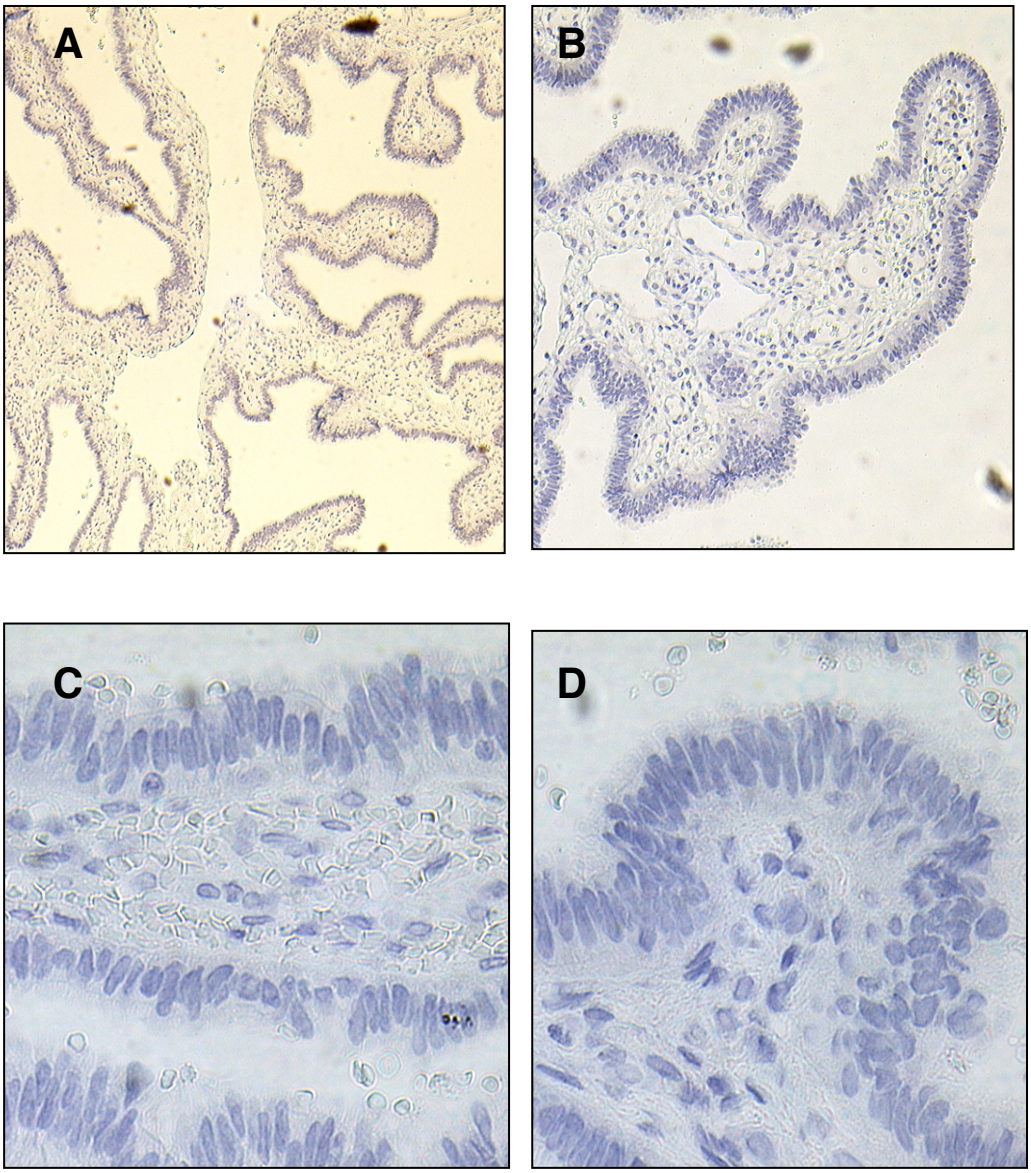


Figure 4-2. A-D: Immunohistochemistry staining (negative control) for 11βHSD2 protein with nuclear counterstaining (blue) in ectopic trophoblast tissue, A: 50x magnification, B: 100x magnification, C & D: 400x magnification of trophoblast villi.

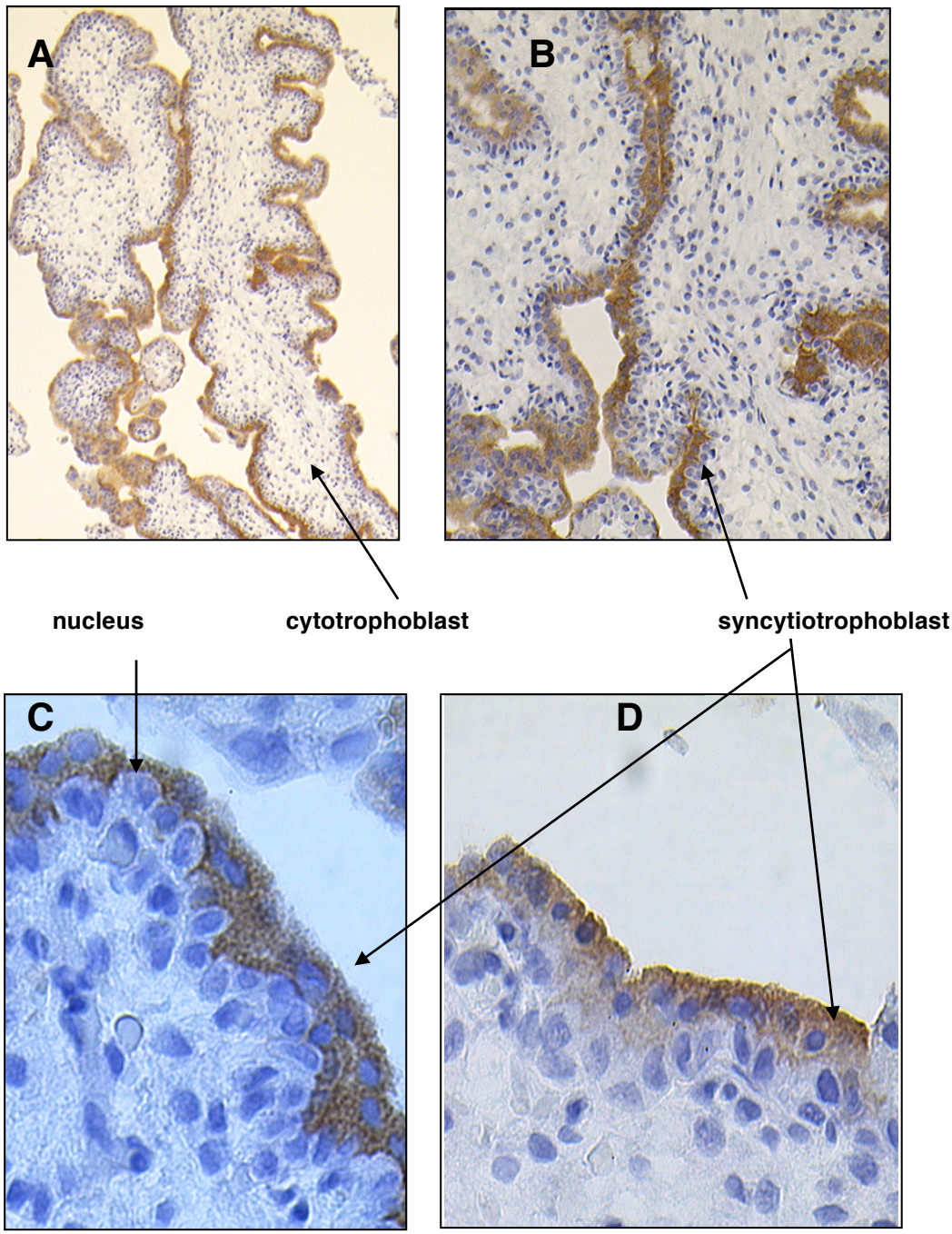


Figure 4-3. A-D: Immunohistochemistry staining for 11βHSD2 protein (brown) with nuclear counterstaining (blue) in first trimester placental trophoblast biopsy tissue, A: 50x magnification, B: 100x magnification, C & D: 400x magnification of placental villi.

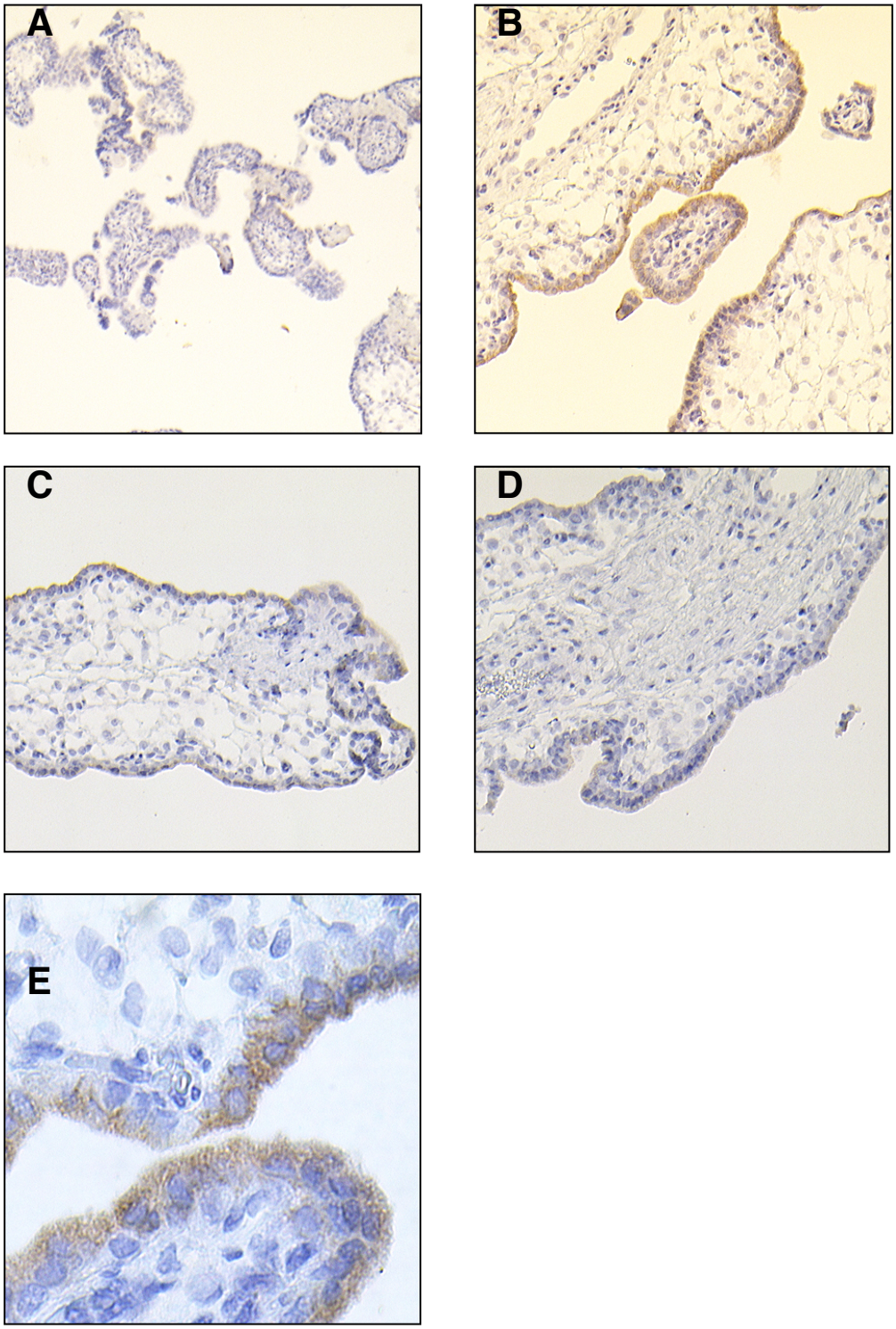


Figure 4-4. A-E: Immunohistochemistry staining for 11βHSD2 protein (brown) with nuclear counterstaining (blue) in second trimester placental trophoblast biopsy tissue, A & B: 50x magnification, C & D: 100x magnification, E: 400x magnification of placental villi.

4.3.2 11βHSD2 Protein in Term Placental Tissue

Term tissues show 11βHSD2 protein that is localized to the syncytiotrophoblast as seen in first and second trimester tissues (see Figure 4-5). The protein appears to be expressed throughout the entirety of the syncytium layer, forming a complete and continuous layer around even the smallest villi (see Figure 4-5B). The villi appear more numerous and more highly branched than at previous time points during pregnancy,. There also appears to be greater vascularisation of the cytotrophoblast, with a number of blood vessels apparent. Finally, the syncytium appears as a very thin monolayer, with characteristic nuclear bulging (see Figure 4-5D).

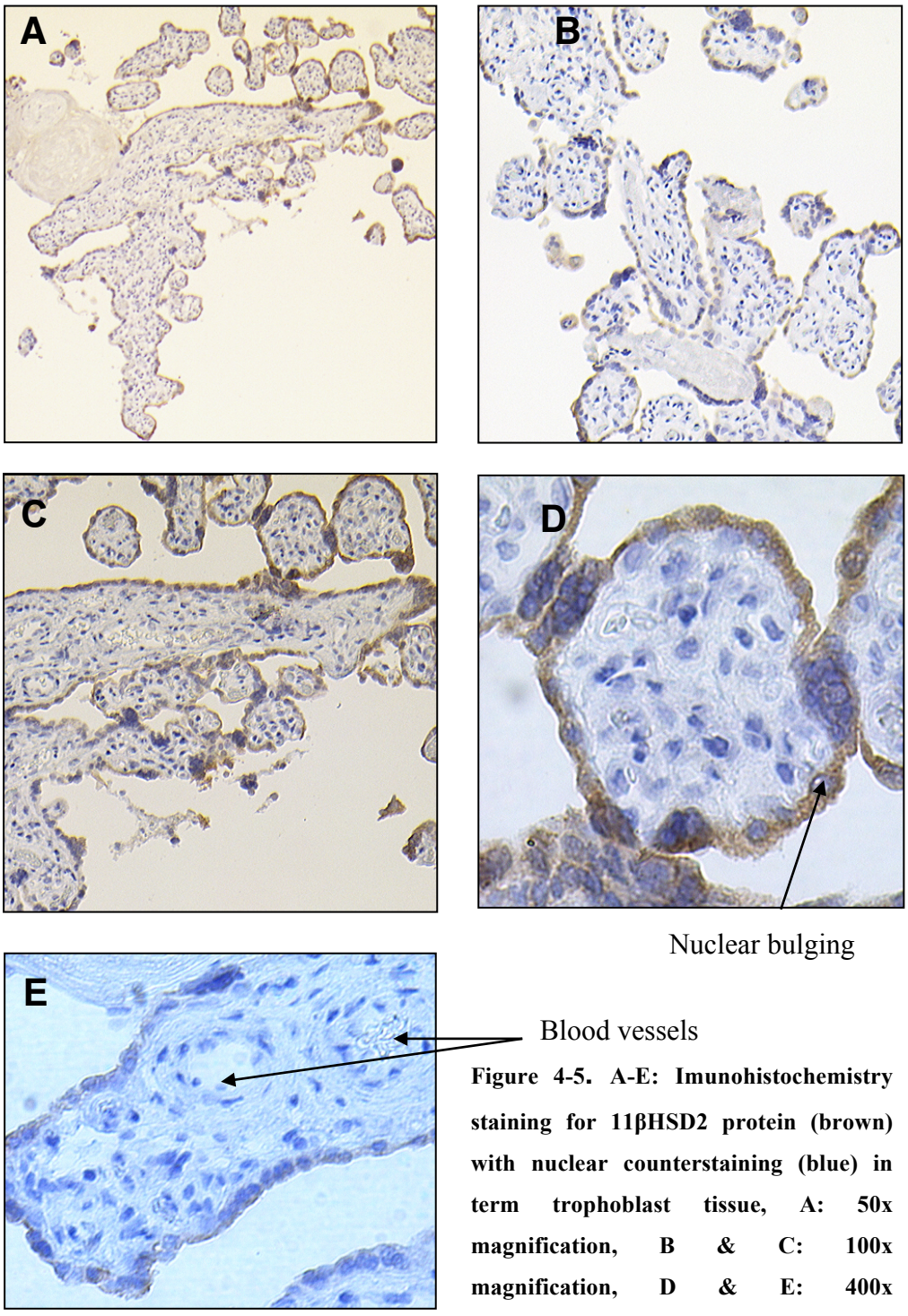


Figure 4-5. A-E: Imunohistochemistry staining for 11βHSD2 protein (brown) with nuclear counterstaining (blue) in term trophoblast tissue, A: 50x magnification, B & C: 100x magnification, D & E: 400x magnification of placental villi.

4.3.3 11βHSD2 Protein Levels

Figure 4-6 shows western blot results for 11βHSD2 protein in placental tissues from a representative sample of first trimester and term samples. Samples A to D are derived from four term placentas, while samples E to H are derived from four first trimester samples. Table 4-1 shows semi-quantitative protein concentrations for the samples relative to α-tubulin protein. Protein levels of 11βHSD2 were significantly higher in first trimester samples compared to term samples ($p<0.005$).

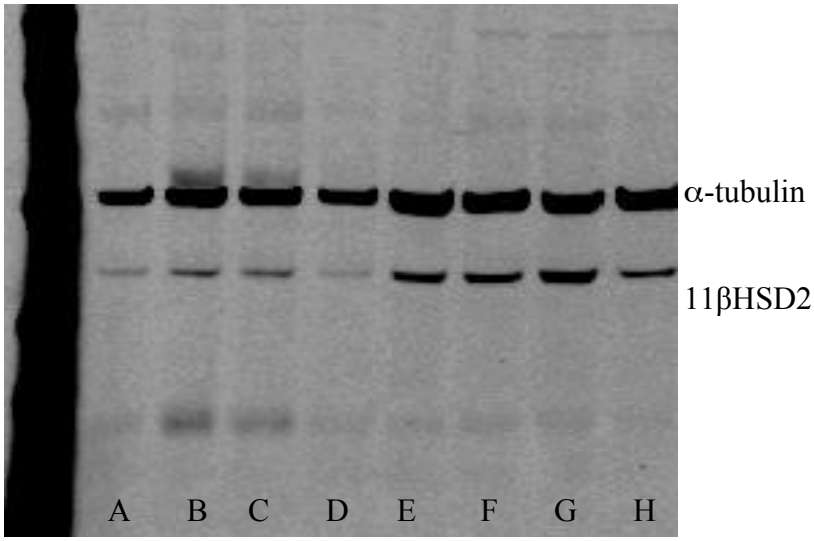


Figure 4-6. Western blot for 11βHSD2 and α-tubulin protein in term (A-D) and first trimester (E-H) placental samples.

Group	Sample	Relative concentration	Mean	p
Term	A	0.35		<0.005
	B	0.61		
	C	0.64		
	D	0.56	0.54	
First Trimester	E	2.10		
	F	1.60		
	G	1.91		
	H	0.98	1.65	

Table 4-1. Quantification of western blot results for 11 β HSD2 protein in term (A-D) and first trimester (E-H) placental samples.

4.3.4 mRNA Transcript Levels

4.3.4.1 First Trimester Placenta Tissue Control Genes

A number of genes were tested for suitability as a control gene for real time PCR in a small sample of first trimester trophoblast tissue (see **Figure 4-**7). *PPIA* transcript was detected in all samples, and was found to have the lowest correlation with BMI (r=0.007, p=0.96) and thus was selected as a control gene for use in real time PCR.

4.3.4.2 Second Trimester Placenta and Liver Tissue control Genes

TBP, *β-ACTIN*, *SDHA*, *GAPDH* and *B2M* were investigated as potential control genes in small samples of second trimester liver and placenta tissue.

In the placenta (Figure 4-8), *TBP* was found to have the lowest correlation with BMI, and thus was selected as a control gene for use in real time PCR. In the liver (Figure 4-9), a mean value of the genes *TBP* and *β-ACTIN* was found to have the lowest correlation with BMI, and was therefore used as a control gene in real time PCR.

4.3.4.3 Term Placenta Tissue Control Genes

Seven control genes, identified as possible candidates through a literature review (Meller *et al.* 2005; Murthi *et al.* 2008; Cleal *et al.* 2009), were measured in a small sample of term placental tissue from lean and obese women (see Figure 4-10). Of the genes investigated, *YWHAZ* was found to have the smallest difference in transcript concentration between the lean and obese groups and thus was selected as a control gene for use in real time PCR.

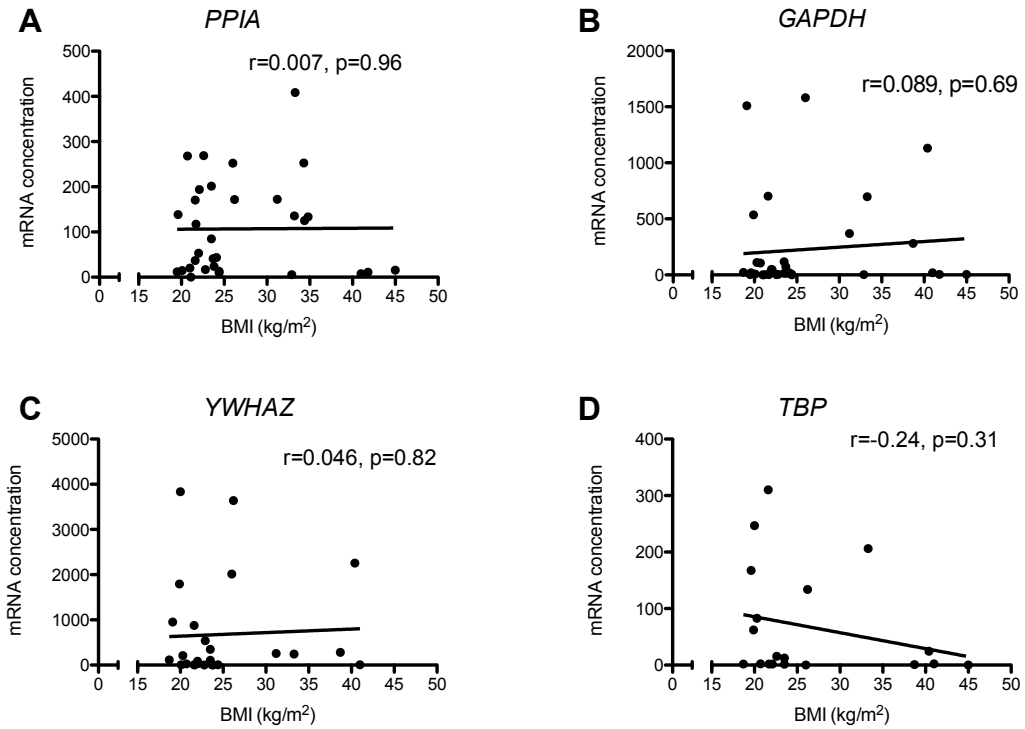


Figure 4-7. Control gene candidates for first trimester placenta samples. However, *YWHAZ* mRNA transcripts were only detected in 26 of the 32 samples and *TBP* transcripts were only detected in 17 samples.

