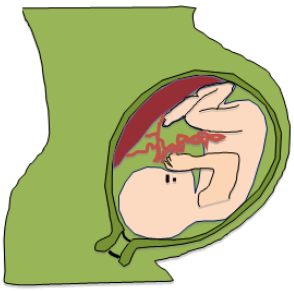

Study of Imprinted Gene Expression in the Placenta

Investigating a role for imprinted genes in pregnancy complications and the possible influence of maternal lifestyle

Anna Bugge Janssen

Submitted for the degree of PhD

2015

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ABSTRACT

Imprinted genes, displaying monoallelic parent of origin specific expression, are known to regulate fetal growth and placental development. Work in animal models also suggests that imprinted genes regulate placental hormone signalling to the mother, which is required for induction of maternal adaptation to pregnancy. Aberrant placental imprinted gene expression may therefore have a causative role in pregnancies characterised by abnormal fetal growth and/or inadequate maternal adaptation to pregnancy. Given these important functions, identifying environmental stimuli responsible for perturbed imprinted gene expression is also of interest.

This thesis examined human placental expression of the imprinted genes *PHLDA2*, *CDKN1C*, *PEG3* and *PEG10* in three independent cohorts, including pregnancies complicated by fetal growth restriction, fetal overgrowth, preeclampsia, gestational diabetes and maternal mood disorders. Placental imprinted gene expression was also analysed in relation to placental hormone (*hPL* and *PGH*) gene expression and in relation to maternal lifestyle factors. The effect of maternal diet on placental imprinted gene expression was further explored in a mouse model to provide evidence for a cause or effect relationship.

Placental *PHLDA2* expression was significantly increased in growth-restricted pregnancies, supporting a role for *PHLDA2* in the negative regulation of fetal growth. In contrast, placental *PEG10* expression was positively associated with fetal growth. This study did not support a role for *PEG3* in the control of fetal growth, but did suggest a role in maternal adaptation to pregnancy with aberrant gene expression observed in pregnancies complicated by maternal depression. Finally, this study provided evidence that *PHLDA2*, *CDKN1C*, *PEG3* and *PEG10* expression is responsive to environmental stimuli, particularly maternal diet, in both human pregnancies and in a mouse model. Thus, this thesis highlights the importance of imprinted genes in achieving a successful pregnancy for both mother and fetus and the possible role of maternal lifestyle in influencing this.

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List of Abbreviations

AC	Abdominal circumference
AGA	Average for gestational age
ART	Assisted reproductive technology
BAC	Bacterial artificial chromosome
Bp	Base pairs
BMI	Body mass index
BW	Birth weight
BWS	Beckwith Wiedemann Syndrome
cDNA	Complimentary DNA
C-section	Caesarean Section
CVS	Chorionic Villus Sampling
DNA	Deoxyribonucleic acid
EMCS	Emergency Caesarean Section
EPDS	Edinburgh Postnatal Depression Scale
EVT	Extravillous cytotrophoblast
FFQ	Food frequency questionnaire
FGR	Fetal growth restriction
FL	Femur length
GDM	Gestational diabetes mellitus
gDNA	Genomic DNA
GH	Growth Hormone
GLUT	Glucose transporter
GSIS	Glucose stimulated insulin secretion
GTT	Glucose tolerance test
HB	Health board
HC	Head circumference
HELLP	Haemolysis, Elevated Liver enzymes and Low Platelets
HFHS	High fat – high sugar diet
hPL	Human placental lactogen
IC2	Imprinting control region 2
IUGR	Intrauterine growth restriction
KO	Knockout

LBW	Low birth weight
LGA	Large for gestational age
LOI	Loss of imprinting
NICE	National Institute for Health and Care Excellence
NICU	Neonatal intensive care unit
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PE	Preeclampsia
PGH	Placental growth hormone
PH	Pleckstrin homology domain
PIH	Pregnancy induced hypertension
qPCR	Quantitative PCR
RFLP	Restriction Fragment Length Polymorphism
RGH	Royal Gwent Hospital
RNA	Ribonucleic acid
RT	Reverse transcription
SGA	Small for gestational age
SNP	Single nucleotide polymorphism
STAI	Spielberger trait anxiety inventory
SSAI	Speilberger state anxiety inventory
SVD	Spontaneous vertex delivery (Vaginal delivery)
Tm	Melting Temperature
UHW	University Hospital Wales
UPD	Uniparental disomy
WHO	World Health Organisation

CHAPTER 1: INTRODUCTION

1.1. Fetal growth

1.1.1. Normal fetal growth

In a healthy pregnancy, a carefully orchestrated series of cell divisions and cell differentiation steps comprise the embryonic period during which the major internal and external structures of the embryo are formed. The human fetal period of development subsequently begins at the end of the first trimester, continuing to delivery and is characterised by fetal and placental growth as well as maturation of the fetal organs. Fetal growth velocity typically peaks at 30 – 36 weeks of gestation, decreasing thereafter till birth in contrast to placental growth, which is relatively consistent from 28 weeks, as shown in Figure 1.1(A) (Johnson 2007).

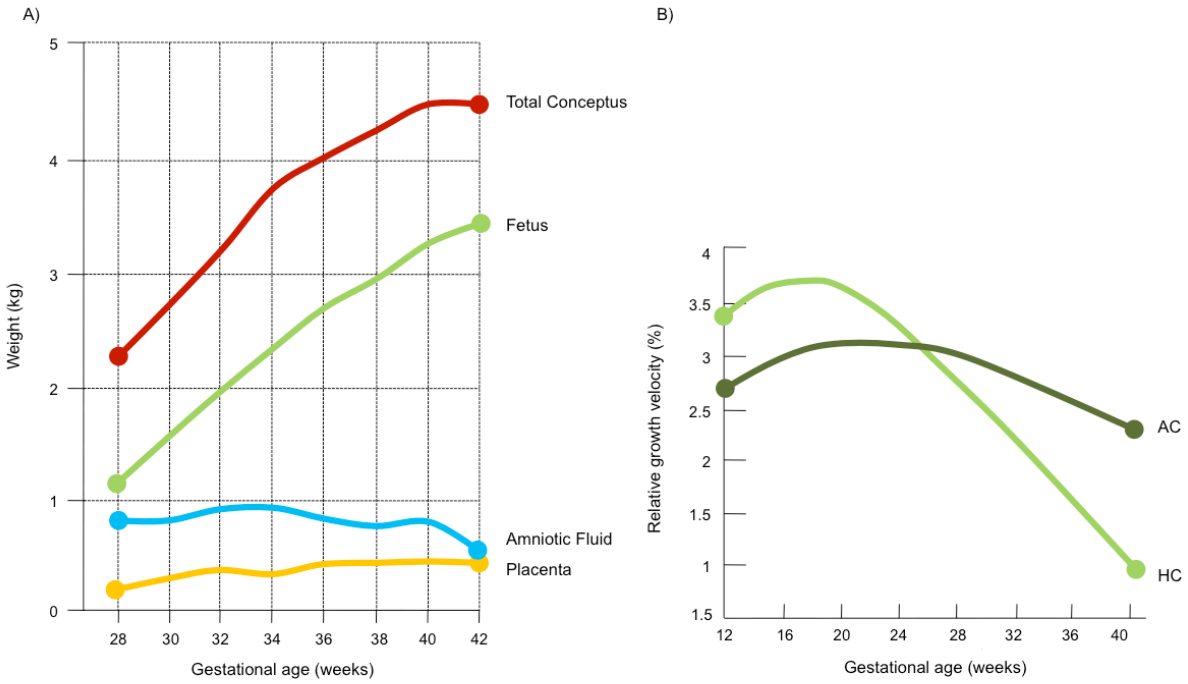


Figure 1.1: Fetal growth in a normal, healthy pregnancy. (A) Weight changes during pregnancy are shown for the total conceptus, fetus, amniotic fluid and placenta. (B) Growth velocity (represented as % of the estimated size at 40 weeks). is shown for head circumference (HC) and abdominal circumference (AC). Adapted from (Johnson 2007) and (Bertino *et al.* 1996).

Head circumference growth velocity peaks earlier in pregnancy (around 17 weeks) compared with abdominal circumference growth velocity (around 22 weeks) likely reflecting the increased deposition of subcutaneous fat towards term (Bertino *et al.* 1996), Figure 1.1(B). The fetal genome and the maternal-placental environment in which the fetus develops (including nutrient and oxygen supply as well as hormonal signalling) determine growth.

1.1.2. Fetal growth restriction

Fetal growth restriction (FGR) or intrauterine growth restriction (IUGR) is the failure of a fetus to achieve its optimal growth potential during gestation (Ergaz *et al.* 2005). Causes of growth restriction (ranging from fetal, placental, maternal and/or environmental) are difficult to measure and therefore growth restriction hard to diagnose (Ergaz *et al.* 2005). Thus, the outcomes of being born low birth weight (LBW) or small for gestational age (SGA) are commonly used to indicate growth restriction (Ergaz *et al.* 2005).

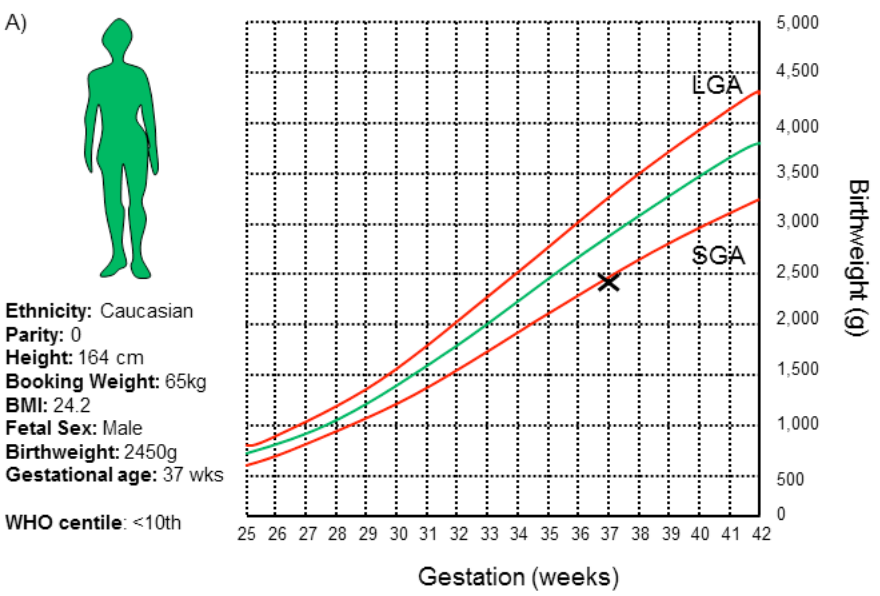
The World Health Organisation defines LBW as birth weight < 2,500g at any gestational age (United Nations Children's Fund and World Health Organization 2004). One issue with the use of LBW as an indicator of growth restriction is that this definition does not distinguish between infants born small due to prematurity (< 37 weeks gestation) and those born small due to growth restriction. Another issue is that birth weight is a continuum with there being little clinical difference between an infant born weighing 2,500g (classified as normal birth weight) and an infant born weighing 2,499g (classified as LBW) (Adams *et al.* 2009). Benefits of using LBW to indicate growth restriction include wide availability of birth weight data, the possibility of comparing international research data and the ability to easily identify a population of high-risk infants (Adams *et al.* 2009).

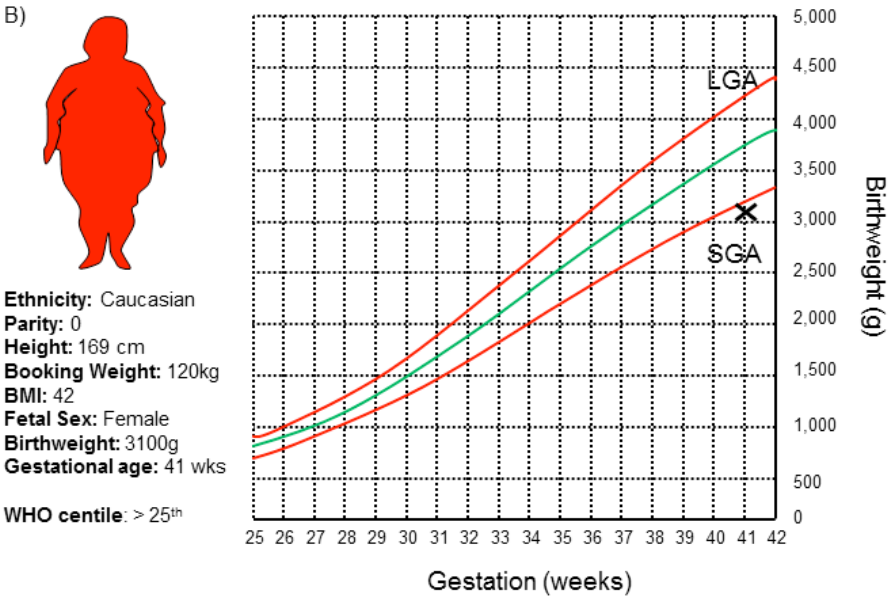
A small for gestational age (SGA) infant is commonly defined as an infant whose birth weight is below the 10th centile relative to a population birth weight standard at a given week of gestation (Zhang *et al.* 2010). In contrast to use of the LBW cut off, this definition takes in to account

gestational age at delivery and can therefore identify those infants born small due to growth restriction rather than prematurity. One concern with the use of the SGA definition to identify fetal growth restriction is that population based centiles do not taken in to account maternal or fetal factors which impact on an individual fetus' growth potential (Zhang *et al.* 2010). Therefore, using population based centiles it is not possible to distinguish between infants born constitutionally small (for example because their mother is short and thin) and those born pathologically small due to *in utero* growth restriction.

Using computer-based software, customised birth weight centiles can be generated that are adjusted for maternal height, weight, ethnicity, parity, fetal sex and gestational age, factors known to affect fetal growth (Gardosi *et al.* 1992). Based on this information, it is possible to predict the optimal weight at term for an individual fetus and therefore also identify cases of fetal growth restriction where the fetus has not achieved its optimal growth potential (Gardosi *et al.* 1992), Figure 1.2 (A).

Use of custom birth weight centiles has been found to identify growth restricted infants not otherwise recognised as SGA by population centiles (de Jong *et al.* 1998; Clausson *et al.* 2001; Gardosi 2009). These include, for example, normal birth weight infants born to obese mothers that while average for gestational age (AGA) by population centiles, are growth restricted relative to the customised optimal term birth weight for that individual fetus, Figure 1.2 (B). In addition, use of custom birth weight centiles has been found to reduce the incidence of false SGA diagnosis (Gardosi 2009). By adjusting for maternal height and weight, custom birth weight centiles are able to distinguish between pathologically small infants and constitutionally small infants such as those born to short, thin mothers (which would otherwise be diagnosed as SGA by population centiles), Figure 1.2 (C). Importantly, use of custom birth weight centiles better predicts adverse outcomes such as stillbirth, fetal distress, delivery complications, low apgar scores and neonatal intensive care unit (NICU) admission (de Jong *et al.* 1998; Clausson *et al.* 2001; Gardosi 2009; Figueras and Gardosi 2011).





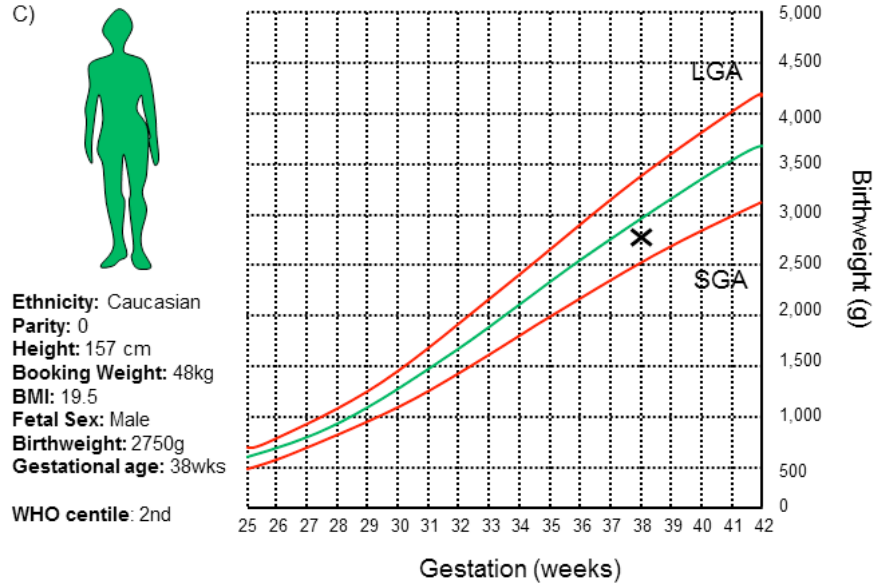


Figure 1.2: The use of custom growth centiles in identifying SGA pregnancies. (A) For a normal BMI woman, an infant born at term weighing ≤ 2.5kg would be considered SGA by custom birth weight centiles and population (WHO) centiles. However, (B) a normal birth weight baby (e.g. 3.1kg) born at term to an obese mother may also be considered SGA by custom birth weight centiles. (C) In contrast, a baby born weighing 2.75kg to a short, thin mother, would be considered AGA for this mother. Custom centiles were generated using GROW version 6.7.5 (Gardosi and Francis 2014). WHO centiles (population centiles) are shown for comparison. Adapted from (Perinatal Institute 2014). Red growth curves indicate the cut off for SGA (< 10th centile) or LGA (> 90th centile). Green growth curves indicate 50th centile.

Growth restricted infants can be further classified as symmetrically or asymmetrically growth restricted, typically based on ultrasound head (HC) and abdominal (AC) circumferences (reviewed in (Monk and Moore 2004; Ergaz *et al*. 2005; Mullis and Tonella 2008; Rosenberg 2008). Symmetrically growth restricted infants (approximately 25% of SGA cases) display proportionally smaller head and abdominal circumferences, which is thought to result from growth restriction in early gestation caused for example by genetic anomalies or viral infection (Rosenberg 2008). In contrast, asymmetrically growth restricted infants (approximately 75% of SGA cases) display reduced length, weight and abdominal circumference but relative sparing of the head resulting in an increased HC:AC ratio (Rosenberg 2008). Asymmetric growth restriction is thought to result from growth restriction occurring in late gestation due to nutrient deficiency and/or placental insufficiency with head sparing resulting from a subsequent preferential allocation of nutrients to the developing fetal brain (Rosenberg 2008). Asymmetrically growth restricted infants are at an increased risk of poor perinatal outcomes (such as low apgar scores, fetal distress and NICU admission) compared with AGA and symmetrically growth restricted infants (Dashe *et al.* 2000).

1.1.3. Prevalence of growth restriction

Prevalence of LBW was 7.1% in England (46,624 births) and Wales (2,378 births) in 2013, affecting a combined total of 49,000 births (Office for National Statistics 2014). LBW prevalence has not changed significantly in Wales between 1993 and 2012 despite health care improvements (Statistics for Wales 2014). Within Wales, Abertawe Bro Morgannwg (7.7%), Cwm Taf (9%) and Aneurin Bevan (7.4%) health boards have higher rates of LBW than the Welsh average (7.1%)(Statistics for Wales 2014), Figure 1.3 (A).

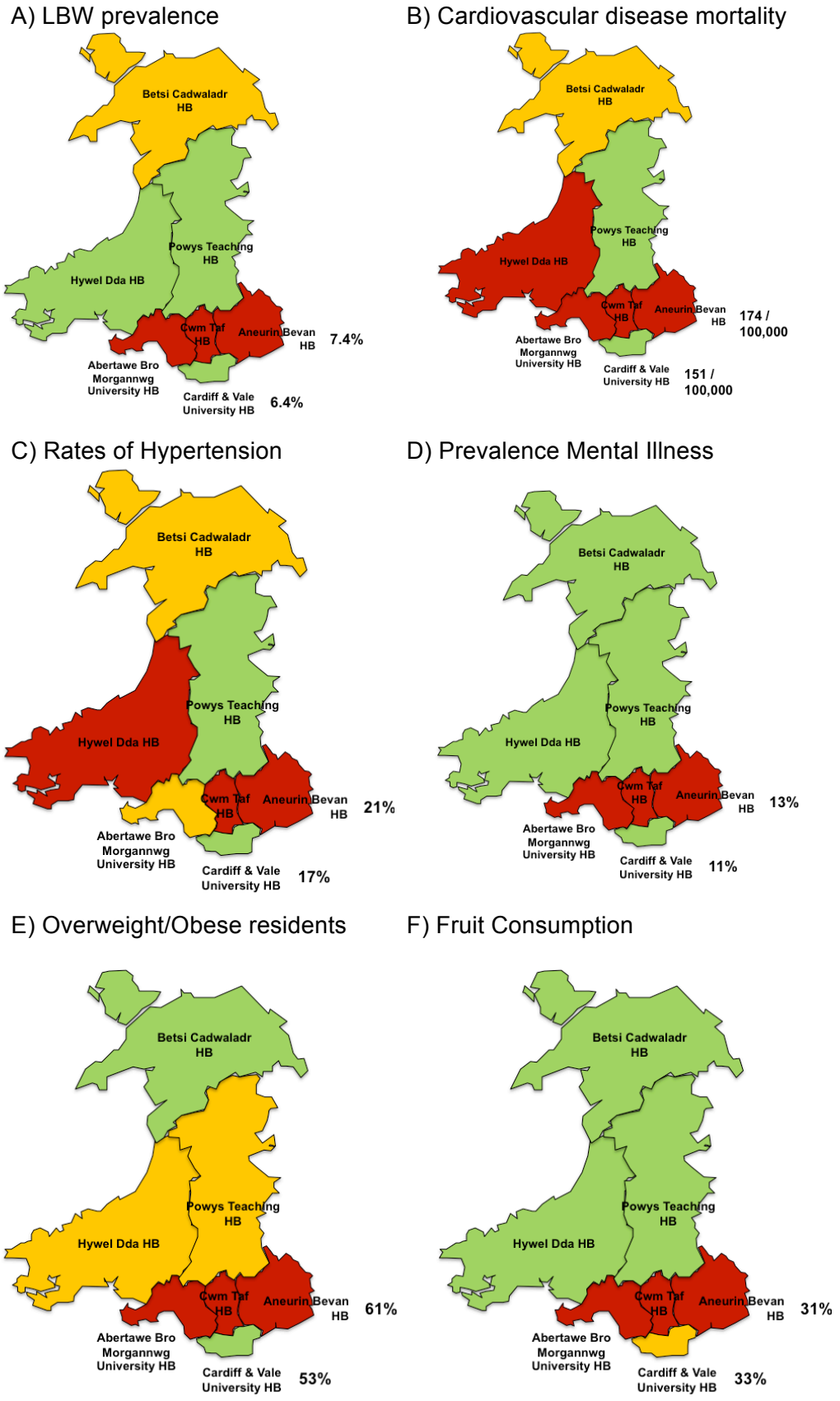


Figure 1.3: Co-occurrence of low birth weight (LBW), adult diseases and poor lifestyle factors in Wales. Colour coding indicates Welsh health boards with prevalence of each factor below (green), average (amber) or above (red) the Welsh Average (with the reverse shown for fruit consumption). Generated based on data from (Statistics for Wales 2013) and (Statistics for Wales 2014). HB = health board.

1.1.4. Consequences of growth restriction

During the perinatal period, growth restricted infants are at an increased risk of morbidity including respiratory complications, hypothermia, hypoglycemia (Rosenberg 2008) and reduced measures of infant wellbeing (such as Apgar Scores and umbilical artery pH) (McIntire *et al.* 1999).

Importantly, these infants are also at an increased risk of perinatal mortality (Dunham and McAlenney 1936; Chase 1969; Saugstad 1981; Wilcox and Russell 1983; McIntire *et al*. 1999; Matthews and MacDorman 2010). This is highlighted in that 2010 UK mortality rates of LBW infants (36.8/1,000 live births) are higher compared with normal BW infants (1.4/1,000 live births) (Office for National Statistics 2011). In particular, there is a strong link between fetal growth restriction and stillbirth with approximately 66% of stillbirths in 2013 occurring in LBW infants (Office for National Statistics 2014). This association has been found to be independent of gestational age, with a 6 fold higher risk of stillbirth at term in infants with fetal growth restriction (M. Kady and Gardosi 2004). Therefore, further understanding of growth restriction is essential in the prevention of infant perinatal morbidity and mortality.

The fetal programming hypothesis proposes that an insult during development, such as fetal growth restriction, can have lasting effects on the individual, increasing their risk of certain diseases in adulthood (Barker 1998). Fetal growth restriction has been associated with decreased fertility (Ibanez *et al.* 2002) and an increased risk of hypertension, cardiovascular disease, stroke, type II diabetes and some types of cancer in adulthood (reviewed in (Godfrey and Barker 2001)). For example, LBW infants are almost seven times more likely to develop type II diabetes in adulthood than normal BW infants (Godfrey and Barker 2001). The fetal programming hypothesis was originally developed based on the observation that geographical differences in coronary heart disease mirrored those of newborn death rates typically associated with LBW (reviewed in (Barker 2007)). Indeed, in Wales, death due to cardiovascular disease and rates of hypertension are highest in health boards previously identified as having higher rates of LBW deliveries, as shown in Figure 1.3 (B) and (C).

Growth restriction may also impact on offspring neurodevelopment, behaviour and mental health in later life (reviewed in (Schlotz and Phillips 2009)). For example, LBW infants are at an increased risk of developing symptoms of ADHD, emotional problems and behavioural problems in childhood and are seven times more likely to suffer from schizophrenia in adulthood (Schlotz and Phillips 2009). Indeed, the percentage of patients being treated for mental illness is greatest in the Welsh health boards also having higher rates of LBW (Statistics for Wales 2013), Figure 1.3 (D).

Recent studies also suggest that being pregnant with an SGA baby can significantly increase the risk of maternal cardiovascular disease and metabolic syndrome in later life (Catov *et al.* 2011; Bukowski *et al.* 2012; Pariente *et al.* 2013; Ngo *et al.* 2015). Importantly, the association remains significant after controlling for confounders such as family history and associated cardiovascular disease risk factors (Bukowski *et al*. 2012) suggesting that the relationship may not be due to shared genetic and/or environmental sources alone. Maternal adaptation to pregnancy includes changes to the cardiovascular system which increase oxygenated blood flow to the fetus (Tan and Tan 2013); it has been suggested that perturbed adaptation of the maternal cardiovascular system to pregnancy, involving maternal, placental and fetal hormones, could underlie the association between fetal growth restriction and an elevated maternal risk of cardiovascular disease in later life (Ngo *et al.* 2015).