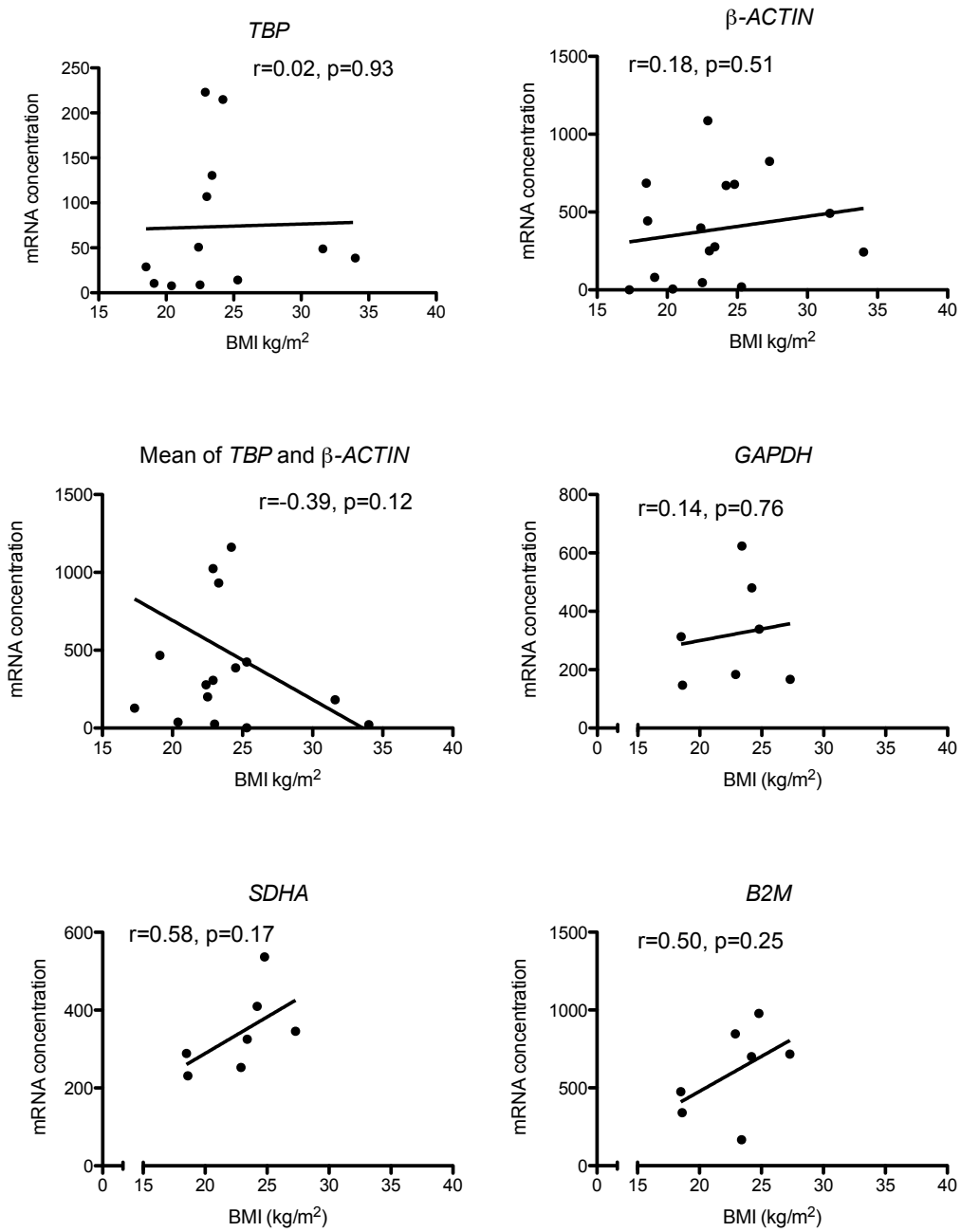


Figure 4-8. Control gene candidates for second trimester placental samples.

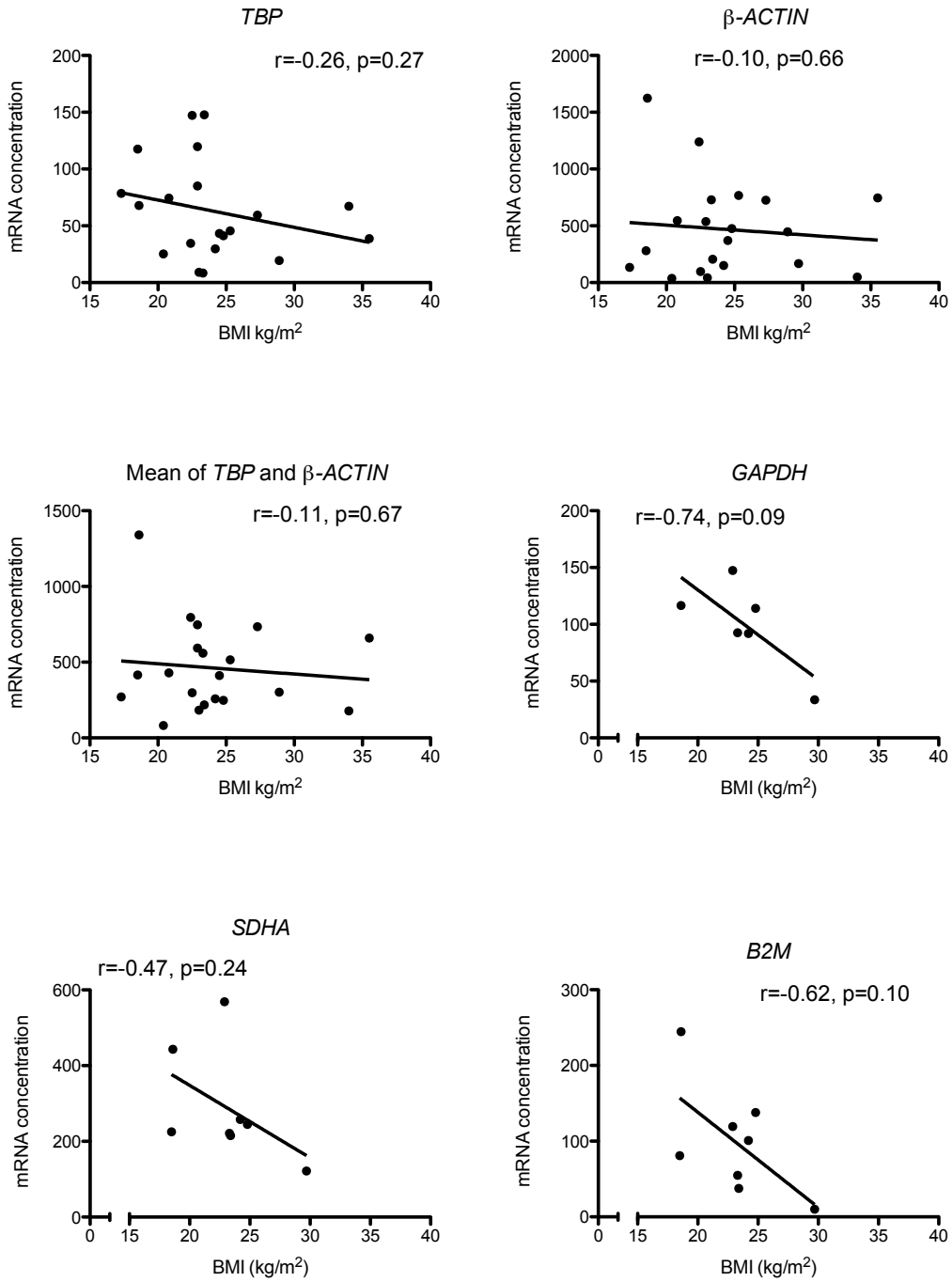


Figure 4-9. Control gene candidates for second trimester liver tissue.

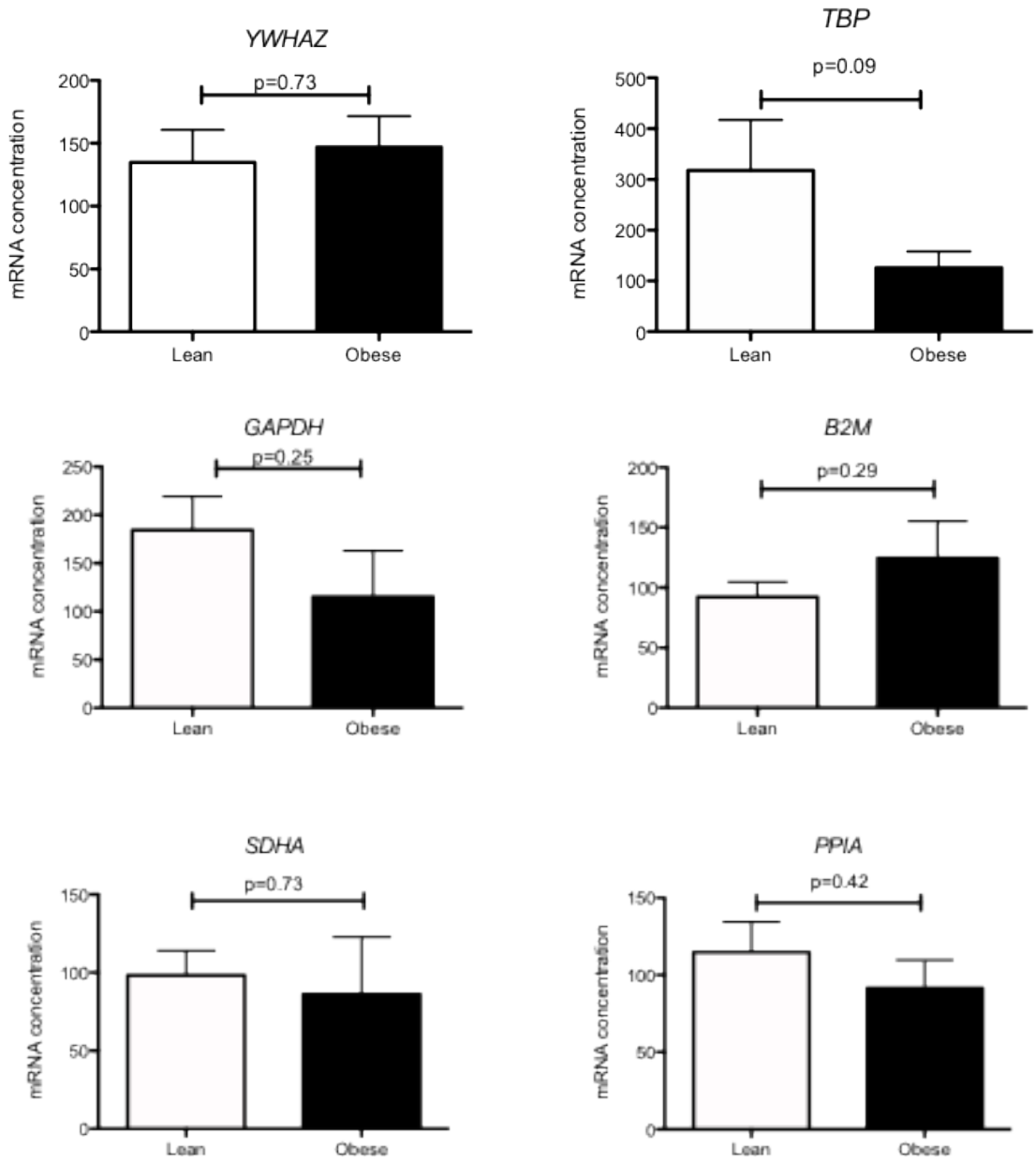


Figure 4-10. Control gene candidates for term placenta samples.

4.3.5 Placental mRNA Transcript Levels During the First Trimester

4.3.5.1 Demographics

Of the 53 first trimester placental tissue samples, RNA of suitable quality for real-time PCR could not be extracted from 21 samples. Table 4-2 shows the characteristics of the 32 women from whom suitable RNA was obtained, including 15 lean women, 5 overweight women, and 12 obese women. BMI did not correlate with length of gestation ($r=0.03$, $p=0.84$) or maternal age ($r=0.30$, $p=0.09$).

	Mean (sem)
BMI (kg/m^2)	26.80 (1.27)
Age (years)	24.75 (1.25)
Gestation (days)	66.12 (1.83)
Smokers*	23 (72%)
Nulliparous*	19 (59%)
Multiparous*	13 (41%)

Table 4-2. Demographic data for first trimester tissue samples (n=32) Data are mean (sem) or *N (%)

4.3.5.2 mRNA Transcript Levels in First Trimester Placental Tissue

There were significant positive correlations between maternal BMI and mRNA transcript levels of *11βHSD2* (r=0.44, p<0.01 see Figure 4-11A), *GR* (r=0.47, p<0.05, see Figure 4-11B) but not *IGF2* (r=0.05, p=0.78, see Figure 4-11C) in trophoblast tissue obtained from terminations of pregnancy conducted during the first trimester. After adjustment for maternal age, parity, smoking status and gestational age, the association between *11βHSD2* transcript levels and BMI remained significant (b=+0.133, adjusted p<0.01,), while the association was weakened for *GR* and BMI (b=+0.052, adjusted p=0.06,).

No significant correlations were observed between maternal BMI and the relative placental mRNA transcript levels of a number of other key genes, including *IGF2R*, *H19* and the GR target genes *PPARγ* and *GILZ* (see Figures 4-12 and 4-13). No significant correlations were observed between mRNA transcript levels and length of gestation or maternal age.

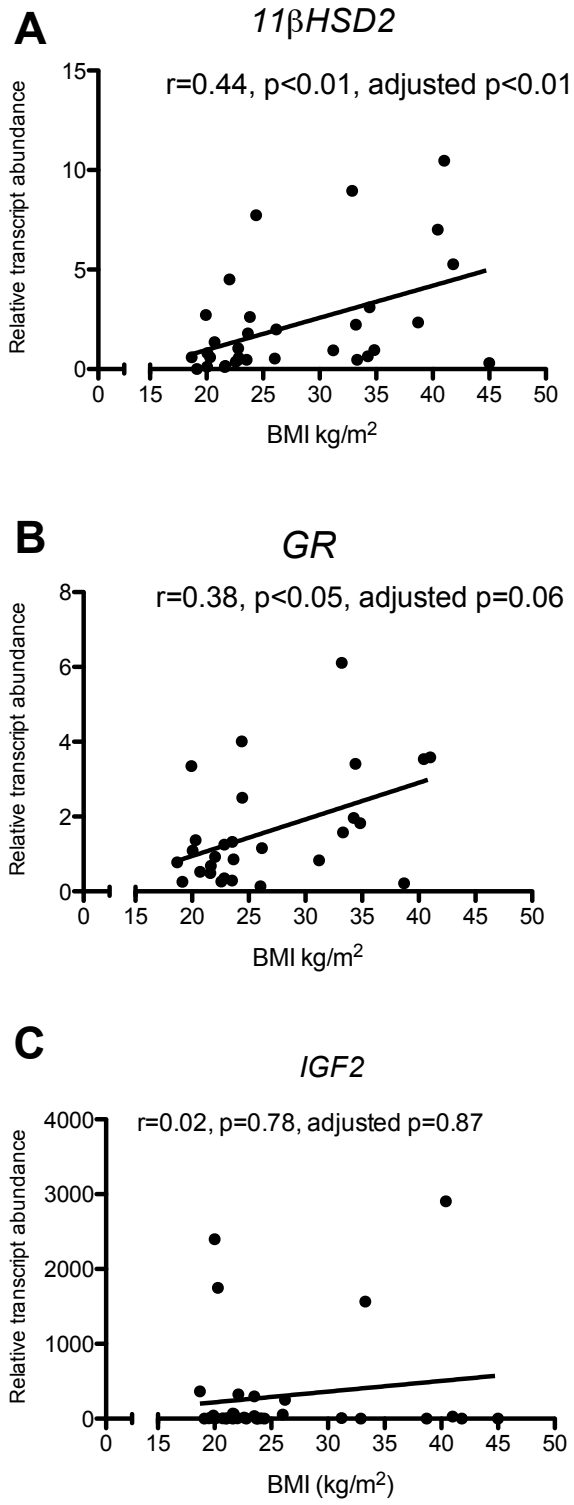


Figure 4-11. mRNA transcript levels of *11βHSD2* (A), *GR* (B) and *IGF2* (C) (all relative to *PPIA*) in first trimester trophoblast tissue plotted against maternal BMI (kg/m^2). The retrospective statistical power of the analysis for *11βHSD2* was 0.8 and for *GR* was 0.81.

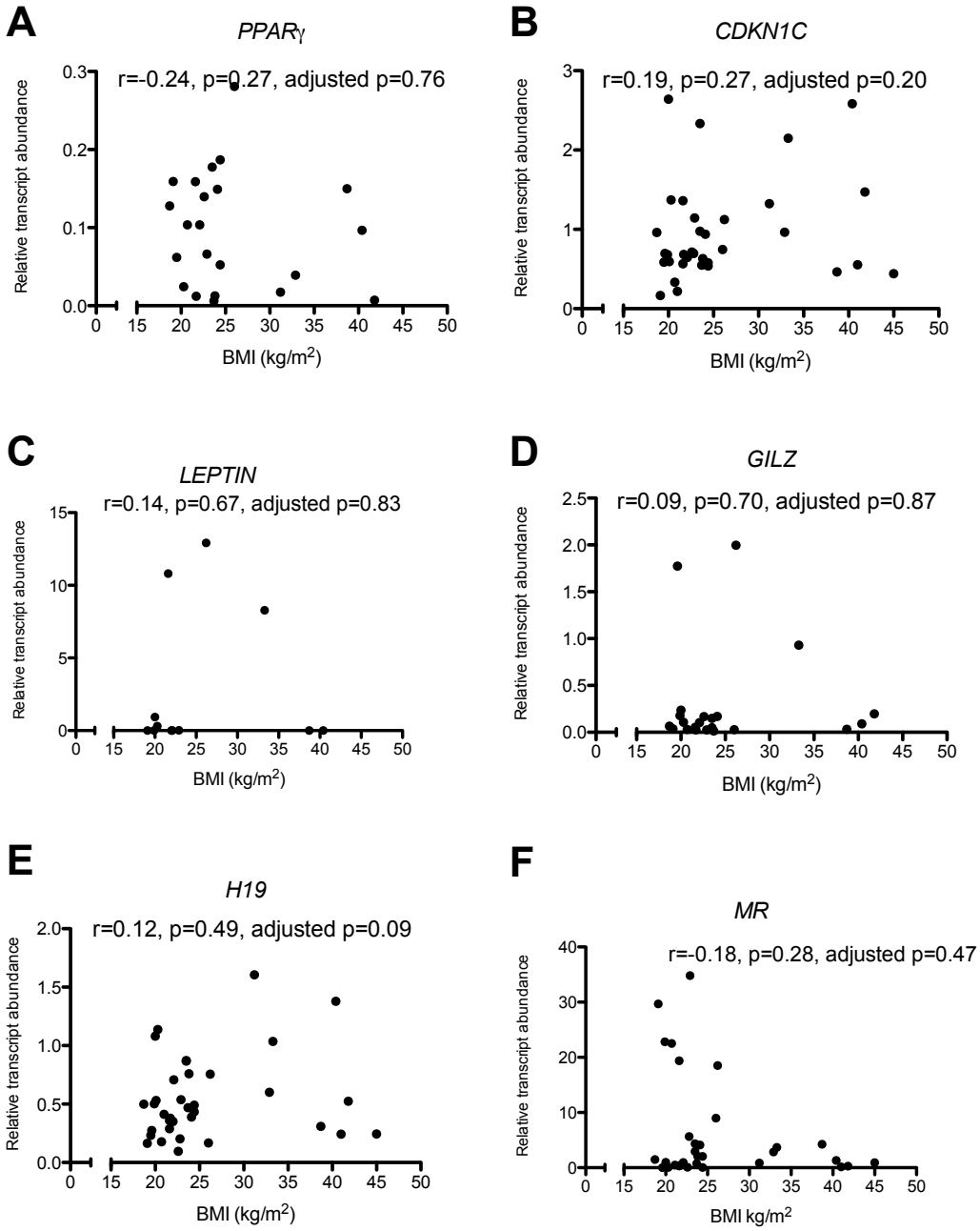


Figure 4-12. mRNA transcript levels of genes in first trimester trophoblast tissue. No significant correlations were observed between maternal obesity and mRNA transcript levels for any of the genes investigated. *LEPTIN* mRNA transcript was only detected in 11 samples and *GILZ* transcripts were only detected in 20 samples.

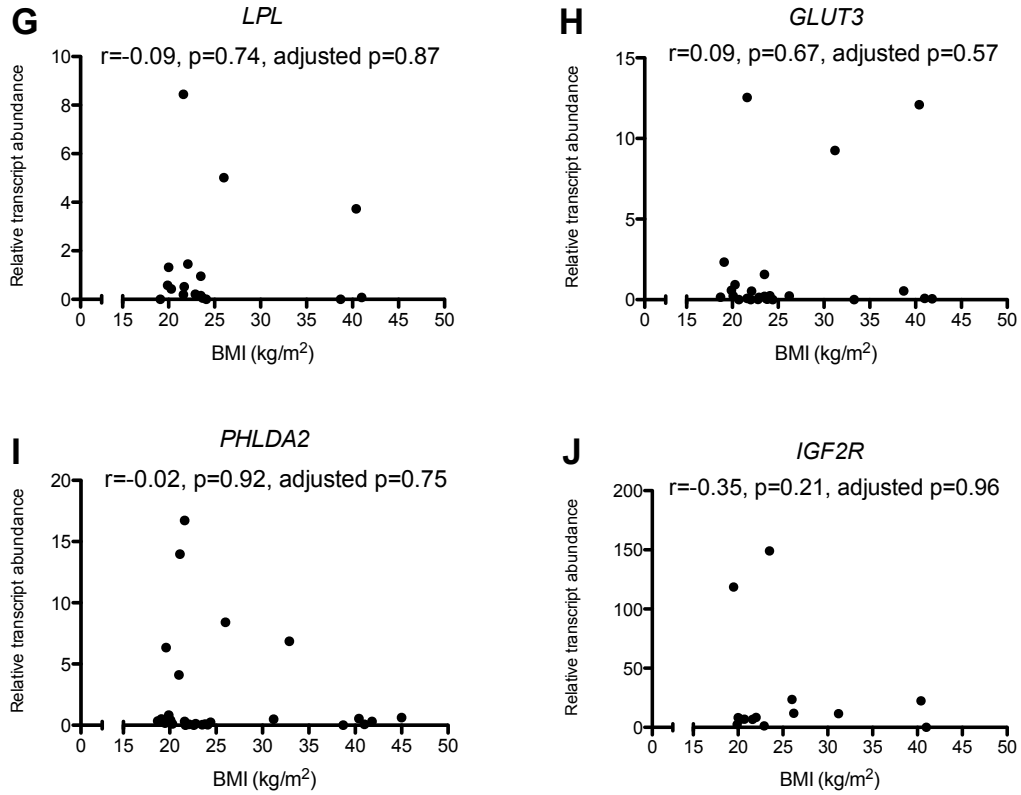


Figure 4-13. mRNA transcript levels of genes in first trimester trophoblast tissue. No significant correlations were observed between maternal obesity and mRNA transcript levels for any of the genes investigated. *LPL* transcripts were detected in 17 samples, *IGF2R* transcripts were only detected in 14 samples and *GLUT3* transcripts were detected in 25 samples.

4.3.6 mRNA Transcript Levels During the Second Trimester

4.3.6.1 Demographics

Tissue samples were collected from a total of 30 pregnancies. Of these, placental tissue samples were collected from 18 pregnancies, while liver tissue samples were collected from 26 pregnancies. BMI data was not available for three women from whom placentas were collected, and seven women from whom fetal liver was collected, and as a result data from these individuals could not be used, leaving a final total of 15 placenta samples and 19 liver samples (see Table 4-3).

	Placenta (n=15)	Liver (n=19)
BMI (kg/m^2)	22.95 (1.6)	24.67 (1.0)
Age (years)	27.35 (0.65)	27.74 (0.88)
Gestation (days)	116.3 (3.12)	115.5 (3.19)
Females *	3 (20%)	3 (15%)
Males *	12 (80%)	16 (85%)

Table 4-3. Demographic data for second trimester tissue samples. All continuous data are mean (±s.e.m), categorical data (*) are n (%).

4.3.6.2 Placental mRNA Transcript Levels During the Second Trimester

In this small sample size (n=15) *IL-1β* mRNA transcript levels positively correlated with maternal BMI (see Figure 4-15), and a similar trend was observed with *IGF2* (see Figure 4-14). There were no other significant correlations between relative mRNA transcript levels and maternal BMI for any of the genes studied in second trimester placental tissue. A number of samples (n=5) failed to amplify for *11βHSD2*, resulting in a reduced group of 10 samples. mRNA transcript of the genes *LEPTIN* was not detected in four samples, resulting in a reduced sample size of 11, while mRNA transcript of *IL-1β* was not detected in one sample.

4.3.6.3 Fetal Liver mRNA Transcript Levels during the Second Trimester

No significant correlations were observed between maternal BMI and mRNA transcript levels of any of the genes studied in second trimester fetal liver tissue (see Figure 4-16 and Figure 4-17). *11βHSD2* mRNA transcript was not detected in 10 samples, leaving a reduced sample size of 9 for this gene. While previous studies have demonstrated expression of *11βHSD2* in ovine liver, little work has been carried out on the fetal hepatic expression of this gene in humans (Langlois *et al.* 1995).