Thus, fetal growth restriction is of clinical and public health relevance due to the negative impacts on offspring health in the perinatal period and in adulthood, as well as long-term effects on maternal health.

1.1.5. Causes of growth restriction

Fetal growth restriction is associated with preterm delivery with the two pregnancy complications sharing several risk factors and consequences (Goldenberg *et al.* 2008). Studying only term infants therefore enables

examination of the specific causes and effects of growth restriction *in utero* independently from those of being born prematurely.

Fetal growth restriction may be driven by fetal, placental, maternal or environmental factors (Sankaran and Kyle 2009). While paternal factors are also known to affect fetal growth, the maternal contribution to FGR risk is estimated to be greater (Magnus *et al.* 2001; Jaquet *et al.* 2005; Mattsson and Rylander 2013). Of note is the inconsistency in definitions of growth restriction used in previous studies investigating causes of FGR, with the risk of delivering a LBW infant typically examined. Fetal causes of growth restriction include genetic and chromosomal abnormalities (e.g. single gene mutations, chromosomal trisomy, triploidy and uniparental disomy), congenital abnormalities, intrauterine infection (most frequently toxoplasmosis and cytomegalovirus) and pregnancies with multiples (Hendrix and Berghella 2008).

Placental causes of growth restriction include placental previa (placental growth over the cervix), tumors in the placenta, placental infarcts (associated with decreased placental blood flow) and cord insertion abnormalities (Hendrix and Berghella 2008). These abnormalities result in placental insufficiency when the placenta is unable to provide the fetus with the optimum nutrients and oxygen needed to support growth (Hendrix and Berghella 2008). Placental insufficiency is recognised as a major cause of FGR (Brodsky and Christou 2004).

Maternal causes of growth restriction are related to maternal demographics, disease, pregnancy complications and/or obstetric history (reviewed in (Valero de Bernabé *et al.* 2004; McCowan and Horgan 2009)). In terms of maternal demographics, advanced maternal age and teenage pregnancy are both associated with an increased risk of LBW (Jacobsson *et al.* 2004; Chen *et al.* 2007). Risk of LBW has also been demonstrated to vary by ethnicity (Valero de Bernabé *et al.* 2004) with the prevalence of LBW births in non-Caucasian women in the UK increased by 62% compared with Caucasian women (Collingwood Bakeo and Clarke 2006). In addition, socio-economic disadvantage in the UK (indicated by e.g. living in a deprived area,

council housing residence and maternal unemployment) significantly increases the risk of LBW (Collingwood Bakeo and Clarke 2006).

Short stature, low pre-pregnancy maternal BMI and poor weight gain during pregnancy are also associated with a significantly increased risk of LBW (Valero de Bernabé *et al.* 2004) . Although maternal obesity is generally thought to be protective against growth restriction, the use of custom birth weight centiles reveals a significantly higher number of SGA infants and increased perinatal mortality rates in obese women compared with normal BMI controls (Gardosi 2009). Although no pregnancy specific data is available, it is estimated that 58% of people in Wales are overweight and 23% obese with rates highest in the Aneurin Bevan and Cwm Taf health boards (Statistics for Wales 2013), Figure 1.3 (E).

Thus, there are a number of maternal demographic factors that affect fetal growth, which must be controlled for in any study looking at environmental causes of fetal growth restriction.

1.2. Environmental causes of FGR

Given that there are currently limited treatment options for FGR, environmental causes of growth restriction are of particular interest as these are most amenable to intervention (Sankaran and Kyle 2009).

1.2.1. Maternal smoking

In Wales, an estimated 33% of women in 2010 smoked before pregnancy and 16% throughout pregnancy (Health and Social Care Information Centre 2012). This was higher than the 12% of mothers smoking during pregnancy in England (Health and Social Care Information Centre 2012). Studies by Pollack *et al.* (2000), Figueras *et al.* (2008) and Rasmussen and Irgens (2006) reported an approximate two fold increase in the risk of LBW in smokers compared with non-smokers. The Pollack *et al.* (2000) study further estimated that 11% of LBW pregnancies in their study population were a

result of maternal smoking thereby highlighting smoking as a significant modifiable risk factor for FGR (Figueras *et al.* 2008).

The mechanisms underlying the association between maternal smoking and fetal growth restriction are not well defined. It has been proposed that growth restriction occurs as a result of carbon monoxide and nicotine restriction of placental oxygen supply (Pollack *et al.* 2000), placental transport of smoking-related toxic compounds to the fetus and/or direct effects of these compounds on placental development and function (Zdravkovic *et al.* 2005). Indeed, the expression of over 200 genes in the placentas of smokers is altered compared to non-smokers (Bruchova *et al.* 2010). However, more research is required to fully determine the role of placental gene expression in mediating the association between smoking and FGR.

1.2.2. Alcohol and illegal drug use

In Wales, 87% of women drank alcohol before pregnancy and 39% during pregnancy in 2010, similar to rates in the rest of the UK (Health and Social Care Information Centre 2012). Of the women drinking alcohol during pregnancy, the majority reported drinking less compared with pre-pregnancy levels, with less than 1 unit consumed per week (Health and Social Care Information Centre 2012).

A review of 28 studies investigating the effect of maternal alcohol consumption before or during pregnancy found a small but non-significant increase in the risk of LBW (Patra *et al.* 2011). The review reported a dose-dependent increase in LBW risk with increasing alcohol consumption, although the risk of LBW only begins to increase above 1-1.5 drinks/day (Patra *et al.* 2011). Thus, although alcohol consumption in high quantities may negatively impact on birth weight, it is unclear whether light to moderate drinking has any effect (Henderson *et al.* 2007; Patra *et al.* 2011).

In terms of illegal drug use, a UK study of women using drugs during pregnancy found an almost 4 fold increase in the risk of LBW and IUGR

compared with controls (Pinto *et al.* 2010). The increased risk of LBW and IUGR was similar among cocaine, heroin and methadone users and those using multiple drugs (Pinto *et al.* 2010). Thus, illegal drug-use significantly increases the risk of growth restriction.

1.2.3. Maternal diet

Several individual components of maternal diet have been demonstrated to negatively or positively impact on birth weight as shown in Table 1. Although no pregnancy specific data is available, it is estimated that only 33% of people in Wales consume the government recommended amount of fruit and vegetables (≥ 5/day)(Statistics for Wales 2013), Figure 1.3 (F). This may have an impact on LBW prevalence given the positive association reported between fruit and vegetable consumption and birth weight (Table 1). In the case of some diet components, e.g. carbohydrate and protein consumption, conflicting results have been reported (Table 1) which may be due to differences in study methodology or population. Micronutrients in the form of maternal folate and iron supplementation are also positively associated with birth weight (Godfrey *et al.* 1996; Mitchell *et al.* 2004).

Birth outcomes associated with maternal diet are related to the timing of exposure (Godfrey *et al.* 1996; Moore *et al.* 2004; Thompson *et al.* 2010). For example, Thompson *et al.* (2010) demonstrated a significant positive effect of a traditional diet (characterised by high intake of fruit, vegetables and lean meat) on SGA risk in early but not late pregnancy. Pre-pregnancy maternal diet may also be important with, for example, maternal pre-conception vegetable consumption positively associated with birth weight (Weisman *et al.* 2011).

Several recent studies of maternal diet have examined the effects of diet patterns on pregnancy outcomes (Crozier *et al.* 2008; Knudsen *et al.* 2008; Thompson *et al*. 2010). Examining diet patterns, in contrast to individual diet components, may provide more information on the interacting

effects of food items and has been suggested to be of greater relevance for designing healthy eating interventions (Crozier *et al.* 2008).

Table 1: Components of maternal diet during pregnancy and their relationship to birth weight. (-) indicates a diet component negatively associated with birth weight and (+) a diet component positively associated with birth weight.

	Maternal Diet Component	Reference
Positively associated with birth weight (+)	Fruit consumption	(Mikkelsen *et al.* 2006) (Rao *et al.* 2001) (Thompson *et al.* 2010) (Mathews *et al.* 1999)
	Vegetable consumption	(Mikkelsen *et al.* 2006) (Rao *et al.* 2001) (Thompson *et al.* 2010)
	Dairy consumption	(Ford 2011) (Xue *et al.* 2008) (Olsen *et al.* 2007) (Ludvigsson and Ludvigsson 2004)
	Fish consumption	(Makrides *et al.* 2006) (Ricci *et al.* 2010) (Rogers *et al.* 2004)
Negatively associated with birth weight (-)	Fat consumption	(Godfrey *et al.* 1996)
	Caffeine consumption	(Santos *et al.* 1998) (Xue *et al*. 2008) (Vik *et al.* 2003)
Conflicting reports	Carbohydrate consumption	(-) (Godfrey *et al.* 1996) (-) (Moore *et al.* 2004) (+) (Mitchell *et al.* 2004) (+) (Thompson *et al.* 2010)
	Protein consumption	(+) (Godfrey *et al.* 1996) (+) (Ford 2011) (+) (Thompson *et al.* 2010) (+) (Moore *et al*. 2004) (-) (Knudsen *et al*. 2008) (-) (Campbell *et al.* 1996) (-) (Ricci *et al.* 2010)

Finally, it is important to note that maternal diet is associated with other lifestyle factors such as smoking and maternal BMI, as well as ethnicity and socioeconimic status (Robinson *et al.* 2000; Northstone *et al.* 2008; Thompson *et al.* 2010) and therefore these potential confounders should be considered in the analysis of maternal diet effects on birth outcomes. Overall,

more research is required into the effect of maternal pre-conception and pregnancy diet on birth weight in the UK, as well as the possible mechanisms mediating these effects.

1.2.4. Maternal exercise

Although no pregnancy specific data is available, it is estimated that 29% of people in Wales are physically active ≥ 5 days/week (Statistics for Wales 2013). Juhl *et al.* (2010) found an inverse association between the frequency of maternal exercise during pregnancy and birth weight, as well as other indicators of fetal growth including abdominal and head circumference. Similarly, Clapp (2003) and Bell *et al.* (1995) reported decreased birth weight of infants whose mothers carried out strenuous exercise during pregnancy. Hopkins *et al.* (2010) even found an effect of modest-intensity exercise on birth weight. One of the mechanisms proposed for the association between exercise during pregnancy and FGR is an exercise associated decrease in placental blood supply and placental weight (Clapp 2003; Juhl *et al.* 2010). However more research is needed into the direct effects of exercise on placental development and function, such as changes in gene expression, and how this may mediate the effect of exercise on fetal growth.

1.3. Role of the placenta

Located at the boundary between the maternal and fetal environment, the placenta controls fetal growth through nutrient and oxygen delivery to the fetus and production and metabolism of growth-related hormones (reviewed in (Fowden *et al.* 2009)). Therefore, alterations in placental structure and function are likely to play a key role in fetal growth restriction.

1.3.1. Placental development and structure

The human blastocyst (the pre-implantation conceptus) is comprised of a layer of trophoblast cells surrounding the blastocoelic cavity and the inner

cell mass. While the inner cell mass will form the embryo proper, the layer of trophoblast cells represents the first extra-embryonic lineage, which facilitates diffusional transport of oxygen and nutrients from maternal uterine secretions to the developing embryo. This histiotrophic nutrition continues throughout the first trimester (Johnson 2007).

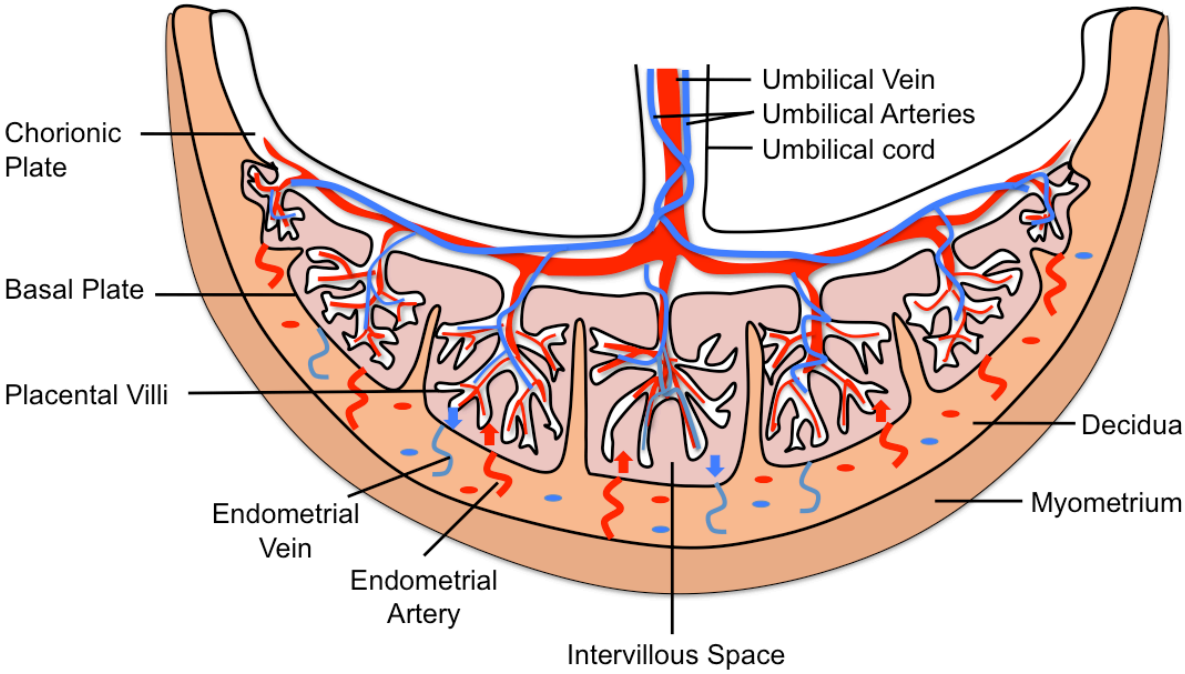


Figure 1.4: Structure of the human term placenta. The major structures of the placenta are the chorionic plate (fetal surface), intervillous space and basal plate (maternal surface). Direction of deoxygenated blood is indicated by blue arrows and oxygenated blood flow by red arrows. Adapted from (Sood *et al.* 2006).

Implantation of the blastocyst in the maternal uterine wall, around 6 -7 days after conception, signifies the beginning of placental development. During implantation, the first trophoblast cells to attach to the uterine epithelium fuse to form an invasive syncytiotrophoblast layer. The remaining trophoblast cells retain their cells walls and form a cytotrophoblast layer. Cytotrophoblast cells undergo constant proliferation and fusion with the syncytiotrophoblast layer, enabling the syncytiotrophoblast layer to expand and further invade the uterine wall (Huppertz 2008).

Within the syncytiotrophoblast layer, fluid filled spaces (lacunae) form surrounded by syncytiotrophoblast (trabeculae). Expansion of the syncytiotrophoblast layer into the endometrium results in erosion of maternal blood vessels such that maternal blood fills the lacunae (although maternal blood flow to the placenta is only established after 10-12 weeks). At this stage, the basic structure of the placenta is apparent, including (1) the chorionic plate (the fetal surface), (2) the intervillous space (syncytiotrophoblast trabeculae separated by lacunae) and (3) the basal plate (the maternal surface) (Huppertz 2008), Figure 1.4.

During placental development, a subset of cytotrophoblast cells migrate through the syncytiotrophoblast layer and into the maternal endometrium. These cytotrophoblast cells differentiate to become extravillous cytotrophoblasts (EVTs) that invade the maternal uterine spiral arteries inducing their remodelling (Huppertz 2008). Maternal spiral artery remodelling results in increased blood flow and reduced resistance in these vessels, thereby maximising placental perfusion (Gude *et al.* 2004). The remodelled spiral arteries deliver oxygenated blood to the intervillous space where it bathes the placental villi (Gude *et al*. 2004), Figure 1.4.

Progressive differentiation and branching of the trabeculae forms the placental villous tree with a large surface area for exchange. Deoxygenated fetal blood is carried via the umbilical arteries and the extensive capillary network within the placental villous tree to the terminal villi (Figure 1.4). The terminal villi extend into the intervillous space and are the main site of exchange between the maternal and fetal circulations, with only a thin layer of syncytiotrophoblast separating the two (Huppertz 2008). The resulting oxygenated blood is transported to the fetus by chorionic veins and the umbilical vein (Gude *et al*. 2004), Figure 1.4.

1.3.2. Placental functions

The placenta supports fetal growth and development through three main functions: (1) transport and metabolism, (2) production and metabolism of hormones and (3) acting as a barrier for fetal protection (Gude *et al.* 2004).

As described, the trophoblast facilitates histiotrophic nutrition of the embryo before maternal blood flow to the placenta is established at 10 - 12 weeks. Once maternal blood flow is established, transfer of oxygen and nutrients to the fetus and removal of carbon dioxide and waste products can occur readily across the syncytiotrophoblast layer of the terminal villi. For example glucose, which acts as a major source of energy for the developing fetus, is transferred from the maternal circulation via glucose transporters (GLUTs) on the syncytiotrophoblast membrane. This transfer of glucose to the fetus is vital as fetal glucose production is limited. In addition to facilitating transport, the placenta itself utilises maternal oxygen and nutrients with the resulting products of metabolism released into the maternal and fetal circulations (Gude *et al.* 2004).

The placenta acts as an endocrine organ during pregnancy, synthesising hormones (mainly in the syncytiotrophoblast), which are released into the maternal and fetal circulations, to bring about a variety of functions. Progesterone produced by the placenta signals the presence of a conceptus (thereby preventing menstruation) and suppresses contractions of the uterus. The placenta is also a source of oestrogen that acts on the mother's breasts, uterus, cervix and vagina inducing adaptations of the maternal reproductive organs to pregnancy (Gude *et al.* 2004). In addition, placental production of human placental lactogen (hPL) and placental growth hormone (PGH) is important in the regulation of fetal growth and maternal adaptation to pregnancy, as is further described in section 1.8.

Finally, the placenta acts to reduce exposure of the fetus to xenobiotics, maternal bacteria and/or viruses. However, it is important to note that this barrier is not complete with substances such as caffeine and alcohol as well as some maternal viruses such as Rubella, still able to cross the placenta (Gude *et al.* 2004).

1.3.3. Placental abnormalities in FGR

Examining the placenta is a practical method for assessing fetal growth as the placenta serves as a record of *in utero* events and samples for research can be obtained non-invasively (Ryan *et al.* 2012). Indeed, it has been proposed that assessment of placental phenotype provides a better indicator of a perturbed *in utero* environment than birth weight, particularly in cases of fetal growth restriction resulting in delivery of a normal birth weight infant (Sibley *et al.* 2005; Jansson and Powell 2007).

There are a number of placental abnormalities associated with FGR, as reviewed by Sibley *et al.* (2005). For example a decreased syncytiotrophoblast surface area for exchange, coupled with an increased exchange barrier has been reported in IUGR placentas, which could affect transfer of nutrients and oxygen to the developing fetus. Increased apoptosis within IUGR placentas has also been noted and is likely to impact on placental size and structure. Furthermore, inadequate uterine spiral artery remodelling resulting in increased vessel resistance and a reduction in uteroplacental blood flow has been demonstrated in IUGR pregnancies. Finally, changes in placental gene expression (for example of nutrient transporters) have been reported highlighting the possibility of identifying a placental biomarker for *in utero* growth restriction (Sibley *et al.* 2005).

1.3.4. The placenta as an environmental sensor

The growth regulating functions of the placenta are susceptible to environmental factors (Fowden *et al.* 2011), providing a possible mechanism by which environmental signals (such as those discussed in section 1.2) could result in fetal growth restriction and program an increased risk of disease in adult life (see Figure 1.5).

An adverse maternal environment may result in changes to the placenta structure and/or function. For example, maternal dietary manipulation (such as calorie restriction or a low protein diet) in an animal model results in a decrease in placental growth preceding any adverse effect

on fetal growth (Jansson and Powell 2007). It has also been proposed that the placenta acts as a nutrient sensor, responding to changes in the maternal environment by altering placental transport (Jansson and Powell 2007). For example, activity of placental amino acid transporters is impaired in IUGR placentas, which has been suggested to slow fetal growth in parallel with changes in maternal nutrient supply (Jansson and Powell 2007).

Imprinted genes expressed in the placenta have been demonstrated to be susceptible to environmental factors (reviewed in (Fowden *et al*. 2011)). As some of these genes regulate fetal growth (see section 1.4), their aberrant expression may play a role in mediating the effects of an adverse environment on fetal growth (Figure 1.5).

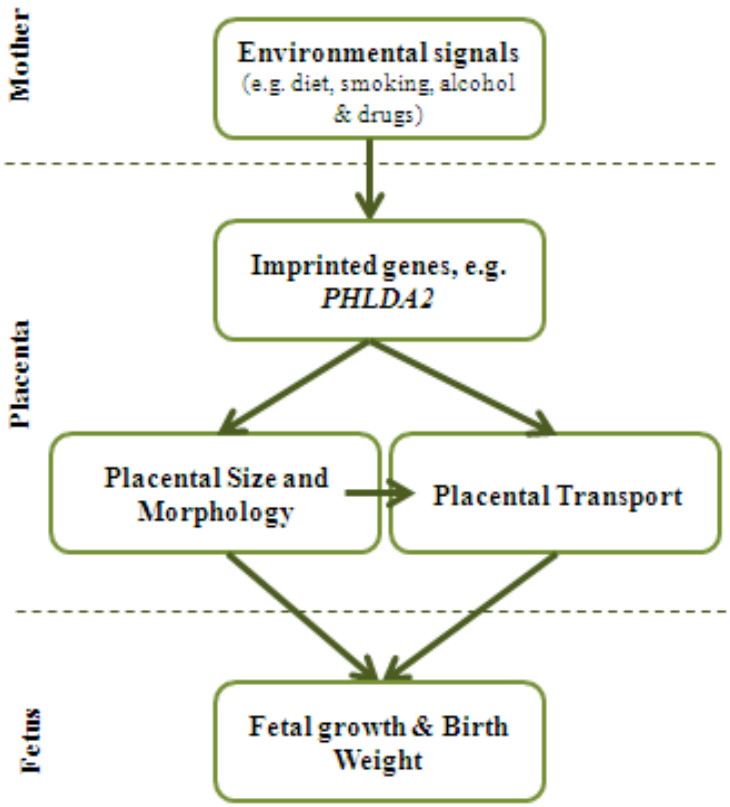


Figure 1.5: Role of imprinted genes and the placenta in mediating the effect of the maternal environment on fetal growth. Adapted from (Fowden *et al*. 2011)

1.4. Imprinted gene control of fetal growth

Imprinting is an epigenetic process, involving DNA methylation and histone modification, resulting in monoallelic expression of a gene, with the active allele depending on the parent of origin (Surani 1998).

Discovery of genomic imprinting arose from studies in which gynogenetic (containing two female pronuclei) and androgenetic (containing two male pronuclei) mouse embryos were created. Although these embryos contained the normal numerical complement of DNA, gynogenetic embryos were found to be unviable, exhibiting growth restriction and inadequate placental development while androgenetic embryos failed to develop properly with excessive placental growth (Kaufman *et al.* 1977; Barton *et al.* 1984; McGrath and Solter 1984; Surani *et al.* 1986).

The growth abnormalities observed in mice with uniparental disomies (UPD) for particular chromosomes, i.e. in which both copies of a chromosome or part of a chromosome are inherited from one parent (reviewed in (Tycko and Morison 2002)) further support a role for imprinted genes in the control of fetal growth. In mice, maternal disomy for proximal chromosome 11 results in growth restriction whereas paternal disomy is associated with over growth (Cattanach and Kirk 1985). It was therefore suggested that genes which are maternally-inherited function differently from those which are paternally-inherited due to epigenetic changes occurring during gametogenesis i.e. imprinting (Tycko and Morison 2002). Thus, normal development is dependent upon both parental genomes and not simply inheritance of a full genetic complement.

A role for imprinted genes in controlling human fetal growth is highlighted by the abnormal growth phenotypes seen in some human disorders resulting from imprinting defects (Lim and Ferguson-Smith 2010). For example, 50% of Beckwith-Wiedemann Syndrome cases, characterised by pre- and postnatal overgrowth, are thought to involve UPD of paternal chromosome 11p15 (Enklaar *et al.* 2006). In contrast, Silver Russell Syndrome, which is characterised by pre-, and postnatal growth restriction, may result from UPD of maternal chromosome 11p15 in up to 63% of

patients (Eggermann *et al.* 2010). Finally, a high frequency of inappropriate imprinted gene expression has been reported in the human placenta of IUGR pregnancies at 17% (of 52 genes) (Diplas *et al.* 2009) and 22% (of 27 genes) (McMinn *et al.* 2006).

There are numerous hypotheses that have been proposed to explain the evolution of genomic imprinting. One theory is that of conflict between the maternal and paternal genome for allocation of maternal resources to the fetus (Moore and Haig 1991). This theory proposes that the paternal genome silences genes that restrict fetal growth in order to maximise allocation of maternal resources to an offspring that shares the paternal genes. In contrast the maternal genome acts to silence genes that promote fetal growth in order to maintain current health and future reproductive fitness and to ensure equal division of resources amongst offspring (Hitchins and Moore 2002). While no hypothesis proposed to explain the evolution of imprinting fully accounts for the numerous functions of the genes known to be subject to this epigenetic process, it is clear that many of these genes have dosage sensitive functions in both the regulation of fetal growth and the development of the placenta. Thus, alterations in their expression may have a causative role in FGR.

1.5. *PHLDA2*

Evidence supporting a role for *PHLDA2* in human growth restricted pregnancies has recently been reviewed by Jensen *et al.* (2014)

PHLDA2 (alternative names: *Ipl*, *Tssc3*, and *Bwr1c*) is a maternally expressed imprinted gene, which is located on human chromosome 11p15.5 and mouse distal chromosome 7. The *PHLDA2* gene is relatively small gene, containing 2 exons and 1 intron (Lee and Feinberg 1998; Frank *et al.* 1999). Imprinting of the *PHLDA2* gene is controlled by the centromeric imprinting control region 2 (IC2 or KvDMR1), which spans the promoter of the long, non-coding RNA *KCNQ1OT1* (Smilinich *et al.* 1999; Fitzpatrick *et al.* 2002; Thakur *et al.* 2004; Mancini-Dinardo *et al.* 2006).