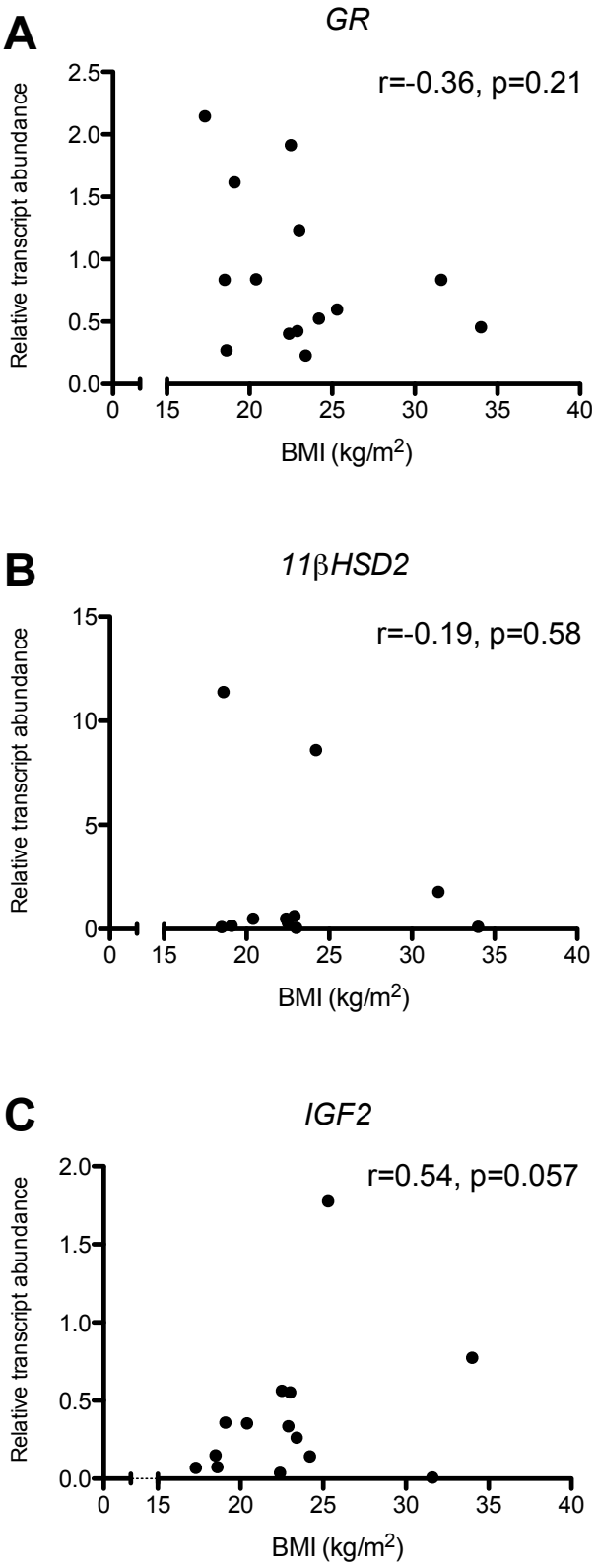


Figure 4-14. mRNA transcript levels of key genes relative to *TBP* in second trimester placental tissue.

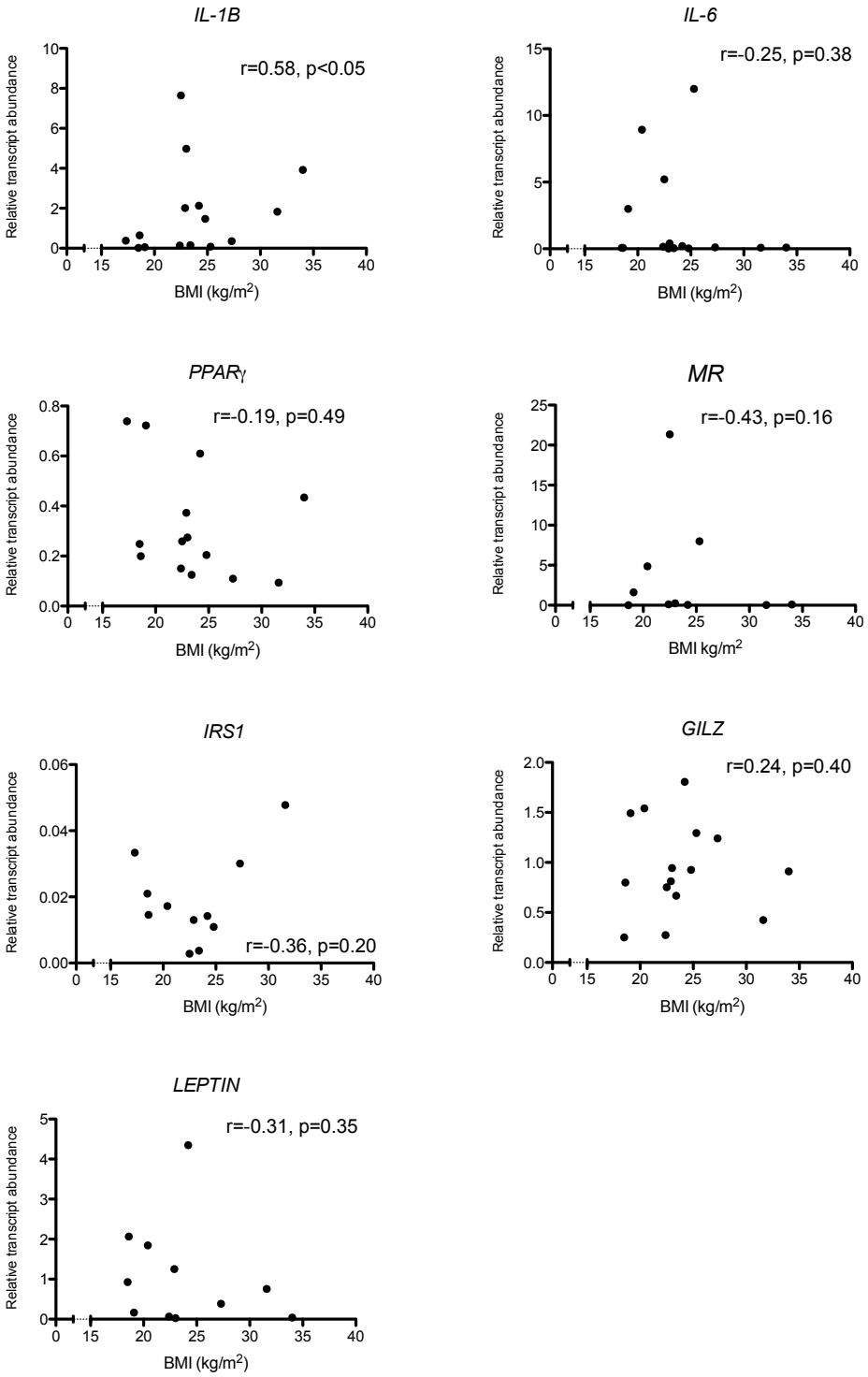


Figure 4-15. mRNA transcript levels (relative to *TBP*) in second trimester placental tissue. No significant correlations were found between relative mRNA transcript levels and maternal BMI. Statistical power was calculated as 0.8 for *IL-1B*.

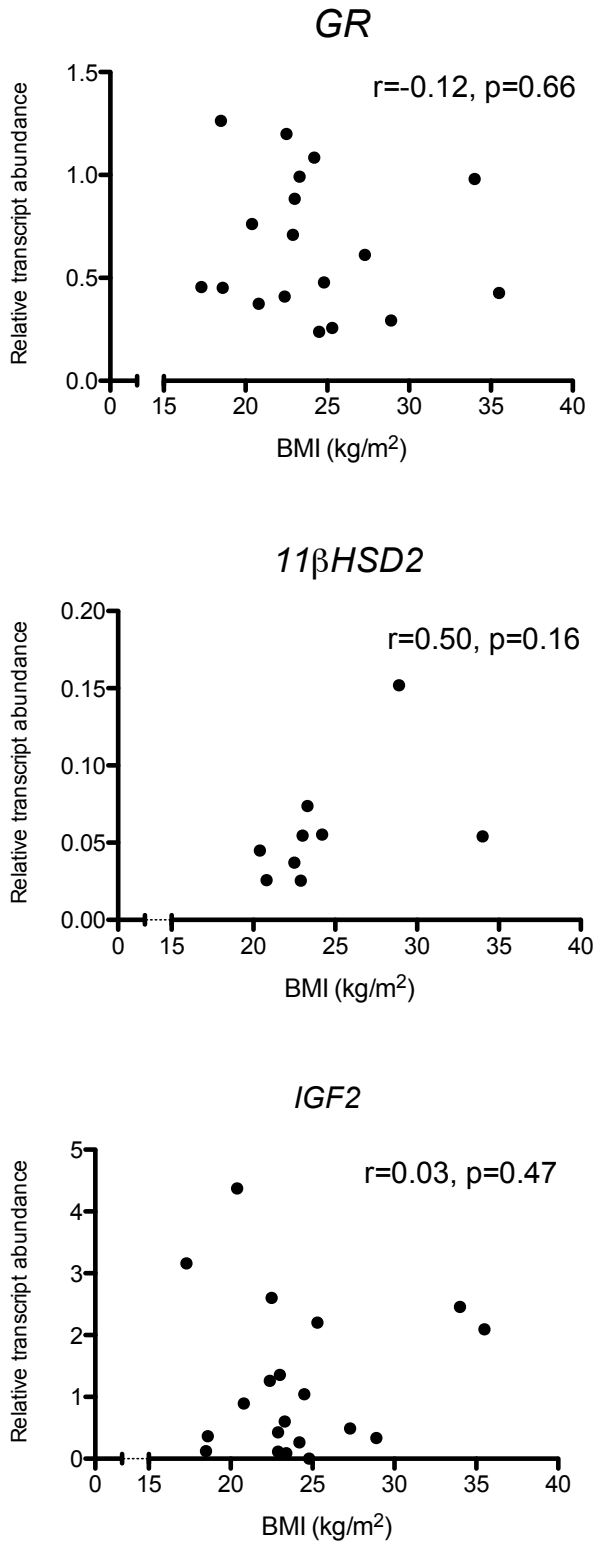


Figure 4-16. mRNA transcript levels of key genes (relative to *B-ACTIN* and *TBP*) in second trimester fetal liver tissue.

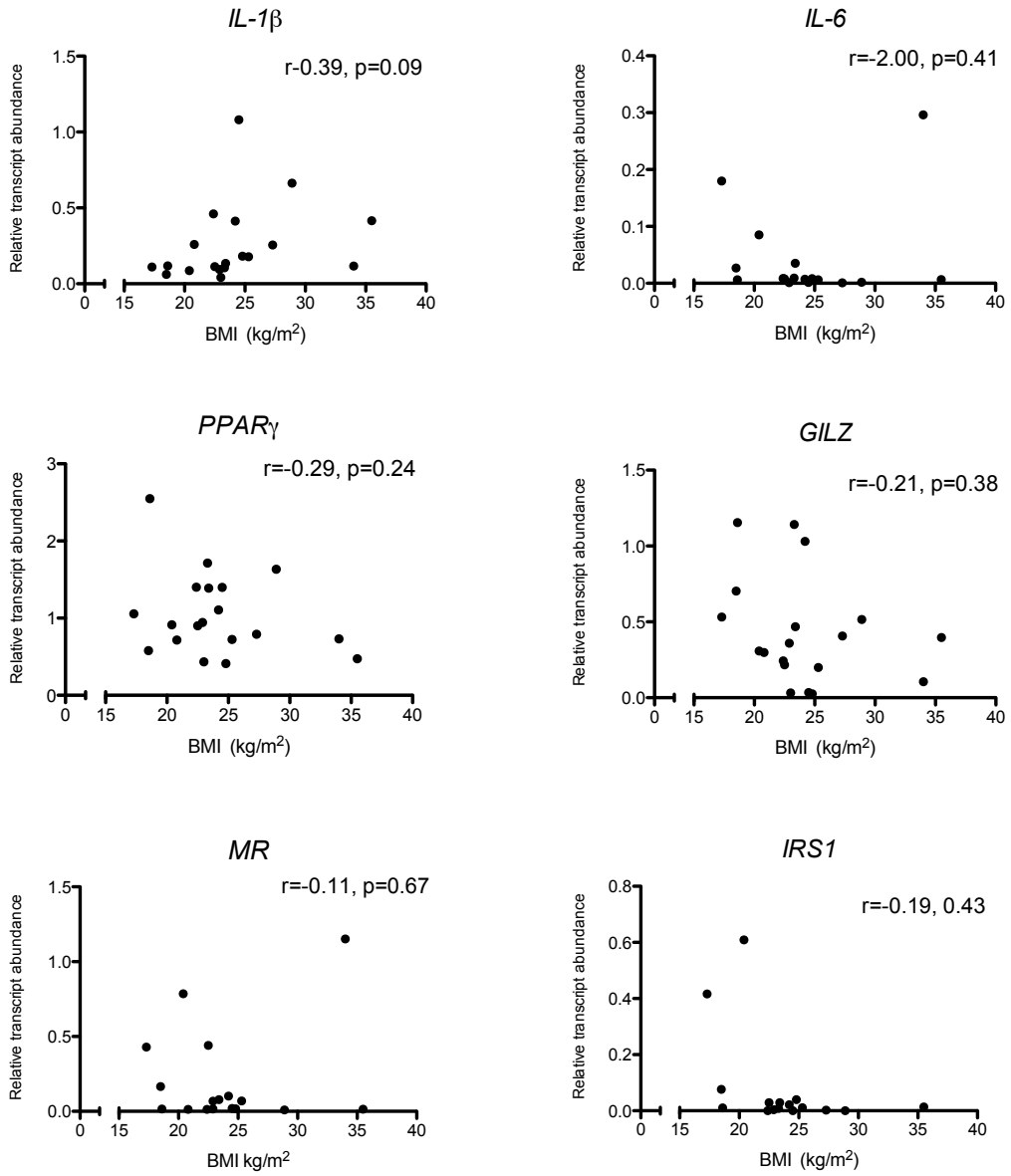


Figure 4-17. mRNA transcript levels (relative to *B-ACTIN* and *TBP*) in second trimester fetal liver tissue.

4.3.7 mRNA transcript levels at term

4.3.7.1 Demographics

Table 4-4 shows the characteristics of 60 women from whom term placental tissue was collected, according to BMI. Of the twenty-eight obese women, twenty had children with birth weights between 2500 grams and 4000 grams ('appropriate for gestational age' [AGA]), while eight delivered children with birth weights greater than or equal to 4000 grams ('large for gestational age' [LGA]). All infants born to lean women were AGA. AGA and LGA descriptions are calculated based on the gestational age.

Lean and obese women did not differ significantly in terms of birth weight or gestation, however the mean age of the lean group was higher than that of the obese group ($p<0.05$). Subsequent analysis showed that mean birth weights did not differ between the lean and obese AGA groups ($p=0.34$), but did differ significantly between lean and obese LGA ($p<0.0001$), and between obese AGA and obese LGA ($p<0.0001$).

Mean length of gestation did not differ between lean and obese AGA groups ($p=0.61$), between lean and obese LGA groups ($p=0.44$) or between obese AGA and obese LGA groups ($p=0.26$).

Mean maternal age did not differ significantly between lean and obese AGA groups ($p=0.16$), or between obese AGA and obese LGA groups ($p=0.47$). Obese LGA tended to be younger than lean ($p=0.058$). Parity did not differ between obese AGA and obese LGA groups ($p=0.415$). However, parity was significantly

higher in the obese AGA compared to lean ($p<0.00001$), and significantly higher in obese LGA compared to lean ($p<0.05$).

Significantly more obese women were of lower socioeconomic status than lean women ($p<0.0005$). No differences in socioeconomic status were observed between lean women and obese women with AGA babies ($p=0.33$), lean women and obese women with LGA babies ($p=0.19$) or obese women with AGA babies and obese women with LGA babies ($p=0.80$).

		All (n=60)	Lean (n=32)	Obese (n=28)	*p*	Obese AGA (n=20)	Obese LGA (n=8)	*p*
BMI (kg/m^2)		31.26 (1.35)	22.63 (0.223)	42.21 (0.93)	<0.0001	42.47 (0.76)	40.61 (2.223)	0.32
Age (years)		32.88 (0.70)	33.91 (0.89)	31.15 (1.10)	<0.05	32.74 (1.13)	29.88 (1.95)	0.19
Higher Socioeconomic Status(DEPCAT 1-3)*		24 (41%)	19 (59%)	5 (19%)	<0.0005	4 (20%)	1 (12%)	0.16
Lower Socioeconomic Status(DEPCAT 5-7)*		34 (59%)	12 (41%)	22 (81%)	<0.0005	16 (80%)	6 (75%)	0.69
Multiparous*		46 (77%)	21 (66%)	25 (79%)	0.22	18 (90%)	7 (88%)	0.88
Smokers*		1 (2%)	1(3%)	2 (7%)	0.21	2 (10%)	0 (0%)	<0.01
Birth weight (g)		3536 (61.66)	3483 (71.90)	3618 (83.3)	0.25	3355 (107.8)	4202 (59.48)	<0.0001
Gestation (days)		277.5 (1.05)	277.6 (1.44)	277.2 (1.50)	0.089	276.4 (1.79)	280.1 (2.61)	0.27
Sex of baby*	Males	32 (53%)	16 (50%)	16 (57%)	0.49	10 (50%)	6 (75%)	<0.05
	Females	28 (47%)	16 (50%)	12 (43%)	0.47	10 (50%)	2 (25%)	<0.05

Table 4-4. Characteristics of subjects from whom term tissues were collected. Socioeconomic status was measured using deprivation category (DEPCAT) scores based on postcode (McLoone 2004). Socioeconomic status data was unavailable for 1 lean woman and 1 obese woman. Data are mean (sem) or *N(%).

4.3.7.2 mRNA Transcript Levels in Term Placental Tissue

There were no significant correlations between maternal age, parity, length of gestation, neonate birth weight or gender and mRNA transcript levels of any of the genes studied.

No differences in placental *11βHSD2* or *GR* transcript levels were observed between lean and obese women when no consideration was given to offspring birth weight in unadjusted analyses, or after adjustment for maternal age, smoking status, length of gestation and neonate gender (*11βHSD2* unadjusted p=0.96, adjusted b=+0.62, p=0.1; *GR* unadjusted p=0.56, adjusted b=-0.58, p=0.22). Placental *IGF2* expression was significantly lower in obese pregnancy compared to lean (see Figure 4-18) and this finding remained significant in regression analyses (unadjusted $p<0.05$, b=-1.11,adjusted $p<0.05$).

However, when the obese group was divided according to offspring birth weight, significant differences in mRNA transcript levels of these three key genes was revealed. Levels of mRNA transcript of *11βHSD2* differed significantly between placentas from lean, obese AGA, and obese LGA pregnancies (one-way ANOVA, $F = 1.473$, $p < 0.005$); mRNA transcript levels were significantly higher in placentas of obese women with LGA babies compared to obese women with AGA babies ($p<0.05$, adjusted $p<0.05$, see Figure 4-19A). mRNA transcript levels of *GR* also differed significantly between placentas from lean, obese AGA, and obese LGA

pregnancies (one-way ANOVA, $F = 2.119$, $p < 0.001$); mRNA transcript levels were significantly lower in placentas of obese women with LGA babies compared to obese women with AGA babies ($p<0.05$, adjusted $p=0.11$, see Figure 4-19B), and tended to be lower in obese women with LGA babies compared to lean women ($p=0.059$). Further, mRNA transcript levels of *IGF2* also differed significantly between placentas from lean, obese AGA, and obese LGA pregnancies (one-way ANOVA, $F = 1.004$, $p < 0.01$); mRNA transcript levels were significantly lower in obese women with LGA babies compared to lean women ($p<0.01$, adjusted $p<0.05$, see Figure 4-19C).

No significant differences in mRNA transcript levels were observed between placental tissues from lean and obese pregnancies for a number of other genes studied, including *11βHSD1*, *IGF2R* and *H19* (see Figure 4-20). Furthermore, when the obese group was divided into those with AGA and LGA babies, no difference was seen between any of the groups.

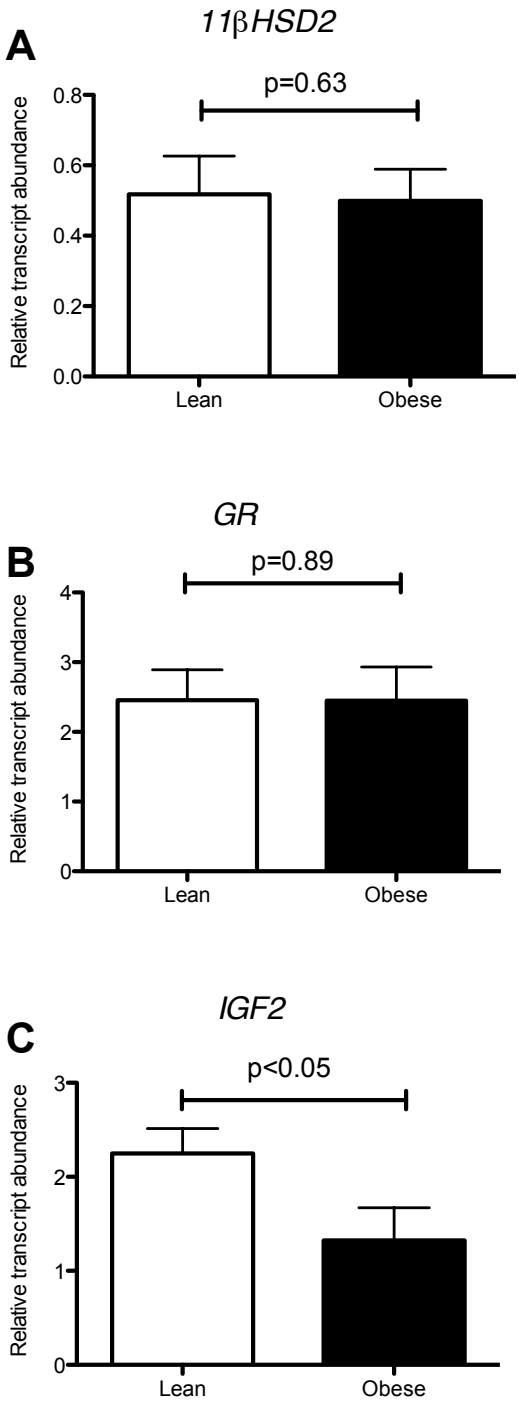


Figure 4-18. mRNA transcript levels of *11βHSD2, GR* and *IGF2* (relative to *YWHAZ*) in term placental tissue from lean (BMI of <25 kg/m^2) and obese (BMI >30 kg/m^2) women. Data are mean (±s.e.m).

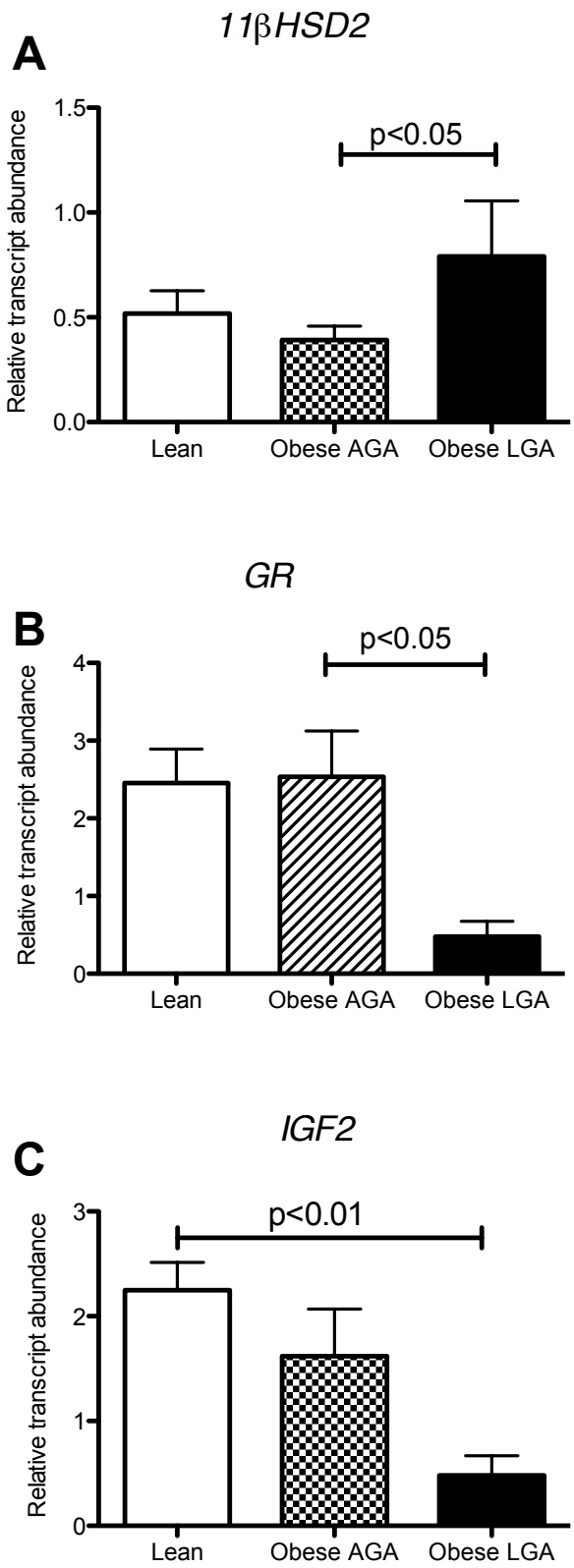


Figure 4-19. A: mRNA transcript levels of *11βHSD2* (relative to *YWHAZ*) in term placental tissue for lean (BMI of <25 kg/m^2, n=30), obese with appropriate for gestational age offspring (maternal BMI >30 kg/m^2, birth weight <4000g, n=20, Obese AGA) and obese with large for gestational age offspring (maternal BMI >30kg/m^2, birth weight >4000g, n=8, Obese LGA). B: mRNA transcript levels of *GR* (relative to *YWHAZ*) in term placental tissue for lean, obese with appropriate for gestational age offspring and obese with large for gestational age offspring. C: mRNA transcript levels of *IGF2* (relative to *YWHAZ*) in term placental tissue for lean, obese with appropriate for gestational age offspring and obese with large for gestational age offspring. Data are mean (±sem).

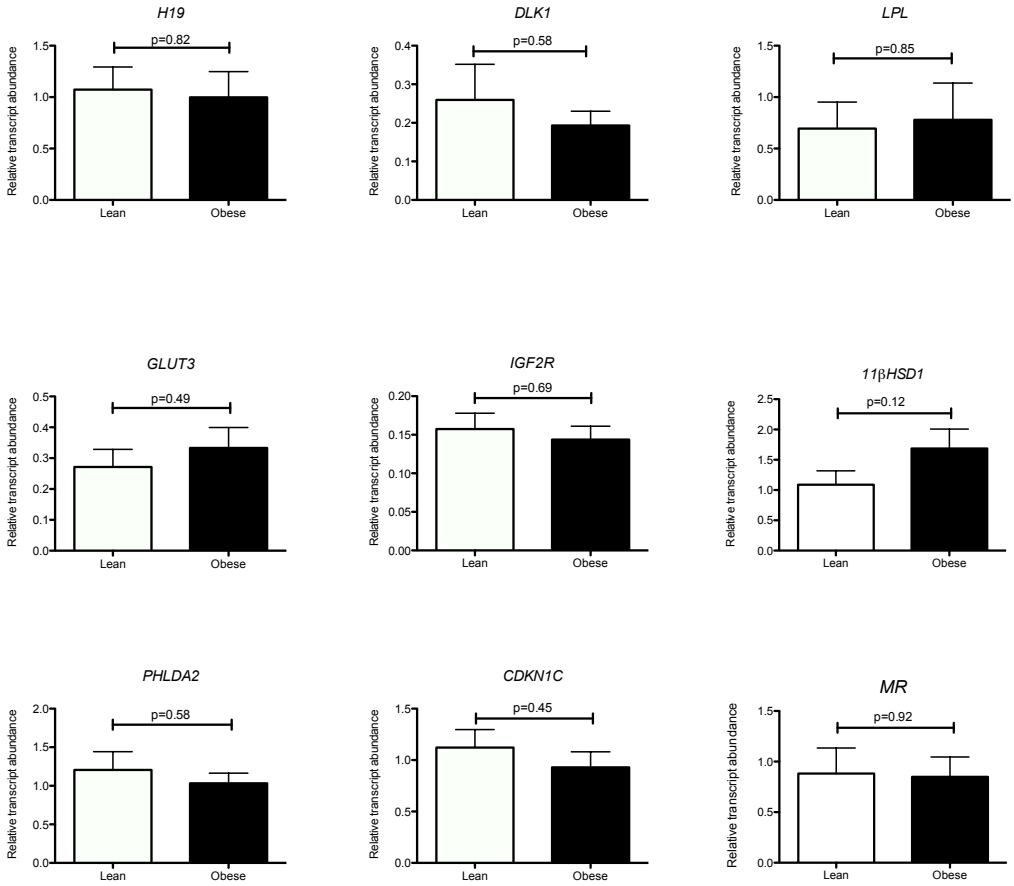


Figure 4-20. mRNA transcript levels of genes in term placental tissue. No significant differences were observed between lean and obese placental transcript levels for any of the genes investigated. Data are mean (±sem).

4.4 Discussion

In this chapter, immunohistochemistry techniques and western blotting were used to analyse the abundance and localization of the placental enzyme 11βHSD2. Additionally, mRNA transcript levels of a number of key genes, including *11βHSD2*, were analysed in human placental tissues obtained from each trimester of pregnancy in both lean and obese women. Finally, mRNA transcript levels of a number of genes in the fetal liver as a representative fetal glucocorticoid sensitive target tissue were also measured during the second trimester of pregnancy. The size of the study was smaller than initially planned due in part to the difficulty in extracting RNA of suitable integrity and in sufficient quantities from the target tissue, despite attempts to minimise the time between tissue collection and mRNA extraction. A number of samples were found to be unsuitable and thus could not be used.

Using immunohistochemistry it was demonstrated that 11βHSD2 protein is present in first trimester, second trimester and term placental tissues, implying continuous presence throughout pregnancy. Furthermore, the protein appears to be localized exclusively to the syncytiotrophoblast, the epithelial lining of the placental villi. This pattern of localisation likely contributes to the formation of a complete and continuous barrier against glucocorticoid transmission between the maternal blood stream and fetal tissues at the point of most intimate contact between the two. This finding reinforces previous literature demonstrating that the placental glucocorticoid barrier (as assessed by mRNA levels of 11βHSD2) is present throughout the entirety of the syncytiotrophoblast for the duration of pregnancy (McTernan *et al.* 2001) and confirms that levels of this protein in human pregnancy differ markedly from the

patterns observed in rodents, where levels are initially high, but subsequently drop mid-gestation (Waddell *et al.* 1998; Thompson *et al.* 2002). Surprisingly, protein levels of 11βHSD2 were found to be higher during the first trimester when compared to term, a finding that contradicts previous studies showing that mRNA transcript levels of *11βHSD2* increase dramatically throughout human pregnancy (McTernan *et al.* 2001). This suggests that post-transcriptional regulation of the enzyme may be occurring, explaining the disparity between gene expression and protein levels. However, the sample size in this part of the current study was small, and further study of post-translational regulation of 11βHSD2 may be an important area of future research.

Excess exposure to glucocorticoids during pregnancy has been associated with reduced fetal growth and lower birth weight, which is in turn associated with increased risk of hypertension, type 2 diabetes and cardiovascular disease later in life (Barker *et al.* 1993a; Moore *et al.* 1996; Rich-Edwards *et al.* 1997). The placental enzyme 11βHSD2 has been hypothesised to protect the developing fetus from the increasing levels of glucocorticoids in maternal blood by converting active cortisol to inactive cortisone, although the barrier is thought to be incomplete (Brown *et al.* 1993; Benediktsson *et al.* 1997). The levels of the enzyme are thought to be an important determinant of its effectiveness in forming a coherent barrier to maternal cortisol and dysfunction of the enzyme has been hypothesised to allow more active glucocorticoids to cross the placenta into the fetal bloodstream, leading to organisational effects on the developing fetus, including neurological and growth

effects (Edwards *et al.* 1993; Welberg *et al.* 2000). In support of this, 11βHSD2 activity has been shown to correlate with birth weight in human pregnancy (Stewart *et al.* 1995; Murphy *et al.* 2002b). The enzymatic activity of 11βHSD2 was not measured in this study, and therefore it is difficult to draw strong conclusions regarding the effects of maternal weight status on 11βHSD2 activity in this cohort.

The determination of useful control (or housekeeping) genes was important to consider before performing real time PCR in placental tissue. While a handful of studies have investigated candidate control genes for placental mRNA transcript levels, the results have often been contradictory, reflecting the diverse nature of the sources of tissues used (Meller *et al.* 2005; Murthi *et al.* 2008; Cleal *et al.* 2009). No studies have been published investigating suitable control genes in an obese pregnancy paradigm, and therefore it was important to establish a suitable candidate. Candidates were initially chosen from a small pool of genes described previously in the literature and the most suitable candidate chosen did differ according to gestation studied.

In the first trimester, increased placental mRNA transcript levels of *11βHSD2* in association with obesity may protect the fetus from excess glucocorticoid exposure and may also contribute ultimately to the higher birth weights observed in obese pregnancy. As described in chapter 3, obese pregnant women have lower circulating levels of glucocorticoids compared to lean women. Lower circulating levels of cortisol coupled with increased levels of placental *11βHSD2* mRNA transcript may lead to a reduced fetal exposure to maternal glucocorticoids and therefore higher birth weight, possibly due to delayed organ maturation. Furthermore, increased

placental mRNA transcript levels of *GR* in early obese pregnancy may lead to an increase in growth of the placenta, potentially leading to higher birth weight. Increased transcript levels of *GR* may also confer immunosuppressive and anti-inflammatory effects, potentially allowing for more effective invasion and growth of the placenta in early pregnancy. These changes in mRNA transcript levels may allow the placenta to maximise its growth in response to increased maternal nutrients early in pregnancy, at a time when placental growth is at a peak. This may in turn allow for a more efficient delivery of nutrients to the fetus throughout pregnancy, particularly during the second and third trimesters, when fetal growth is maximal. This arm of the study was somewhat limited by the relatively small sample size used. While preliminary power calculations suggested that the intended sample population was sufficiently powered, difficulties in obtaining viable mRNA samples from placental biopsies reduced the number of samples available for analysis. Furthermore, assays for some genes did not produce sufficient signal strength for all samples, again significantly limiting the sample size for some analyses. These limitations are likely to have impacted the power of the study. Nevertheless, it is possible that this work will be an important basis for future experiments involving a larger population of patients from whom first trimester placental tissue biopsies can be obtained.

As low birth weight has previously been linked to reduced placental *11βHSD2* levels, it was hypothesised that maternal obesity would be associated with increased placental *11βHSD2* near term, leading to a higher birth weight through an increased placental barrier to maternal glucocorticoids. While no significant differences in placental gene transcript levels were observed between lean and obese pregnancies,

when individuals were separated into groups by birth weight, levels of *11βHSD2* mRNA transcript were significantly higher in obese women who gave birth to LGA babies when compared to those who gave birth to AGA babies. Thus, higher levels of *11βHSD2* in the placentas of some obese pregnant women may protect the developing fetus more effectively, allowing greater levels of growth *in utero* and thus a higher birth weight. This may reflect an adaptation to excess nutrients, delaying maturation in order to maximise growth in a nutritionally rich environment. Additionally, no difference in mRNA transcript levels of *GR* at term was observed between lean pregnancies and obese pregnancies with appropriate for gestational age offspring. However, when the obese group were divided by offspring birth weight placental GR mRNA transcript levels were lower in obese women with LGA babies compared to obese women with AGA babies. As glucocorticoids, acting via GR, are fundamental regulators of decidualisation, implantation, placental development and angiogenesis, this may reflect a difference in placental growth and function between obese women with AGA and LGA pregnancies (Korgun *et al.* 2012). However, it is important to note that GR target genes such as *GILZ* did not appear to be affected by maternal BMI, suggesting that the biological significance of the altered gene expression of *GR* may be questionable. It will be important to examine the effects of maternal obesity on other GR target genes.

IGF2 transcript levels were lower in obese pregnancy compared to lean and were lowest in the in obese women with LGA babies. This apparent incongruity, that the expression of this growth-promoting gene is lower in pregnancies resulting in larger offspring, is not easy to explain. However, it is possible that this pattern represents

placental-specific down-regulation of this gene and that expression in the fetus remains unchanged at a time when placental growth is less important.

The planned sample population for the term arm of this study was adequately powered based on initial power calculations. However, difficulties encountered in obtaining mRNA of suitable quality from placental samples led to a reduction in the sample size. This may have limited the power of the study. Nevertheless, the final sample size was calculatd to be sufficiently powered, although will be important for future studies to utilise larger populations in order to validate the preliminary findings presented herein.

The results of this study suggest that the changes in placental mRNA transcript levels of these three key genes (*11βHSD2*, *GR* and *IGF2*) may be, at least, useful markers of underlying processes or even contributory factors in the determination of offspring birth weight and pregnancy outcome in obese pregnancy. Unanswered questions remain to be explored as to whether the two birth weight groups differ in terms of vulnerability to later life disease, and whether low birth weight in obese pregnancy is more indicative (and predictive) of future pathology than high birth weight. It seems possible that, as a state of nutrient excess, one may expect obese pregnancy to result in higher birth weight as the fetus maximises growth in response to the increased availability of nutrients. Alternatively, higher birth weight may represent an aberrant outcome of obese pregnancy, an inappropriate overgrowth response to excess nutrients. Whatever the case may be, alterations to the mRNA transcript levels of the genes *11βHSD2*, *GR* and *IGF2* represent a potential mechanism by which changes in fetal-placental growth, and therefore birth weight, may be mediated.

The results of a small-scale exploratory study suggested that no effect of obesity was observed on either placental or fetal liver mRNA transcript levels during the second trimester of pregnancy, with the exception of placental mRNA levels of the pro-inflammatory cytokine gene *IL-1β*. The positive association between *IL-1β* and BMI may indicate increased levels of placental invasion in obese individuals, as placental *IL-1β* production has been shown to correlate with invasive potential in cytotrophoblast cells (Librach *et al.* 1994). Alternatively, the increased levels of *IL-1β* may reflect a pro-inflammatory environment in obese pregnancy, as *IL-1β* is primarily produced by infiltrating inflammatory cells (Ikoma *et al.* 2003). Increasing concentrations of IL-1β have been associated with the onset of labour, suggesting a role in the timing of parturition (Romero *et al.* 1989). While the lack of observed effects may indicate changing vulnerability to maternal obesity during the course of pregnancy, indicating a protective response to the maternal environment at a time when growth and organ development are occurring and are potentially most vulnerable to excess nutrients, it may also be the result of the relatively small number of samples obtained at this time point. Second trimester tissues are difficult to obtain, and thus numbers were limited for this study. This analysis was likely insufficiently powered to adequately determine whether obesity significantly affects, and potentially increased the risk of a type II error. As a pilot study, however, this analysis may serve as a useful basis for future work utilizing larger sample sizes in order to draw firmer biological conclusions.

Furthermore, the use of liver tissue, rather than other fetal tissues (e.g. brain) may be an important limitation, as the liver may not be susceptible to such insults at this time

point during human pregnancy. Finally, mRNA transcript level is not necessarily directly reflective of protein levels and post-translational regulation may affect the level of active protein, independently of mRNA transcription.

A summary of the BMI-associated changes in mRNA transcript levels of key genes at each time point during pregnancy is presented in table 4-5.

Alterations in mRNA transcript levels of *11βHSD2* in obese pregnancy, particularly the increased mRNA transcript levels observed in early gestation, may help explain the reduced levels of total circulating cortisol in obese pregnant women observed in Chapter 3. While urinary clearance of cortisol is elevated in non-pregnant obesity, it is possible that placental clearance of cortisol through inactivation by the barrier enzyme 11βHSD2 may contribute to changes observed in the blood measure of HPA axis activity in obese pregnant women. Testing such a relationship experimentally may be difficult, however, particularly in a clinical setting. It may be possible to perform animal experiments in which *11βHSD2* is knocked out in a tissue specific manner in the placenta, allowing for the investigator to track changes in maternal glucocorticoids throughout pregnancy in the absence of placental *11βHSD2*; likewise, it may also be possible to perform experiments wherein placental *11βHSD2* is overexpressed.

The results of this study suggest that maternal obesity may be associated with changes in the placental expression of key genes. The mechanisms that are thought to influence gene expression are complex. The effects of obesity on one such mechanism, DNA methylation, will be explored in the next chapter.

Gene	First trimester placenta	Second trimester placenta	Second trimester liver	Term placenta
11βHSD2	Increased with increased BMI	No difference	No difference	Increased in obese LGA pregnancy relative to obese AGA
GR	Increased with increased BMI	No difference	No difference	Decreased in obese LGA pregnancy relative to obese AGA
IGF2	No difference	Trend for increase with increasing BMI ($p = 0.057$)	No difference	Decreased in obese pregnancy relative to lean pregnancy; decreased in obese LGA pregnancy relative to obese AGA

Table 4-5. Summary of BMI-associated changes in mRNA transcript levels of key genes at three time points during pregnancy.

Chapter 5:

Methylation

5.1 Introduction

The mechanisms by which adverse early-life conditions affect the fetus remain unclear. There is a growing body of evidence to suggest that epigenetic processes, the modification of DNA and histones without alterations to the nucleotide sequence, may be an important mechanism underlying fetal and early life programming (Waterland and Jirtle 2004; Mathers and McKay 2009) (see Introduction section 1.9).

In chapter 4 the roles of a number of key placental genes were explored in an obese pregnancy paradigm, including *GR* and *11βHSD2,* key genes involved in the regulation of fetal glucocorticoid exposure. These genes have been suggested to be regulated by epigenetic modifications, specifically DNA methylation, and their methylation status has been associated with programming effects (Heijmans *et al.* 2008; Oberlander *et al.* 2008; Wright *et al.* 2008). Human and animal studies have suggested that the prenatal environment, particularly maternal stress, can alter methylation of the *GR* promoter (Mueller and Bale 2008; Oberlander *et al.* 2008; Turner *et al.* 2008; Turner *et al.* 2010; Cao-Lei *et al.* 2011). Animal work has suggested that IUGR is associated with increased methylation of *11βHSD2* in the kidney, and methylation of the genes *11βHSD2* and *GR* in blood is positively associated with increased adiposity and blood pressure later in life in human offspring exposed to an unbalanced maternal diet prenatally (Baserga *et al.* 2010) (Drake *et al.* 2012). Maternal depression is associated with increased methylation of *GR* in neonatal cord blood (Oberlander *et al.* 2008).

Imprinted genes, including the *IGF2-H19* locus, have been suggested to act as nutrient sensors, optimising fetal nutrient supply via modulation of their epigenetic changes, ultimately leading to changes to the placental phenotype; alterations in the methylation state of such genes may have consequences for placental and fetal growth, reflecting the potent level of influence that these genes exert over development (Fowden *et al.* 2011). Accordingly, methylation of *IGF2* in placental samples is associated with changes in birth weight in humans, and altered methylation of this gene has been shown to be altered in individuals exposed to famine prenatally (Heijmans *et al.* 2008; St-Pierre *et al.* 2012).

The placenta is crucial to the maintenance of pregnancy and the development of the offspring. Thus, changes in epigenetic profiles within the placenta may contribute directly to programming effects through alterations in the transcriptional activity of genes involved in nutrient supply to the fetus, as well as placental function and growth. By studying the methylation status of genes such as *11βHSD2* and *GR* in the placenta, one may potentially gain an insight into the mechanisms underlying the differences in gene expression observed in obese and lean pregnancy. By studying the changes in DNA methylation at the imprinted growth factor *IGF2* and its neighbour *H19*, insight may be gained into mechanisms that finely regulate placental growth and development. Furthermore, as the placenta is of partly fetal origin, such study may potentially provide clues as to the effects of maternal obesity on methylation in other fetal tissues.

An increasing number of animal studies (and a smaller number of clinical studies) have explored the role of altered DNA methylation in obese pregnancy (Gemma *et*

al. 2009; Milagro *et al.* 2009; Plagemann *et al.* 2009; Vucetic *et al.* 2010), although no studies have examined the effects of maternal obesity on the methylation status of *IGF2*, *GR* or *11βHSD2* in human pregnancy. In this chapter, DNA methylation of these genes was studied in both first trimester and term placental tissues in samples collected from obese and lean women.

5.1.1 Hypothesis

It was hypothesized that changes described in Chapter 4 in the mRNA transcript levels of the genes *11βHSD2*, *GR* and *IGF2* associated with maternal obesity in first trimester and term placental tissues would be associated with alterations in DNA methylation status in regions of these genes involved in transcriptional regulation.

5.1.2 Aims

This pilot study aimed to investigate whether maternal obesity is associated with changes in the DNA methylation of key genes in placentas collected in the first trimester of pregnancy, as well as at term, and whether DNA methylation of these genes correlates with mRNA levels.

5.2 Methods

5.2.1 Tissues

First trimester tissue was collected from 22 women at time of termination of pregnancy and term placental tissue was collected at elective caesarean section from 55 women as described in methods section 2.1.2. The samples were from the same women as the samples used for gene expression in chapter 3.

5.2.2 DNA Extraction

DNA purification was performed using a phenol/chloroform extraction method as described in Section 2.3.2.2. DNA quantity and integrity were measured using a Nanodrop spectrophotometer (Thermo Scientific, DE, USA) and by running on TBE/agarose gels.

5.2.3 Bisulfite Treatment

DNA extracted from placental samples was bisulfite treated using Epitect Bisulfite Kits (Qiagen, Hilden, Germany).

5.2.4 PCR

PCR for *IGF2* (DMR0 and DMR2), *GR* and *11βHSD2* was performed on bisulfite-treated DNA as described in methods section 2.3.2.4. DMR 2 of *IGF2* was assayed using two previously published assays covering separate regions of the DMR, these assays will be referred to as DMR2.1 and DMR2.2.

5.2.5 Pyrosequencing

Methylation of DNA was measured by pyrosequencing as described in methods section 2.3.2.6.

5.2.6 Statistical Analysis

Normal distribution of methylation data was assessed visually using histograms, and statistically using the D'Agostino & Pearson omnibus normality test. Correlations between methylation levels and maternal BMI in first trimester tissues were analysed using Pearson correlation. Adjustments for confounding factors (including maternal age, BMI, smoking status, parity and gestational age) were performed using multiple linear regression. Differences between methylation levels between obese AGA, obese LGA and lean groups at term were compared using independent t-tests.

All data are presented as mean (±s.e.m), and p values of less than 0.05 were considered significant. For a statistical power of 0.8 and an alpha of 0.05, a sample

size of 23 biopsies was determined to be necessary for correlation analyses ($r \geq 0.4$), while a sample size of 12 was determined to be necessary to detect a 5% difference (sigma 6%) between groups for grouped analyses.

Analysis was performed using Minitab (Minitab Inc, PA, USA) and Statistica (StatSoft Inc, OK, USA). Graphs were prepared using GraphPad Prism (GraphPad Software Inc, CA, USA).

5.3 Results

5.3.1 First Trimester Placenta

5.3.1.1 Demographics

Table 5-1 shows the characteristics of the 22 women from whom first trimester placental tissue samples were collected. The characteristics of this subset of women involved did not differ significantly from the larger cohort (see chapter 4).

	Mean (sem) or N (%)
BMI (kg/m^2)	28.05 (1.59)
Age (years)	24.25 (1.43)
Gestation (days)	69.24 (1.90)
Smokers*	9 (41%)
Nulliparous*	11 (50%)
Multiparous*	11 (50%)

Table 5-1. Characteristics of first trimester study participants. Data are mean (s.e.m) or *N(%).

5.3.1.2 Methylation

IGF2 DMR0 had a mean methylation of 51.34%, DMR2 had a mean methylation of 44.93%, *H19* had a mean methylation of 45.50%, while the mean methylation of *11βHSD2* promoter region was 9.79%, and *GR* promoter 1C had a mean methylation of 2.67%.

No associations were observed between maternal BMI and methylation of any of the regions studied in either unadjusted analysis or when adjusting for maternal age, parity, smoking and gestational age (see Figure 5-1).

A positive trend was observed between methylation of DMR 0 and transcript abundance of *IGF2* in unadjusted analysis and a negative trend between methylation of DMR 2.1 and transcript abundance of *IGF2*, the latter association was significant when adjusted for maternal age, BMI, smoking status, parity and gestational age (see Figure 5-2). No other associations were observed between methylation and transcript concentration for any of the genes studied in either unadjusted or adjusted analysis.