PHLDA2 encodes the pleckstrin homology-like domain family A member 2 protein, the biochemical function of which has not yet been determined. The 144 amino acid long protein (Frank *et al.* 1999) is comprised of a highly conserved pleckstrin-homology (PH) domain, flanked by short N and C-terminal extensions (Saxena *et al.* 2002). PHLDA2 is able to bind to phosphatidlyinositol lipids with moderate affinity via its PH domain (Saxena *et al.* 2002). This suggests that PHLDA2 may be active in intracellular trafficking, cell signalling and membrane-cytoskeletal interactions, characteristic functions of proteins with PH domains (Saxena *et al.* 2002). PHLDA2 has also been suggested to play a role in apoptosis or growth restriction based on homology and co-expression of PHLDA2 to TDAG51, involved in Fas-mediated apoptosis (Hu *et al.* 1997; Qian *et al.* 1997) and the induction of protein kinase B mediated apoptosis by the closely related *Phlda3* gene (Kawase *et al.* 2009). However, these suggested functions of PHLDA2 have yet to be determined experimentally.

1.5.1. Animal models investigating *Phlda2* and fetal growth

There is high sequence conservation between the mouse *Phlda2* and human *PHLDA2* genes (Qian *et al.* 1997). Maternal monoallelic gene expression has been demonstrated in both the mouse and human placenta (Qian *et al.* 1997) demonstrating conservation of the direction of imprinting. In the mouse, *Phlda2* expression has been demonstrated in the fetal liver, lung, limb and kidney in addition to the adult kidneys and prostate (Qian *et al.* 1997; Frank *et al.* 1999). Highest *Phlda2* expression is seen in the extraembryonic tissues including the placenta and yolk sac (Qian *et al.* 1997; Frank *et al.* 1999). Placental mRNA and protein is localised to the labyrinth zone of the mouse placenta, which is the main site of nutrient exchange (Frank *et al.* 1999; Frank *et al.* 2002). A gradual decrease in placental *Phlda2* expression is observed towards the end of gestation (Frank *et al*. 1999; Frank *et al*. 2002).

As reviewed by Jensen *et al.* (2014), a number of studies in mice support a role for *Phlda2* in the control of placental development. Frank *et al.* (2002) examined fetal and placental growth in *Phlda2* knockout (KO) mice.

These mice exhibited increased placental weights, attributed to a specific increase in the junctional zone of the placenta (Frank *et al.* 2002). Importantly, the increased placental growth observed was shown to be independent of any effects on expression of other imprinted genes within the same imprinting cluster, such as *Igf2* (Frank *et al.* 2002). Loss of function of *Phlda2* was not associated with increased fetal weight at birth, as predicted, but instead transient fetal growth restriction was observed from mid to late gestation (Frank *et al*. 2002; Salas *et al.* 2004).

Effects of *Phlda2* over expression on fetal growth have been examined by adding extra copies of the *Phlda2* gene via a bacterial artificial chromosome (BAC) transgene (John *et al.* 2001; Andrews *et al.* 2007; Tunster *et al.* 2010). Two fold *Phlda2* expression, modelling loss of imprinting, resulted in significantly decreased placental weight (Tunster *et al.* 2010). This could be rescued by combining the transgene with a maternally inherited targeted deletion of *Phlda2*, which normalised *Phlda2* expression levels (Tunster *et al.* 2010). *Phlda2* over expression resulted in a specific 60% reduction in the junctional zone of the placenta (Tunster *et al.* 2010). Within the junctional zone, expression of a spongiotrophoblast marker was reduced suggesting a decrease in these cells (Tunster *et al.* 2010). In addition, there was a decrease in glycogen storage within the placenta and migration of glycogen cells into the maternal decidua impaired (Tunster *et al.* 2010). *Phlda2* transgenic embryos carrying three copies of the transgene were 13% lighter at birth indicating a gradual decrease in fetal growth velocity during gestation (Tunster *et al.* 2010). A more recent study, focused on a single copy line, demonstrated that a double dose of *Phlda2* was sufficient to induce FGR and importantly, that growth restriction was asymmetric with evidence of brain sparing and was followed by rapid postnatal catch-up growth (Tunster *et al.* 2014). These characteristics are typical of human pregnancies complicated by placental insufficiency.

1.5.2. Human studies of *PHLDA2* and fetal growth

PHLDA2 is highly expressed in the human placenta (Qian *et al.* 1997; Piedrahita 2011) with expression highest in the first trimester, decreasing thereafter till term (Saxena *et al.* 2003; Kumar *et al.* 2012; Sitras *et al.* 2012). In particular, cytotrophoblast cells of the placental villi are sites of localised high *PHLDA2* expression (Saxena *et al.* 2002; McMinn *et al.* 2006). Relatively lower levels of *PHLDA2* expression have also been demonstrated in the fetal brain, liver, lung and kidney, as well as the adult liver, lung, kidney, pancreas, prostate and colon (Qian *et al.* 1997; Lee and Feinberg 1998; Piedrahita 2011).

Monoallelic expression of *PHLDA2* from the maternal allele has been demonstrated in the human placenta (Qian *et al.* 1997; Frank *et al.* 1999) and the kidney, intestine, limb, liver and lung of the human fetus (Lee and Feinberg 1998). Another line of evidence demonstrating *PHLDA2* as a maternally expressed imprinted gene is the absence of *PHLDA2* expression in human complete hydatidiform moles, which contain only paternal chromosomes (Saxena *et al*. 2003; Thaker *et al.* 2004).

Studies investigating the role of aberrant *PHLDA2* expression in human growth restricted pregnancies have been reviewed by Jensen *et al*. (2014), as summarised in Table 2. Thus far, three studies have reported increased placental *PHLDA2* expression in IUGR compared with control pregnancies (McMinn *et al.* 2006; Diplas *et al.* 2009; Kumar *et al*. 2012). Diplas *et al.* (2009) observed three fold increased *PHLDA2* expression, with McMinn *et al.* (2006) estimating that 25% of the placentas examined demonstrated aberrant placental *PHLDA2* expression.

Placental *PHLDA2* expression has also been demonstrated to be significantly inversely associated with birth weight in a cohort of term, normal birth weight infants (Apostolidou *et al.* 2007). Examination of a *PHLDA2* single nucleotide polymorphism (SNP) in the placenta and parental DNA demonstrated that *PHLDA2* was expressed from the maternal allele only and therefore that loss of imprinting (LOI) did not underlie the observed association between gene expression and birth weight (Apostolidou *et al.*

2007). Similarly, Guo *et al.* (2008) reported monoallelic maternal expression of *PHLDA2* in all SGA placentas examined, although gene expression was not analysed with respect to birth weight. A significant inverse association between *PHLDA2* expression and birth weight, in the absence of LOI, has also been demonstrated in umbilical cord tissue (Lim *et al.* 2012).

Table 2: Previous studies analysing the association between human fetal growth and placental *PHLDA2* expression. IUGR = Intrauterine Growth Restriction, SGA = Small for Gestational Age, AGA = Appropriate for Gestational Age, LGA = Large for Gestational Age, BMI = Body Mass Index, CVS = Chorionic Villus Samples. (Jensen *et al.* 2014).

Study	Cohort	Factors controlled for	Results
(McMinn *et al.* 2006)	38 IUGR 75 Controls	Placental weight, Gestational age	Increased *PHLDA2* expression in IUGR placentas
(Apostolidou *et al.* 2007)	200 term placentas	Gender, Parity, Gestational age, Maternal Weight	Significant inverse association between *PHLDA2* expression and birth weight
(Diplas *et al.* 2009)	7 IUGR 10 Controls	None described	Increased *PHLDA2* expression in IUGR placentas
(Kumar *et al.* 2012)	10 IUGR 10 Controls	Maternal age, Gender, Pregnancy induced hypertension, Ethnicity	Increased *PHLDA2* expression in IUGR placentas
(Lambertini *et al.* 2012)	33 SGA 51 AGA 22 LGA	Maternal age, Ethnicity, Insurance, Gestational age, Mode of Delivery, Gender	No significant association between *PHLDA2* expression and birth weight
(Lewis *et al.* 2012)	102 term placentas	Gender	Significant inverse association between *PHLDA2* expression and fetal skeletal growth
(Demetriou *et al.* 2014)	260 first trimester CVS	Gestational age, Gender, Parity, Maternal age, Smoking, Maternal BMI	No significant association between *PHLDA2* expression and birth weight

A study by Ishida *et al.* (2012) demonstrated that maternal inheritance of a variant of a promoter sequence upstream of the *PHLDA2* transcription start site (RS1) was associated with significantly increased birth weight. The RS1 variant was demonstrated *in vitro* to decrease *PHLDA2* promoter activity, which was suggested to be due to a reduced number of transcription factor binding sites (Ishida *et al.* 2012). Thus, the RS1 variant (conferring reduced *PHLDA2* promoter activity) was positively associated with birth weight, further supporting a role for *PHLDA2* in the negative regulation of fetal growth.

Three studies have reported no significant association between placental *PHLDA2* expression and birth weight (Lambertini *et al.* 2012; Lewis *et al.* 2011; Demetriou *et al.* 2014). These conflicting results may be due to differences in study methodology or population. For example, Lambertini *et al.* (2012) analysed *PHLDA2* expression in relation to birth weight within a cohort including AGA, SGA and LGA infants, in contrast to previous studies comparing IUGR and control placentas only. In addition, the study by Demetriou *et al.* (2014) examined *PHLDA2* expression in chorionic villus samples from the first trimester. The authors proposed that the absence of an association between first trimester *PHLDA2* expression and birth weight demonstrates that *PHLDA2* regulates fetal growth in late pregnancy only (Demetriou *et al.* 2014). Although placental *PHLDA2* expression was not significantly associated with birth weight, Lewis *et al.* (2012) did demonstrate an inverse association between growth velocity of the fetal femur between 19 and 34 weeks gestation and placental *PHLDA2* expression. There was also an association between increased *PHLDA2* expression and decreased bone mineral content, bone area and bone mineral density at 4 years (Lewis *et al.* 2012).

Thus, a number of studies have demonstrated increased human placental *PHLDA2* in FGR pregnancies, with work in animal models supporting a causal role for *PHLDA2* in the negative control of fetal growth (Jensen *et al.* 2014). However, as shown in Table 2, the number of growth restricted placentas examined in these studies has been relatively small prompting further investigation in large cohort studies. In addition, participant

demographics have not been clearly described and few confounders known to be associated with growth restriction (section 1.2) have been controlled for, Table 2.

Importantly, previous studies did not distinguish between infants born SGA due to growth restriction from those born constitutionally small. Comparing placental *PHLDA2* expression between these two groups may indicate whether placental gene expression can specifically identify infants that have been growth restricted *in utero* with the aim of optimising postnatal care (Jensen *et al.* 2014). Similarly, although animal models suggest a role for *Phlda2* in asymmetric growth restriction (Tunster *et al.* 2014) previous human studies have not examined placental *PHLDA2* expression in symmetric compared with asymmetric growth restricted pregnancies.

No study to date has examined placental *PHLDA2* expression in relation to birth outcomes. Increased expression of placental *PHLDA2* has been reported in cases of spontaneous miscarriage or fetal death (Doria *et al.* 2010) and more specifically in cases of fetal death attributed to fetal growth restriction (Cordeiro *et al.* 2014). However, little is known about infant outcomes in pregnancies resulting in a live birth but characterised by abnormal *PHLDA2* expression. Investigating placental *PHLDA2* expression in relation to infant outcomes will establish whether *PHLDA2* could serve as a biomarker to identify at risk infants (Jensen *et al.* 2014).

In conclusion, previous studies have demonstrated an inverse association between placental *PHLDA2* and fetal growth. The mouse model of *Phlda2* over expression suggests that aberrant *PHLDA2* expression is causal of fetal growth restriction, rather than a consequence of an abnormal intrauterine environment. Jensen *et al.* (2014) propose that altered *PHLDA2* expression may result in fetal growth restriction through direct effects on the fetus and/or placenta. *Phlda2* over expression in a mouse model is associated with perturbed placental growth and development, which could act to limit fetal growth (Tunster *et al.* 2010). For example, glycogen storage required for fetal growth in late gestation was reduced and expression of glucose transporters altered in *Phlda2* transgenic placentas (Tunster *et al.*

2010). In addition, *Phlda2* has been demonstrated to regulate the endocrine lineage of the mouse placenta (the spongiotrophoblast cells of the junctional zone) thereby indirectly controlling production of placental hormones, which are known to regulate fetal growth (reviewed in (John 2013)), as further described in section 1.8.

1.5.3. Factors affecting *PHLDA2* expression

Given the association between perturbed *PHLDA2* and fetal growth restriction, it is important to determine factors responsible for altered *PHLDA2* expression (Jensen *et al.* 2014).

In terms of maternal demographics, advanced maternal age in mice is associated with fetal growth restriction and increased *Phlda2* expression in ovarian tissue (Paczkowski *et al.* 2015). In addition, Moore *et al.* (2015) reported no significant association between pre-pregnancy maternal weight or BMI and placental *PHLDA2* expression in two large independent cohorts.

Shukla *et al*. (2011) demonstrated significantly increased placental *Phlda2* expression in response to maternal ethanol consumption in pregnant rat dams. The same study also showed increased placental *Phlda2* expression in response to a calorie-restricted diet (Shukla *et al*. 2011). In contrast Lillycrop *et al*. (2010) reported no significant difference in *Phlda2* expression in the liver following protein restriction in pregnant rat dams but did note a significant increase in hepatic *Phlda2* expression following folic acid supplementation. A maternal high sugar/high fat diet has been demonstrated not to be associated with significantly altered *Phlda2* expression in the mouse placenta (Sferruzzi-Perri *et al.* 2013). Thus, it is possible that human placental *PHLDA2* expression is affected by maternal diet during pregnancy, e.g. calorie restriction or folate supplementation.

A significant increase in placental *PHLDA2* expression has been reported in response to maternal smoking during pregnancy (Bruchova *et al*. 2010). However, Moore *et al.* (2015) observed no significant correlation

between placental *PHLDA2* expression and maternal smoking in two independent cohorts. Finally, Lewis *et al*. 2012 demonstrated significantly increased placental *PHLDA2* expression in mothers undertaking strenuous exercise.

In terms of pregnancy specific factors, placental *PHLDA2* expression was demonstrated to be reduced in association with a severe fetal abnormality (McMinn *et al*. 2006). In addition, studies suggest an effect of twin pregnancy on placental *PHLDA2* expression with differential expression in IUGR twin pregnancies compared with IUGR singleton pregnancies (Roh *et al.* 2005; McMinn *et al.* 2006). Finally, Feng *et al.* (2011) demonstrated decreased *PHLDA2* expression, in the absence of LOI, in the umbilical cord blood of children conceived using assisted reproductive technology (ART). Thus studies analysing placental *PHLDA2* expression in human pregnancies should exclude or control for ART pregnancies, pregnancies with multiples and those in which the fetus suffers from a severe abnormality.

In summary, these studies suggest that placental *PHLDA2* expression may be altered in response to environmental signals. However, the majority of previous studies examining factors affecting *Phlda2* expression have been carried out in animal models and not always in the placenta. Thus, further research is required into factors affecting human placental *PHLDA2* expression.

1.6. Other imprinted genes of interest

Three additional imprinted genes were identified as being of interest with respect to human FGR based on animal data or preliminary human studies: the maternally expressed imprinted gene *CDKN1C* and the paternally expressed imprinted genes *PEG3* and *PEG10*.

1.6.1. *CDKN1C*

CDKN1C (alternative names: *p57*, *Kip2*) is a maternally expressed imprinted gene nearby to *PHLDA2* on human chromosome 11p15.5 and under control of the same centromeric imprinting control region 2 (IC2 or KvDMR1). *Cdkn1c* encodes the cyclin-dependent kinase inhibitor 1C protein, which is a negative regulator of cell proliferation (reviewed in (Coan *et al.* 2005)).

Cdkn1c is imprinted in the mouse placenta with expression from the maternal allele only (Wang *et al.* 2011). *Cdkn1c* is expressed predominantly in the glycogen cells, spongiotrophoblast and giant cells of the mouse placenta (reviewed in (Tunster *et al.* 2013)). Tunster *et al.* (2011) demonstrated overgrowth of *Cdkn1c* deficient embryos from mid to late gestation, although there was no effect on pup weight at birth. This may be due to the significant placental abnormalities associated with loss of *cdkn1c*, in the context of intrauterine competition. Impaired vascularisation in these *Cdkn1c* deficient placentas has been attributed to excessive proliferation of labyrinth trophoblasts and spongiotrophoblast cells (Takahashi *et al.* 2000; Kanayama *et al.* 2002). In contrast, increasing *Cdkn1c* expression using a BAC transgene approach (modelling loss of imprinting) resulted in fetal and postnatal growth restriction (Andrews *et al.* 2007).

CDKN1C is expressed in the human heart, lung, brain, kidney, pancreas, testis, skeletal muscle and placenta (Eggerman *et al.* 2014). Monoallelic expression from the maternal allele has been confirmed in the human placenta (Monk and Moore 2004). Mutations in *CDKN1C* have been reported in human cases of BWS (characterised by pre- and postnatal overgrowth) (Engel *et al.* 2000) and in a family displaying IUGR and short stature and type II diabetes in adulthood (Kerns *et al.* 2014).

Few studies, often with conflicting results, have examined placental *CDKN1C* expression in relation to growth restriction. In a relatively small study (10 normal and 7 IUGR placentas) Diplas *et al.* (2009) reported no significant difference in *CDKN1C* expression in IUGR placentas. Similarly, Moore *et al.* (2015) found no significant association between placental *CDKN1C* expression and placental weight, birth weight or head

circumference in 81 participants (across a normal birth weight range). In contrast, McMinn *et al.* (2006) report a three-fold increase in *CDKN1C* expression in IUGR placentas. Rajaraman *et al.* (2010) found significantly reduced *CDKN1C* expression and protein in growth restricted placentas associated with placental insufficiency. Thus, further research is needed to fully clarify the relationship between fetal growth and human placental *CDKN1C* expression.

Little is known about whether maternal lifestyle factors influence placental *CDKN1C* expression. Shukla *et al.* (2010) found increased placental *Cdkn1c* expression in response to ethanol exposure and calorie restriction in pregnant rat dams. In contrast no significant effect of maternal high fat – high sugar diet on placental *Cdkn1c* expression was observed in a mouse model (Sferruzi-Perri *et al.* 2013). Finally, human placental *CDKN1C* expression was demonstrated not to be significantly associated with maternal pre-pregnancy weight or smoking during pregnancy (Moore *et al.* 2015). Thus, further research is required into factors affecting human placental *CDKN1C* expression.

1.6.2. *PEG3*

The paternally expressed imprinted gene *Peg3* (alternative names: *Pw1, Znf904, Zscan24, Zkscan22*) encodes the paternally expressed gene 3 protein, which is thought to play a role in cell proliferation and p53-mediated apoptosis (reviewed in Coan *et al.* 2005). In humans, the *PEG3* gene is located on chromosome 19q13.4 and in mice on proximal chromosome 7. *Peg3* is imprinted in both the mouse (Wang *et al.* 2011) and human (Hiby *et al.* 2001) placenta with expression from the paternal allele only.

Loss of function of *Peg3* results in fetal growth restriction with *Peg3* mutants being 10% smaller than wild type littermates at birth. This suggests a role for *Peg3* in the positive control of fetal growth (Li *et al.* 1999; Kim *et al.* 2013). Furthermore, these *Peg3* mutants become obese in adulthood, showing increased body fat despite reduced food intake (Curley *et al.* 2005).

Peg3 deficient placentas were significantly smaller and displayed significant changes in gene expression with differentially expressed genes including those involved in regulating cell proliferation, behaviour, and metabolism (Kim *et al.* 2013a). The *Peg3* mutation was also demonstrated to affect the maternal reproductive organs and lactation (Li *et al.* 1999; Kim *et al.* 2013). Finally, the mouse model of *Peg3* loss of function demonstrated a role for *Peg3* in the induction of nurturing and nest building behaviour in rodents (Li *et al.* 1999; Curley *et al.* 2004; Champagne *et al.* 2009; Chiavegatto *et al.* 2012) a role which is further described in section 1.9.3.

Four previous studies have found no association between placental *PEG3* expression and measures of fetal growth. Moore *et al.* (2015) found no significant correlation between placental *PEG3* expression and placental weight, birth weight or head circumference, although it should be noted that all placentas were from normal birth weight deliveries. Similarly, McMinn *et al.* (2006), Diplas *et al.* (2009) and Kumar *et al.* (2012) reported no significant difference in *PEG3* expression between SGA and AGA placentas. However, the largest of these studies examined only 15 SGA placentas and therefore the association between *PEG3* and birth weight needs to be further explored in a larger cohort.

A number of studies have analysed the effect of the maternal environment on mouse placental *Peg3* expression. Broad and Keverne (2011) demonstrated a significant 35% decrease in *Peg3* expression following maternal starvation for 24 hours. In contrast, Radford *et al.* (2012) reported increased *Peg3* expression in response to maternal calorie restriction. Maternal high fat diet (Gallou-Kabani *et al.* 2010) and high fat – high sugar diet (Sferruzzi-Perri *et al.* 2013) during pregnancy do not result in altered placental *Peg3* expression. In terms of human studies, Moore *et al.* (2015) found no significant correlation between placental *PEG3* expression and maternal smoking or pre-pregnancy weight. Similarly, umbilical cord *PEG3* expression is not significantly altered in obese mothers (Thakali *et al.* 2014).

1.6.3. *PEG10*

The paternally expressed imprinted gene *Peg10* (alternative names: *EDR, HB-1, Mar2, MEF3L, Mart2, RGAG3*) encodes the retrosponson-derived protein PEG10 of unknown function (Coan *et al.* 2005). In humans, the *PEG10* gene is located on chromosome 7q21 (Ono *et al.* 2001) and in mice on proximal chromosome 6 (Ono *et al.* 2006). *Peg10* is imprinted in both the mouse (Ono *et al.* 2006) and human (Ono *et al*. 2001; Daelemans *et al.* 2010) placenta with expression from the paternal allele only.

Peg10 expression is extensive throughout the trophoblast lineage of the mouse placenta (Tunster *et al.* 2013). Loss of function of *Peg10* results in embryonic lethality before E10.5, in association with growth retardation and an absent heart beat (Ono *et al.* 2006). Embryonic lethality was attributed to a severe placental defect with perturbed labyrinth development and an absence of spongiotrophoblast cells (Ono *et al.* 2006).

PEG10 is expressed in the human testis, brain, lung and placenta (Smallwood *et al.* 2003). In the human placenta, *PEG10* begins to increase around 11 weeks of gestation (coinciding with establishment of maternal blood supply to the placenta) and continues to increase till term (Smallwood *et al.* 2003). Dowregulation of *PEG10* expression (by 85%) has been demonstrated in third trimester placentas from fetal deaths, although this was not analysed in relation to fetal growth restriction (Doria *et al.* 2012). Three studies have examined placental *PEG10* expression in relation to fetal growth with conflicting results. Diplas *et al.* (2009) reported an almost three fold increase in *PEG10* expression in placentas of IUGR pregnancies, with monoallelic expression maintained. In contrast, umbilical cord *PEG10* expression was reported to be significantly positively correlated with birth weight in a study of Chinese infants (Lim *et al.* 2012). In this study, *PEG10* expression was significantly decreased by 50% and *PEG10* methylation significantly increased in LBW placentas (Lim *et al.* 2012). Finally, Moore *et al.* (2015) found no significant correlation between placental *PEG10* expression and birth weight, placental weight or head circumference in a cohort of 110 infants of normal birth weight range.

Few studies have examined the effect of maternal lifestyle on placental *PEG10* expression. Placental *Peg10* expression was not significantly altered in response to a maternal high fat diet in a mouse model (Gallou-Kabani *et al.* 2010). Similarly, there was no significant effect of maternal antibiotic use (Vidal *et al.* 2013) or parental obesity (Soubry *et al.* 2013) on *PEG10* methylation in umbilical cord blood. More research is needed to determine the association between placental *PEG10* expression and fetal growth, and the possible influence of maternal lifestyle factors.

1.7. Fetal overgrowth

Given the role of imprinted genes in the control of fetal growth, it is possible that fetal overgrowth is associated with aberrant imprinted gene expression.

Excessive growth of a fetus during pregnancy can result in the birth of a macrosomic (≥4,000g) and/or large for gestational age (LGA) infant (≥ 90th birth weight centile). In 2013, 11% and 11.8% of infants were born macrosomic in England and Wales respectively (Office for National Statistics 2014).

Fetal overgrowth is of clinical relevance due to the increased risk of adverse outcomes observed in these infants and their mothers (Zhang *et al.* 2008; Larkin *et al.* 2011; Pasupathy *et al.* 2012). For example, mothers of LGA or macrosomic infants have an increased risk of prolonged labour, postpartum haemorrhage and birth trauma (such as third or fourth degree tearing) (Jolly *et al.* 2003; Henriksen 2008; Larkin *et al.* 2011; Pasupathy *et al.* 2012). In addition, these infants have reduced apgar scores at delivery and are more likely to be admitted to NICU (Pasupathy *et al.* 2012). Zhang *et al.* (2008) also reported an increased risk of perinatal mortality in infants born weighing ≥ 4,500g. Importantly, the association between fetal overgrowth and adverse birth outcomes is most pronounced in pregnancies where the infant is LGA by custom birth weight centiles, i.e., in which the infant has exceeded its genetic growth potential (Pasupathy *et al.* 2012).

Advanced maternal age and multiparity have both been demonstrated to be associated with macrosomia (Jolly *et al.* 2003; Orskou *et al.* 2003). In terms of modifiable risk factors, maternal pre-pregnancy BMI and weight, gestational weight gain and subcutaneous fat are associated with an increased risk of delivering an LGA or macrosomic infant (Jolly *et al.* 2003; Orskou *et al.* 2003; Voldner *et al.* 2008; Henriksen *et al.* 2008). Low physical activity (< 1 hr / week) has been demonstrated to increase LGA risk (Voldner *et al.* 2008). Finally, pregnancies complicated by gestational diabetes are 3 times more likely to result in delivery of an LGA infant (Surkan *et al.* 2004).