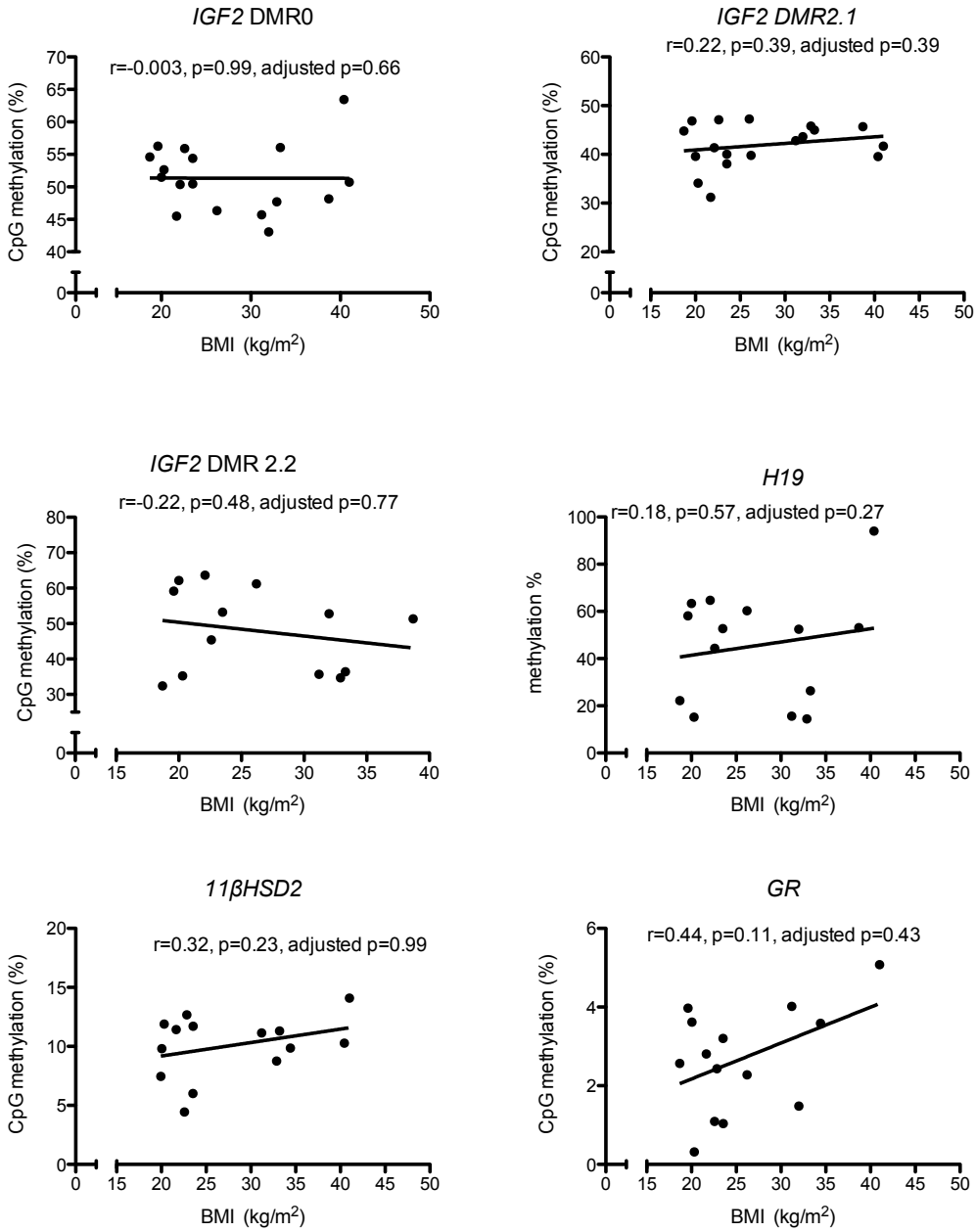


Figure 5-1. Methylation of differentially methylated regions of *IGF2*, *H19* ICR, *11βHSD2* promoter region and *GR* exon 1C in trophoblast samples collected during the first trimester.

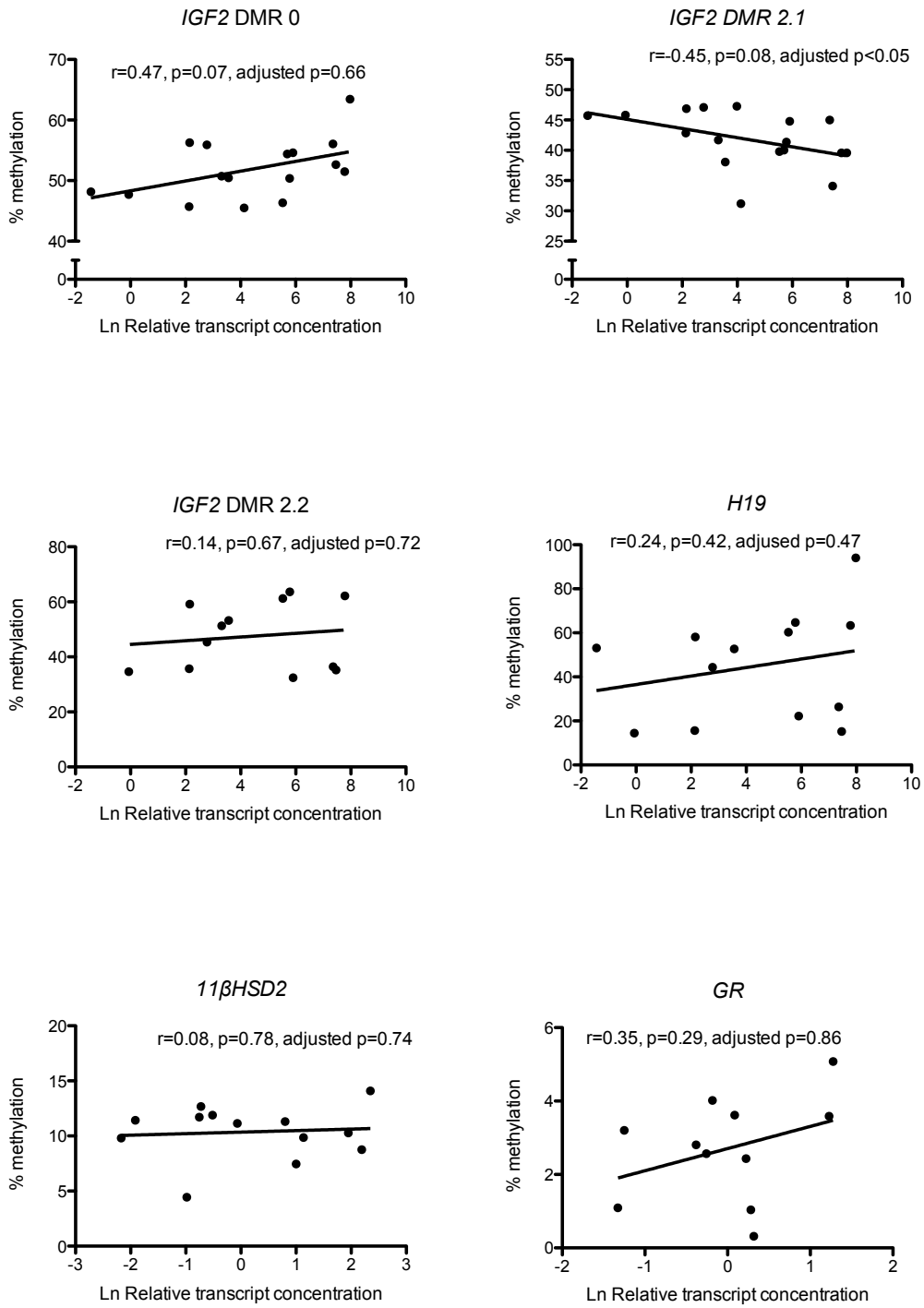


Figure 5-2. Associations between methylation and transcript abundance of *IGF2*, *11βHSD2* and *GR* in first trimester placenta.

5.3.2 Term Placenta

5.3.2.1 Demographics

Table 5-2 shows the characteristics of 55 women from whom term placental tissue was collected, according to BMI. Of the twenty-four obese women, sixteen had children with birth weights under 4000 grams ('appropriate for gestational age' [AGA]), while eight delivered children with birth weights greater than or equal to 4000 grams ('large for gestational age' [LGA]). All infants born to lean women were AGA. A greater proportion of obese women were of lower socioeconomic class (and fewer were of higher socioeconomic class) when compared to the lean group, and the obese group also had a higher proportion of male offspring compared to the lean. The subset of women involved in this study were similar to the larger cohort in terms of BMI, age, smoking status, birth weight, gestation and sex of baby. However, the proportion of women who were multiparous and who had lower socioeconomic status was lower when compared to the larger cohort (both $p<0.05$).

Obese women with LGA babies were significantly more likely to be multiparous ($p<0.05$) and less likely to be smokers ($p<0.05$) compared to obese women with AGA babies. No other differences were observed between the characteristics of obese women with LGA babies and those with AGA babies.

5.3.2.2 Methylation

Mean methylation of each region was similar to levels observed in first trimester tissue. No differences in methylation were observed between lean and obese women for any of the regions studied (see Figure 5-3).

When the obese group was subdivided into women with AGA babies and women with LGA babies, region 1C of *GR* was found to have significantly lower methylation in obese women with LGA babies compared to lean women (see Figure 5-4). No other differences in methylation were observed.

No associations were observed between methylation and transcript concentrations at term in either unadjusted analysis or when adjusting for maternal age, parity, BMI and length of gestation (see Figure 5-5).

Methylation of *GR* was found to negatively associate with birth weight at term in unadjusted analysis ($p<0.05$), but not after adjustment for maternal BMI, parity, neonate gender and gestational age (see Figure 5-6). No other associations with birth weight were observed.

		All (n=55)	Lean (n=31)	Obese (n=24)	*p*	Obese AGA (n=16)	Obese LGA (n=8)
BMI (kg/m^2)		30.84 (1.35)	22.71 (0.25)	41.35 (1.12)	<0.0001	41.95 (1.11)	40.13 (2.58)
Age (years)		32.65 (0.75)	33.73 (4.50)	31.20 (1.34)	0.09	32.99 (1.76)	27.84 (1.42)
Higher Socioeconomic Status (DEPCAT 1-3)*		20 (36%)	15 (48%)	5 (21%)	<0.05	3 (19%)	2 (25%)
Lower Socioeconomic Status (DEPCAT 5-7)*		20 (36%)	7 (22%)	13 (54%)	<0.0005	9 (56%)	4 (50%)
Multiparous*		28 (51%)	14 (45%)	14 (58%)	0.20	8 (50%)	6 (75%)
Smokers*		2 (4%)	1 (3%)	1 (4%)	0.71	1 (6%)	0 (0%)
Birth weight (g)		3542 (71.54)	3465 (85.11)	3643 (120.7)	0.22	3321 (107.9)	4287 (68.6)
Gestation (days)		275.90 (1.07)	277.3 (1.56)	274.2 (1.37)	0.16	272.1 (1.45)	278.4 (2.39)
Sex of baby*	Males	33 (60%)	15 (48%)	18 (75%)	<0.05	12 (75%)	6 (75%)
	Females	22 (40%)	16 (52%)	6 (25%)	<0.05	4 (25%)	2 (25%)

Table 5-2. Characteristics of study participants. Data are mean (s.e.m), or *N(%). Socioeconomic status was measured using deprivation category (DEPCAT) scores based on postcode (McLoone 2004). DEPCAT information was unavailable for 9 lean woman and 6 obese women. 'Smokers' are defined as those who considered themselves as current smokers. Parity data was unavailable for 8 lean women and 4 obese women.

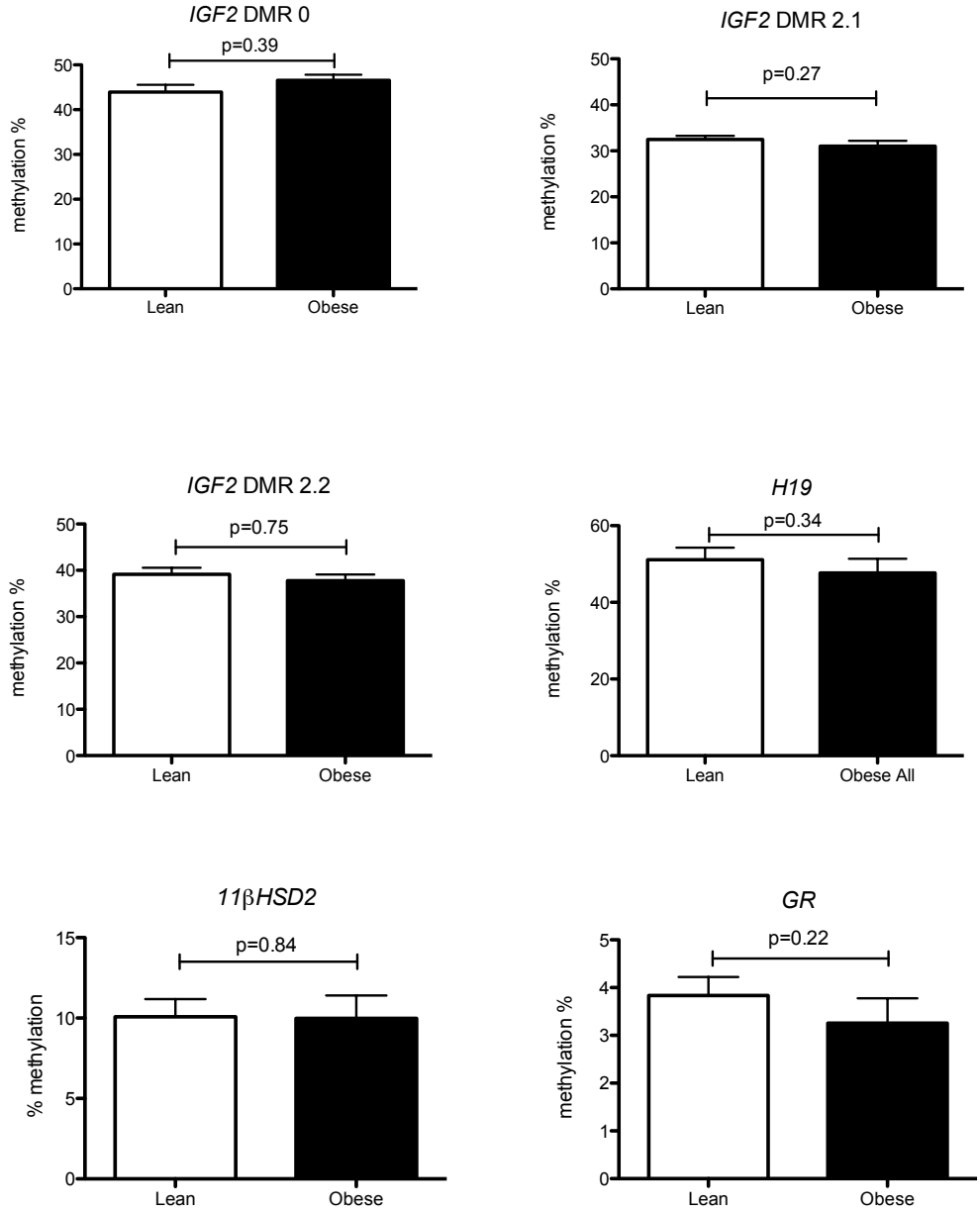


Figure 5-3. Methylation of differentially methylated regions of *IGF2*, *H19* ICR, *11βHSD2* promoter region and *GR* exon 1C in placenta samples collected from lean and obese women at term.

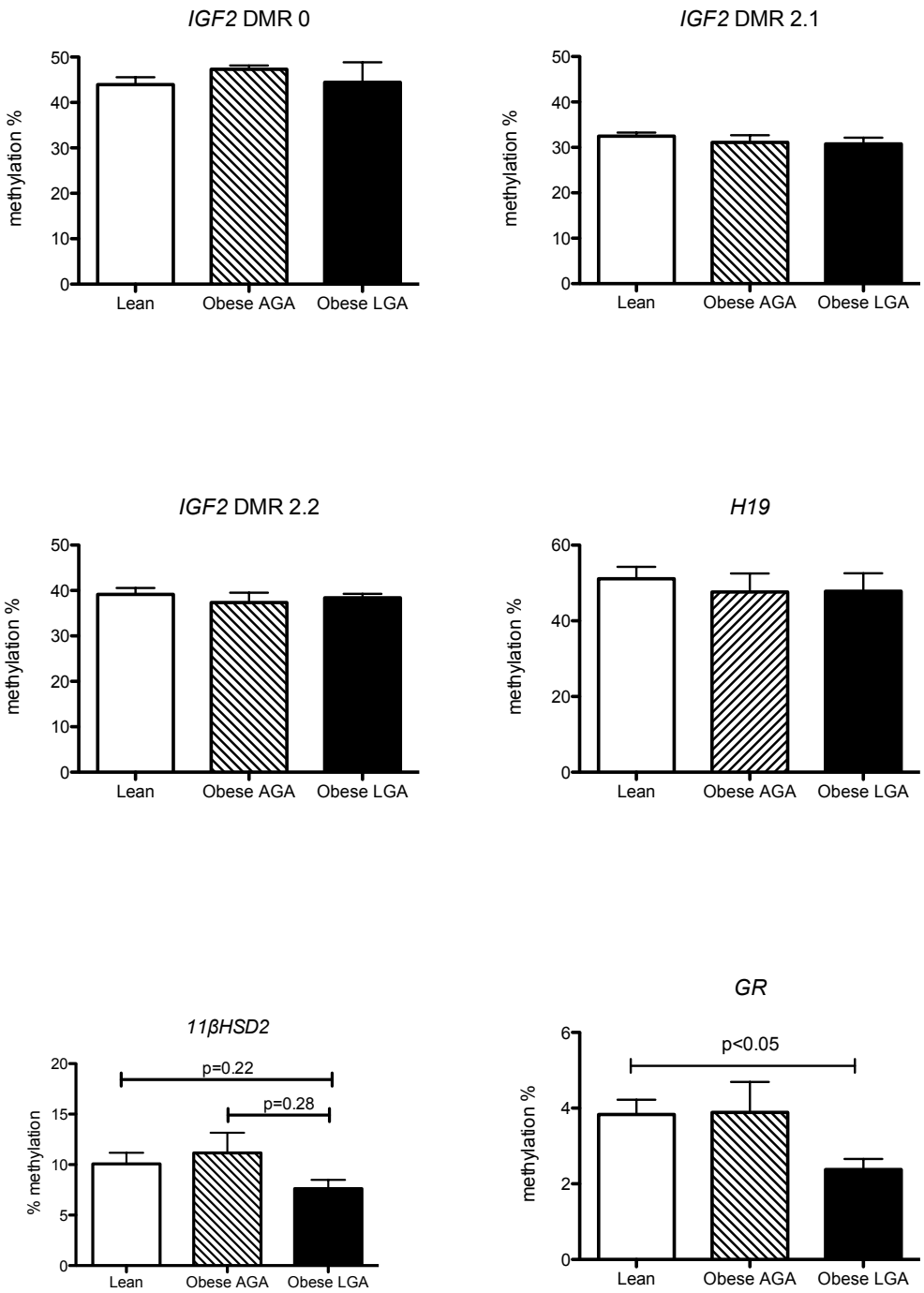


Figure 5-4. Methylation of differentially methylated regions of *IGF2*, *H19* ICR, *11βHSD2* promoter region and *GR* exon 1C in placenta samples collected from lean women, obese women with AGA babies and obese women with LGA babies at term.

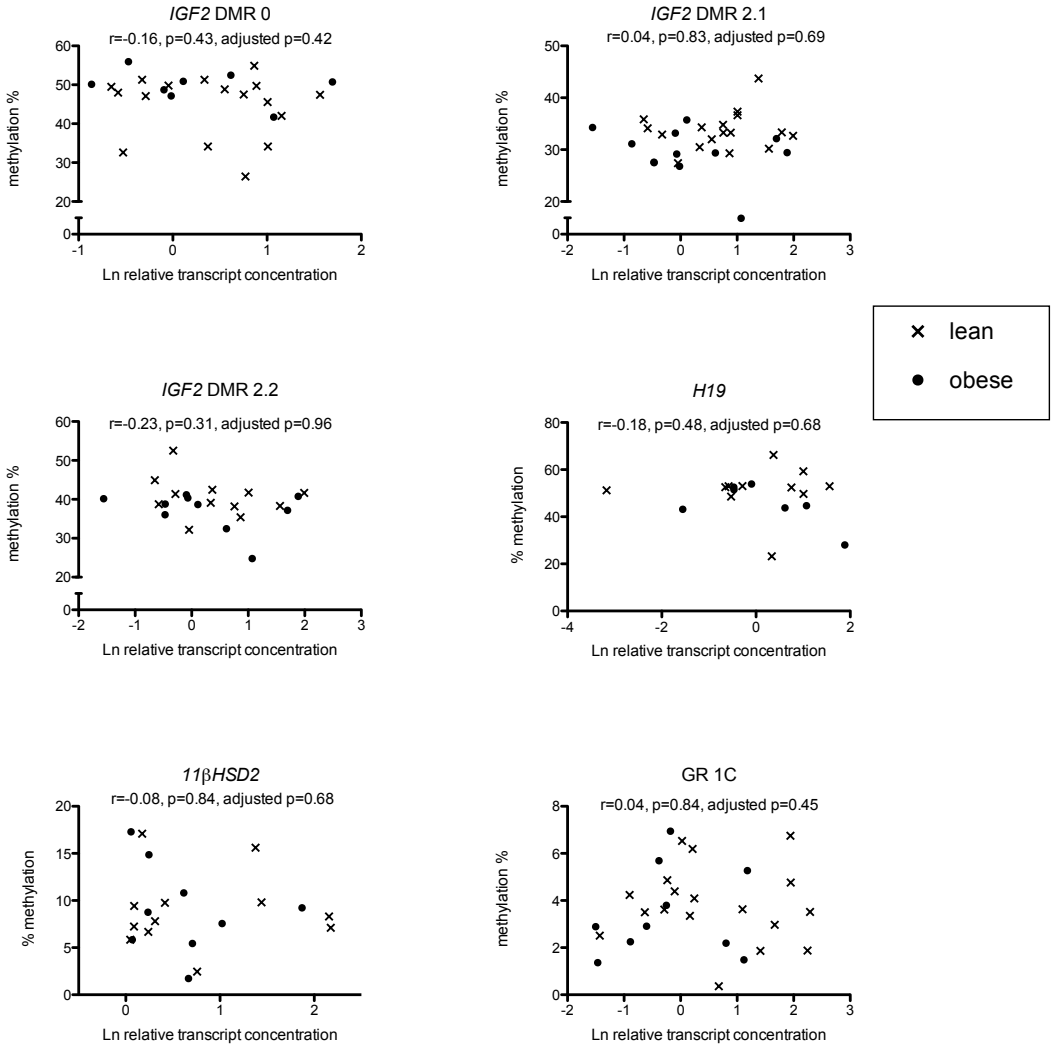


Figure 5-5. Associations between methylation and transcript concentration of *IGF2*, *11βHSD2* and *GR* in placental samples collected from lean and obese women at term.

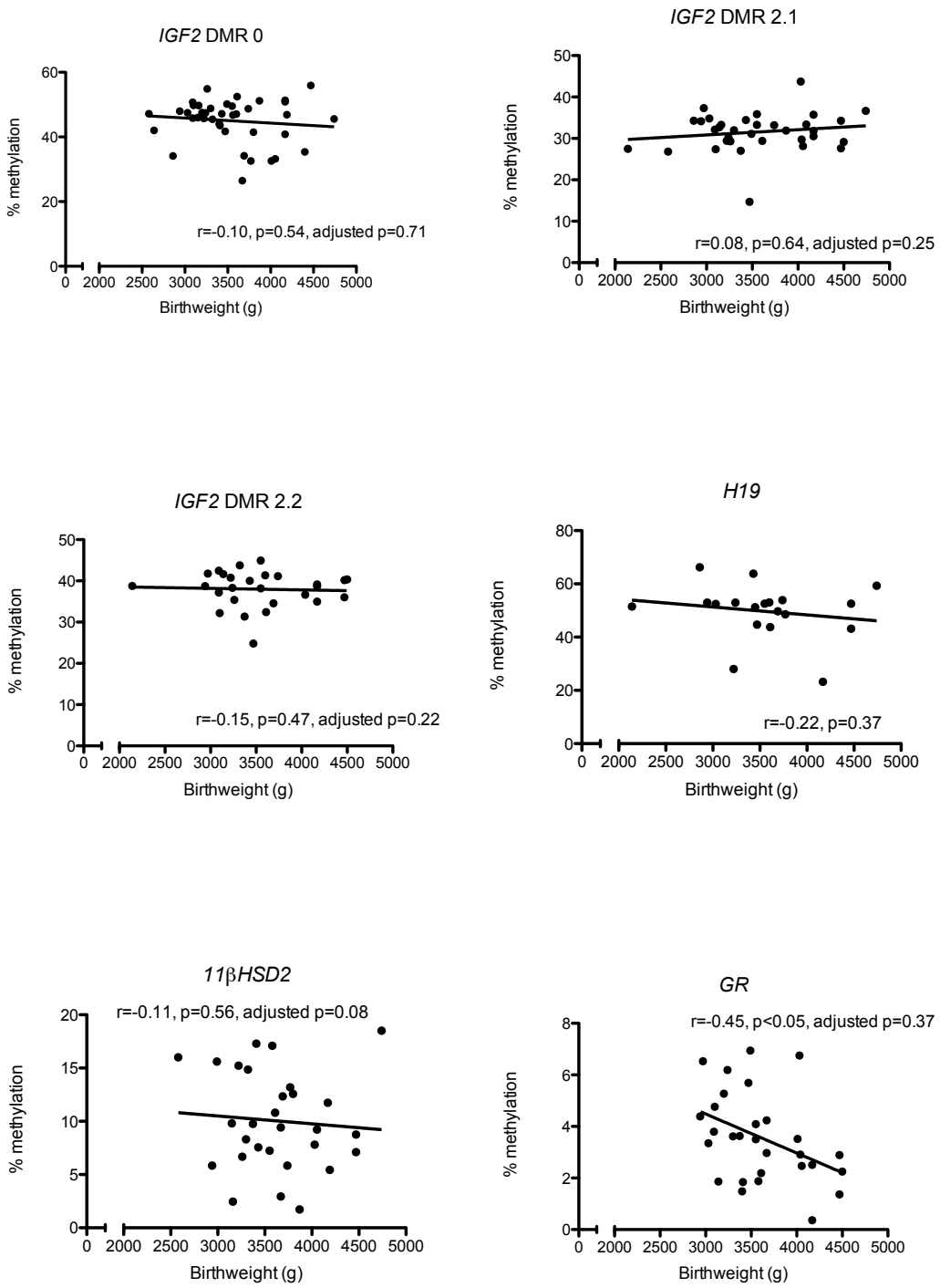


Figure 5-6. Associations between birth weight and placental methylation of differentially methylated regions of *IGF2*, *H19* ICR, *11βHSD2* promoter region and *GR* exon 1C collected from lean and obese women at term.

5.4 Discussion

A growing body of evidence is emerging to suggest that epigenetic mechanisms are an important contributor to the phenomenon of fetal programming of adult disease, with a number of animal studies in particular demonstrating an association between maternal nutrition and alterations in DNA methylation of a number of genes involved in growth and metabolism, including *IGF2* and *PPARγC1A* (Waterland and Jirtle 2004; Gemma *et al.* 2009; Mathers and McKay 2009; Vucetic *et al.* 2010). To date however, the effects of maternal obesity on gene methylation have not been extensively studied. This is due in part to the tissue specific nature of DNA methylation patterns. While ethical and practical considerations often necessitate the use of peripheral tissues (e.g. umbilical cord blood) for epigenetic studies in humans, the data derived from these studies may not necessarily reflect DNA methylation patterns in tissues more directly involved in the disease process. In spite of this, a number of studies have suggested a role for epigenetic mechanisms, in particular methylation, in fetal programming (Oberlander *et al.* 2008; Devlin *et al.* 2010; Turan *et al.* 2012). The use of peripheral tissues, along with the development of increasingly robust methods of assaying DNA methylation, has allowed for an increasing number of clinical epigenetic studies to be undertaken.

In chapter 4, the expression of two genes involved in the regulation of glucocorticoid effects, *GR* and *11βHSD2*, were found to be altered in obese pregnancy compared to lean controls in both first trimester and term placental samples. Regulation of 11βHSD2 transcriptional activity has been suggested to be an important factor in pregnancy outcome, influencing variables such as birth weight; furthermore,

methylation of the gene is thought to be important, as increased methylation of the 11βHSD2 promoter is associated with lower transcription, and increased methylation of this gene in the placenta has also been shown to associate with lower birth weight (Alikhani-Koopaei *et al.* 2004; Marsit *et al.* 2012).

A handful of studies have suggested that methylation of the GR promoter is affected by maternal stress in the early-life environment (Mueller and Bale 2008; Oberlander *et al.* 2008; Turner *et al.* 2008; Turner *et al.* 2010; Cao-Lei *et al.* 2011; Hompes *et al.* 2013). There is also evidence of a role for GR methylation in predicting pregnancy outcome, as increased methylation of the GR promoter in the placenta has been linked to higher birth weight (Filiberto *et al.* 2011). Furthermore, a study by Drake *et al* found that methylation of the genes *11βHSD2* and *GR* positively associated with increased adiposity and blood pressure later in life (Drake *et al.* 2012).

In this study, methylation of the *GR* 1C promoter in term placenta negatively correlated with birth weight in unadjusted analysis, and was found to be lower in placentas of obese women with LGA pregnancy than lean women at term. This is counter to expectations, as mRNA levels of this gene were found to be lower in LGA obese pregnancy compared to lean. It is worth noting, however, that the association was found to be not significant in multivariate analysis. This may indicate that other epigenetic changes, including changes in the methylation of other *GR* promoters, may be more important regulators of transcriptional activity of this gene. Despite the link between maternal obesity and later adiposity in offspring, no associations were found between maternal BMI and methylation of *GR* 1C in the first trimester or the *11βHSD2* promoter region at either time point. Furthermore, no associations were

observed between methylation and mRNA transcript levels for *GR* or *11βHSD2* in first trimester and term tissues, indicating that other mechanisms may be regulating gene transcription in this population. This calls into question the significance of the difference in methylation seen between groups for *GR*. If levels of methylation do not associate with expression, it seems unlikely that changes in the methylation at the sites analysed has biological significance.

When considering the signifiance of differences in methylation, it is worth noting that while pyrosequencing is a robust method of measuring levels of methylation, it is limited by an inability to distinguish between methylation and 5-hydroxymethylation (Radford *et al.* 2012). 5-hydroxymethylation involves the substitution of the C5 hydrogen in cytosine with a hydroxymethyl group. While the function of this form of methylation is unclear, it is currently thought to act either as an intermediate in DNA demethylation pathways, or as a novel form of epigenetic regulation (Guo *et al.* 2011; Bhutani *et al*. 2011; Kim *et al*. 2013). If 5-hydroxymethylation reflects a step in a process of demethylation, it may be inferred to function in a manner antagonistic to methylation. Methods such as pyrosequencing that do not distinguish between the two forms may therefore be of limited use until improvements in detection are made. In order to fully elucidate the influence of methylation on gene expression, and by extension, pregnancy outcomes, it will be necessary to utilise novel methods that can distinguish between the different forms of methylation. Significantly, the inability to differentiate between methylation and hydroxymethylation may explain the lack of associations observed between methylation and gene expression in this study.

This study was also limited by the relatively small number of samples used, as good quality DNA proved difficult to extract from both first trimester and term tissues despite optimisation. Both phenol/chloroform and column-based methods of DNA extraction were attempted, and extractions were attempted a number of times for each sample. In addition, a number of samples did not generate sufficient fluorescence when assayed to effectively quantify DNA methylation, despite repeated attempts. This further decreased the effective sample size, significantly limiting the conclusions that can be drawn from this analysis. While the intended sample population size was determined to be adequately powered, the difficulties encountered with DNA extraction and assay efficiency may have detrimentally affected the statistical power of the analyses. However, despite the relatively small size used in this study when compared with some larger analyses (e.g. Heijmans *et al*. 2008; Filiberto *et al*. 2011), this work is comparable in size to a number of other clinical methylation studies in which significant effects were observed (McMinn et al. 2006; Guo et al. 2008; Oberlander et al. 2008; Gemma et al. 2009; Bourque et al. 2010; Drake et al. 2012; Marsit et al. 2012).

Additionally, studies involving pyrosequencing are often limited by the relatively poor lower limit of detection inherent to the method. Particularly small changes in methylation may fall below the lower limit of detection for pyrosequencing assays, generally considered to be approximately 5% (Tsiatis *et al.* 2010). In this study, the relatively low levels of methylation of both *GR* and *11βHSD2* may have had an effect: methylation levels of both *GR* and the *11βHSD2* promoter were found to be low (less than 20%), a finding which is similar to other studies, raising the question

of whether small changes in methylation can have significant biological effects (Oberlander *et al.* 2008; Drake *et al*. 2012). Similarly low levels of methylation have been shown to be functionally relevant in another hormone receptor, ER (approximately 0.5% change) (Champagne *et al.* 2006). Even in the case of highly methylated genes, such as *IGF2*, the difference in methylation levels seen between groups in this study was rarely greater than 10%. More precise measurements of methylation may be possible in the future, as current methods are refined, or new techniques are developed.

Finally, while this study provides some insight into the epigenetic processes at work in the placenta, it remains to be seen whether similar patterns are present in fetal tissues. It will be important to follow up any findings in the placenta with similar studies involving fetal tissues, where possible, in order to confirm whether epigenetic changes are similar in the placenta and the fetus.

In chapter 4, mRNA transcript levels of the gene *IGF2* were found to be significantly lower in obese LGA placentas than in placentas from lean pregnancy at term; however, no differences were observed in methylation of the gene. This agrees with the findings of a previous study, where *IGF2* methylation was found to have no association with prenatal maternal BMI; however methylation of *IGF2* was not found to be associated with birth weight, a finding which contrasts with previous work and may reflect the relatively small size of this study (St-Pierre *et al.* 2012). Furthermore, methylation of *IGF2* did not appear to be associated with mRNA transcript levels at either time point studied. This suggests that other mechanisms may be regulating the activity of this gene in the placenta such as histone modifications. No obesity-related

changes were observed in *H19* ICR methylation in either first trimester or term placental tissues. Furthermore, *H19* ICR methylation did not correlate with *IGF2* mRNA levels at either of the time points studied.

In summary, this study suggests that maternal obesity may not significantly affect methylation of the genes *GR*, *11βHSD2*, *IGF2*, or *H19* in placenta despite associating with altered transcriptional activity of these genes. Further work is necessary, and should focus on the role of other mechanisms regulating gene transcription (such as histone modification, microRNAs, and hydroxymethylation) in the regulation of gene transcription in the placentas of obese women. If alterations in other epigenetic marks are found in peripheral tissues such as the placenta, they may prove to be useful biomarkers of programming effects induced by maternal obesity.

Obesity may be considered a type of physiological stress due to its metabolic, inflammatory and HPA axis effects. In the next chapter, the effects of psychological stress and mood during pregnancy on placental gene expression in two cohorts, one from Edinburgh and another from Finland, will be investigated.

Chapter 6:

Maternal Stress During Pregnancy and Placental Gene Regulating Fetal Glucocorticoid Exposure

6.1 Introduction

In Chapter 4, it was shown that the physiological stressor of maternal obesity has effects on placental mRNA concentration for a number of key genes regulating glucocorticoid metabolism. Psychological stress during pregnancy has been recognised to have programming effects on the developing offspring, with increased risk of anxiety and ADHD postnatally (O'Connor *et al.* 2003; O'Connor *et al.* 2005; Phillips *et al.* 2005; O'Donnell *et al.* 2009; Motlagh *et al.* 2010). It has been suggested that this may be to prepare the unborn child for survival in a hostile postnatal environment (Glover 2011). A retrospective study by O'Donnell *et al*. demonstrated that placental mRNA concentration of *11βHSD2* is down-regulated by maternal anxiety, as measured by the State/Trait Anxiety Index on the day before caesarean section (O'Donnell *et al.* 2012). The authors suggested that this may contribute to increased exposure of the fetus to maternal glucocorticoids.

The aim of the work in this chapter was to investigate the effects of maternal anxiety on placental levels of key genes involved in glucocorticoid metabolism in a prospective study, in a cohort of pregnant Finnish women in whom maternal stress was evaluated throughout pregnancy. We then aimed to evaluate whether the findings could be replicated in another cohort using samples collected from the very severely obese and lean Scottish women (described in Chapters 2 and 3) in whom stress and anxiety had been assessed during pregnancy.

6.1.1 Hypothesis

It was hypothesised that maternal stress/anxiety during pregnancy would be associated with altered levels of key genes regulating glucocorticoid metabolism including decreased mRNA levels of the gene *11βHSD2* in the placenta at term.

6.1.2 Aims

1. To determine the effects of psychological stress on term placental mRNA transcript levels of key genes, particularly those involved in regulating glucocorticoid transfer to the fetus, in a cohort of pregnant women in whom stress and anxiety levels were assessed during each trimester.
2. To test whether any of the findings observed in aim 1) were replicated in the subset of obese and lean women described in Chapter 4 who had also completed stress and anxiety questionnaires during pregnancy.

6.2 Methods

6.2.1 Helsinki Cohort

6.2.1.1 Subject Recruitment

Participants for this study were recruited as part of the PREDO-Project (Prediction and Prevention of Pre-Eclampsia) between September 2005 and December 2010 in Finland, as described in Methods section 2.1.5.1 (Villa *et al.* 2013). Participants attended ultrasound screening between 12 and 14 weeks of gestation in one of ten hospital maternity clinics.

6.2.1.2 Assessing Maternal Mood During Pregnancy

All participants of the PREDO were invited to complete a set of standardized and validated questionnaires, aimed at measuring stress, depression, anxiety and anger throughout pregnancy at two-week intervals between 12 and 14 weeks of gestation and ending two weeks postpartum. Maternal anxiety was measured using the State-Trait Anxiety Inventory (STAI), while maternal personality traits were measured using the NEO Personality Inventory as described in Methods section 2.1.5.2.

6.2.1.3 Tissues

Placenta samples (n = 67) were collected from healthy singleton term (37 to 42 weeks of gestation) pregnancies a maximum of 90 minutes (median=45.9, standard deviation=21.2 minutes) after vaginal (n=48) or caesarean delivery (n=19). Given the

heterogeneity of the placenta, biopsies were obtained avoiding areas of obvious infarction or damage. Placenta samples were collected in RNAlater solution and stored at -20C.

6.2.2 Edinburgh Cohort

The Edinburgh arm of the study was a case-control study comparing lean and obese pregnant women who had been recruited among participants of a larger prospective cohort study, as described in chapter 4. Participants were recruited as part of a between April 2008 and September 2012.

Term placental tissue was collected at elective caesarean section as described in methods section 2.1.4.

6.2.2.1 Assessing Maternal Anxiety during Pregnancy

Participants were asked to complete validated questionnaires to assess mood (including stress at home, work, stress over money, stress over life events, satisfaction with life and anxiety), Spielberger State and Trait Anxiety Inventory and the Hospital Anxiety and Depression Subscale (HADS). Questionnaires were completed at ~16 weeks gestation and at ~28 weeks gestation, as described in Methods section 2.1.1.2.

6.2.3 RNA Extraction

Tissues stored in RNALater were used for RNA purification and extraction as described in Section 2.3.1.1.

6.2.4 Reverse Transcription and Real Time PCR

RNA successfully extracted from tissues was converted to cDNA using Roche Reverse Transcription kits as described in Section 2.3.1. cDNA was used to perform real time PCR in order to estimate mRNA levels encoding for the genes *GR*, *11βHSD1*, *11βHSD2* and *MR* as described in detail in Section 2.3.1.5 Samples in the PREDO cohort were corrected to *TBP*.

6.2.5 Statistical Analyses

Normal distribution of data was assessed visually using histograms. mRNA transcript data were not normally distributed were normalised using natural log transformation. Correlations between mRNA transcript levels and maternal mood assessment scores were analysed using Pearson rank correlation. Adjustments for confounding factors (in the Edinburgh cohort: maternal age, smoking status, parity, DEPCAT category, and length of gestation; in Helsinki cohort: maternal age, smoking status, parity, education, mode of delivery, length of gestation, birth weight and time between sampling and placental birth) were performed using multiple linear regression. Lean and obese women were analysed separately in the Edinburgh cohort. As each of the

measures used tested a different psychological component of stress and anxiety, the a priori hypothesis was to test each stress and anxiety questionnaire individually. In order to control for the effects of multiple testing, the Bonferroni correction was carried out for all analyses. The Bonferroni correction can aid in controlling for the increased probability of a type I error (i.e. falsely rejecting the null hypothesis) that can occur with multiple testing. All data are presented as mean (±s.e.m), and overall p values of less than 0.05 were considered significant.

Statistical power calculations were performed to determine the sample size required for these analyses. For correlation analyses where alpha = 0.05 and statistical power = 0.8, a sample size of n = 70 was required to detect a correlation of r = 0.3, n = 30 for a correlation of r = 0.4, and n = 23 for a correlation of r = 0.5. For multiple regression analyses using 8 predictors (e.g. correcting for maternal age, parity, education, mode of delivery, neonate birthweight, gestational age, and time to sampling) with an alpha of 0.05, a sample size of 45 was calculated as being necessary to detect an $r^2 \geq 0.3$.

Analysis was performed using Minitab (Minitab Inc, PA, USA), SPSS (IBM, NY, USA) and Statistica (StatSoft Inc, OK, USA). Graphs were prepared using GraphPad Prism (GraphPad Software Inc, CA, USA).

6.3 Results

6.3.1 Helsinki Cohort

6.3.1.1 Demographics

Demography of the Helsinki Stress in Pregnancy cohort (n=67) is detailed in Table 6-1.

6.3.1.2 *11βHSD1*

Associations between stress evaluation results and placental mRNA transcript levels of the gene *11βHSD1* are shown in Table 6-2. The majority of associations were with first trimester anxiety measures: *11βHSD1* was found to positively correlate with NEO personality inventory scores for neuroticism at 12 weeks. This association remained significant after adjustment for maternal age, length of gestation, birth weight, smoking status, parity, education and mode of delivery. *11βHSD1* positively correlated with Spielberger state anxiety scores in the first trimester of pregnancy in adjusted analysis and was found to associate with the score for Spielberger state anxiety negative anxiety subscale in first trimester in both univariate and adjusted analysis. *11βHSD1* did not associate with Spielberger state anxiety curiosity subscale score in either univariate or adjusted analysis. For trait anxiety there were only trends for correlations of *11βHSD1* and total trait score in adjusted analysis with a similar pattern if only the negative anxiety subscale effects (i.e., high negative affect) were considered. After adjusting for the effects of multiple testing, however, only negative

anxiety in the first trimester was found to significantly correlate with placental *11βHSD1* expression.

While a trend was observed for Spielberger state anxiety (negative anxiety subscale) in adjusted analysis during the second trimester, the association was no longer present by the third trimester; no associations were observed between *11βHSD1* and Spielberger state anxiety score in the second and third trimesters of pregnancy, or the mean state anxiety score over pregnancy.

Age (years)		31.45 (0.51)
Parity*	Nulliparous	29 (43%)
	Multiparous	38 (57%)
Education*	Lowest	3 (4%)
	Intermediate	23 (34%)
	Highest (university level)	41 (62%)
Length of Gestation (days)		280.41 (0.81)
Birth weight (g)		3440.74 (45.07)
Birth length (cm)		49.86 (0.22)
Gender*	Male	32 (48%)
	Female	35 (52%)
NEO personality inventory	Anxiety (12 weeks)	12.62 (5.14)
Spielberger Trait Anxiety	Total (12 weeks)	38.73 (6.65)
	Negative Trait Anxiety (12 weeks)	16.92 (3.77)
	Positive Trait Anxiety (12 weeks)	28.77 (3.54)
Spielberger State Anxiety	1st trimester	29.79 (7.76)
	2nd trimester	33.24 (7.79)
	3rd trimester	33.52 (8.21)
	Mean	33.06 (7.45)
Spielberger negative anxiety subscale	1st trimester	13.54 (3.04)
	2nd trimester	14.48 (3.35)
	3rd trimester	14.43 (3.49)
Spielberger curiosity subscale	1st trimester	33.75 (5.25)
	2nd trimester	31.23 (5.07)
	3rd trimester	30.90 (5.14)

Table 6-1. Demographic data for Helsinki cohort. All data are mean (±s.e.m), Categorical data (*) are expressed as n (%).

11βHSD1		**Unadjusted model**		**Adjusted model**	
Predictor variable		**β**	**P**	**β**	**P**
NEO personality inventory	Anxiety (12 weeks)	2.84	<0.01	4.18	<0.01
Spielberger Trait Anxiety	Total (12 weeks)	0.27	0.06	0.31	0.05
	Negative Trait Anxiety (12 weeks)	0.24	0.08	0.26	0.09
	Positive Trait Anxiety (12 weeks)	-0.13	0.38	-0.27	0.11
Spielberger State Anxiety	1st trimester	0.27	0.057	0.3	<0.05
	2nd trimester	0.05	0.72	0.22	0.16
	3rd trimester	-0.03	0.81	0.06	0.69
	Mean	0.04	0.79	0.16	0.29
Spielberger negative anxiety subscale	1st trimester	0.39	<0.005	0.35	<0.05
	2nd trimester	0.15	0.28	0.28	0.054
	3rd trimester	0.05	0.72	0.10	0.48
	Total	0.13	0.34	0.21	0.14
Spielberger curiosity subscale (high score indicates low anxiety)	1st trimester	-017	0.24	-0.24	0.12
	2nd trimester	0.01	0.93	-0.15	0.33
	3rd trimester	0.09	0.54	-0.02	0.90
	Total	0.02	0.87	-0.11	0.48

Table 6-2 Associations between *11βHSD1* and stress measures in a cohort of pregnant Finnish women by Pearson correlation. Linear regression adjusted for maternal age, smoking status, parity, education, mode of delivery, length of gestation, birth weight and time between sampling and placental birth. Using Bonferroni correction, p values > 0.003 were considered significant.

6.3.1.3 *11βHSD2*

No associations were observed between *11βHSD2* mRNA transcript levels and any of the stress measures examined at any time point during pregnancy (see Table 6-3).

6.3.1.4 *GR*

In adjusted analyses, there was a positive association between *GR* and total Spielberger trait anxiety score at 12 weeks, (see Table 6-4) and a negative association with Spielberger trait anxiety score when considering only positively-worded anxiety items (i.e., low positive affect) at 12 weeks. After adjusting for the effects of multiple testing, however, no associations were found to be significant.

GR did not associate with Spielberger trait anxiety score at 12 weeks when considering negative anxiety subscale influences only. Similarly, no associations were observed between *GR* and NEO personality index neuroticism anxiety score at 12 weeks, in either unadjusted or adjusted analysis. No associations were observed between *GR* and state anxiety in the first trimester.

There were trends for an association between *GR* and state anxiety in the second trimester although after adjustment the association was no longer significant. Similar trends were observed between *GR* and state anxiety in the third trimester as well as between *GR* and mean state anxiety throughout pregnancy in unadjusted and adjusted analysis. *GR* was not found to associate with either Spielberger State anxiety subscale or curiosity subscale scores at any time point in pregnancy.

11βHSD2		**Unadjusted model**		**Adjusted model**	
Predictor variable		**B**	**P**	**β**	**P**
NEO personality inventory	Anxiety (12 weeks)	-0.04	0.77	-0.15	0.38
Spielberger Trait Anxiety	Total (12 weeks)	0.11	0.48	0.09	0.61
	Negative Trait Anxiety (12 weeks)	0.17	0.25	0.16	0.33
	Positive Trait Anxiety (12 weeks)	0.01	0.92	0.07	0.71
Spielberger State Anxiety	1st trimester	0.09	0.54	0.06	0.73
	2nd trimester	0.07	0.65	0.08	0.63
	3rd trimester	0.14	0.34	0.09	0.60
	Mean	0.09	0.49	0.08	0.63
Spielberger negative anxiety subscale	1st trimester	0.08	0.58	0.11	0.46
	2nd trimester	0.11	0.47	0.13	0.44
	3rd trimester	0.11	0.46	0.04	0.82
	Total	0.11	0.47	0.08	0.61
Spielberger curiosity subscale (high score indicates low anxiety)	1st trimester	-0.08	0.57	-0.01	0.94
	2nd trimester	-0.04	0.79	-0.04	0.81
	3rd trimester	-0.14	0.32	-0.11	0.52
	Total	-0.09	0.56	-0.70	0.68

Table 6-3 Associations between *11βHSD2* and stress measures in a cohort of pregnant Finnish women by Pearson correlation. Linear regression adjusted for maternal age, smoking status, parity, education, mode of delivery, length of gestation, birth weight and time between sampling and placental birth. Using Bonferroni correction, p values > 0.003 were considered significant.

GR		**Unadjusted model**		**Adjusted model**	
Predictor variable		**β**	**P**	**β**	**P**
NEO personality inventory	Anxiety (12 weeks)	0.05	0.74	0.13	0.42
Spielberger Trait Anxiety	Total (12 weeks)	0.22	0.12	0.30	<0.05
	Negative Trait Anxiety (12 weeks)	0.15	0.29	0.15	0.32
	Positive Trait Anxiety (12 weeks)	-0.27	0.052	-0.37	<0.05
Spielberger State Anxiety	1st trimester	0.13	0.36	0.096	0.51
	2nd trimester	0.24	0.09	0.23	0.11
	3rd trimester	0.25	0.07	0.27	0.054
	Mean	0.25	0.072	0.27	0.056
Spielberger negative anxiety subscale	1st trimester	0.09	0.51	0.05	0.74
	2nd trimester	0.17	0.24	0.20	0.15
	3rd trimester	0.19	0.19	0.19	0.16
	Total	0.18	0.20	0.19	0.16
Spielberger curiosity subscale (high score indicates low anxiety)	1st trimester	-0.14	0.33	-0.12	0.42
	2nd trimester	-0.25	0.07	-0.23	0.13
	3rd trimester	-0.26	0.06	-0.23	0.13
	Total	-0.26	0.06	-0.23	0.13

Table 6-4 Associations between natural logged *GR* and stress measures in a cohort of pregnant Finnish women by Pearson correlation. Linear regression adjusted for maternal age, smoking status, parity, education, mode of delivery, length of gestation, birth weight and time between sampling and placental birth. Using Bonferroni correction, p values > 0.003 were considered significant.