Whilst the main focus of research on placental abnormalities has been in relation to fetal growth restriction, fetal overgrowth has also been associated with altered placental structure and function. Increased placental weight and volume are typically observed in LGA pregnancies with differences apparent from as early as the first trimester (Schwartz *et al.* 2014). In terms of placental abnormalities, Evers *et al.* (2003) reported increased markers of chronic hypoxia in macrosomic placentas. There is also evidence to suggest that glucose transport by the placenta is altered in cases of fetal overgrowth (reviewed in (Desforges and Sibley 2010)).

Although a number of studies have examined the relationship between *PHLDA2*, *CDKN1C*, *PEG3* and *PEG10* expression and fetal growth (section 1.5 and 1.6), no study to date has determined whether expression of these genes is altered in macrosomic or LGA placentas.

1.8. Imprinted genes and placental hormone production

During pregnancy, women undergo a number of physiological and hormonal adaptations required to support the optimal development of the fetus, many of which are driven by placental hormones (reviewed in Tan and Tan 2013). Imprinting has been proposed to have evolved and subsequently conserved due to a conflict between the maternal and paternal genome for allocation of maternal resources to the fetus (Moore and Haig 1991). It is therefore possible that imprinted genes regulate fetal growth through control of

mechanisms driving maternal adaptation to pregnancy (Haig 1993), such as placental hormone production (John 2013). The two related genes placental growth hormone (*PGH*) and human placental lactogen (*hPL*) encode placental hormones with established roles in maternal adaptation to pregnancy and control of fetal growth (Newbern and Freemark 2011).

1.8.1. Placental growth hormone (PGH)

The placental growth hormone gene (PGH/GH2/GH-V) is located within a growth hormone/placental lactogen gene cluster on human chromosome 17q.22-24 (Baumann 2009). The gene encodes a 191 amino acid protein (placental growth hormone), which is highly related to pituitary growth hormone (GH) and binds to the same growth hormone receptor (Lacroix *et al.* 2002). *PGH* is expressed in the syncytiotrophoblast layer of the human placenta, the main site of placental growth hormone production (Scippo *et al.* 1993) and in extravillous cytotrophoblast cells (Lacroix *et al.* 2002).

PGH is released in to the maternal circulation and between 10 – 20 weeks of gestation replaces pituitary GH as the primary form of growth hormone (Lacroix *et al.* 2002). PGH levels in the maternal circulation continue to rise till term, peaking around 34-37 weeks gestation (Lacroix *et al.* 2002). Production of PGH is positively associated with placental size and maternal glucose levels (Newbern and Freemark 2011).

One maternal adaptation to pregnancy with consequences for fetal growth is changes to maternal metabolism that prioritise transfer of glucose to the developing fetus (Tan and Tan 2013). Insulin production is increased by approximately 60% resulting in increased fat accumulation in the second trimester (Newbern and Freemark 2011). Despite the increase in insulin production, in mid to late pregnancy peripheral insulin resistance develops with insulin sensitivity decreased by approximately 70% (Newbern and Freemark 2011).

The rise in PGH production around 20 weeks gestation coincides with the appearance of maternal insulin resistance (Newbern and Freemark

2011). PGH promotes fetal growth by acting as an insulin antagonist, preventing uptake of glucose by the maternal muscles (Newbern and Freemark 2011). In addition, PGH promotes maternal lipolysis in order to utilise the accumulated maternal fat as an energy source (Newbern and Freemark 2011). Finally, PGH stimulates glucose production by the liver (Newbern and Freemark, 2011). Thus, the actions of PGH (Figure 1.6) ensure that glucose present in the maternal circulation is preferentially transported to the developing fetus. Another growth promoting function of PGH is in the positive regulation of maternal serum levels of insulin-like growth factor -1 (IGF-1) (Mirlesse *et al.* 1993), which stimulates maternal tissue growth and transfer of nutrients and oxygen to the fetus (Newbern and Freemark 2011). Presence of growth hormone receptors in the developing placenta, further suggest a growth-promoting role of PGH on the placenta (Lacroix *et al.* 2002). Finally, PGH may be of importance in response to nutrient deficiency as hormone levels are negatively associated with maternal glucose levels; this has been suggested to reflect an adaptation by the placenta to ensure delivery of nutrients to the fetus in an adverse intrauterine environment (Newbern and Freemark 2011).

A number of studies have demonstrated decreased PGH levels and/or gene expression in growth restricted pregnancies. Mannik *et al.* 2010 reported a significant correlation between term placental *PGH* expression and birth weight but not placental weight. However, *PGH* expression was only reduced by 10% in SGA compared with AGA placentas (Mannik *et al.* 2010). Koutsaki *et al.* (2011) similarly demonstrated a modest reduction in placental *PGH* expression in FGR placentas. In another study, maternal serum PGH levels were significantly reduced by 44% in IUGR pregnancies (Mirlesse *et al.* 1993). Finally, McIntyre *et al.* (2000) found significantly reduced serum PGH levels in IUGR pregnancies at both 28 and 36 weeks gestation.

Thus, PGH promotes fetal growth through induction of peripheral insulin resistance in mid to late pregnancy as part of the normal adaptation of a mother's body to pregnancy. The subsequent development of glucose intolerance is prevented by actions of human placental lactogen (section

1.8.2). However, in some women with genetic and/or environmental risk factors, gestational diabetes may develop as a result of inadequate adaptation to pregnancy (section 1.9.2).

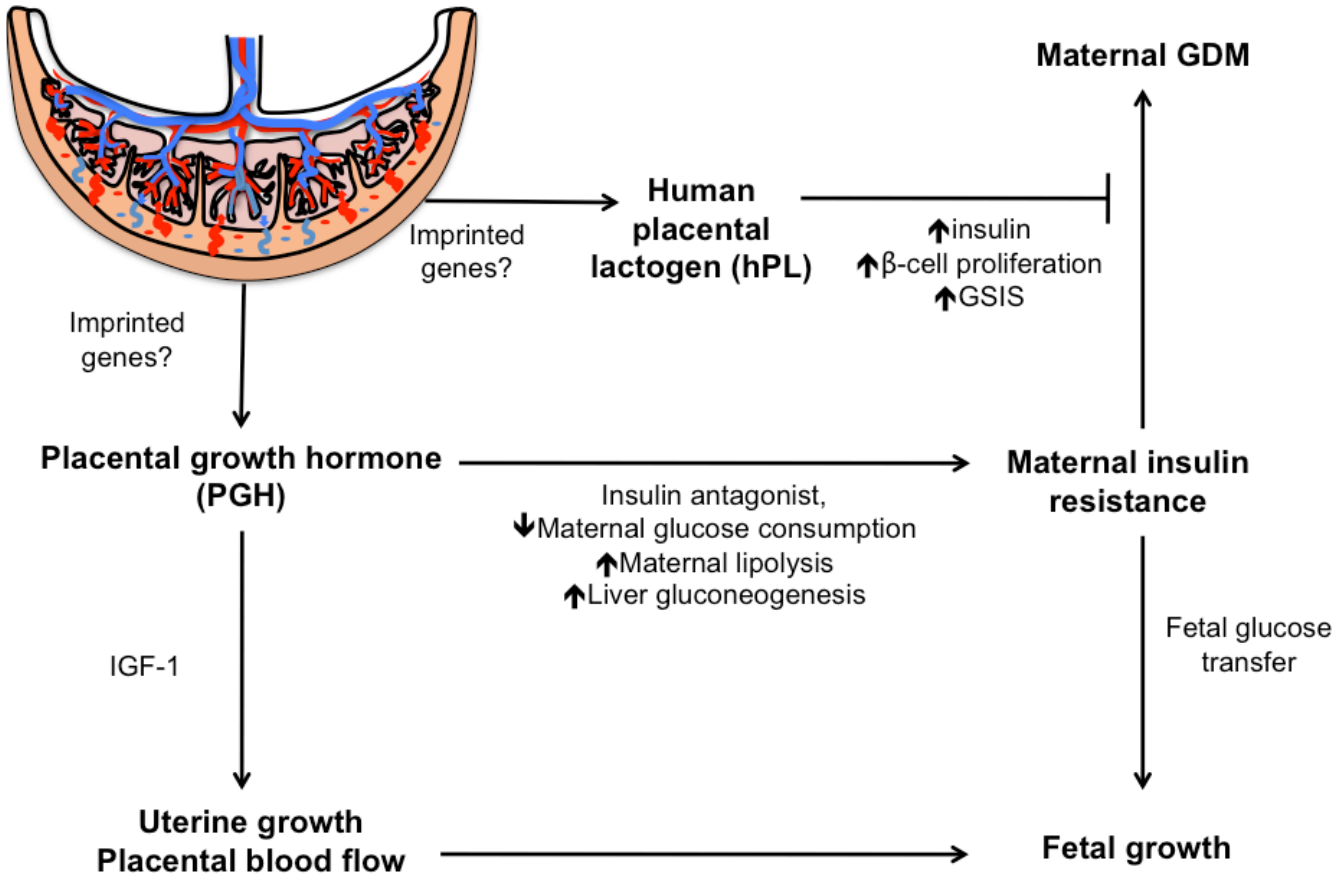


Figure 1.6: Placental hormone control of fetal growth and adaptation of maternal metabolism to pregnancy. A possible role for imprinted genes in regulating placental hormone production is shown. GDM = gestational diabetes, GSIS = glucose stimulated insulin secretion. Adapted from Newbern and Freemark (2011).

1.8.2 Human placental lactogen (hPL)

Human placental lactogen (also known as human chorionic somatomammotropin hormone (CSH)) is encoded by three genes *hPL-A*, *hPL-B* and *hPL-L* located within the growth hormone/placental lactogen gene cluster on human chromosome 17q.22-24. Although highly related and

structurally similar to GH, hPL is a lactogenic hormone that binds to the prolactin receptor (PRLR) and shares many similar functions with prolactin (Newbern and Freemark 2011).

hPL is expressed in the syncytiotrophobast of the human placenta (Newbern and Freemark 2011). hPL is secreted in to the maternal and fetal circulations, with levels rising during gestation and peaking at 32 – 35 weeks (Newbern and Freemark 2011). Around 13 weeks of gestation hPL replaces prolactin as the main lactogenic hormone (Newbern and Freemark 2011). As with PGH, serum hPL levels are positively associated with placental size (Newbern and Freemark 2011). Although maternal serum levels of hPL are associated with PGH levels, they are significantly higher (Fuglsang and Ovesen 2006).

Maternal secretion of insulin increases during pregnancy as a result of increased insulin production, proliferation of β-cells and glucose-stimulated insulin production (GSIS) an adaptation that is mediated by hPL (Newbern and Freemark 2011), Figure 1.6. Prolactin and placental lactogens stimulate pancreatic β-cell expansion and GSIS both *in vivo* and *in vitro* (Kim *et al.* 2010; Arumugam *et al.* 2011). The action of placental lactogen on β-cell proliferation was demonstrated to be via effects on serotonin synthesis, which when blocked resulted in reduced β-cell expansion and glucose intolerance in pregnant mice (Kim *et al*. 2010). In addition, PRLR KO mice in which the actions of placental lactogens and PRL are inhibited, demonstrate a reduction in pancreatic islet density and size (Freemark *et al.* 2002). Insulin production was also reduced in the pancreas of these mice and GSIS was inhibited both *in vivo* and in isolated islets *in vitro* (Freemark *et al.* 2002). Adult PRLR KO mice also displayed symptoms of impaired glucose tolerance (Freemark *et al.* 2002). The effects of loss of function of PRLR were similar in pregnant mice (Huang *et al.* 2009). Thus, these studies support a role for hPL in the control of maternal insulin secretion, counteracting the effects of PGH in the regulation of fetal growth, Figure 1.6.

Mannik *et al.* (2010) found no significant difference in placental *hPL* expression between SGA and AGA pregnancies. In contrast, Dutton *et al.*

(2012) demonstrated significantly reduced maternal serum hPL levels (but not placental *hPL* expression) in pregnancies with a poor perinatal outcome including FGR. Roh *et al.* (2005) examined placental *hPL* expression in twin pregnancies where only one twin suffered from IUGR. *hPL* expression was significantly decreased by at least 50% in IUGR compared with control placentas (Roh *et al*. 2005).

Thus, hPL and PGH control fetal growth through opposing actions on maternal metabolism, such that preferential transfer of nutrients to the fetus occurs while maintaining maternal metabolic homeostasis. It is possible that inadequate maternal adaptation to pregnancy, due to aberrant placental hormone signalling, could result in fetal growth restriction as well as other complications of pregnancy (section 1.9).

1.8.3 Imprinted gene control of placental hormone production

A number of imprinted genes have been demonstrated to regulate the endocrine lineage of the mouse placenta (the spongiotrophoblast cells of the junctional zone) thereby indirectly controlling production of placental hormones (reviewed in John 2013). For example, the junctional zone of the mouse placenta is increased in *Phlda2* KO mice (Frank *et al.* 2002). In contrast, two-fold expression of *Phlda2* resulted in a significantly decreased junctional zone with a specific loss of spongiotrophoblast cells (Tunster *et al.* 2010). In addition, there was a three-fold decrease in expression of the placental lactogen *Prl8a8* (Tunster *et al.* 2010). *Cdkn1c* loss of function in a mouse model resulted in a reduction in spongiotrophoblast cells and decreased expression of the placental lactogens *Prl8a8* and *Prl3a1* (Tunster *et al.* 2011). Loss of function of *Peg3* in the mouse placenta results in a myriad of gene changes in the placenta including a marked alterations in the expression of several mouse placental lactogens as gestation proceeds (Broad and Keverne 2011;Kim *et al.* 2013). Finally, *Peg10* KO mice show a severe placental defect caused by an absence of spongiotrophoblast cells (Ono *et al.* 2006). Thus, these studies provide evidence for imprinted gene control of the endocrine lineage of the mouse placenta, in particular

production of placental lactogens. Perturbed imprinted gene expression could therefore, via changes in placental hormone production, result in inadequate maternal adaptation to pregnancy and subsequent fetal growth restriction (John 2013), Figure 1.6.

The human placenta differs in many respects to the rodent placenta, not in the least with respect to the cell types and placental hormones that they manufacture (Carter 2012). Therefore, it is important to obtain evidence as to whether these observations on imprinted gene control of placental hormone production in a mouse model have relevance to humans. Thus far, no study has reported on the relationship between imprinted gene expression and hormone production by the human placenta.

1.9. Imprinted genes and other complications of pregnancy

Imprinted genes have been hypothesised to play a role in the control of maternal adaptations to pregnancy, as supported by evidence of imprinted gene regulation of the placental endocrine lineage (John 2013). It is therefore possible that aberrant imprinted gene expression is associated with other pregnancy complications characterised by inadequate maternal adaptation to pregnancy. In this study, placental imprinted gene expression was analysed in pregnancies complicated by preeclampsia, gestational diabetes and maternal mood disorders.

1.9.1. Preeclampsia

Preeclampsia (PE) is defined as hypertension during pregnancy developing after 20 weeks gestation (≥ 90 – 99 mm Hg diastolic blood pressure and ≥ 140 – 149 mm Hg systolic blood pressure) in association with significant proteinuria (300 mg protein in 24 hr urine test) (NICE 2010) . Preeclampsia may precede development of eclampsia which is characterised by seizures (Hutcheon *et al.* 2011). Preeclampsia differs from pregnancy-induced hypertension (PIH), which is defined as new-onset hypertension during

pregnancy that is not associated with proteinuria (Hutcheon *et al.* 2011). There is debate as to whether PE and PIH are separate pregnancy complications resulting in similar symptoms or whether PIH is an earlier, milder form of PE (Villar *et al.* 2006).

Preeclampsia is associated with maternal morbidity and mortality as well as adverse short and long-term outcomes for the infant. It is estimated that hypertensive disorders of pregnancy underlie 18% of maternal deaths worldwide (Abalos *et al.* 2013). Women with a previous PE pregnancy are also at increased risk of cardiovascular and metabolic disease in later life (Hutcheon *et al.* 2011). Importantly, PE pregnancies are associated with an increased risk of infant perinatal mortality, preterm birth and reduced measures of infant wellbeing at delivery (such as low apgar scores and NICU admission) (Villar *et al.* 2006; Hutcheon *et al.* 2011). These infants are also more likely to develop hypertension and cardiovascular disease in later life although it is not certain whether this is due to shared genetic or environmental risk factors (Hutcheon *et al.* 2011).

Estimates of PE prevalence vary but it is thought that approximately 4.6% of pregnancies worldwide and 2.5% in the UK are affected by PE (Abalos *et al.* 2013). A previous PE pregnancy or a family history of PE is associated with an increased risk of developing PE (Duckitt and Harrington 2005). Similarly, first-time mothers have a three fold increased risk of PE and mothers over the age of 40 have a two fold increased risk (irrespective of parity)(Duckitt and Harrington 2005). In terms of maternal lifestyle factors, a raised maternal BMI is associated with PE (Duckitt and Harrington 2005). Several studies have examined the effect of micronutrient supplementation (e.g. calcium, vitamin C, folate) on reducing PE risk, with limited success (Sibai *et al.* 2005; Oken *et al.* 2007). Brantsaeter *et al.* (2009) found the risk of PE was increased in women with a high-energy diet rich in sugar and polyunsaturated fatty acids. In contrast, a diet rich in milk and vitamin D was associated with a reduced risk of PE (Brantsaeter *et al.* 2009).

Although the exact cause of preeclampsia is unknown, abnormal placentation is a characteristic feature of this pregnancy complication (Noris

et al. 2005). During placental development, extravillous cytotrophoblasts invade the maternal uterine spiral arteries inducing their remodelling (Huppertz 2008). This increases blood flow and reduces resistance in these vessels, thereby maximising placental perfusion (Gude *et al*. 2004). Poor trophoblast invasion and inadequate uterine spiral artery remodelling is a common feature of PE pregnancies and results in increased vessel resistance and a reduction in uteroplacental blood flow (Noris *et al.* 2005).

Examination of a role for imprinted genes in the pathogenesis of preeclampsia is suggested by two observations. Firstly, there is an increased prevalence of fetal growth restriction in PE pregnancies; Villar *et al.* (2006) estimated that IUGR occurred in 22% of women with PE and in 13% of women with PIH. Similarly, women with PE are up to 4 times more likely to deliver an SGA infant (Hutcheon *et al.* 2011). Given the established role of imprinted genes in the control of fetal growth, it is possible that the co-morbidity between FGR and PE can be explained by aberrant imprinted gene expression.

Secondly, some studies suggest a role for the maternally expressed imprinted gene *CDKN1C* in preeclampsia. Loss of *Cdkn1c* expression in a mouse model resulted in preeclampsia-like symptoms including increased blood pressure and proteinuria during pregnancy (Kanayama *et al.* 2002). Importantly, preeclampsia-like symptoms were present in wild type mice carrying mutant pups suggesting a feto-placental cause (Kanayama *et al.* 2002). Loss of function of *Cdkn1c* was associated with increased spongiotrophoblast and labyrinth trophoblast proliferation resulting in a narrowed intervillous space (Takashashi *et al.* 2000; Kanayama *et al.* 2002). It was suggested that a narrowed intervillous space could impede uteroplacental blood flow, which combined with the shallow trophoblast invasion observed, could contribute to the development of preeclampsia-like symptoms (Kanayama *et al*. 2002). Given the role of the imprinted genes *Phlda2*, *Peg3* and *Peg10* in the regulation of the spongiotrophoblast cell lineage (section 1.8.3), it is possible that these genes also play a role in preeclampsia.

Few studies have examined imprinted gene expression in relation to preeclampsia in human pregnancies. As with the animal data, most studies thus far have focused on *CDKN1C*, with conflicting results. Women carrying infants with BWS due to a *CDKN1C* mutation have been reported to develop a severe form of preeclampsia known as HELLP (haemolysis, elevated liver enzymes and low platelets) (Romanelli *et al.* 2009). However, Bourque *et al.* (2010) found no significant difference in placental *CDKN1C* expression or methylation in PE pregnancies. In contrast, Enquobahrie *et al.* (2008) reported a significant 59% increase in *CDKN1C* expression in PE placentas. *PHLDA2* expression has similarly been shown to be upregulated in PE placentas, although this was not statistically significant (McMinn *et al.* 2006). Lambertini *et al.* (2008) found LOI of *PEG3* in 50% of the human preeclamptic placentas examined, although gene expression was not analysed. Finally, analysis of *PEG10* expression in relation to preeclampsia have yielded conflicting results with Chen *et al.* (2012) reporting increased expression and Liang *et al.* (2014) decreased *CDKN1C* expression in PE placentas.

In summary, the role of imprinted genes in preeclampsia is intriguing but, as yet, not well established.

1.9.2. Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is defined as new-onset glucose intolerance during pregnancy (Buchanan *et al.* 2007; Hartling *et al.* 2014; Mitanchez *et al.* 2014). WHO criteria for the diagnosis of GDM is fasting glucose ≥ 6.1 mmol/L or ≥7.8 mmol/L at 2 hours following a 75g oral glucose tolerance test (GTT). In gestational diabetes, maternal β-cell function and therefore insulin secretion is reduced (Buchanan *et al.* 2007). As this occurs in conjunction with normal pregnancy-induced insulin resistance, hyperglycaemia results (Buchanan *et al.* 2007). GDM is typically managed with diet and exercise, however in up to 20% of cases metformin or insulin treatment is necessary (NICE 2008).

GDM has consequences for both the mother and her offspring. GDM is associated with increased risk of birth trauma, hypertension and depression in the mother (Hartling *et al.* 2014). In addition, GDM women are almost 6 times more likely to subsequently develop type II diabetes after pregnancy (Hartling *et al.* 2014). GDM is also associated with fetal overgrowth. This has been proposed to occur as maternal hyperglycaemia stimulates increased transfer of glucose to the fetus, fetal insulin production and therefore also increased fat accumulation (Mitanchez *et al.* 2014). Consistent with this hypothesis is the 2 fold increased risk for GDM mothers of delivering an LGA or macrosomic infant (Hartling *et al.* 2014). Catalano *et al*. (2003) also reported significantly increased neonatal body fat in infants of GDM mothers. Fetal overgrowth has consequences for the offspring, as discussed in section 1.7, and may underlie the 2 fold increased risk of shoulder dystocia during delivery of a GDM infant (Hartling *et al.* 2014). It is important to note that the association between GDM and adverse infant outcomes is strongest in obese women (Makgoba *et al.* 2012; Mitanchez *et al.* 2014). Long-term consequences of GDM on the offspring include an increased risk of obesity in childhood (Hartling *et al.* 2014) as well as symptoms of neurodevelopmental delay (Ornoy 2011).

2 – 5% of pregnancies in England and Wales are complicated by maternal diabetes, of which 88% is gestational diabetes (NICE 2008). Some ethnicities, e.g. women of Asian origin, are at an increased risk of developing GDM (Teh *et al.* 2011). In addition, women who are overweight or obese before pregnancy are more likely to develop GDM, for example women with a BMI ≥ 35 are at a 6 fold increased risk (Teh *et al.* 2011). One study reported that raised maternal BMI accounted for 46% of GDM cases (Mitanchez *et al.* 2014). Maternal lifestyle factors affecting GDM risk are less well understood. Bowers *et al.* (2012) reported an increased risk of GDM in women consuming a diet rich in animal fat and cholesterol. In terms of GDM prevention, maternal exercise during pregnancy (up to 2 hours per week) was found to significantly reduce GDM risk (Dempsey *et al.* 2004).

Placental abnormalities associated with GDM have recently been reviewed (Gauster *et al.* 2012). Oxygen supply to the fetus has been

demonstrated to be compromised in GDM pregnancies, possibly due to increased fetal aerobic metabolism in response to hyperinsulinemia (Gauster *et al.* 2012). The increased placental weight and enhanced villous branching (despite relative villous immaturity) observed in GDM placentas was therefore suggested to be an adaptation to fetal hypoxia (Gauster *et al.* 2012). Genes differentially expressed in the GDM placentas include genes involved in apoptosis and inflammation (Radaelli *et al.* 2003; Enquobahrie *et al.* 2009; Magee *et al.* 2014).

A role for imprinted genes in the pathogenesis of GDM is suggested by two observations. Firstly, GDM pregnancies are typically associated with fetal overgrowth (Hartling *et al.* 2014). Given the established role of imprinted genes in the control of fetal growth, it is possible that the co-morbidity between GDM and fetal overgrowth can be explained by aberrant imprinted gene expression. Secondly, imprinted genes are known to control the endocrine lineage of the mouse placenta, particularly expression of placental lactogens known to prevent glucose intolerance in normal pregnancies (section 1.8). Indeed, maternal serum levels and placental gene expression of *hPL* and *PGH* have been found to be altered in pregnancies complicated by diabetes (Ursell *et al.* 1973; McIntyre *et al*. 2000; Mannik *et al.* 2012). While Mao *et al.* (2012) reported significantly increased risk of GDM in women inheriting a genetic variant of *KCNQ1*, the imprinting cluster in which *PHLDA2* and *CDKN1C* are located, no study to date has examined placental *PHLDA2*, *CDKN1C*, *PEG3* or *PEG10* gene expression in GDM pregnancies.

1.9.3. Maternal mood disorders

Women are particularly prone to developing symptoms of depression and anxiety during and after pregnancy, which may be a consequence of the numerous physiological and hormonal changes that are required to support the optimal development of the fetus and to prepare the mother for the postnatal care of her offspring (Tan and Tan 2013). A 2001 UK study reported depressive symptoms in 11.8% (second trimester) to 13.6% (third trimester) of the 12,059 women studied (Evans *et al.* 2001). Interestingly,

Evans *et al.* (2001) also reported that prenatal depression was in fact more frequent than postnatal depression. Prevalence of anxiety disorders during pregnancy is estimated to be around 6% (Andersson *et al.* 2004).

Maternal symptoms of depression and anxiety, exposure to adverse life events and acute disasters are all associated with adverse long term consequences for the child including an increased risk of fetal growth restriction (Steer *et al.* 1992; Pritchard and Teo Mfphm 1994; Paarlberg *et al.* 1999; Berkowitz *et al.* 2003; Khashan *et al.* 2008; Henrichs *et al.* 2010; Uguz *et al.* 2013), emotional and behavioural problems, learning difficulties, cognitive impairment and psychopathology in adulthood (reviewed in (Van den Bergh *et al.* 2005; Talge *et al.* 2007)).

Maternal prenatal stress, anxiety and depression have previously been associated with altered placental function in both animal models (Mairesse *et al.* 2007; Jensen Pena *et al.* 2012) and in humans (O'Donnell *et al.* 2012; Blakeley *et al.* 2013). This has primarily been interpreted as an effect of maternal mood on placental function. However, the placenta is a significant source of hormones that act on the maternal brain priming the mother for pregnancy and postnatal care (Glynn and Sandman 2011). It is therefore possible that aberrant placental function may influence maternal mood (Glynn and Sandman 2011).

The placenta produces large quantities of hPL (section 1.8.2), a lactogenic hormone that has been demonstrated to induce maternal behaviour in animal studies (Bridges *et al.* 1985; Bridges *et al.* 1990; Bridges and Freemark 1995; Bridges *et al.* 1997). hPL is closely related to the pituitary hormone prolactin which stimulates maternal neurogenesis (Bridges and Grattan 2003; Shingo *et al.* 2003; Walker *et al.* 2012). Prolactin also contributes to a suppression of anxiety related behaviours during pregnancy via binding to prolactin receptors, which also bind placental lactogens (Torner *et al.* 2001). Previous studies have demonstrated decreased serum prolactin levels in mothers with postnatal depression symptoms (Abou-Saleh *et al.* 1998; Ingram *et al.* 2003; Groer and Morgan 2007) and increased levels in mothers with low anxiety scores during pregnancy (Asher *et al.* 1995).

Given that imprinted genes have been reported to functionally converge on the endocrine lineages of the murine placenta to regulate placental signalling (John 2013), particularly of placental lactogens, it is possible that imprinted genes play a role in the adaptation of maternal psychology to pregnancy.

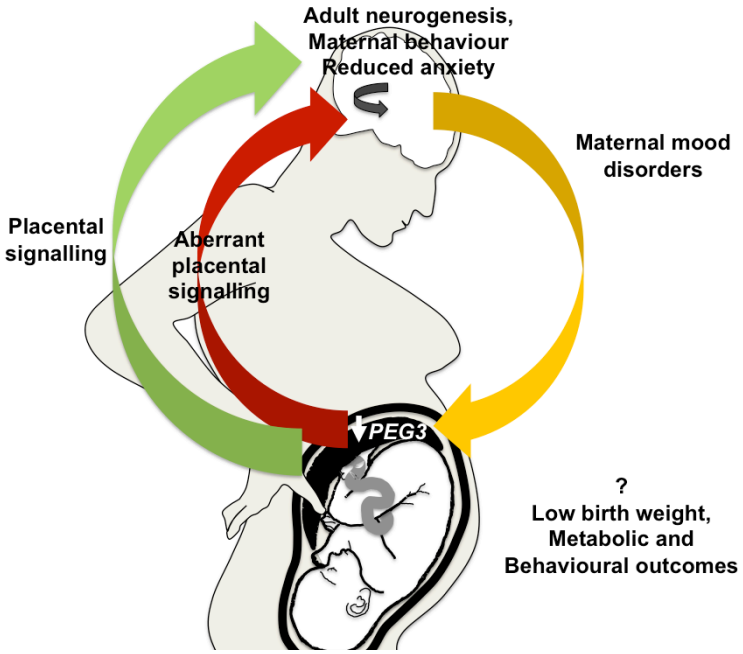


Figure 1.7: Placental signalling and maternal psychological adaptation to pregnancy. Animal studies have identified a role for placental hormones (e.g. placental lactogen) in inducing maternal behaviour during pregnancy (green arrow). It is therefore possible that aberrant placental signalling could contribute to inadequate adaptation of maternal psychology to pregnancy, manifesting as maternal mood disorders. In particular, loss of expression of the imprinted gene *Peg3* has been associated with impaired maternal behaviour in a mouse model and is known to regulate placental hormone production. Aberrant hormonal signalling may also occur as a consequence of prenatal stress (yellow arrow) thereby establishing a cycle of aberrant placental gene expression, placental signalling and maternal stress. Impaired placental signalling may therefore underlie maternal mood disorders and associated outcomes in the offspring.

Via a direct action on the maternal brain, the paternally expressed imprinted gene *Peg3* has been demonstrated to be required for the induction of nurturing and nest building behaviour in primiparous rodent mothers (Li *et al.* 1999; Curley *et al.* 2004; Champagne *et al.* 2009; Chiavegatto *et al.* 2012). It is also possible that *Peg3* modulates maternal psychological adaptation to pregnancy via indirect actions on placental hormone

production, Figure 1.7. Indeed, loss of function of *Peg3* in the mouse placenta results in marked alterations in the expression of several mouse placental lactogens as gestation proceeds (Broad and Keverne 2011; Kim *et al*. 2013a). Since loss of *Peg3* in an animal model also results in offspring fetal growth restriction, later life obesity and abnormal behaviour (Li *et al*. 1999; Curley *et al*. 2005; Champagne *et al*. 2009; Chiavegatto *et al*. 2012), aberrant placental *Peg3* expression could potentially explain the co-occurrence of inadequate adaptation of the maternal brain to pregnancy (manifesting as altered maternal mood), fetal growth restriction and poor outcomes (Figure 1.7).

No maternal behaviour phenotypes have as yet been reported for aberrant *Phlda2*, *Cdkn1c* or *Peg10* expression, but male mice carrying a *Cdkn1c*-BAC transgene display aberrant social behaviour (Thesis of G McNamara) and wild type female mice carrying *Phlda2* mutant pups display altered maternal behaviours (RMJ lab, unpublished data). Given their role in the control of the endocrine lineage of the mouse placenta, particularly expression of placental lactogens, it is possible that imprinted genes contribute to maternal psychological adaptation to pregnancy. Finally, no study has examined human placental imprinted gene expression in relation to maternal behaviour or maternal mood disorders of pregnancy.

1.10. Study aims and hypotheses

As discussed above, experimental data from both human and mouse studies suggest a role for the imprinted genes *PHLDA2*, *CDKN1C*, *PEG3* and *PEG10* in the control of fetal growth and other pregnancy complications. The aims of this study was to:

1) Quantify target imprinted gene expression in the human placenta of growth restricted pregnancies
2) Quantify target imprinted gene expression in the human placenta of pregnancies complicated by fetal overgrowth, preeclampsia, gestational diabetes and maternal mood disorders
3) Identify environmental factors associated with reduced birth weight and altered target imprinted gene expression in the human placenta
4) Identify environmental factors associated with altered imprinted gene expression in the mouse placenta

The hypotheses to be tested were as follows:

1) Growth restriction will be associated with increased expression of the maternal expressed imprinted genes (*PHLDA2* and *CDKN1C*) and decreased expression of the paternally expressed imprinted genes (*PEG3* and *PEG10*) in the human placenta.

2) Fetal overgrowth will be associated with decreased expression of *PHLDA2* and *CDKN1C* but increased expression of *PEG3* and *PEG10* in the human placenta.

3) Preeclampsia, gestational diabetes and maternal mood disorders will be associated with aberrant imprinted gene and placental hormone gene expression.

4) Adverse maternal lifestyles (such as smoking, alcohol and poor diet) will be associated with aberrant target gene expression in the human and mouse placenta.

CHAPTER 2: MATERIALS AND METHODS

In order to examine placental imprinted gene expression in human fetal growth restriction (Chapter 3) and pregnancy complications such as preeclampsia, gestational diabetes and maternal mood disorders (Chapter 4), the Wales Cohort was established (section 2.1). To provide further validation for findings from the Wales Cohort, placental gene expression was also analysed in the Manchester (section 2.2) and Imperial (section 2.3) Cohorts.

The effect of maternal lifestyle factors on human placental target gene expression (Chapter 5) was also analysed in the Wales Cohort. These findings were further explored in a mouse model (section 2.4) to provide evidence for a cause or effect relationship between maternal lifestyle, fetal growth and placental gene expression (Chapter 6).

2.1. Wales cohort

2.1.1 Study design

The Wales Cohort was established as part of this PhD with the aim of determining the association between placental target gene expression and complications of pregnancy such as fetal growth restriction, fetal overgrowth, preeclampsia and gestational diabetes.

The study design is shown in Figure 2.1. The study was approved by the South East Wales Research Ethics Committee Panel B (REC number: 10/WSE02/10) and by University Hospital Wales (UHW) and Royal Gwent Hospital (RGH) Research and Development.

2.1.2. Participant recruitment

A total of 271 participants were recruited for the study. Participants were recruited at two research sites: 176 at University Hospital Wales in Cardiff (Cardiff and Vale University Health Board) and 98 at the Royal Gwent Hospital in Newport (Aneurin Bevan Health Board). Recruitment was carried

out at these two sites based on anticipated differences in LBW prevalence and maternal lifestyle factors, as exemplified in Figure 1.3. At both sites, participants were recruited any time from 20 weeks gestation (for example during antenatal assessment) up till delivery (for example when admitted for elective caesarean section or induction of labour). Dr Richard Penketh at UHW, Dr Sajitha Parveen at RGH and trained midwives at both sites recruited participants.

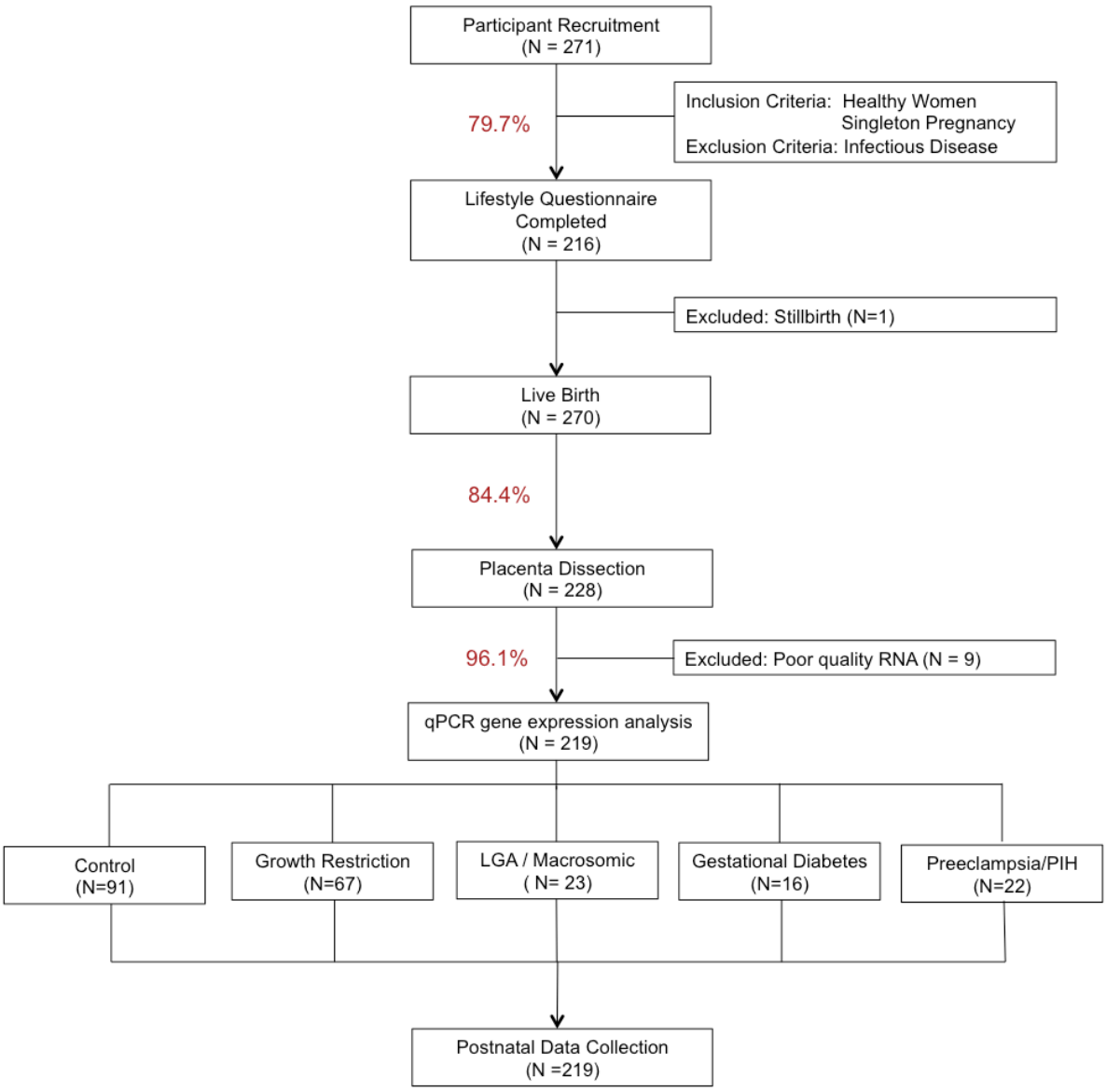


Figure 2.1: Wales cohort study design. Retention rates are shown as percentages in red. LGA = large for gestational age, PIH = pregnancy induced hypertension. Growth restriction was defined as birth weight < 10th centile by custom birth weight centiles.

A participant information sheet (see Appendix 1) was used to explain the study to participants. Subsequently, written informed consent (see Appendix 2) was obtained from otherwise healthy women with singleton pregnancies. Exclusion criteria included mothers with infectious diseases or those whose infants had known congenital or chromosomal abnormalities.

2.1.3. Participant questionnaire

A self-administered participant questionnaire (Appendix 3) was completed by 216 participants representing approximately 80% of the study population. The questionnaire was designed to collect information on physical (maternal BMI, age, and birth weight) and socioeconomic characteristics (education, family income) of the mother and information about her pregnancy (gestational age, IVF or natural conception). The questionnaire was also designed to collect information on maternal lifestyle factors before and during pregnancy including smoking, alcohol, drugs, exercise and diet. Variables chosen for inclusion in the questionnaire were based on factors previously demonstrated to be associated with fetal growth restriction (section 1.2) and/or aberrant *PHLDA2* gene expression (section 1.5.3). Questions on maternal lifestyle in the three months before conception were included as preconception maternal health is known to affect fetal development and growth (Ford 2011, Weisman *et al.* 2011). In addition, questions regarding maternal smoking and alcohol during pregnancy were divided into exposure in the first compared with the second and third trimesters combined. Finally, an important consideration in the questionnaire design was that it should be feasible to complete within a short period of time.

A food frequency questionnaire (FFQ) was chosen to assess maternal habitual diet before and during pregnancy as this method is easily self-administered and is associated with high return rates (Cade *et al.* 2004; Crozier *et al.* 2008; Thompson and Subar 2008). A FFQ is comprised of a list of foods with which the participant indicates how frequently they consumed each food item over a specified period of time (Cade *et al.* 2004). FFQs are frequently used in the study of diet during pregnancy (Erkkola *et al.* 2001;

Rao *et al.* 2001; Moore *et al.* 2004; Baer *et al.* 2005; Mouratidou *et al.* 2006; Venter *et al.* 2006). However, no pre-existing FFQ was considered appropriate for use in this study due to issues of length, target population and/or diet components of interest and therefore a new FFQ was designed specifically for the purpose of the current study. The FFQ was designed based on food items previously demonstrated to be associated with fetal growth restriction (section 1.2.3) and the typical diet of the target population.

A Cardiff-based nutritionist, J Crovini with extensive practical experience of the typical diet of pregnant women living in Cardiff, evaluated the FFQ with the aim of ensuring that a sufficient range of food items was included on the basis of which diet quality could be simply assessed.

In addition, 10 women (of which 4 were pregnant and 6 had recently given birth) reviewed the draft questionnaire. The women chosen to review the questionnaire were of different ages (23-45), either primiparous or multiparous (up to 4 children) and included those with and without a scientific background. All women were UK based to exclude cultural differences in diet. The women were asked to comment on the following questions:

1. Are there any questions that you feel that you would not have liked to answer?
2. Are there any questions that you feel were not clear?
3. Do you have any comments on the length of the questionnaire?
4. Would reading through the questionnaire discourage you from taking part in the study?

Based on the feedback from the questionnaire trial and the nutritionist, the final questionnaire (Appendix 3) was submitted to South East Wales Research Ethics Committee Panel B as a significant amendment to the study.

2.1.4. Obstetric covariates

Further information was obtained from the participant's medical notes using a data collection proforma (see Appendix 4) to ensure consistency across study sites. N Savory, A Holmes and personnel from the National Institute for Social Care and Health Research Clinical Research Centre (NISCHR CRC)

at UHW and J Beasley at RGH carried out data collection. Obstetric history was noted with respect to parity, previous stillbirths and previous low birth weight or macrosomic pregnancies. Information was also obtained on the current pregnancy including prescribed medication, intrauterine infection or antepartum haemorrhage. Maternal smoking, alcohol consumption and drug use before and during pregnancy was recorded from participant's medical notes and compared with self-report measures from the participant questionnaire. Information on birth outcomes was also obtained including mode of delivery (and indication), complications of delivery, birth weight, fetal sex and gestational age. Finally, measures of infant wellbeing such as Apgar scores, arterial cord blood pH and NICU admission were also recorded.

2.1.5. Measures of fetal growth

In addition to birth weight, a number of growth parameters were recorded from the participant's medical notes including antenatal concern over fetal growth (such as static growth, SGA abdominal or head circumference measurements on scan and/or low fundal height), IUGR diagnosis, amniotic fluid index (AFI), Doppler anomalies and ultrasound growth centiles during pregnancy. Custom birth weight centiles were calculated based on maternal height, weight, parity and ethnicity as well as infant birth weight, gestational age and gender using the GROW bulk centile calculator (UK), version 6.7.5 (Gardosi and Francis 2014).

2.1.6. Gestational diabetes information

Information on GDM was recorded from the participant's medical notes including gestational age at which the first abnormal glucose tolerance test (GTT) was observed and when GDM was diagnosed. A previous GDM pregnancy was also noted. Finally, the form of GDM management (diet, exercise and/or medication) was recorded. At UHW and RGH, women are not routinely screened for GDM unless they have one or more risk factors such as previous history, family history of diabetes, or BMI ≥ 30. Therefore, in

addition to the GDM participants recruited, the study cohort included a number of control participants with a confirmed normal GTT (at ≥ 28 weeks).

2.1.7. Preeclampsia information

A diagnosis of Preeclampsia and the gestational age at diagnosis was recorded from the participant's medical notes. In addition, previous history of a PE pregnancy was noted. Gestational age at first occurrence of hypertension and proteinurea were recorded to distinguish between pregnancy induced hypertension (hypertension without proteinurea) and preeclampsia (presence of both hypertension and proteinurea). Finally, the treatment for preeclampsia e.g. labetalol was noted as well as the gestational age at which treatment began.

2.1.8. Placental Dissection

Placental dissection was carried out for 228 participants (approximately 84% of the study population) with 74% sampled by A Janssen and the remaining by trained midwives. The most frequent cause of failing to dissect a study participant's placenta was complications during or immediately following delivery including infant distress and NICU admission, maternal post-partum haemorrhage and/or emergency C-section.

Placentas were collected and dissection carried out within 2 hrs of delivery, as determined by protocol optimisation experiments (section 2.1.9). Placentas were weighed and both the fetal and maternal surfaces checked for gross abnormalities. Tissue was taken from the maternal side of the placenta (Figure 2.2) midway between the cord and distal edge. To exclude contamination of the sample with maternal decidual cells, the uppermost cotyledon surface was removed and the villous tissue underneath samples (Figure 2.2). To remove excess maternal blood and amniotic fluid, samples were washed in phosphate buffered saline, PBS (Life Technologies, Paisley, UK) and then stored in RNA*later* (Sigma, Dorset, UK) at 4ºC overnight. Approximately 100mg of tissue was stored in each 1.5ml microcentrifuge tube containing 1 ml RNA*later* (Sigma). This process was repeated for a total

of 5 sampling sites distributed evenly across the maternal surface of the placenta (Figure 2.2).

Samples were subsequently removed from RNA*later* and finely dissected in ice cold PBS (Life Technologies) to remove vessel parts. Finally, placental tissue was stored at -80ºC. Details of each placental dissection were recorded on the placenta proforma (see Appendix 5).

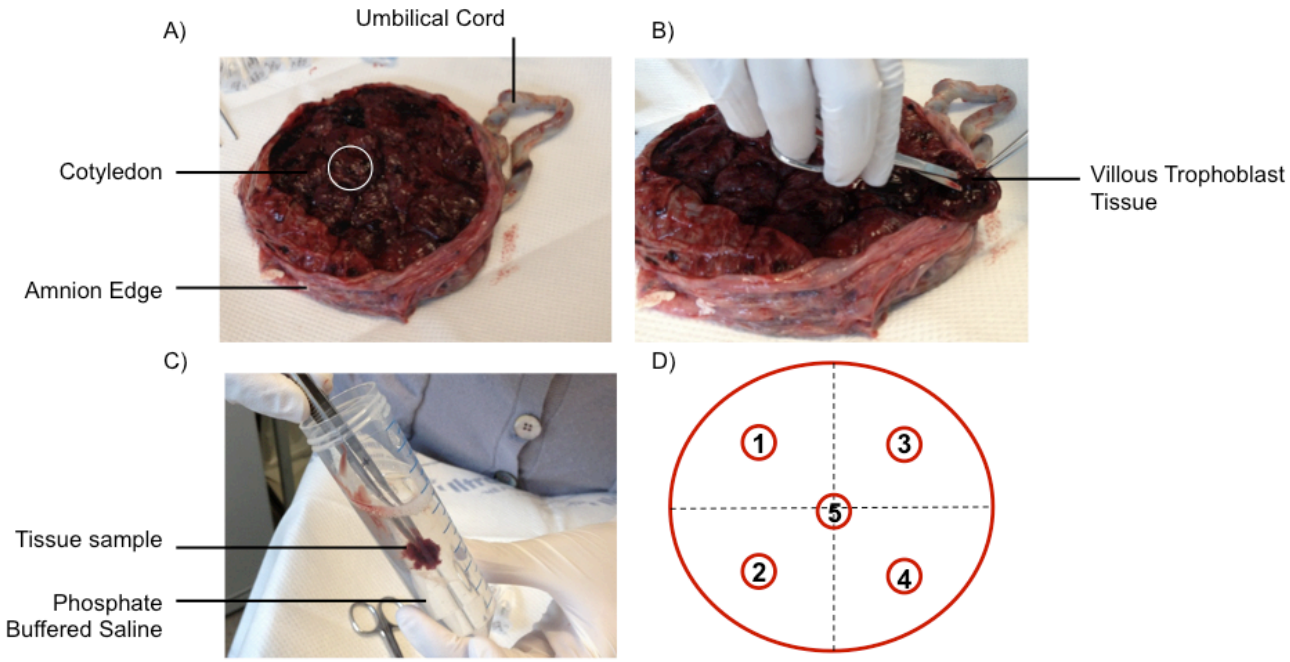


Figure 2.2: Placenta dissection protocol. (A) Maternal placental surface showing tissue sampling site. (B) Dissection of villous trophoblast tissue directly under the uppermost cotyledon surface. (C) Washing in phosphate buffered saline to remove maternal blood and amniotic fluid. (D) Process repeated for five evenly spaced sites across the placenta.

2.1.9. Protocol optimisation

In order to control for any effects of time to sampling on RNA concentration and quality as well as placental gene expression, for three participants placental tissue samples were taken at the same sampling site within 30 minutes, 1 hour, 1.5 hours and 2 hours of delivery. RNA was extracted from all time points and RNA concentrations compared. In addition, qPCR analysis of housekeeping and target gene expression was carried out.

Possible differences in placental target gene expression depending on tissue sampling site were also analysed. Following the protocol of Wyatt *et al.* (2005) three samples (close to the cord insertion site, middle and distal edge) were taken from each of the fetal, middle and maternal layers of the placenta as shown in Figure 2.3. This was carried out for 3 placentas from elective C-sections with normal birth weight infants.

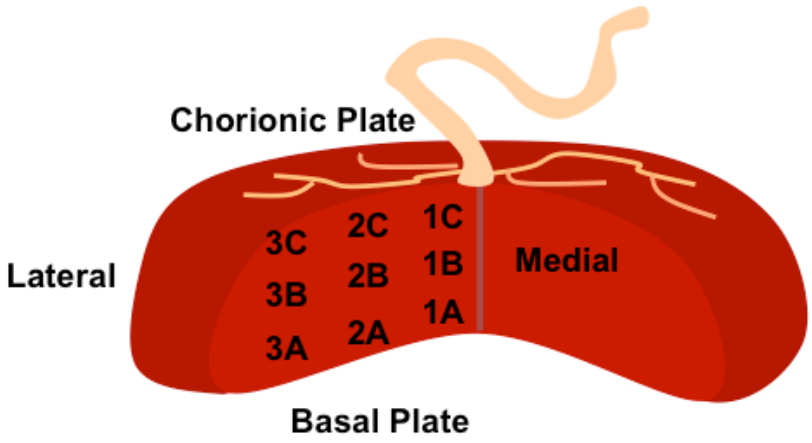


Figure 2.3: Controlling for placental sampling site. For three participants, tissue samples were taken close to the cord insertion site (1 A – C), middle (2 A – C) and distal edge of the placenta (3 A – C). Adapted from Wyatt *et al.* (2005).

In addition, placental gene expression was compared between early term (37 – 38 weeks), full term (39 – 40 weeks) and late term (≥ 40 weeks) gestational age groups for 19 control participants to determine the effect of gestational age on target gene expression. Importantly, all infants were delivered normal birth weight by elective caesarean section. In order to examine the effect of fetal sex on placental target gene expression, gene expression was also compared between 11 male and 11 female control placentas from normal birth weight, elective C-section deliveries. Finally, placental gene expression was compared between 29 participants delivering by elective C section (N = 21), emergency C section (N = 4) or vaginal delivery (N = 4) in order to examine the effect of labour or mode of delivery on placental target gene expression. All participants delivered normal birth weight infants and had no known complications of pregnancy.

2.1.10 RNA extraction and DNase treatment

RNA was extracted using the Sigma GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Dorset, UK) according to the manufacturers instructions. Briefly, 40mg of placental tissue per sample was homogenized in 500μl of a 1:100 dilution of 2-mercaptoethanol and lysis solution. The resulting mix was added to a GenElute Filtration column and centrifuged at 13,000 rpm for 2 minutes. The filtration column was subsequently discarded and 500μl 70% ethanol added to the filtered lysate. The lysate was thoroughly vortexed then added in 700μl aliquots to a GenElute Binding Column. The Binding column was centrifuged at 13,000 rpm for 15 seconds.