6.3.1.5 *MR*

No associations were observed between *MR* and NEO personality inventory anxiety score at 12 weeks (see Table 6-5).

No associations were observed between *MR* and total Spielberger trait anxiety score at 12 weeks, or with either the positive or negative trait anxiety subscales.

MR positively correlated with Spielberger state anxiety scores in the first trimester, and was found to negatively associate with Spielberger state anxiety curiosity subscale (i.e., low curiosity) score measured in the first trimester in adjusted analysis. *MR* also positively correlated with Spielberger state anxiety scores in the second trimester and was also found to negatively associate with Spielberger state anxiety curiosity subscale score in adjusted analysis. *MR* was found to positively correlate with Spielberger State negative anxiety subscale score in adjusted analysis in the second trimester. A positive correlation was also observed between *MR* and Spielberger state anxiety scores measured in the third trimester, as well as with the mean anxiety score for the entirety of pregnancy. *MR* was also found to negatively associate with Spielberger state anxiety curiosity subscale score in adjusted analysis in the third trimester. *MR* was found to correlate positively with mean Spielberger state negative anxiety and negatively with mean Spielberger state curiosity subscale scores measured over the entirety of pregnancy.

After adjusting for the effects of multiple testing, *GR* expression was found to negatively associate with scores for state anxiety curiosity in the second trimester only; other observed associations found to be weakened.

MR		**Unadjusted model**		**Adjusted model**	
Predictor variable		**β**	**P**	**β**	**P**
NEO personality inventory	Anxiety (12 weeks)	-0.004	0.97	0.07	0.69
Spielberger Trait Anxiety	Total (12 weeks)	0.19	0.20	0.33	0.09
	Negative Trait Anxiety (12 weeks)	0.11	0.46	0.22	0.21
	Positive Trait Anxiety (12 weeks)	-0.22	0.14	-0.323	0.085
Spielberger State Anxiety	1st trimester	0.22	0.13	0.3	0.054
	2nd trimester	0.27	0.058	0.46	<0.01
	3rd trimester	0.22	0.13	0.36	<0.05
	Mean	0.26	0.069	0.42	<0.01
Spielberger State negative anxiety subscale	1st trimester	0.07	0.51	0.13	0.41
	2nd trimester	0.22	0.13	0.35	<0.05
	3rd trimester	0.20	0.17	0.29	0.07
	Total	0.22	0.13	0.33	<0.05
Spielberger curiosity subscale (low score refers to high anxiety)	1st trimester	-0.27	0.06	-037	<0.05
	2nd trimester	-0.28	0.05	-0.47	<0.005
	3rd trimester	-0.21	0.15	-0.38	<0.05
	Total	-0.26	0.07	-0.45	<0.01

Table 6-5 Associations between natural logged *MR* and stress measures in a cohort of pregnant Finnish women by Pearson correlation. Linear regression adjusted for maternal age, smoking status, parity, education, mode of delivery, length of gestation, birth weight and time between sampling and placental birth. Using Bonferroni correction, p values > 0.003 were considered significant.

6.3.2 Edinburgh Obesity in Pregnancy Study

6.3.2.1 Demographics

The demographics of a subset of participants in a longitudinal study of the effects of obesity during pregnancy who self-reported their stress throughout gestation and for whom term placental samples were available are reported in Table 6-6. Obese women had higher reported stress at home at visit 2, stress over money at visit 2, state anxiety at visits 1 and 2, and lower satisfaction with life at visit 2. Obese women were also more likely to be of a lower socioeconomic status compared to lean women. Baseline characteristics of subjects who completed stress questionnaires did not differ from the rest of the cohort (data not shown).

6.3.2.2 *11βHSD1*

In lean women, *11βHSD1* correlated with a number of measures of anxiety at visit 1, including Spielberger state and trait anxiety, with trends for associations with anxiety measured at the same time point (see Table 6-7). However, only financial stress in the second trimester was found to correlate with placental *11βHSD1* expression after correcting for the effects of multiple testing. Additionally, many of these associations were weakened when adjusting for confounding factors. At visit 2, *11βHSD1* correlated with money-related stress in both univariate and adjusted analysis. In univariate analysis, placental *11βHSD1* was also found to positively correlate with measures of anxiety measured at visit 2, while there was a negative trend with

satisfaction with life, although the association was not significant in multivariate analysis.

In obese women, *11βHSD1* was found to correlate with the participants' reported satisfaction with life and there was a trend with Spielberger state anxiety at visit 2 in univariate analysis, but neither association was significant with adjustment. No other associations were observed.

		Lean (n=39)	Obese (n=36)	P
BMI		23.08 (0.33)	41.57 (0.95)	<0.0001
Age (years)		33.88 (0.75)	31.87 (1.06)	0.12
Birthweight (g)		3490 (69.47)	3635 (94.4)	0.21
Gestational Age (days)		276.54 (1.28)	276.58 (1.25)	1.00
Parity*	Nulliparous	13 (33%)	10 (28%)	0.52
	Multiparous	17 (44%)	21 (59%)	0.14
Depcat category*	0	17 (44%)	8 (23%)	<0.05
	1	12 (31%)	21 (59%)	<0.05
Smokers*		9 (23%)	5 (14%)	0.14
Non-smokers*		21 (53%)	23 (64%)	0.31
Ethnicity*	White	16 (41%)	25 (70%)	<0.01
	Non-white	1 (3%)	1 (3%)	1.00
Stress measures:				
Stress at work	Visit 1	1.33 (0.14)	1.43 (0.19)	0.83
	Visit 2	1.53 (0.27)	1.58 (0.26)	0.89
Stress at home	Visit 1	0.94 (0.17)	1.0 (0.19)	0.81
	Visit 2	0.67 (0.16)	1.21 (0.15)	<0.05
Stress over money	Visit 1	0.31 (0.12)	0.73 (0.18)	0.56
	Visit 2	0.40 (0.13)	0.79 (0.14)	<0.05
Stress events	Visit 1	0.56 (0.20)	0.67 (0.41)	0.62
	Visit 2	0.067 (0.067)	0.68 (0.20)	<0.01
Satisfaction with life	Visit 1	27.75 (1.37)	26.13 (1.10)	0.26
	Visit 2	28.27 (1.33)	24.11 (1.47)	<0.05
Anxiety	Visit 1	5.33 (0.97)	5.87 (1.04)	0.71
	Visit 2	5.60 (1.01)	6.95 (0.95)	0.34
State Anxiety	Visit 1	29.19 (2.03)	36.33 (2.92)	<0.05
	Visit 2	30.33 (2.44)	38.47 (2.92)	<0.05
Trait Anxiety	Visit 1	34.06 (2.84)	34.53 (2.80)	0.89
	Visit 2	34.20 (3.11)	40.42 (3.32)	0.18

Table 6-6. Demographics of lean and obese cohorts. All continuous data are mean (±s.em.). Categorical data (*) are expressed as percentages. Socioeconomic status was measured using deprivation category (DEPCAT) scores based on postcode, with depcat category 0 indicating low deprivation, and 1 indicating higher levels of deprivation (McLoone 2004).

11βHSD1		Lean				Obese			
		Unadjusted		Adjusted		Unadjusted		Adjusted	
Predictor variable		r	p	b	p	R	p	b	p
Stress work	Visit 1								
	Visit 2	0.33	0.27	0.14	0.63	0.28	0.28	0.12	0.27
Stress home	Visit 1	0.23	0.43	0.10	0.66	0.45	0.12	0.25	0.11
	Visit 2	0.20	0.51	0.09	0.68	0.25	0.33	0.07	0.33
Stress money	Visit 1	0.29	0.31	0.002	0.99	0.27	0.37	0.14	0.33
	Visit 2	0.73	<0.005	0.24	<0.05	0.01	0.97	0.03	0.79
Stress events	Visit 1	-0.6	<0.05	-0.41	0.06	0.14	0.65	-0.007	0.98
	Visit 2	0.11	0.71	0.003	0.96	-0.09	0.7	-0.07	0.48
Satisfaction with life	Visit 1	-0.39	0.16	-0.45	0.76	0.03	0.93	0.03	0.98
	Visit 2	-0.55	0.052	-1.58	0.24	0.53	<0.05	1.44	0.11
Anxiety	Visit 1	0.54	0.55	1.38	0.1	0.07	0.82	0.12	0.9
	Visit 2	0.62	<0.05	1.79	0.09	-0.26	0.32	-0.25	0.73
State	Visit 1	0.62	<0.05	1.68	<0.05	-0.28	0.36	-1.95	0.11
	Visit 2	0.68	<0.05	1.94	0.11	-0.42	0.09	-2.65	0.12
Trait	Visit 1	0.63	<0.05	2.37	0.15	-0.02	0.95	0.05	0.98
	Visit 2	0.72	<0.01	2.64	0.08	-0.3	0.24	-1.44	0.50

Table 6-7. Associations between natural logged *11βHSD1* and stress measures in a cohort of obese and lean pregnant women by Pearson correlation. Linear regression adjusted for maternal age, smoking status, parity, DEPCAT category, and length of gestation. Using Bonferroni correction, p values > 0.003 were considered significant.

6.3.2.3 *11βHSD2*

In lean women, no associations were observed between *11βHSD2* mRNA levels and maternal pre-pregnancy anxiety (see Table 6-8).

In obese women, there were trends for *11βHSD2* to be associated with increased Spielberger trait anxiety at visit 1 in univariate analysis, with self-reported stress at work at visit 2 in both univariate and multivariate analysis and with decreased satisfaction with life reported at visit 2 in univariate analysis.

6.3.2.4 *GR*

Stress at work at visit 1 was positively associated with expression of GR in adjusted analysis. However, this association was not found to be significant after adjustment for multiple testing. No other associations were observed between *GR* transcript levels and maternal pre-pregnancy anxiety in either lean or obese women (see Table 6-9).

11βHSD2		Lean				Obese			
		Unadjusted		Adjusted		Unadjusted		Adjusted	
Predictor variable		**r**	**p**	**b**	**p**	**r**	**p**	**b**	**p**
Stress work	Visit 1	-0.42	0.35	1.64	0.35	-0.26	0.42	-1.10	0.32
	Visit 2	-0.39	0.51	2.56	0.66	0.59	0.06	0.97	0.06
Stress home	Visit 1	0.42	0.35	0.45	0.35	0.31	0.38	0.43	0.32
	Visit 2	-0.39	0.51	-0.56	0.54	0.43	0.18	0.06	0.91
Stress money	Visit 1	0.06	0.89	0.30	0.53	0.23	0.53	0.05	0.92
	Visit 2	-0.39	0.51	1.28	0.66	0.1	0.76	0.22	0.69
Stress events	Visit 1	0.22	0.63	1.05	0.5	-0.19	0.59	-0.54	0.46
	Visit 2	0.20	0.74	1.21	0.62	0.21	0.54	0.05	0.89
Satisfaction with life	Visit 1	0.2	0.67	-3.25	0.49	-0.46	0.18	-2.98	0.09
	Visit 2	-0.3	0.62	7.36	0.35	-0.58	0.06	-3.44	0.15
Anxiety	Visit 1	-0.68	0.09	-0.62	0.54	0.49	0.15	2.72	0.28
	Visit 2	-0.59	0.29	4.43	0.53	0.29	0.39	0.95	0.68
State	Visit 1	-0.24	0.59	1.15	0.38	0.18	0.62	0.37	0.95
	Visit 2	-0.14	0.83	4.45	0.68	0.04	0.92	2.71	0.69
Trait	Visit 1	-0.29	0.52	4.55	0.85	0.58	0.08	3.65	0.12
	Visit 2	-0.43	0.47	19.27	0.53	0.25	0.46	8.32	0.51

Table 6-8. Associations between natural logged *11βHSD2* and stress measures in a cohort of obese and lean pregnant women by Pearson correlation. Linear regression adjusted for maternal age, smoking status, parity, DEPCAT category, and length of gestation. Using Bonferroni correction, p values > 0.003 were considered significant.

		Lean				Obese			
GR		**Unadjusted**		**Adjusted**		**Unadjusted**		**Adjusted**	
Predictor variable		**r**	**p**	**b**	**p**	**r**	**p**	**b**	**p**
Stress work	Visit 1	0.44	0.12	2.46	<0.05	0.15	0.62	0.04	0.97
	Visit 2	0.41	0.16	0.29	0.052	0.05	0.86	0.02	0.89
Stress home	Visit 1	0.07	0.81	0.09	0.52	0.11	0.75	-0.03	0.66
	Visit 2	0.25	0.4	0.09	0.5	0.28	0.31	0.04	0.67
Stress money	Visit 1	0.44	0.11	0.11	0.07	0.38	0.24	0.15	0.46
	Visit 2	0.35	0.24	0.08	0.31	0.13	0.65	-0.04	0.70
Stress events	Visit 1	-0.26	0.37	0.18	0.18	0.07	0.83	-0.26	0.45
	Visit 2	-0.02	0.95	0.00001	0.99	0.15	0.61	-0.26	0.51
Satisfaction with life	Visit 1	-0.19	0.5	-0.82	0.41	0.17	0.62	1.39	0.07
	Visit 2	-0.11	0.73	-0.27	0.75	0.23	0.42	1.05	0.22
Anxiety	Visit 1	0.16	0.61	0.57	0.29	0.22	0.52	0.50	0.58
	Visit 2	0.24	0.43	0.48	0.48	-0.08	0.77	-0.15	0.82
State	Visit 1	0.19	0.51	1.02	0.43	-0.14	0.68	-0.61	0.85
	Visit 2	0.33	0.27	1.41	0.37	-0.07	0.79	-1.18	0.61
Trait	Visit 1	0.24	0.42	1.91	0.30	-0.03	0.94	0.01	0.99
	Visit 2	0.27	0.38	1.57	0.44	-0.003	0.99	-0.76	0.73

Table 6-9. Associations between natural logged *GR* and stress measures in a cohort of obese and lean pregnant women by Pearson correlation. Linear regression adjusted for maternal age, smoking status, parity, DEPCAT category, and length of gestation. Using Bonferroni correction, p values > 0.003 were considered significant.

6.3.2.5 *MR*

In the lean group, increased stress over money at visit 1 was found to associate with reduced *MR* transcript levels (see Table 6-10), although the association was weakened with multivariate analysis. No other associations were observed.

In the obese group, increased *MR* was found to correlate with a number of measures of increased maternal stress at visit 1 although neither association was significant after adjustments for other factors or after adjustment for the effects of multiple testing. At visit 2, measures of maternal stress and anxiety positively associated with *MR* in univariate analysis only.

		Lean				Obese			
MR		**Unadjusted**		**Adjusted**		**Unadjusted**		**Adjusted**	
Predictor variable		**r**	**p**	**b**	**p**	**r**	**p**	**b**	**p**
Stress work	Visit 1	-0.35	0.21	0.97	0.36	0.33	0.23	-1.05	0.33
	Visit 2	-0.35	0.24	0.06	0.81	0.35	0.2	0.31	0.42
Stress home	Visit 1	0.28	0.34	0.37	0.05	0.86	<0.005	0.38	0.11
	Visit 2	-0.23	0.45	0.11	0.28	0.68	<0.01	0.22	0.18
Stress money	Visit 1	-0.55	<0.05	-0.1	0.41	0.77	<0.01	0.21	0.44
	Visit 2	-0.37	0.22	-0.1	0.53	0.62	<0.05	0.27	0.06
Stress events	Visit 1	0.21	0.48	0.29	0.26	0.68	<0.05	0.27	0.2
	Visit 2	-0.18	0.55	0.04	0.34	0.43	0.11	0.27	0.06
Satisfaction with life	Visit 1	0.03	0.93	0.1	0.96	-0.62	0.06	-1.75	0.53
	Visit 2	0.1	0.74	0.48	0.74	-0.46	0.09	-1.79	0.13
Anxiety	Visit 1	-0.31	0.3	0	0.99	0.76	<0.05	0.99	0.13
	Visit 2	-0.47	0.1	-0.64	0.62	0.62	<0.05	0.9	0.24
State	Visit 1	-0.22	0.45	-0.03	0.99	0.02	0.96	0.73	0.46
	Visit 2	-0.35	0.24	-1.34	0.64	0.29	0.29	0.66	0.82
Trait	Visit 1	-0.19	0.52	-0.59	0.86	0.66	<0.05	1.99	0.67
	Visit 2	-0.34	0.26	-2.41	0.57	0.56	0.03	2.67	0.41

Table 6-10. Associations between natural logged *MR* and stress measures in a cohort of obese and lean pregnant women by Pearson correlation. Linear regression adjusted for maternal age, smoking status, parity, DEPCAT category, and length of gestation. Using Bonferroni correction, p values > 0.003 were considered significant.

6.4 Discussion

Maternal stress and anxiety have been associated with an increased risk of adverse postnatal outcomes in the child; these include autism, schizophrenia and behavioural problems (Van den Bergh and Marcoen 2004; Gutteling *et al.* 2005; Rodriguez and Bohlin 2005a; van den Bergh *et al.* 2006; Khashan *et al.* 2008a; Kinney *et al.* 2008; Van den Bergh *et al.* 2008). Over-exposure of the fetus to excess glucocorticoids through alterations in the maternal HPA axis, and particularly the placental barrier enzyme 11βHSD2, has been the main focus of research to date into the programming effects of maternal stress and anxiety.

6.4.1 *11βHSD1* is up-regulated with maternal anxiety

The results of these analyses suggest that increased levels of mRNA encoding the glucocorticoid-activator *11βHSD1* may associate with increased maternal anxiety during pregnancy in both cohorts, and also negatively correlated with satisfaction with life. However, these associations were limited to specific components of anxiety and stress in the first trimester, and were significantly weakened when the potential effects of multiple testing were controlled for. It will therefore be important for the results of this preliminary analysis to be validated in a larger cohort with a specific focus on anxiety in the first trimester.

NADP-dependent 11βHSD1 is predominantly localised to the maternal compartment of the placenta, whereas 11βHSD2 is predominantly localised to the fetal compartment (McMullen *et al*., 2004). 11βHSD1 acts to increase local levels of

glucocorticoids, in contrast to 11βHSD2. The increased levels of glucocorticoids on the maternal side of the placenta that result are thought to aid in the inhibition of inflammatory and immune responses to the fetal allograft and placental invasion (McMullen *et al*., 2004). In both rodents and humans, rising mRNA concentrations of *11βHSD1* in the placenta during the last week of pregnancy may augment the surge in glucocorticoids at that time (Burton *et al.* 1996; Murphy and Clifton 2003). This is particularly important in rodents where both *11βHSD2* mRNA levels and activity drop at the end of pregnancy. In humans, *11βHSD2* activity drops between weeks 38 and 40, producing a synergistic increase in placental cortisol concentration (Murphy and Clifton 2003). Thus, increasing *11βHSD1* gene expression may represent, in tandem with lowered *11βHSD2* enzyme activity, an important mechanism by which fetal and placental exposure to maternal cortisol may be regulated, particularly at term. Previous studies have suggested that placental 11βHSD1 and 11βHSD2 work together to control the concentration of maternal glucocorticoids reaching the fetus (Sun *et al.* 1997). Maternal anxiety may lead to programming effects through an up-regulation of *11βHSD1* and an alteration of the finely-controlled glucocorticoid concentration at the placenta (Sun *et al.* 1997). This in turn could potentially lead to increased fetal exposure to maternal glucocorticoids, and thus hasten fetal maturation, potentially leading to programming effects.

These data suggest that the effects of maternal stress on placental *11βHSD1* may be less pronounced in obese women than their lean counterparts. This may indicate that the placentas of obese women are somewhat protected against the effects of maternal stress and anxiety, or that the adverse effects of obesity may override any adverse

effects of anxiety. The HPA axis of obese women may be less sensitive to stress as a result of attenuation of the normal negative feedback of cortisol on the higher centres of the system. In baboons, increased estrogen production has been linked to a changed placental preference from cortisone reduction to cortisol oxidation in mid-gestation (Pepe *et al.* 1988). It is possible that increased adipose tissue estrogen production in obese women decreases the reactivity of *11βHSD1* to other forms of regulation. This may explain the ceiling effect seen within this group when compared to their lean counterparts. However, placental *11βHSD1* mRNA transcript levels did not differ between obese and lean women (as described in chapter 4).

Furthermore, placental mRNA levels of *11βHSD1* at term appear to be more sensitive to stresses in early and mid-pregnancy, with third trimester stressors ultimately having little apparent effect on transcript levels in the Helsinki cohort. This suggests that the timing of maternal stress and anxiety is critical. The finding may also imply that the chronic effects of maternal anxiety are important, and that stressors experienced earlier in pregnancy may produce effects on placental gene activity that persist until term, even if the stressor is no longer present.

Due to the large number of tests carried out, and the relatively low number of significant associations found, it will be important to validate the findings of this study. However, this study may prove a useful starting point for furture investigations into the relationship between term placental *11βHSD1* expression and anxiety during early pregnancy,

6.4.2 *11βHSD2* did not respond to maternal anxiety

11βHSD2, the cortisone-reductive counterpart of *11βHSD1*, and the main component of the placental barrier to maternal glucocorticoids, was not associated with maternal anxiety during pregnancy in either cohort, other than weak trends in the obese women. This stands in contrast to the findings of a study by O'Donnell *et al*, in which prenatal maternal anxiety was associated with a down-regulation of placental *11βHSD2* (O'Donnell *et al.* 2012). This anxiety-associated down-regulation has been suggested to be indicative of increased fetal exposure to maternal cortisol, potentially leading to programming effects.

This discrepancy may be due to the different approach to measuring maternal anxiety. In the study by O'Donnell *et al*, stress questionnaires were administered on the day before caesarean section, rather than throughout pregnancy. It seems plausible that the anxiety associated with the impending operation and delivery may have affected maternal responses in a manner absent from prospective testing. Pregnant women in the study by O'Donnell *et al* generally scored more highly on both state and trait anxiety scores (mean state anxiety of 43) when compared to participants in this study (mean state anxiety 39 in the obese and mean 30 in the lean) or the Helsinki study (mean state anxiety 33). It seems plausible that the impending operation may have influenced self-reported anxiety.

6.4.3 Glucocorticoid and Mineralocorticoid Receptors

Placental *GR* mRNA transcript levels appeared to be positively associated with some measures of maternal anxiety, particularly trait anxiety, in the Helsinki cohort only. This may indicate effects on placental growth and hormone production in response to chronic stress. This stands in apparent contrast to the findings of previous studies, which suggest that increased exposure to glucocorticoids results in a decrease in *GR* transcription in human placenta (Johnson *et al.* 2008b). Up-regulation of placental *GR* may increase placental susceptibility to maternal glucocorticoids.

MR appeared to be positively associated with a number of measures of maternal stress and anxiety, with the strongest associations being observed in obese women. 11βHSD2, high levels of which are found in the placenta, normally confers specificity on MR by conversion of active glucocorticoids to inactive forms, allowing the much lower concentrations of aldosterone to bind to the non-specific MR (Edwards *et al.* 1988; Funder *et al.* 1988). Aldosterone is important for placental growth and function, and deficiency of this substance has been linked to pre-eclampsia; aldosterone concentration has also been positively linked with placental size in humans (Brown *et al.* 1997; Gennari-Moser *et al.* 2011). The anxiety-induced up-regulation of *11βHSD1* may weaken this conferred specificity by increasing the concentration of active cortisol in the placenta. Thus, the up-regulation of *MR* transcription may be a compensatory response to the anxiety-induced increase in mRNA levels of *11βHSD1*; i.e. as baseline activation of *MR* increases, greater number of receptors are required in order to compensate for the altered glucocorticoid signal to noise ratio. However, while *11βHSD1* has been shown to be

expressed predominantly in the chorion and the intermediate trophoblasts, *MR* is thought to be expressed in the syncytiotrophoblast, cytotrophoblasts, and the villous core, suggesting that the proposed interaction may not occur (Hirasawa *et al.* 2000).