The flow through was discarded and 250μl Wash Solution 1 added to the column before centrifuging at 13,000 rpm for 15 seconds. DNase treatment was subsequently carried out by adding 10μl DNase I and 70μl DNase Digest Buffer to the column and incubating at room temperature for 15 minutes. 250μl of Wash Solution 1 was then added to the column, which was centrifuged at 13,000 rpm for 15 seconds before transfer to a clean collection tube.

500μl Wash solution 2 was next added to the column before centrifugation at 13,000 rpm for 15 seconds. The flow through was discarded and another 500μl Wash solution 2 added to the column before centrifugation at 13,000 rpm for 2 minutes. After the resulting flow through had been discarded, the column was centrifuged for a further 1 minute at 13,000 rpm. Finally, the binding column was transferred to a new collection tube and 30μl Elution Solution added to the column which was centrifuged at 13,000 rpm for 1 minute. The flow through (containing the eluted RNA) was nanodropped using a Nanodrop ND-1000 spectrophotometer in order to determine RNA concentration and purity. The 260/280 ratio was used to assess RNA purity, with a ratio of 1.8 to 2.1 considered acceptable.

2.1.11 Reverse transcription

For each DNase treated RNA sample, approximately 5μg of RNA was added to 1μl (0.5μg/μl) random hexamers (Promega, Southampton, UK) and the

volume made up to 11μl with RNase free water (Ambion, Paisley, UK). Samples were incubated at 70°C for 10 minutes before transfer to ice. 8μl of master mix (Table 2.1) was added to each sample before incubation for 2 minutes at 37 °C. 1μl MMULV (Promega) was subsequently added to each RT+ (but not RT-) reaction and the incubation continued at 37°C for a further 2 hours. The samples were then diluted 1:10 with 10mM Tris (pH 8) (Sigma) for use in PCR and 1:50 for qPCR. PCR was performed on all –RT samples to check for presence of genomic DNA (protocol described in section 2.1.13) with a lack of product in the RT – reactions confirming the absence of any genomic DNA contamination.

Table 2.1: Master mix (1X) components for reverse transcription

Reagent	**Volume (μl)**
5 X First strand synthesis buffer	4μl
10mM dNTPs	1μl
RNase free water	3μl

2.1.12. Primer design

Sequences for target gene primers are shown in Table 2.2. Primers included those previously published (as detailed in Table 2.2) and those designed for the current study using Primer3 (Untergasser *et al.* 2012). Criteria for design of these primers were a product size between 100 – 250 bp, an approximate melting temperature (T_m) of 60 and that forward and reverse primers were either side of an exon-exon boundary (to prevent amplification of genomic DNA). A blast search of the NCBI database was also performed for all primers to avoid non-target amplification of a PCR product of a similar size.

Table 2.2: Primers used for qPCR analysis of gene expression.

Target gene	Primer sequence	Product size	Reference
Human			
YWHAZ	Forward: TTCTTGATCCCCAATGCTTC Reverse: AGTTAAGGGCCAGACCCAGT	212bp	Own design
PHLDA2	Forward: GAGCGCACGGGCAAGTA Reverse: CAGCGGAAGTCGATCTCCTT	68bp	(Apostolidou *et al*. 2007)
CDKN1C	Forward: CCCATCTAGCTTGCAGTCTCTT Reverse: CAGACGGCTCAGGAACCATT	106bp	(Diplas *et al.* 2009)
PEG3	Forward: CTCACAACACAATCCAGGAC Reverse: TAGACCTCGACTGGTGCTTG	152bp	Own design
PEG10	Forward: AAATTGCCTGACATGAAGAGGAGTCTA Reverse: AAGCCTAGTCACCACTTCAAAACACACTAAA	158bp	(Diplas *et al.* 2009)
hPL	Forward: CATGACTCCCAGACCTCCTTC Reverse: TGCGGAGCAGCTCTAGATTG	97bp	(Dutton *et al.* 2012)
PGH	Forward: GTTTGAAGAAGCCTATATCCTG Reverse: TCACCCTGTTGGAAGGTGTT	107bp	(Vakili *et al.* 2013)
Mouse			
β-actin	Forward: CCTGTATGCCTCTGGTCGTA Reverse: CCATCTCCTGCTCGAAGTCT	260bp	Designed by S Tunster
Phlda2	Forward: TCAGCGCTCTGAGTCTGAAA Reverse: TCCTGGGCTCCTGTCTGAT	124bp	Designed by S Tunster
Cdkn1c	Forward: AGAGAACTGCGCAGGAGAAC Reverse: TCTGGCCGTTAGCCTCTAAA	141bp	Designed by S Tunster
Peg3	Forward: AAAACTCACCACTCCGTTGG Reverse: GTCTCGAGGCTCCACATCTC	190bp	Designed by S Tunster
Peg10	Forward: GGGTAGATAATCATAAGTATTTTGGGC Reverse: CAACATTCTAAACTTTATTCCAGCAAC	526bp	(Ogawa *et al.* 2009)

In addition, PCR was carried out (section 2.1.13) to ensure amplification of a single product at the expected product size. Melt curves produced during qPCR were also examined to confirm the presence of a single peak (section 2.1.14).

2.1.13. PCR

24μl of PCR master mix (Table 2.3) was added to 1μl of template cDNA (diluted 1:10 with 10mM Tris (pH 8)). Primer combinations were made up by combining 25μl 100μM forward primer, 25μl 100μM reverse primer and 50μl 10mM Tris (Sigma). PCR was carried out using thermocycler conditions: 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds and finally 72°C for 3 minutes. PCR products were run on a 1% Tris-acetate EDTA (TAE) agarose gel containing 0.5μg/ml ethidium bromide (Sigma) and visualised under UV. Products were compared against a 100bp DNA ladder (Norgen Biotek, Lichfield, UK) to confirm correct product size for RT+ reactions.

Table 2.3: Master mix (1X) components for PCR

Reagent	**Volume (μl)**
10X Buffer (Sigma)	2.5μl
25mM $MgCl_2$	2μl
10mM dNTPs	0.5μl
Primer Combination	1μl
RNase free water	17.84μl
Taq (Fermentas)	1μl

2.1.14 qPCR

cDNA samples were diluted 1:50 with 10mM Tris (pH 8) (Sigma). Each sample was assayed in triplicate and duplicate plates run to confirm gene

expression results. 15μl qPCR master mix (Table 2.4) was added to 5μl cDNA in each well of a 96 well qPCR plate. Primer combinations were made up as described for PCR (section 2.1.13).

Table 2.4: Master mix (1X) components for qPCR

Reagent	**Volume (μl)**
RNase free water	10.9μl
10 X Buffer (Sigma)	2μl
25mM $MgCl_2$	1.6μl
10mM dNTPs	0.4μl
Primer Combination	0.8μl
Taq (Fermentas)	0.13μl
Sybr Green (Invitrogen)	0.16μl

qPCR was performed using Chromo4 Continuous Fluorescence Detector mounted on a PTC 200 Thermocycler (MJ Research) and results analysed using Opticon 3 software. The thermocycler conditions were as follows: 94°C for 15 minutes, then 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds followed by an anti dimer step at 75°C for 30 seconds. Melt curves were produced by incubating at a further 70°C to 94°C and reading at every 0.5°C. Melt curves produced by qPCR were examined to confirm the presence of a single peak, suggesting no primer dimers or non-specific amplification.

Expression data is presented as the ΔCT (target gene expression relative to the housekeeping gene expression) and as the fold change in expression. The fold change was calculated using the $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001), where the ΔΔCT is the target gene expression relative to expression in a defined control group. To ease interpretation of gene

expression data, ΔCT values were inverted (χ(-1)) such that lower ΔCT values represent decreasing expression.

2.1.15 Statistical analysis

All statistical analysis was carried out using IBM SPSS Statistics for Windows (version 20.0, 2011). All variables were analysed for outliers. P-P plots were generated and a Shapiro-Wilk test carried out to test for normal distribution of the data. Normally distributed data, either untransformed or following ln transformation, was analysed using appropriate parametric tests. Data that remained non-normally distributed after transformation was analysed using appropriate non-parametric tests. Chapter specific statistical analyses are further detailed in each individual chapter.

2.2. Manchester cohort

To provide further validation for results reported in the Wales Cohort with respect to fetal growth, gene expression was further analysed in placentas from the Manchester Cohort. Study design was as shown in Figure 2.4. Participants were recruited as part of a larger study investigating the association between reduced fetal movements, placental structure and function (Warrander *et al.* 2012) and infant outcomes (Dutton *et al.* 2012). Written informed consent was obtained from mothers and the study approved by Oldham and Greater Manchester North Research Ethics Committees (REC no. 08/1011/83 and 11/NW/0664). Villous trophoblast samples (n = 110) were obtained from participants delivering within one week of clinical assessment for reduced fetal movements in the third trimester as previously described in (Dutton *et al*. 2012; Warrander *et al*. 2012). All placentas were dissected within 30 minutes of delivery. Placental sampling procedure was directly comparable to that carried out in the Wales Cohort study.

In addition to birth weight, a number of growth parameters were available for these participants. Firstly, antenatal assessment of fetal growth such as ultrasound measures of femur length, abdominal circumference and head circumference were documented. Umbilical Artery Doppler

measurements, mean placental diameter on scan and amniotic fluid levels were recorded, factors that are used to ascertain the presence and severity of growth restriction. As with the Wales Cohort, custom birth weight centiles were calculated using the GROW bulk centile calculator (UK), version 6.7.5 (Gardosi and Francis 2014). Information on infant outcomes around the time of delivery, such as apgar scores, NICU admission and umbilical artery cord blood pH was also available. Finally, information on maternal lifestyle during pregnancy and a diagnosis of depression during pregnancy (including any treatment prescribed) was recorded from the participant's medical notes.

Following transfer to Cardiff University, samples were processed for qPCR analysis as described in section 2.1.10 – 2.1.15.

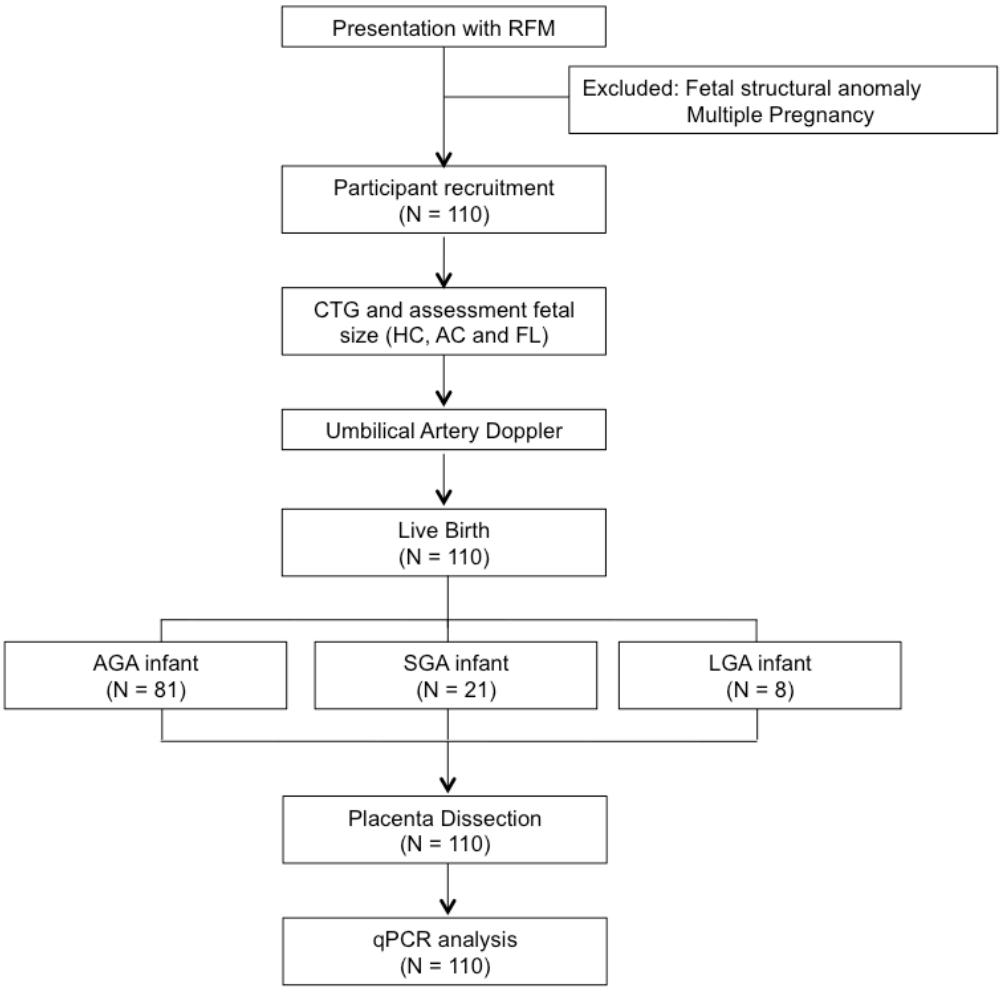


Figure 2.4: Study design for Manchester cohort. CTG = cardiotocograph, HC = head circumference, AC = abdominal circumference, FL = femur length, AGA = average for gestational age, SGA = small for gestational age, LGA = large for gestational age. Adapted from Dutton *et al.* (2012).

2.3. Imperial cohort

2.3.1. Study design

In order to analyse target imprinted gene expression in relation to maternal mood during pregnancy, gene expression was further analysed in placentas of the Imperial Cohort. Study design was as shown in Figure 2.5.

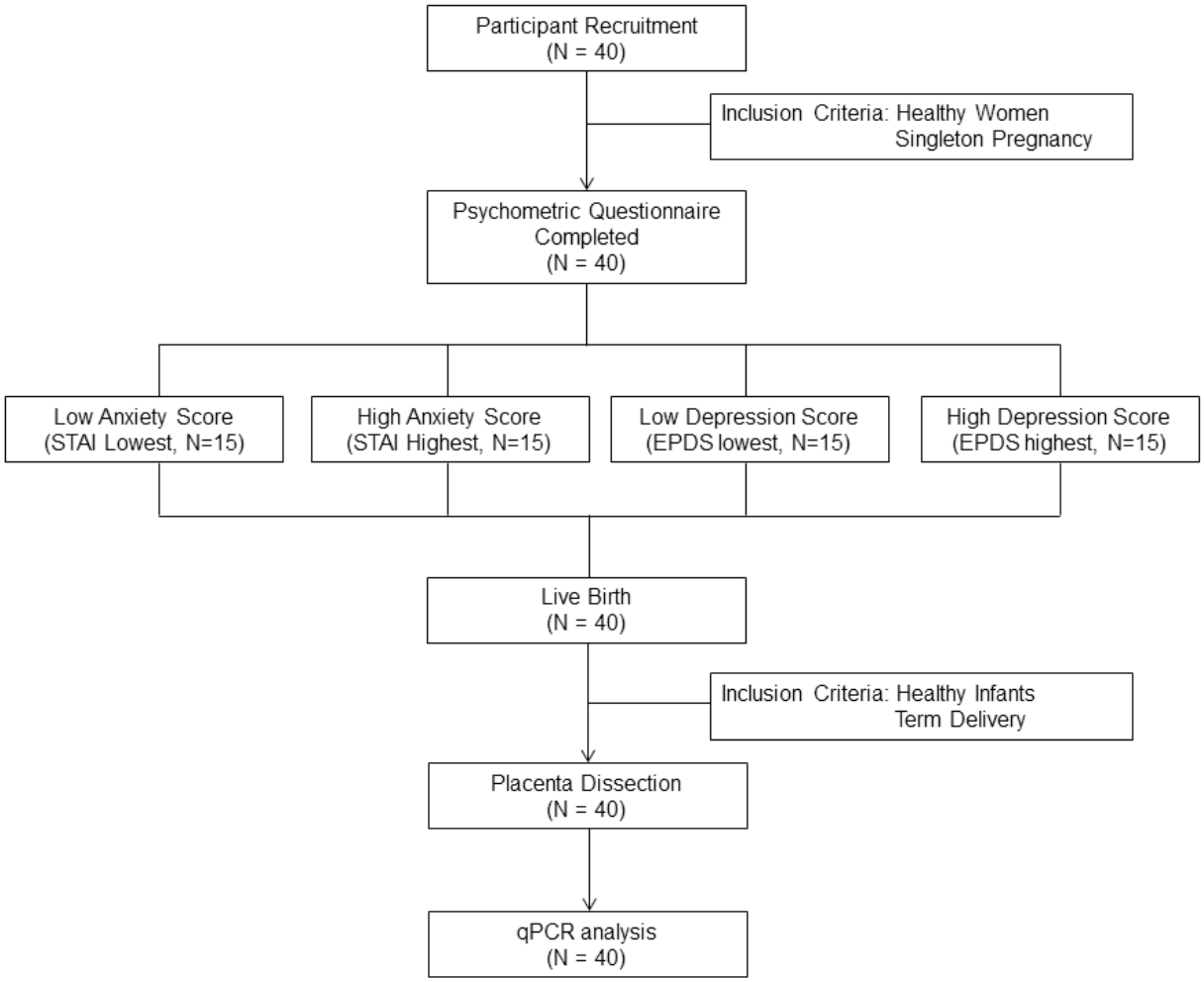


Figure 2.5: Study design for Imperial cohort. EPDS = Edinburgh Postnatal Depression Scale. STAI = Spielberger Trait Anxiety Inventory. Adapted from O'Donnell *et al.* (2012) and Blakeley *et al.* (2013).

Healthy women with singleton pregnancies were recruited one day prior to their elective caesarean sections at Queen Charlotte's and Chelsea Hospital, Hammersmith (London) as described in O'Donnell *et al.* (2012) and Blakeley

et al. (2013). Written informed consent was obtained and the study was approved by the Ethics Committee of Hammersmith and Queen Charlotte's Hospital, London (REC no. 08/H0708/126). All participants were recruited by V. Glover's research group at Imperial College London as part of a larger cohort study (N=65) examining the effects of prenatal stress on the placenta. 40 placenta samples from this cohort were available for analysis of placental hormone and imprinted gene expression.

2.3.2. Participant psychometric assessment

Maternal anxiety was measured using the Spielberger Trait Anxiety Inventory (STAI), see Appendix 6. The STAI is a comprehensively validated self-report questionnaire which assesses how the participant "generally feels". The questionnaire is comprised of twenty 4-point questions with total scores ranging from 20 (low anxiety) to 80 (high anxiety), with higher scores indicating higher levels of anxiety. An anxiety disorder is indicated by an STAI score ≥ 40 (Grant *et al.* 2008). Participants were divided into those 15 with lowest STAI scores and those 15 with highest STAI scores.

Maternal depression was measured using the Edinburgh Postnatal Depression Scale (EPDS), see Appendix 7. The EPDS has been validated for use during pregnancy (Cox *et al.* 1996) and is a self-report questionnaire comprised of ten questions. Total EPDS scores range from 0 (low depression) to 30 (high depression). Depression is indicated by an EPDS score of ≥13 (Cox *et al.* 1987). Participants were divided into those 15 with lowest EPDS scores and those 15 with highest EPDS scores.

In addition, maternal demographics including prescribed medication, occupation, household income, education and lifestyle during pregnancy (e.g. alcohol and smoking) were recorded using a self-report questionnaire. Birth outcomes including gestational age, fetal sex, birth weight and apgar scores were recorded from the participant's medical notes.

2.3.3. Placental sample processing

Placental dissection was carried out as described in O'Donnell *et al.* (2012) and Blakeley *et al.* (2013). Placental sampling procedure was directly comparable to that carried out in the Wales Cohort study. Briefly, a villous trophoblast tissue sample was taken from the central part of the placenta (just underneath the maternal surface) within one hour of delivery. Samples were washed in PBS before overnight storage in RNA*later* at 4°C. RNA*later* was then removed and the placental sample stored at -80°C until RNA extraction was carried out. RNA was extracted by A Janssen and P Blakeley (Imperial College London) using RNeasy Mini Kits (Qiagen, Crawley, UK) and according to the manufacturer's instructions. Subsequently, RNA integrity was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Stockport, UK) and samples with a RIN ≥ 5 used for gene expression analysis. Finally, 2 µg of RNA was reverse transcribed using Superscript III first strand cDNA synthesis system (Invitrogen, Paisley, UK), according to manufacturer's instructions. Following transfer of cDNA samples to Cardiff University, gene expression was analysed by qPCR as described in section 2.1.14.

2.4. Animal model

In order to investigate the effects of maternal pre-conception and prenatal diet on allelic expression of imprinted genes within the distal chromosome 7 region, *Mus mus domesticus* C57BL/6 mice carrying the *Mus spretus* region of distal chromosome 7 were used. Cdkn1c-RFLP mice were originally generated by crossing a *M.m* spretus male with a C57BL/6J female and selecting for the *Cdkn1c Aval* RFLP for > 8 generations. To generate placental and fetal material exposed to a variety of maternal diets, wildtype C57BL/6 female mice (aged 7 – 10 weeks) were mated with Cdkn1c-RFLP males. RFLP analysis of the *Cdkn1c Aval* polymorphism was used to determine which fetuses inherited the *Spretus* allele. In this way, RFLP analysis was used to determine the parent of origin of imprinted gene expression.

Animals were housed following Home Office Regulations and under the project licence 30/2600 (until November 2013) and 30/3134 (from November 2013). Females were checked daily for the presence of a vaginal plug, indicating a successful mating. The day on which a vaginal plug was observed was designated as embryonic day 0.5 (E0.5), and females were subsequently re-housed singly. Generation of and care for experimental mice was carried out by G. McNamara and S. Tunster.

2.4.1. Maternal diet

For the first experiment, the effect of maternal low protein or high fat diet during pregnancy was examined. Control females were fed a basal diet (diet energy from; protein 18.3%, fat 22.1%, carbohydrate 59.6%) (Test Diet, St. Louis, MO) before and during pregnancy. Both low protein and high fat diet females were similarly fed a basal diet before pregnancy. However, from E0.5 low protein diet females were placed on a low protein diet (diet energy from; protein 8.1%, fat 21.8%, carbohydrate 70.1%) (Test Diet, St. Louis, MO) whereas high fat diet females were placed on a high fat diet (diet energy from; protein 18.1%, fat 46.1%, carbohydrate 35.8%) (Test Diet, St. Louis, MO).

The second experiment design is shown in Figure 2.6. Before pregnancy females were placed on a basal (15% sugar content and diet energy from; protein 17.7%, fat 10%, carbohydrate 61%) (Test Diet, St. Louis, MO) or high fat - high sugar (HFHS) diet (34% sugar content and diet energy from; protein 17.8%, fat 20.0%, Carbohydrate 49.9%) (Test Diet, St. Louis, MO). Females were on the pre-pregnancy diet for 12 weeks and weighed every week to assess pre-pregnancy weight gain. At week 12 females were mated and from E0.5 till E18.5 females were fed either the basal or HFHS diet during pregnancy, as shown in Figure 2.6. Control-HFHS females were used to model effects of over nutrition specific to pregnancy. In addition, comparison of the HFHS-Control with the HFHS-HFHS females was used to model effectiveness of an intervention to improve maternal diet

during pregnancy. Mice were weighed at E0.5 and E18.5 to determine maternal weight gain during pregnancy.

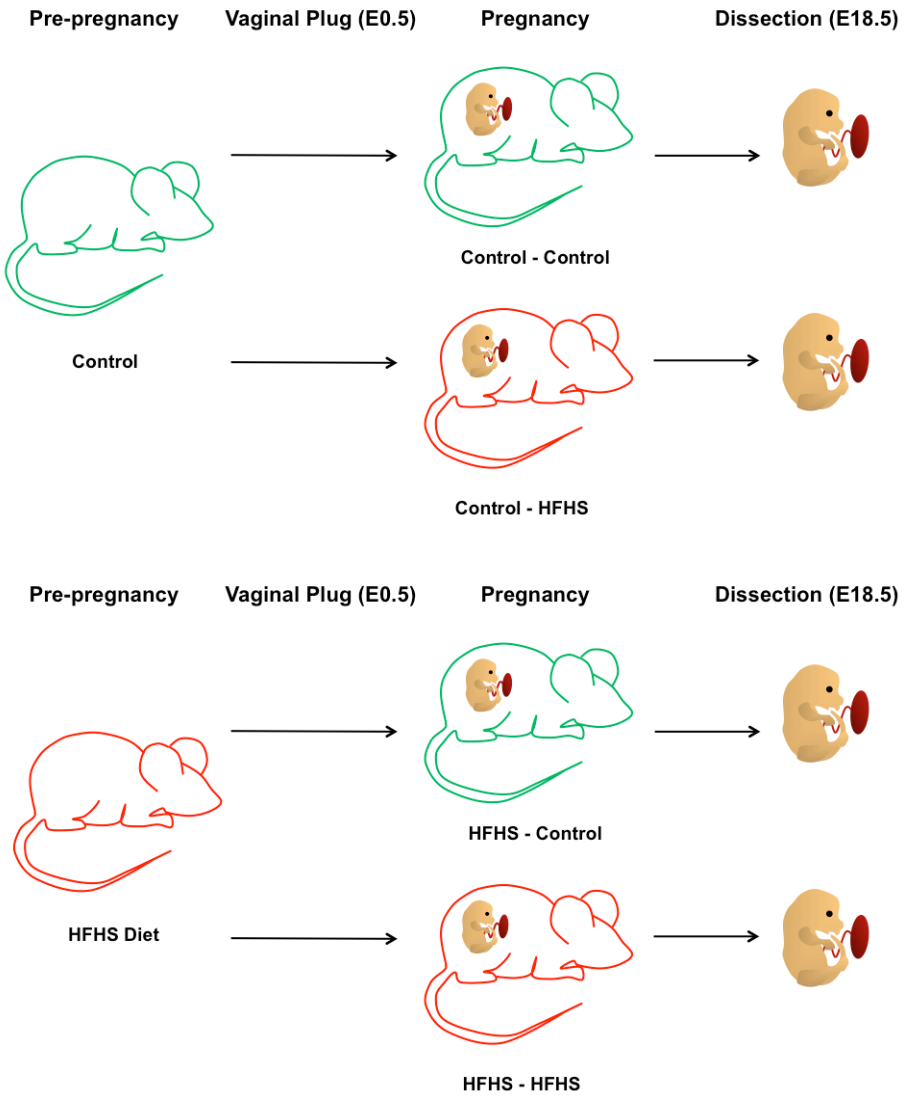


Figure 2.6: Study design for a mouse model of maternal high fat-high sugar diet before and during pregnancy. HFHS = high fat-high sugar diet. E = embryonic day.

Of note was the relative difficulty in achieving and maintaining pregnancy in the HFHS-Control and HFHS-HFHS females. 75% of control-control females and 57% of control-HFHS females mated produced a litter at E18.5. In contrast, although all 6 females in the HFHS-control group plugged, only 2 successfully delivered a litter (33% success rate), of which one

included three reabsorptions. Similarly, of the 5 females on the HFHS-HFHS diet that were mated, only 2 became pregnant (40% success rate) with both delivering litters including 2-3 reabsorptions. This contributed to significantly lower numbers of placentas for analysis in these two groups.

2.4.2. Dissection

Dissection was carried out at E13.5 and E18.5 for the low protein diet experiment in order to examine time specific effects of maternal protein restriction. For all other mice, dissection was carried out at E18.5. Females were killed by cervical dislocation at E18.5 by G McNamara and S Tunster. The uterus was placed in ice cold PBS and the embryos killed under schedule 1 by separation from the yolk sac (ASPA 1986). Dissection was carried out by A Janssen and G McNamara. The embryos and placentas were weighed. Embryo and placental weight was adjusted to account for differences in litter size using the following formula:

$$\frac{\text{Mean weight for diet}}{\text{Mean weight for litter}} \times \text{individual weight}$$

The kidneys were dissected out of the embryos and stored at - 80°C. The placentas were then frozen immediately at - 80°C until processing for qPCR (as described in section 2.1.10 – 2.1.15). Yolk sacs were stored at -20°C for RFLP and sex typing (Section 2.4.3).

2.4.3. RFLP and sex typing

RFLP analysis was performed by G McNamara and R John. Yolk sacs were digested in 300µl lysis buffer (0.1M Tris.HCl pH 8.5, 0.005 M EDTA pH 8.0, 0.02% SDS, 0.2M NaCl, 100 µg/ml Proteinase K (Promega, UK)) at 55-60°C overnight. Samples were then vortexed and centrifuged at 13,000 rpm for 14 minutes before transfer of the supernatant to a new 1.5ml Eppendorf. A 1X volume of isopropanol was added and the samples mixed thoroughly before incubation at 4°C for 30 minutes. After vortexing briefly, samples were

centrifuged at 13,000 rpm for 12 minutes with the gDNA forming a pellet. The supernatant was discarded and the resulting pellet air dried at room temperature for 10 minutes. The pellet was then resuspended in 80 µl TE buffer (10mM Tris pH8, 0.1mM EDTA pH8). PCR was carried out using 1µl of the resulting solution as the PCR template.

Table 2.5: Master mix (1X) PCR components for RFLP and sex typing

Reagent	**Volume (µl)**
Buffer 10X with 15 mM $MgCl_2$ (Qiagen)	2.5
$MgCl_2$ (25 mM)	2
dNTPs (4 mM)	2
Forward (25 µM) and Reverse (25 µM) Primers	1
HotStart Taq (Qiagen)	0.25
Template DNA	1
dH_20	16.25 µl

Table 2.5 shows the master mix volumes for PCR. For sex typing, PCR conditions were as follows; 94°C for 15 minutes, 94°C for 45 seconds, 61°C for 45 seconds, 72°C for 45 seconds, repeat 35 times then 72°C for 5 minutes. Primer sequences for sex typing were Ssty forward: 5'-CT GGAGCTCTACAGTGATGA-3' and Ssty reverse: 5'-CAGTTAC CAATCAACACATCAC-3', Om1a forward: 5'-TTACGTCCATCGTGGAC AGCAT-3' and Om1a reverse: 5'-TGGGCTGGGTGTTAGTCTTAT-3'. PCR products were run on a 1% TAE agarose gel with ethidium bromide and visualised under UV. Products were compared against a DNA ladder to confirm correct product size. Primers used for sex typing amplified an autosomal linked gene (*Om1a*) and a Y linked gene (*Ssty*), with the presence of two bands indicating male sex and a single band female sex.

For the RFLP analysis, PCR conditions were as described above (Table 2.5) for *Phlda2* and *Cdkn1c* with Tm of 58°C and 64.5°C, respectively. Primers for *Cdkn1c* RFLP were 5'-GGCTTCAGATCTGACCTCAG-3' and 5'-AGAGAGGCTGGTCCTTCAGC-3'. For the *Phlda2* RFLP, Primer E36 was 5'-

TCAGCGCTCTGAGTCTGAAA-3' and primer R647 was 5'-TCCTGGGCTCCTGTCTGAT-3' (Figure 2.7). Samples amplified by PCR from either genomic DNA or cDNA were digested to show the presence of the different alleles. For the *Cdkn1c* digest, 10 µl *Cdkn1c* PCR product was added to 3 µl 10X Buffer 4, 0.5 µl *AvaI* enzyme (NEB) and 16.5 µl H_20. The samples were then digested for at least 8 hours at 37 °C. The RFLP for *Cdkn1c* is further described in Li *et al*. (2005).

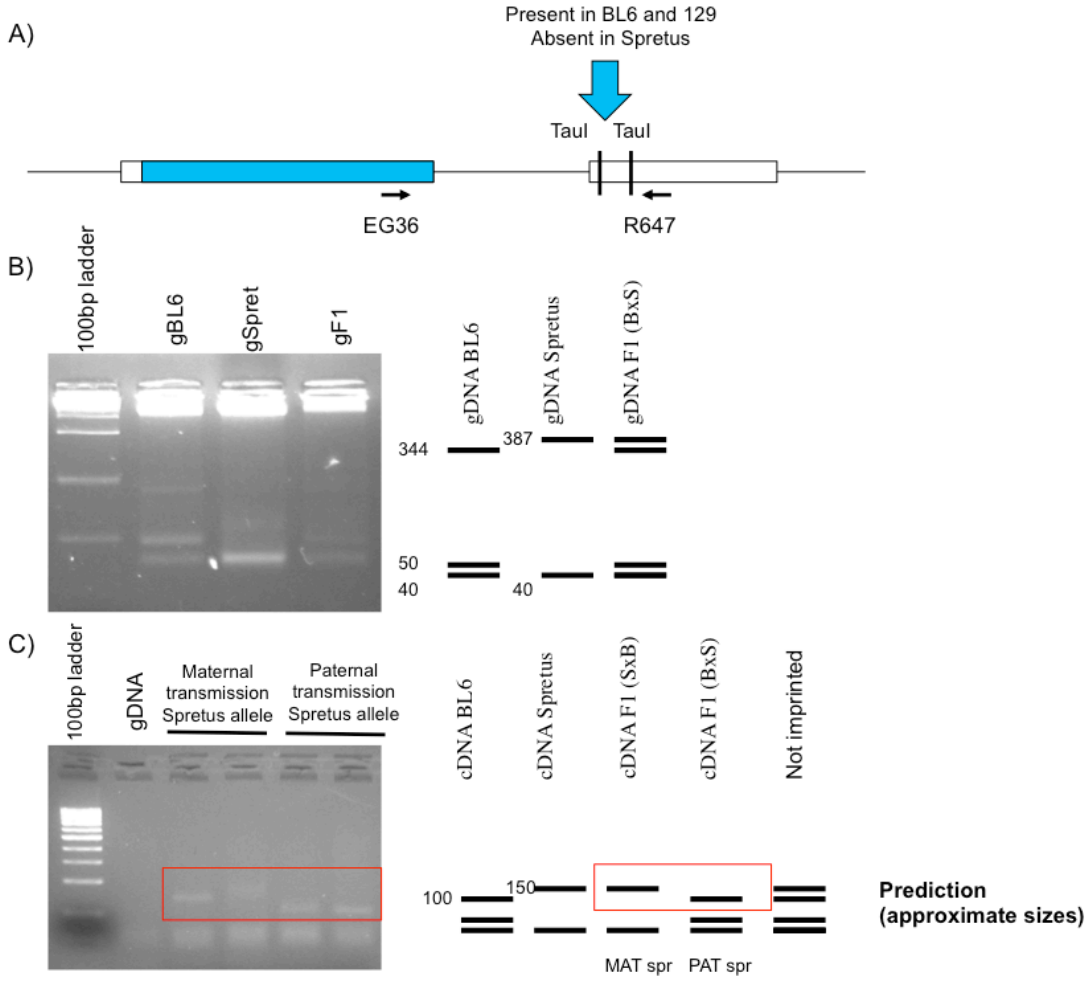


Figure 2.7: *Phlda2* RFLP. (A) *Phlda2* genomic locus. Arrows indicate position of primers EG36 and R647 used to in PCR and boxes indicate exons. TaqI restriction enzyme cut sites are also shown. (B) Validation of *Phlda2* RFLP using genomic DNA with predicted band sizes shown on the right. (C) RFLP analysis using cDNA with predicted band sizes shown on the right. Red box indicates position of predicted bands on gel image.

For the *Phlda2* digest, 10 µl *Phlda2* PCR product was added to 3 µl 10X Buffer B (ThermoScientific 10X Buffer B with BSA), 0.5 µl *TaqI* enzyme

(ThermoScientific *TauI* enzyme 3U/µl) and 16.5 µl H_20. The samples were then digested for 8 hours at 55 °C. A sample of genomic DNA was used as a control in both cases and to validate the *Phlda2* RFLP (Figure 2.7). The products were analysed by gel electrophoresis as described in section 2.1.13. The *TauI* restriction enzyme recognises a sequence on the C57BL/6 *Phlda2* allele and cuts the cDNA, resulting in the presence of two bands on the gel approximately 100bp and 50bp in length respectively (Figure 2.7). In contrast, a polymorphism in the *Spretus* sequence means that the *TauI* enzyme cannot recognise the TauI cut sites, resulting in the presence of a larger (undigested) band of 150bp in length (Figure 2.7).

In this way, it is possible to identify the parental origin of the *Phlda2* allele; because *Phlda2* is a maternally expressed imprinted gene, inheritance of the maternal *Spretus* allele by the F1 offspring will result in the presence of one larger undigested band of 150bp in length. In contrast, inheritance of the paternal *Spretus* allele by the F1 offspring (which when imprinted is not expressed) will result in the presence of two (digested) bands of 100bp and 50bp in length. Thus, loss of imprinting (resulting in both maternal and paternal expression of *Phlda2*) will be indicated by the presence of two bands at 150bp and 100bp in length as shown in Figure 2.7.

CHAPTER 3: IMPRINTED GENES AND FETAL GROWTH

3.1. Introduction

Fetal growth restriction (FGR) is the failure of a fetus to achieve its optimal growth potential during gestation (Ergaz *et al.* 2005), and is associated with an increased risk of adverse infant outcomes around the time of delivery as well as diseases such as type II diabetes and cardiovascular disease in adulthood (Godfrey and Barker 2001). Further understanding of the causes of growth restriction is therefore essential for the prevention of adverse short and long term effects on offspring health. It is possible that imprinted genes have a causative role in fetal growth restriction, given their well-established functions in the control of fetal growth. Thus, the main aim of this chapter was to explore a possible association between aberrant placental imprinted gene expression and FGR.

In particular, a number of animal and human studies have investigated the role of the maternally expressed imprinted gene *PHLDA2* in fetal growth restriction (reviewed in Jensen *et al.* 2014). Two fold over expression of *Phlda2* in a mouse model resulted in stunted placental growth combined with asymmetric fetal growth restriction in late gestation (Tunster *et al.* 2010; Tunster *et al.* 2014). Increased placental *PHLDA2* expression has similarly been reported in human growth restricted pregnancies (McMinn *et al.* 2006; Diplas *et al.* 2009; Kumar *et al.* 2012), with an estimated 25% of the IUGR placentas analysed displaying aberrant *PHLDA2* expression (McMinn *et al.* 2006). However, a relatively small number of placentas were examined in these studies, with the largest study analysing only 38 IUGR placentas (Diplas *et al.* 2009). Thus, larger cohort studies are required to fully determine whether aberrant placental *PHLDA2* expression plays a role in fetal growth restriction. In addition, previous studies have not distinguished between asymmetric and symmetric growth restriction. Tunster *et al.* (2014) observed asymmetric growth restriction as a result of *Phlda2* over expression in a mouse model, however it is not certain whether placental *PHLDA2* expression is similarly associated with asymmetric growth restriction in human pregnancies. Finally, no study to date has analysed placental *PHLDA2* expression in relation to birth outcomes. This would determine

whether placental *PHLDA2* expression could serve as a biomarker to identify at risk infants (Jensen *et al.* 2014).

The imprinted genes *Cdkn1c*, *Peg3* and *Peg10* have also been demonstrated to regulate fetal growth in an animal model. Overexpression of the maternally expressed imprinted gene *Cdkn1c* was associated with pre- and postnatal growth restriction (Andrews *et al*. 2007). Loss of function of the paternally expressed imprinted gene *Peg3* was also associated with fetal growth restriction and impaired placental growth (Li *et al.* 1999; Kim *et al.* 2013). Finally, loss of function of the paternally expressed imprinted gene *Peg10* resulted in embryonic lethality attributed to a severe placental defect (Ono *et al.* 2006). Despite convincing data in an animal model, the expression of *CDKN1C*, *PEG3* and *PEG10* in the placenta of human growth restricted pregnancies has been less extensively researched and with conflicting results. Both increased (McMinn *et al.* 2006), decreased (Rajaraman *et al.* 2010) and unaltered (Diplas *et al*. 2009) *CDKN1C* expression has been reported in IUGR placentas. Placental *PEG3* expression was not significantly altered in growth restricted pregnancies in three small studies (McMinn *et al.* 2006; Diplas *et al.* 2009; Kumar *et al.* 2011). Finally, placental *PEG10* expression was significantly increased in IUGR placentas (Diplas *et al.* 2009). Thus, further research is required as to the role of aberrant placental *CDKN1C*, *PEG3* and *PEG10* expression in human FGR pregnancies.

Importantly, differences in placental dissection protocol may account for the conflicting results reported in previous studies, particularly of human placental *CDKN1C* expression in relation to fetal growth. A considerable number of factors have been reported to affect placental gene expression such as time to sampling, sampling site, fetal sex and mode of delivery (Burton *et al.* 2014). Increased time between delivery and sampling of the placenta has previously been demonstrated to result in increased RNA degradation and subsequent effects on placental gene expression (Avila *et al.* 2010). In terms of sampling site, a number of studies have demonstrated significant intraplacental variation in gene expression (Hempstock *et al.* 2003; Pidoux *et al.* 2004; Wyatt *et al*. 2005; Sood *et al*. 2006) and thus consistency

in placental sampling is of key importance. Fetal sex is another important consideration in study design, given the sexual dimorphism observed in placental gene expression, particularly of genes related to fetal growth, both under normal conditions (Buckberry *et al.* 2014) and in response to an adverse intrauterine environment (Clifton 2010). Mode of delivery represents another potential confounding factor in analysis of placental gene expression. Decreased placental blood supply during contractions, exposure to hormones associated with labour as well as differences in pain relief (Burton *et al.* 2014) may account for differences in gene expression observed between labouring and non labouring placentas (Cindrova-Davies *et al.* 2007; Sitras *et al.* 2008; Lee *et al.* 2010; Peng *et al.* 2011; Kim *et al.* 2013b). Gestational age differences in infant morbidity and mortality exist within term pregnancies (Crump *et al.* 2013) and the incidence and type of placental pathologies has also been demonstrated to vary between different term gestational ages (Stanek 2014). Therefore, although not previously examined, it is possible that gestational age differences, even within term pregnancies, may affect placental gene expression. While no previous study has examined placental imprinted gene expression in relation to these potential confounding factors, this is likely to be of relevance in the design of future studies.

It has been proposed that imprinted genes, such as *PHLDA2*, may regulate fetal growth through control of mechanisms driving maternal adaptation to pregnancy (Haig 1993), such as placental hormone production (John 2013). Indeed, the imprinted genes *Phlda2*, *Cdkn1c*, *Peg3* and *Peg10* have been demonstrated to regulate the endocrine lineage of the mouse placenta, thereby indirectly controlling production of placental hormones such as placental lactogens (reviewed in John 2013). The human placenta differs in several ways from the rodent placenta (Carter 2012) and therefore it is important to investigate whether the observations of imprinted gene control of placental hormone production in a mouse model has relevance to humans. In humans, the placental hormones hPL and PGH control fetal growth by driving maternal metabolic adaptations to pregnancy such that preferential transfer of nutrients to the fetus occurs while maintaining maternal homeostasis (Newbern and Freemark 2011). It is therefore possible that aberrant

imprinted gene expression could result in FGR via negative effects on placental production of hormones such as hPL and PGH. No previous study has analysed the relationship between placental imprinted gene expression and expression of the placental hormones *hPL* and *PGH*, in the context of growth restriction.

Fetal overgrowth, resulting in delivery of an LGA or macrosomic infant, is also of clinical relevance given the increased risk of adverse outcomes in these infants around the time of delivery, as well as the increased risk of associated birth traumas for the mothers (Zhang *et al.* 2008; Larkin *et al.* 2011; Pasupathy *et al*. 2012). As imprinted genes have a well-established role in the control of fetal growth, it is possible that aberrant placental imprinted gene expression also underlies cases of fetal overgrowth. As discussed above, previous studies of placental imprinted gene expression in relation to fetal growth have focussed on FGR and therefore no study to date has examined placental imprinted gene expression in macrosomic or LGA placentas.

In summary, while results from previous animal and human studies suggest a role for placental imprinted gene expression in the control of human fetal growth, further research is warranted in larger cohorts to confirm these findings. This chapter therefore aimed to examine placental imprinted gene and placental hormone gene expression in pregnancies complicated by growth restriction or fetal overgrowth.

3.2. Chapter specific methods

Methods were as described in Chapter 2. For ease of interpretation of the results presented in this chapter, methods related to the study of placental target gene expression in pregnancies complicated by FGR and fetal overgrowth are summarised below. In this Chapter, gene expression was analysed in placentas from two cohorts, the Wales and Manchester Cohorts.

3.2.1. Placental dissection protocol optimisation

To control for any effect of time to sampling on RNA concentration and placental target gene expression, placental samples were taken at 30 minutes, 1 hour, 1.5 hours and 2 hours after delivery for three participants delivering AGA infants by elective C-section. RNA was extracted from placental samples taken at all time points and RNA concentrations compared. In addition, placental target gene expression was compared between time points.

In order to analyse intraplacental variation in target gene expression, three samples (close to the cord insertion site, middle and distal edge) were taken from each of the fetal, middle and maternal layers of the placenta as described in Wyatt *et al.* (2005). This was carried out for 3 placentas from elective C-sections.

Placental target gene expression was also compared between early term (37 – 38 weeks), full term (39 – 40 weeks) and late term (≥ 40 weeks) gestational age groups for 19 control participants to determine the effect of gestational age on gene expression. In order to examine the effect of fetal sex on placental target gene expression, gene expression was also compared between 11 male and 11 female control placentas from normal birth weight, elective C-section deliveries. Finally, placental gene expression was compared between 29 participants delivering by elective C section (N = 21), emergency C section (N = 4) or vaginal delivery (N = 4) in order to examine the effect of labour or mode of delivery on placental target gene expression.

3.2.2. Statistical analysis

All statistical analysis was carried out using IBM SPSS statistics for Windows (version 20.0, 2011) with a p value <0.05 considered statistically significant. Normal distribution was assessed using P-P plots and a Shapiro-Wilk test. For normally distributed data, associations between placental gene expression and measures of fetal growth were assessed using a Pearson correlation test while differences in placental gene expression were analysed using an independent samples T tests (to compare e.g. control and IUGR pregnancies) or one-way ANOVA with a Tukey post hoc test (to compare e.g. AGA, symmetric SGA and asymmetric SGA pregnancies). Any significant associations with gene expression were confirmed with and without the addition of preterm deliveries. Linear regression analysis was used to determine the proportion of variation in fetal growth measures accounted for by differences in target gene expression. A Bonferroni correction was also used to control for multiple comparisons in target gene expression between SGA and AGA placentas ($\alpha/6 = 0.008$).

Only placental *PHLDA2* expression in the Manchester cohort remained non-normally distributed (also after ln transformation); In this cohort, a Spearman rank order correlation test was used to determine the association between placental *PHLDA2* gene expression and measures of fetal growth while differences in *PHLDA2* expression between groups was analysed using a Mann Whitney U test.

For all protocol optimisation experiments, a small number of matched control participants delivering normal birth weight infants were chosen for gene expression analysis to control for variation due to differences in maternal demographics or birth outcomes. In order to examine intraplacental variation in target gene expression, gene expression was first analysed relative to sampling site 1A (on the basal surface close to the cord insertion site) to determine the variation in gene expression across all sampling sites as described in Wyatt *et al.* (2005). Subsequently, gene expression was compared between different regions of the placenta (basal versus chorionic sampling sites and cord versus distal sampling sites).

3.3. Results

3.3.1. Protocol optimisation

3.3.1.1. Time to sampling

For three control participants, placental tissue samples were taken at the same sampling site at four time points; 30 minutes, 1 hour, 1.5 hours and 2 hours after delivery in order to determine the effect of time to sampling on RNA concentration and placental target gene expression.

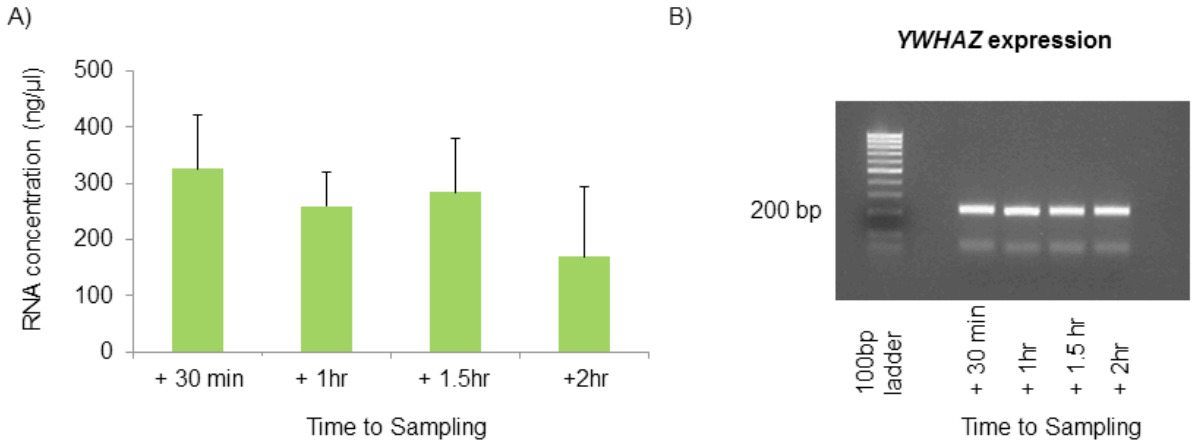


Figure 3.1: Effect of time to sampling on (A) RNA concentration and (B) *YWHAZ* expression. (A) There was no significant effect of time to sampling on RNA concentration as determined by one-way ANOVA. (B) PCR analysis revealed RNA of sufficient quality at all sampling time points as indicated by consistently high expression of the housekeeping gene *YWHAZ*. Error bars represent SD.

There was no significant effect of time to sampling on placental RNA concentration as determined by a one-way ANOVA ($F(3,8) = 0.46$, $p = 0.72$), Figure 3.1. There was also no significant correlation between expression of the housekeeping gene *YWHAZ* and time to sampling ($r = 0.43$, $p = 0.16$, $n = 12$), as confirmed by PCR analysis demonstrating sufficient quality RNA for gene expression analysis from placental samples taken at all time points (Figure 3.1). Finally, there was no significant effect of time to sampling on placental *PHLDA2* ($F(3,8) = 0.65$, $p = 0.61$), *CDKN1C* ($F(3,8) = 0.94$, $p = 0.47$), *PEG3* ($F(3,8) = 0.74$, $p = 0.56$), *PEG10* ($F(3,8) = 0.53$, $p = 0.68$), *hPL*

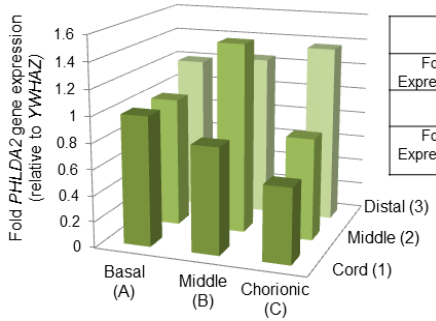
(F(3,8) = 0.02, p = 0.99) or *PGH* (F(3,8) = 0.05, p = 0.98) expression as determined by qPCR. These results therefore suggest that gene expression analysis can be carried out for samples taken up to 2 hours after delivery without a significant effect on RNA concentration or target gene expression.

3.3.1.2. Intraplacental variation in gene expression

For three AGA participants, tissue samples were taken at different sites across the placenta and gene expression compared to determine the effect of sampling site on target gene expression. Variation in target gene expression with sampling site is shown in Figure 3.2, relative to sampling site 1A (close to the cord insertion and on the basal surface of the placenta).

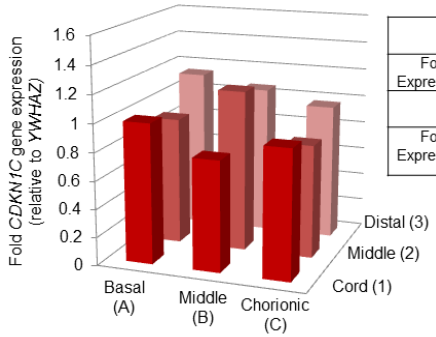
As shown in Figure 3.2, placental *PHLDA2* expression was significantly increased by 59% in samples taken at distal sites (3 A – C) compared with sites close to the cord insertion (1 A – C) (p = 0.048, n = 18). In contrast no significant difference in placental *PHLDA2* gene expression was seen between basal (1 – 3 A) and chorionic sites (1 – 3 C) (p = 0.52, n = 18). No significant difference was observed in placental *CDKN1C*, *PEG3* or *PEG10* expression relative to sampling site 1A (Figure 3.2). Similarly, there was no significant difference in expression at distal sites compared with sites close to the cord insertion or between basal and chorionic sites. There was a significant 52% increase in placental *hPL* expression at site 3B (distal, middle layer) compared with site 1A (cord insertion site, basal layer) (p = 0.002, n = 18) Figure 3.2. However, there was no significant difference in placental *hPL* expression between site 1A and any other sampling site, or between distal sites compared with sites close to the cord insertion or between basal and chorionic sites. Finally, no significant difference in placental *PGH* expression was observed relative to sampling site 1A (see Figure 3.2) and expression did not differ significantly between distal sites and sites close to the cord insertion or between basal and chorionic sites.