6.4.4 Limitations

This study was limited by the use of multiple testing when considering the associations between maternal anxiety and placental gene expression. While the indices used measure different components of stress and anxiety, it is important to acknowledge the potential effects of multiple testing. When performing a large number of analyses on a single dataset, the probability of errors of inference (i.e. erroneously rejecting the null hypothesis, or a type I error) increases. This is due to the fact that when performing a single test at the 5% level (i.e. accepting p values < 0.05 to be significant), one accepts that there is a 5% chance that an association will be seen by chance alone, and the null hypothesis will be incorrectly rejected. When performing multiple tests on the same dataset, the number of expected incorrect rejections increases. For example, when performing 100 tests, one will expect 5 incorrect rejections of the null hypothesis to occur. Techniques such as the Bonferroni correction can help to minimize this effect by allowing the statistician to revise the significance level, or alpha, of the tests performed. Nevertheless, it will be important for future investigations concerning anxiety during pregnancy to control for the effects of multiple testing. This may be accomplished by focusing on specific anxiety criteria, or on a specific timepoint during pregnancy. In the case of *11βHSD1* for example, it may be illuminating to concentrate on the effects of stress during early pregnancy.

6.4.5 Conclusions

Similar findings were observed in both the Helsinki and Edinburgh cohorts. By using two cohorts, it was hoped that the effects of multiple statistical testing would be mitigated, however when correcting for multiple testing many of the observed associations were found to be no longer significant. The observed replication of findings lends confidence to the conclusion that *11βHSD1* may play an important role in the regulation of placental glucocorticoid metabolism, however it will be important to validate these findings in a larger single cohort focusing on anxiety during early gestation. Whether changes in *11βHSD1* mRNA transcript levels lead to programming effects, presumably via alterations in fetal exposure to maternal cortisol, remains unclear. This enzyme, while often overlooked in studies, may prove to be an important component of the programming effects of maternal stress.

Interestingly, the admittedly limited effects of maternal stress and anxiety on placental mRNA transcript levels of *11βHSD1* and *MR* appeared to differ between lean and obese women. While placentas from obese women did not appear to have significant changes in *11βHSD1* transcription, strong associations were seen between maternal stress and *MR* transcript levels. This may indicate that the placentas of obese women are more protected against placental effects of maternal stress, specifically minimising the conversion of inactive cortisone to cortisol and through the increased availability of *MR* for activation by aldosterone. However, as *MR* and *11βHSD1* do not appear to be co-localised in placental cells, the nature of such an interaction, if it occurs at all, remains unclear.

Chapter 7:

Discussion

The work in this thesis aimed to test the hypothesis that the programming effects of maternal obesity and maternal stress are mediated by altered action of glucocorticoids on the developing fetus. This was carried out by examining the relationship between maternal obesity and a number of pregnancy parameters, including the mother's HPA axis activity and placental gene expression.

Obesity is an increasing problem, of global pandemic proportions (WHO 2013). Rates of obesity during pregnancy have increased dramatically throughout the world, with developed and developing countries alike affected (Prentice *et al.* 2005; Heslehurst *et al.* 2007a; Heslehurst *et al.* 2007b; Prentice 2009; Heslehurst *et al.* 2010). As maternal obesity increases the risk of childhood and adulthood obesity amongst the offspring of obese women, obesity may be to some extent self-perpetuating and cyclical.

With the increasing rates of obesity during pregnancy, and the programming effects of this state, it is becoming increasingly important to identify the mechanisms through which obesity affects pregnancy health, pregnancy outcome, and the long-term health of the offspring. It will also be important to establish potential biomarkers for future disease risk in this rapidly growing section of the population.

7.1 Obesity and the Maternal HPA Axis

It was shown that maternal obesity has significant effects on the maternal HPA axis. As glucocorticoids are involved in fetal-placental growth and maturation, and excess

fetal exposure to glucocorticoids has been implicated in a number of programming effects, this observation is of great importance.

Total circulating cortisol levels were found to be significantly lower throughout pregnancy in obese women when compared to their lean peers. Similarly, circulating levels of CBG and CRH, two important regulators of cortisol activity and production, were also found to be lower in obese pregnant women compared to lean counterparts. This suggests that maternal HPA axis activity may be reduced in obese pregnancy, potentially due to a reduction in cortisol production as a result of lower levels of placental CRH. As excess glucocorticoids are associated with reduced birth weight, this finding may help to explain the increased incidence of macrosomia amongst the offspring of obese women. A logical next step would be to more closely examine the effects of maternal obesity on placental CRH production. This may involve measurements of CRH gene expression, and investigation of factors influencing both expression and post-transcriptional regulation.

Significantly, CRH levels were found to predict length of gestation in obese pregnancy. This finding is in agreement with previous studies, and adds weight to the hypothesis that CRH acts as a 'placental clock' regulating the timing of parturition (Sandman *et al.* 2006). Obesity-related reductions in CRH may explain the longer gestations that are generally observed in obese pregnancy, a phenomenon that may also help explain the increased incidence of macrosomia among children of obese mothers (Challis *et al.* 2001; Sandman *et al.* 2006).

7.1.1 Free cortisol

While total cortisol was found to be lower in obese women compared to lean women during pregnancy, calculated levels of free cortisol were found to be higher in obese women by term, due largely to correspondingly lower levels of CBG. This may have important ramifications when considering the effects of maternal obesity on fetal glucocorticoid exposure. However, one must bear in mind that the method of calculating free cortisol represents an approximation, and does not necessarily reflect the actual circulating levels of unbound cortisol. Therefore, it will be an important goal of follow-up studies to confirm these findings through direct measurement of free cortisol.

7.1.2 Other Regulators of the HPA Axis

Future work may be also carried out to investigate the role of AVP as a regulator of HPA-axis function during obese pregnancy. As CRH levels increase throughout gestation, AVP is thought to increasingly regulate stress response and other HPA axis functions (Chowdrey *et al.* 1995). Furthermore, the roles of estradiol, estriol, fetal DHEA, and insulin as regulators of CBG production and activity may also be explored in follow-up studies. Measurements of these substances, particularly estrogen, were unavailable for the cohort studied, however it will be important for future studies to take them into consideration as potential mediators of the effects of obesity on the HPA axis during pregnancy, particularly when considering the effects of maternal obesity on the levels of free cortisol.

While this study attempted to investigate the effects of maternal obesity on diurnal rhythms of cortisol production, the small number of samples obtained limited interpretation of results. As changes in the diurnal production profile potentially influence overall glucocorticoid exposure, repeating this experiment with a larger cohort may be greatly beneficial.

Overall, this section of the study highlighted the significant effect of maternal obesity on HPA axis activity, as well as the potential importance of HPA axis activity as a potential biomarker of maternal obesity-related effects on pregnancy outcome, including length of gestation. With further work, a more complete understanding of this relationship may be established, including the mechanisms by which maternal obesity alters CRH and cortisol production, whether at the placenta or other sites.

7.2 Obesity and the Placenta

The placenta is intimately involved in the regulation of fetal growth and development thanks to its role as a regulator of nutrient transfer, hormone production and its function as a glucocorticoid barrier.

Of the many genes involved in placental function, genes involved in the regulation of fetal glucocorticoid exposure, and fetal and placental growth were studied. Of these genes, three were found to be affected by maternal obesity. The gene encoding the placental glucocorticoid barrier enzyme 11βHSD2 was more highly expressed in

obese pregnancy during the first trimester. This suggests that the offspring of obese pregnant women may be better protected from circulating maternal glucocorticoids compared to the offspring of lean women. Intriguingly, this pattern was also partially present at term, with placental *11βHSD2* expression found to be highest in the placentas of obese women who gave birth to macrosomic offspring. Given the negative relationship between excess glucocorticoid exposure and birth weight reported in other studies (Reinisch *et al.* 1978; French *et al.* 1999; Bloom *et al.* 2001), an increased placental barrier to cortisol may help contribute to increased fetal growth. In combination with the observed reduction in total cortisol levels in obese pregnancy, it seems possible that the offspring of obese women may be exposed to a significantly lower level of maternal glucocorticoids compared to those of lean women.

As with *11βHSD2*, *GR* expression was found to be higher in obese compared to lean pregnancy in the first trimester. This is suggestive of increased placental sensitivity to glucocorticoids. As glucocorticoids are important regulators of growth and development, this finding may predict altered in placental growth and maturation in obese pregnancy. Interestingly, both *GR* and *IGF2*, an important growth factor, were less highly expressed in placentas from macrosomic obese pregnancies, suggesting that by late pregnancy placental growth has slowed in these individuals. This may represent a shift in balance from placental to fetal growth, again helping to explain the observed macrosomia.

7.3 Maternal Stress and the Placenta

Contrary to the findings in maternal obesity, maternal stress and anxiety were found to be associated with increased expression of the gene *11βHSD1*, a gene encoding an enzyme principally involved in reactivation of glucocorticoids. This finding is novel and previously unreported, and suggests that in pregnancies complicated by stress and anxiety, the fine control by *11βHSD1* and *11βHSD2* over fetal glucocorticoid exposure may be disrupted (Sun *et al.* 1997). Importantly, *11βHSD2* did not appear to be affected by maternal stress, a finding that conflicts with previous studies (O'Donnell *et al.* 2012). This conflict may be due to the different nature of the stressors involved, and highlights the importance of effective study design, as well as the complex relationship between mood and placental gene expression. Taken together with the findings for maternal obesity however, it seems clear that the placental control over glucocorticoid exposure is particularly sensitive to maternal influences. As a result, it may be beneficial to further explore the use of *11βHSD1* and *11βHSD2* as biomarkers of potential programming effects in a maternal obesity or maternal stress context.

7.4 DNA Methylation was not Affected by Maternal Obesity

As the expression of a number of genes was associated with maternal obesity, consideration was given to how such changes may be regulated. One mechanism that

has come under scrutiny in a number of studies is DNA methylation. This epigenetic mark can be analysed relatively quickly and easily through the use of techniques such as pyrosequencing. Maternal obesity does not appear to significantly affect methylation of any of the genes studied. Other transcription regulating mechanisms may be involved, including micro-RNAs and histone modification; these may prove useful in future studies. Epigenetic marks are excellent candidates as biomarkers of future disease, representing as they do a likely mechanism by which gene expression is altered in maternal obesity. While DNA methylation does not appear to be affected by maternal overnutrition, other mechanisms may be more sensitive to such influences.

Problems were encountered in this study with DNA extraction from placental tissues, leading to a smaller than planned sample size. This represents a potentially significant limitation of the study, and further work using a larger cohort would be extremely beneficial.

7.5 Proposed Future Work

This study was limited in a number of key ways, particularly by sample size, particularly for the maternal CRH and placental DNA methylation arms. These limitations were due to a number of factors, including costs and difficulties with DNA and RNA extraction. Data was also unavailable for some of the variables that may have otherwise proven useful to study, including estrogen levels during pregnancy. Ideally, the first step in creating follow-up studies would be to repeat the key experiments with a larger number of women.

The study was also limited by the absence of a long-term follow-up arm. While significant insight has been gained into some of the effects of maternal stress and obesity on placental and HPA-axis function during gestation, the long-term programming effects remain to be fully explored. Ideally, the effects of stress and obesity on health at offspring middle-age could be studied, with particular focus on risk of obesity, cardiovascular disease, anxiety and metabolic disorders; in doing so, the usefulness of potential biomarkers including *11βHSD2* may be validated.

7.6 Conclusions

Maternal obesity appears to have significant effects on the glucocorticoid milieu during pregnancy. Generally, maternal obesity lowers circulating total cortisol and CRH levels, as well as being associated with increased expression of the glucocorticoid inactivating enzyme *11βHSD2*. This suggests an overall reduction of fetal glucocorticoid exposure. This may help to explain the increased length of gestation and birth weights observed in obese pregnancy, as well as the associated increased risk of programming effects such as adiposity and obesity.

Importantly, this study also highlighted the potential role of the enzyme *11βHSD1* in mediating the effects of maternal stress on fetal development. While *11βHSD2* has been widely studied, *11βHSD1* has received relatively little focus. The reported finding may contribute to further exploration of the role of this enzyme in the fine control of fetal-placental glucocorticoid exposure, and its disruption in response to maternal stress and anxiety.

Appendix I: Suppliers' Addresses

Agilent Technologies: 610 Wharfedale Road, Wokingham, RG41 5TP, UK

Anachem Ltd: 1 & 2 Titan Court, Laporte Way, Luton, LU4 8EF, UK

Applied Biosystems: 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, UK

Bio-Rad Laboratories: Maxted Road, Hemel Hemstead, HP2 7DX, UK

Biotage AB: Box 8, SE-751 03 Uppsala, Sweden

Biotium, Inc: Munro House, Trafalgar Way, Cambridge, CB23 8SQ, UK

Contained Air Solutions: Unit 4, Greengate, Middleton Junction, Manchester M24 1RU, UK

Dako: Cambridge House, St Thomas Place, Ely, Cambridgeshire, CB7 4EX, UK

DIASource Immunoassays SA: Rue du Bosquet, 2 1348 Louvain-La Neuve, Belgium

Eppendorf: Arlington Business Park, Stevenage, SG1 2FP, UK

Invitrogen: 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, UK

GE Healthcare: Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK

GraphPad Software, Inc: 2236 Avenida de la Playa, La Jolla, CA 92037, USA

G-Storm Ltd: Somerton Biotechnology Centre, Ricksey Lane, Catcombe, Somerton, Somerset, TA117JH

Li-Cor Biosciences: St. John's Innovation Centre, Cowley Road, Cambridge CB4 0WS, UK

Lonza: 228 Bath Road, Slogh, Berkshire, SL1 4DX, UK

Millipore: Croxley Green Business Park, Watford, WD18 8YH, UK

Minitab Ltd: Brandon Court, Unit E1-E2, Progress Way, Coventry, CV3 2TE, UK

Nanodrop Technologies: 3411 Silverside Rd, Wilmington, DE 19810, USA

New England BioLabs: 75/77 Knowl Piece, Wilbury Way, Hitchin, SG4 0TY, UK

Promega: Southampton Science Park, Southampton, SO16 7NS, UK

Qiagen: Fleming Way, Crawley, West Sussex, RH10 9NQ, UK

Roche Applied Science: Charles Avenue, Burgess Hill, RH15 9RY, UK

Sarstedt: 8 Boston Road, Beaumont, LE4 1AW, UK

Sigma Aldrich: The Old Brickyard, New Road, Gillingham, Dorset SP8 4XT, UK

Swann Morton: Owlerton Green, Sheffield S6 2BJ, UK

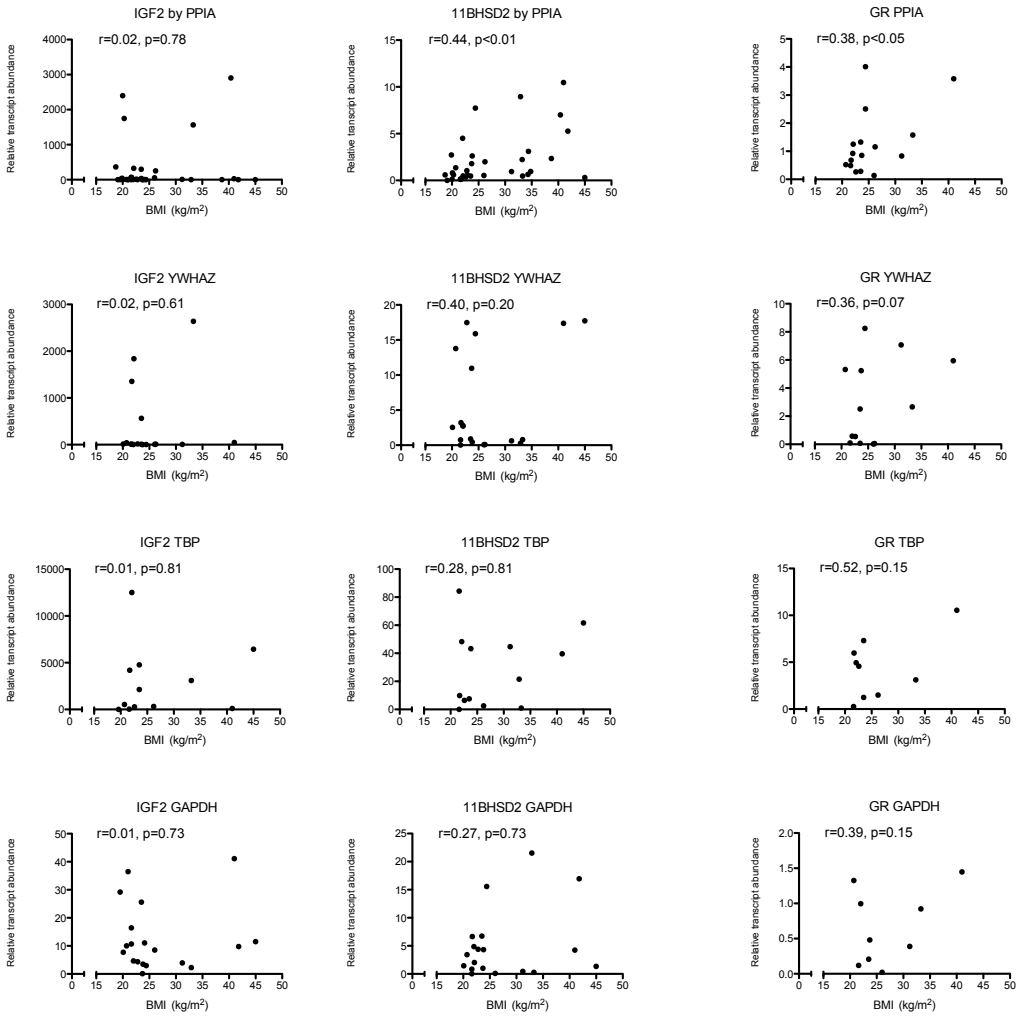
Thermo Fisher: Blenheim Road, Epsom, Surrey, KT19 9AP, UK

UVITec Ltd: Unit 36, St John's Innovation Centre, Cowley Road, Cambridge C54 0WS, UK

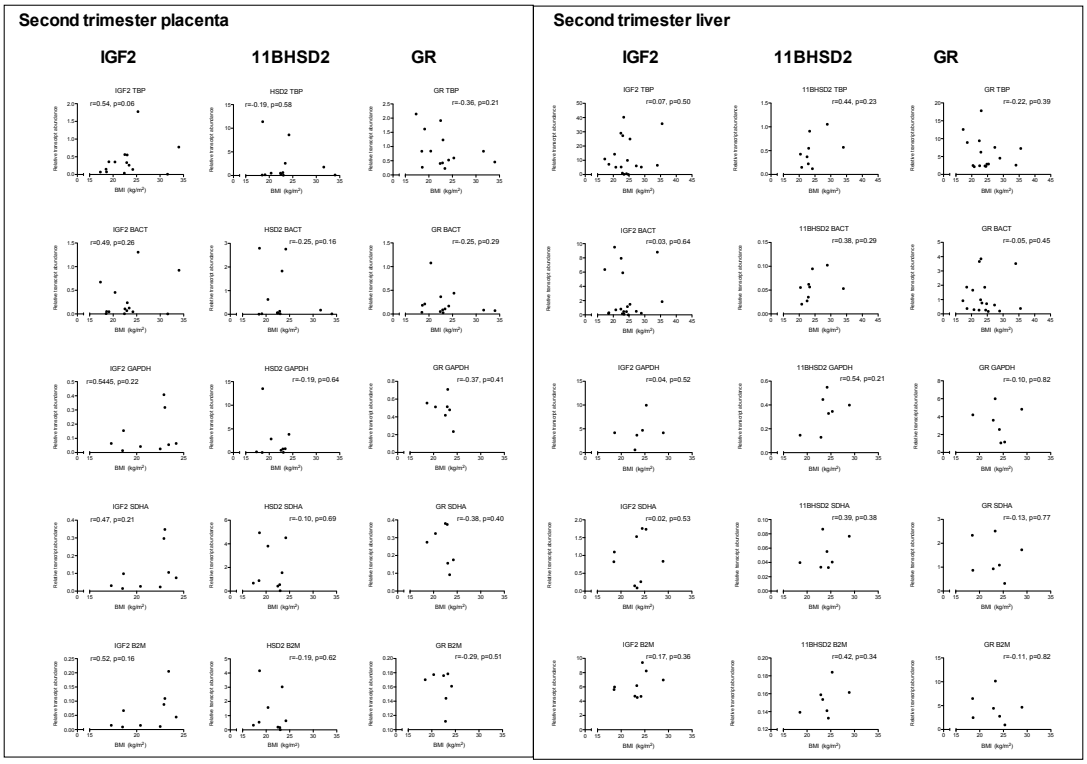
Zeiss: 509 Coldhams Lane, Cambridge, CB1 3JS, UK

Appendix II: Placental expression of key genes using various housekeeping genes

First trimester placenta

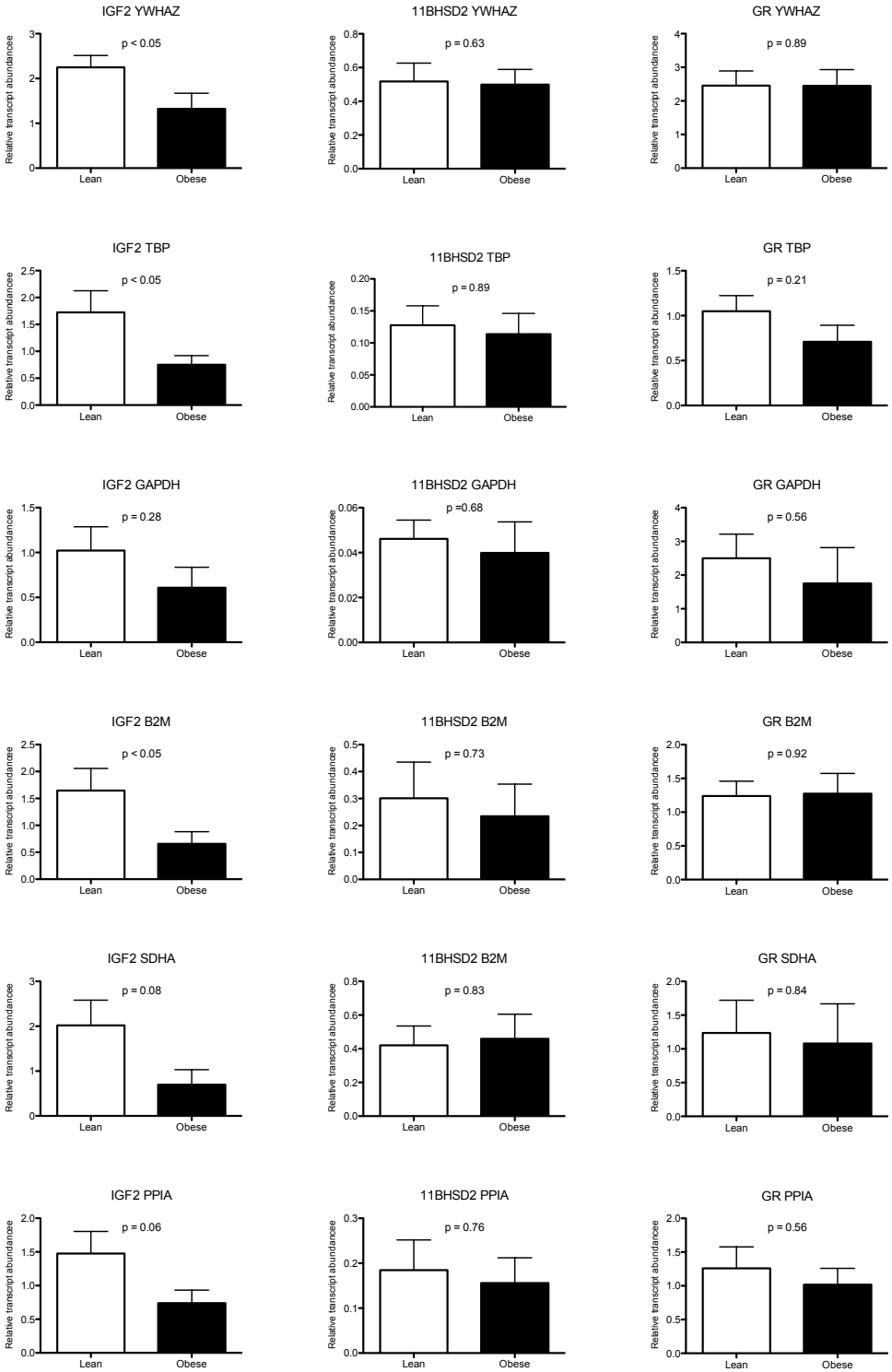


First trimester placental expression of key genes using various housekeeping genes



Second trimester placental and liver expression of key genes using various housekeeping genes

Term placenta



Expression of key genes in term placental tissue using various housekeeping genes

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