A)



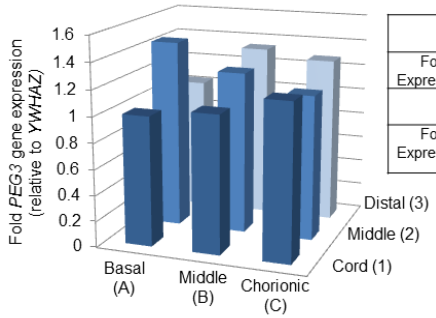
	Basal (1A – 3A)	Chorionic (1C – 3C)	P value
Fold Expression	1.00	0.85	0.52
	Cord (1A – 1C)	**Distal (3A – 3C)**	**P value**
Fold Expression	1.00	1.59	< 0.05*

B)



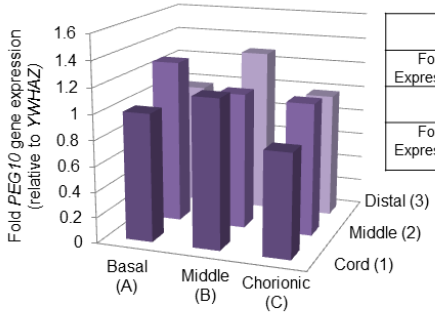
	Basal (1A – 3A)	Chorionic (1C – 3C)	P value
Fold Expression	1.00	0.89	0.47
	Cord (1A – 1C)	**Distal (3A – 3C)**	**P value**
Fold Expression	1.00	0.85	0.52

C)



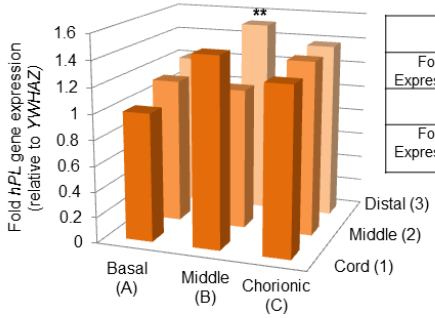
	Basal (1A – 3A)	Chorionic (1C – 3C)	P value
Fold Expression	1.00	1.04	0.83
	Cord (1A – 1C)	**Distal (3A – 3C)**	**P value**
Fold Expression	1.00	1.12	0.51

D)



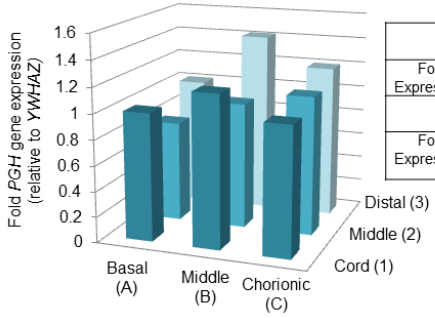
	Basal (1A – 3A)	Chorionic (1C – 3C)	P value
Fold Expression	1.00	0.87	0.43
	Cord (1A – 1C)	**Distal (3A – 3C)**	**P value**
Fold Expression	1.00	1.10	0.66

E)



	Basal (1A – 3A)	Chorionic (1C – 3C)	P value
Fold Expression	1.00	1.21	0.09
	Cord (1A – 1C)	**Distal (3A – 3C)**	**P value**
Fold Expression	1.00	1.10	0.51

F)



	Basal (1A – 3A)	Chorionic (1C – 3C)	P value
Fold Expression	1.00	1.18	0.17
	Cord (1A – 1C)	**Distal (3A – 3C)**	**P value**
Fold Expression	1.00	1.13	0.48

Figure 3.2: Effect of sampling site on placental target gene expression. Samples were taken from the cord insertion site to distal edge (1-3) and from the basal to chorionic surface (A – C). Graphs show mean fold gene expression, relative to sampling site 1A (Cord, Basal). Tables summarize differences in gene expression between basal and chorionic sites and between cord and distal sites. * $p \leq 0.05$, ** $p \leq 0.001$.

3.3.1.3. Effect of gestational age on placental gene expression

Placental gene expression was compared between gestational age groups for 19 controls participants. Birth outcomes for early term (37 – 38 weeks), full term (39 – 40 weeks) and late term (≥ 40 weeks) deliveries are shown in Table 3.1. Aside from gestational age, there was no significant difference in any other birth outcome between these groups.

There was no significant correlation between gestational age at delivery and placental *PHLDA2* (r = 0.24, p = 0.33, n = 19), *CDKN1C* (r = 0.21, p = 0.39, n = 19), *PEG3* (r = 0.35, p = 0.14, n = 19) or *PEG10* expression (r = 0.34, p = 0.15, n = 19). Similarly, there was no significant difference in expression between early term, full term and late term deliveries as determined by a one-way ANOVA (Figure 3.3). *PHLDA2* and *PEG3* expression was greatly increased in the late term placentas examined but due to the small number of participants and the large variation in gene expression in this group, these differences were not statistically significant.

Table 3.1: Birth Outcomes of early, full and late term participants used for methods optimisation. Mean (SD)/Range or Number (%) is shown. Groups were compared with early term deliveries using a one-way ANOVA or Chi squared test as appropriate. *** p < 0.001.

	Early Term (37 – 38 weeks)	**Full Term (39 – 40 weeks)**	**Late Term (>40 weeks)**	**P value**
Gestational Age (days)	266 (4.6) / 259 - 272	279 (4.6) / 273 - 285	290 (3.6) / 287-294	**< 0.001** ***
Birth Weight (g)	3398 (112) / 3020 - 3920	3488 (159) / 3180 - 3740	3823(284) / 3500 - 4030	0.10
Placental Weight (g)	651(112) /510 – 835	760 (159) /531 - 970	666 (135) /580 - 822	0.40
Fetal Sex				
Male	4 (44%)	4 (57%)	1 (57%)	0.76
Female	5 (56%)	3 (53%)	2 (53%)	

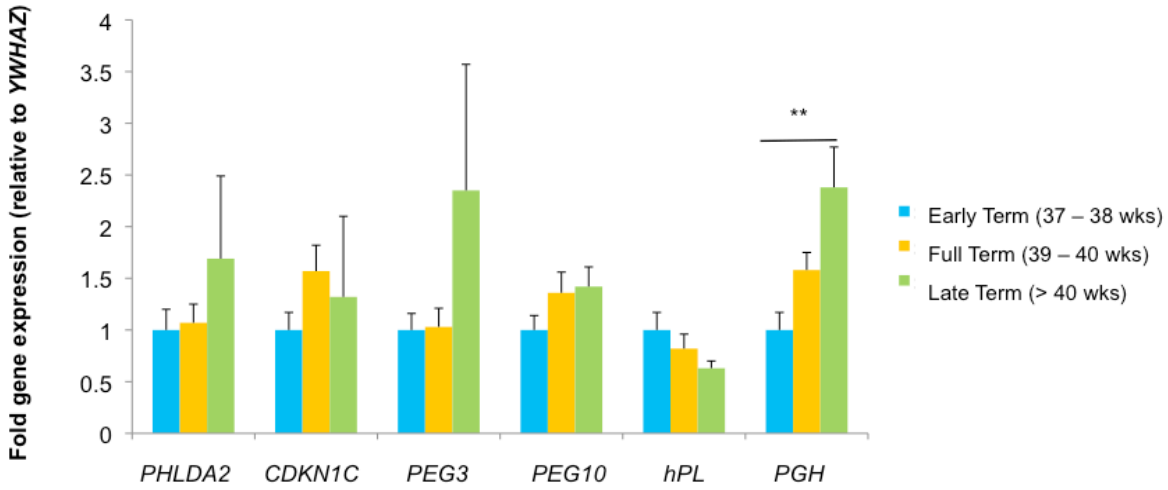


Figure 3.3: Effect of gestational age on target gene expression. Fold gene expression is shown relative to early term deliveries. Differences in fold gene expression were analysed using a one-way ANOVA and Tukey Post hoc test. ** $p < 0.01$. Error bars represent SEM.

There was no significant correlation between gestational age at delivery and placental *hPL* expression (r = - 0.32, p = 0.18, n = 19) and no significant difference in gene expression between gestational age groups (Figure 3.3). However, there was a significant positive correlation between gestational age at delivery and placental *PGH* expression (r = 0.70, p = 0.001, n = 19). Placental *PGH* was also significantly different between gestational age groups (F (2,16) = 8.41, p = 0.003, n = 19); a Tukey post hoc test revealed a significant increase in *PGH* expression in late term compared with early term deliveries (Figure 3.3).

3.3.1.4. Effect of fetal sex on placental gene expression

Placental gene expression was compared between 11 male and 11 female AGA infants to determine the effect of fetal sex on target gene expression. There was no significant difference in birth outcomes as shown in Table 3.2. Placental target gene expression did not differ significantly between male and female placentas as shown in Figure 3.4.

Table 3.2: Birth outcomes of male and female infants used for methods optimisation. Mean (SD)/Range is shown. Birth outcomes for female infants were compared with those for male infants using an independent samples T-test with resulting P values shown.

	Male (N = 11)	Female (N = 11)	P value
Gestational Age (days)	274 (8.2) / 263 - 289	274 (11.4) / 259 - 274	0.85
Birth Weight (g)	3380 (215) / 3100 - 3780	3600 (305) / 3020 - 4030	0.07
Placental Weight (g)	663 (114) /531 – 859	742 (143) /510 - 970	0.17

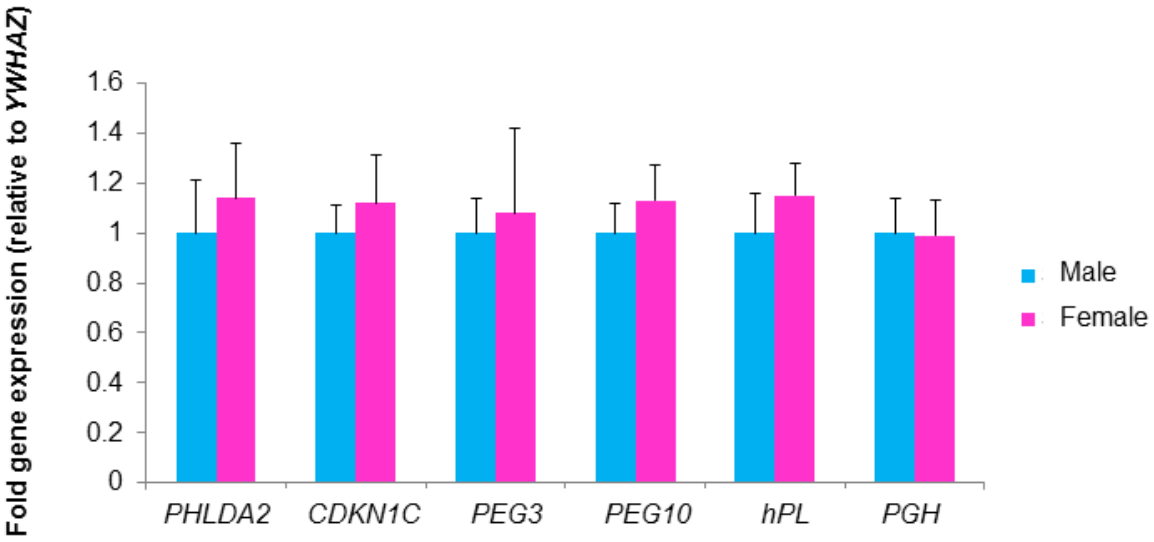


Figure 3.4: Sex differences in placental target gene expression. Fold gene expression is shown relative to expression in male placentas. Error bars represent SEM. Differences in gene expression were not statistically significant using a t-test.

3.3.1.5. Effect of mode of delivery on placental gene expression

Placental gene expression was compared between 29 participants delivering by elective C section (ELCS), emergency C section (EMCS) or vaginal delivery (SVD) to determine the effect of mode of delivery on target gene

expression. There was no significant difference in birth outcomes between different modes of delivery, as shown in Table 3.3.

Table 3.3: Birth outcomes by mode of delivery. Mean (SD)/Range or Number (%) is shown. Groups were compared using a one-way ANOVA or Chi squared test as appropriate.

	Elective C-section (N = 21)	Emergency C-section (N = 4)	Vaginal Delivery (N = 4)	P value
Gestational Age (days)	273 (9.5) / 259 - 294	280 (13.5) / 266 – 296	273 (8.5) / 266-285	0.91
Birth Weight (g)	3469 (269) / 3020 - 4030	3532 (381) / 3160 - 3940	3502 (284) / 3290- 3850	0.42
Placental Weight (g)	707 (134) /510 – 970	657 (98) /552 - 744	633 (149) /434 - 788	0.51
Fetal Sex				
Male	11 (52%)	1 (25%)	2 (50%)	0.60
Female	10 (48%)	3 (75%)	2 (50%)	

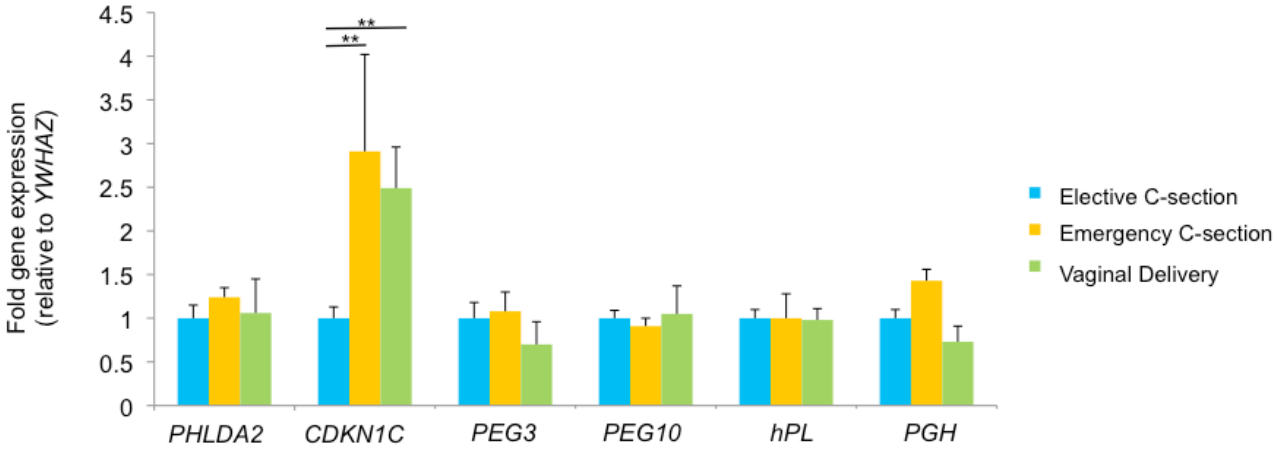


Figure 3.5: Effect of mode of delivery on target gene expression. Fold gene expression is shown relative to elective c section deliveries. Differences in fold gene expression were analysed using a one-way ANOVA and Tukey Post hoc test. ** $p < 0.01$. Error bars represent SEM.

As shown in Figure 3.5, there was no significant difference in placental *PHLDA2* (p = 0.32, n = 29), *PEG3* (p = 0.79, n = 29), *PEG10* (p = 0.77, n = 29), *hPL* (p = 0.96, n = 29) or *PGH* (p = 0.69, n = 29) expression between varying modes of delivery. However, *CDKN1C* expression was significantly 2.9 fold higher in placentas of women delivering by EMCS and 2.5 fold higher in women delivering by SVD (Figure 3.5).

3.3.1.6. Methods optimisation summary

A summary of the changes in target gene expression according to time to sampling, sampling site, gestational age, fetal sex and mode of delivery is shown in table 3.4. There was no significant effect of time to sampling or fetal sex on expression of any target gene. There was however a significant effect of sampling site on *PHLDA2* expression, a significant positive association between *PGH* expression and gestational age and a significant increase in *CDKN1C* expression in emergency caesarean and vaginal delivery placentas.

Table 3.4. Summary of methods optimisation results. Percentage change in expression is shown relative to controls. EMCS = emergency caesarean section, SVD = spontaneous vertex delivery (vaginal delivery).

Gene	Time to Sampling	Sampling site	Gestational age	Fetal sex	Mode of delivery
PHLDA2	-	↑ 59% at distal sites	-	-	-
CDKN1C	-	-	-	-	↑ 190% in EMCS, ↑ 150% in SVD
PEG3	-	-	-	-	-
hPL	-	-	-	-	-
PGH	-	-	Positive association	-	-

3.3.2. Fetal growth restriction: Wales cohort

3.3.2.1. SGA participant demographics

The characteristics of the Wales Cohort participants (N=219) are shown in Table 3.5. The cohort included 67 participants delivering SGA infants (with no other pregnancy complication) of which 34 were also LBW infants (< 2,500g).

Participants delivering SGA infants did not differ significantly in terms of ethnicity ($p = 0.40$, $n = 158$) compared with controls, with 90% and 96% of participants, respectively, being Caucasian. There was also no significant difference in maternal age between groups ($p = 0.27$, $n = 158$).

Maternal BMI at booking was significantly reduced in participants with SGA infants (24.5 v. 26.7, $p = 0.02$, $n = 219$). This reduction was significant for both maternal weight (64.1 kg v. 71.4 kg, $p = 0.003$, $n = 138$) and height (1.61m v. 1.64 m, $p = 0.01$, $n = 115$) independently. Similarly, the participants own birth weight (self-reported) was significantly lower for SGA participants (3.03kg v. 3.29kg, $p = 0.02$, $n = 83$).

Birth outcomes for SGA and AGA participants are shown in Table 3.6. Due to sampling bias, a significantly smaller number of SGA participants delivered by elective c-section highlighting the need to control for mode of delivery in target gene expression analysis. In addition to the hypothesised differences in birth weight, placental weight and head circumference, gestational age was also significantly reduced in SGA participants (Table 3.6). This likely reflects the early delivery of severe IUGR cases. Finally, the number of infants admitted to NICU on delivery was significantly higher for SGA participants compared with controls (Table 3.6).

Table 3.5: Characteristics of Wales cohort participants (N=219). Mean (SD)/Range or Number (%) is shown.

	Mean (SD)/Range or Number (%)		Mean (SD)/Range or Number (%)
Birth Outcomes		**Maternal characteristics**	
Fetal Sex		*Ethnicity*	
Male	115 (52.5%)	Caucasian	207 (94.5%)
Female	104 (47.5%)	African/Afro-Caribbean	2 (1%)
		Indian/Pakistani/Bangladeshi	6 (2.7%)
Gestational age *(weeks)*	39 (1.40) / 29 – 42	Middle Eastern	1 (0.5%)
		Other	3 (1.3%)
Birth weight *(g)*	3214 (678) / 730 - 4990		
Custom birth weight centile	41 (32) / 0 - 100	Age (years)	30 (5.5) / 17 – 43
Placental Weight *(g)*	632 (160) / 226 – 1064	Maternal BMI	27 (6.06) / 17 – 49
Apgar Scores (1 min)	9 (1.37) / 0 - 10	Parity	1 (1.02) / 0 – 6
Apgar Scores (5 min)	10 (0.81) / 3 – 10		
		Smoking during Pregnancy	
Birth weigh Classification		Yes	52 (24%)
AGA only	91 (42%)	No	153 (70%)
SGA only	67 (31%)	Not reported	14 (6%)
LGA only	23 (11%)		
		Alcohol consumption	
NICU Admission		None	140 (64%)
Yes	7(3%)	1-5 units / week	69 (32%)
No	212 (97%)	Not reported	10 (4%)
Mode of Delivery		*Obstetric complications of Pregnancy*	
SVD	45 (20%)		
ELCS	113 (61%)	Preeclampsia/PIH	22 (7%)
EMCS	24 (11%)	Gestational diabetes	16 (10%)
Instrumental	17 (8%)		

Table 3.6: Comparison of birth outcomes between AGA and SGA participants. Mean (SD)/Range or Number (%) is shown. Differences were analysed using an independent samples T test or Chi – square test where appropriate.

	AGA Participants (N = 91)	**SGA participants** (N = 67)	**P Value**
Birth Outcome			
Mode of Delivery:			
Vaginal	7 (8%)	28 (42%)	
Elective C section	78 (86%)	22 (33%)	
Emergency C section	4 (4%)	8 (12%)	
Instrumental	2 (2%)	9 (13%)	**P < 0.001*****
Birth weight (g)	3480 (277)/ 2830 - 4190	2448 (446)/ 730 - 3250	**P < 0.001*****
Custom birth weight centile	53 (22) / 10 - 89	7 (7)/ 0 - 10	**P < 0.001*****
Head circumference (cm)	35 (1) / 32 - 39	33 (2) / 28 - 35	**P < 0.001*****
Gestational age (weeks)	39 (1) / 37 - 42	38 (2) / 29 - 41	**P < 0.001*****
Placental weight (g)	703 (124) / 309 - 905	500 (110) / 226 - 832	**P < 0.001*****
Gender			
Male	44 (48%)	32 (48%)	
Female	47 (52%)	35 (52%)	P = 0.80
Apgar score (1 min)	9 (1) / 4 - 10	8 (2) / 0 - 10	P = 0.07
Apgar score (5 min)	10 (0.6) / 7 – 10	9 (1)/ 3 - 10	P = 0.16
Arterial cord blood pH	7.3 (0.07) / 7.1 – 7.4	7.3 (0.08) / 7.1 – 7.5	P = 0.36
NICU admission			
No	91 (100%)	63 (94%)	**P = 0.01****
Yes	0 (0%)	4 (6%)	
Fetal distress during delivery			
No	77 (85%)	49 (73%)	
Yes	4 (4%)	4 (8%)	P = 0.32

3.3.2.2 Prenatal growth restriction and *PHLDA2* expression

Prenatal growth information was available for 136 AGA or SGA participants. Placental *PHLDA2* expression was significantly increased by 60% in participants where concern over fetal growth had been documented prenatally, including static growth, SGA centiles and/or low fundal height (Figure 3.6). However, there was no significant correlation between placental *PHLDA2* expression and week of first documented concern over fetal growth (r = 0.06, p = 0.67, n = 45).

Placental *PHLDA2* expression was also significantly increased in those participants where infants measured SGA by abdominal and head circumference on ultrasound scan (< 10th centile), Figure 3.6. However, there

was no significant correlation between placental *PHLDA2* expression and week of first reported SGA abdominal (r = - 0.08, p = 0.60, n = 48) or head (r = - 0.16, p = 0.28, n = 48) centile.

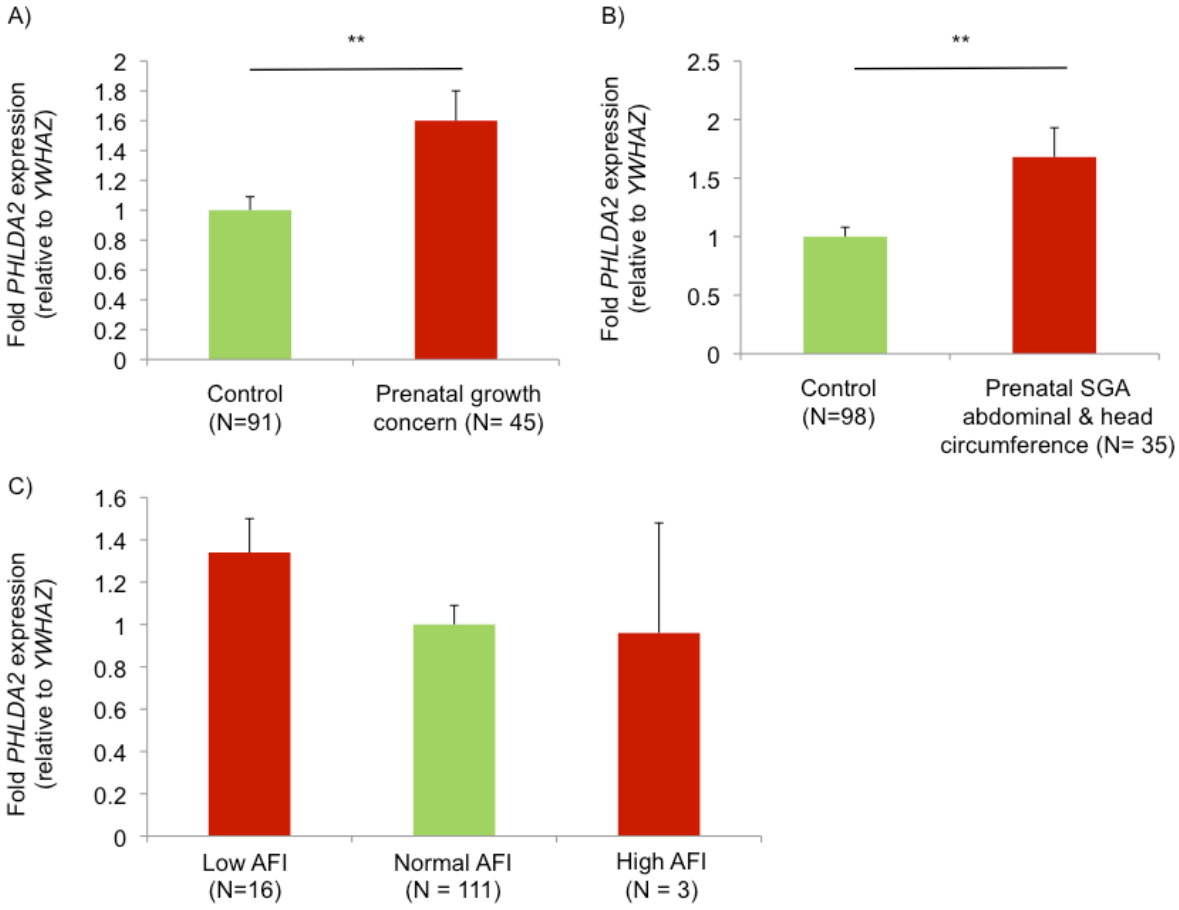


Figure 3.6: Placental *PHLDA2* expression and prenatal growth restriction. Placental *PHLDA2* expression was significantly increased in participants with prenatal growth concerns (A) and in infants measuring SGA by abdominal and head circumference on scan (B). Gene expression was not significantly altered in participants with low or high AFI (C). ** p < 0.01. AFI = amniotic fluid index. Error bars represent SEM.

Another prenatal indicator of fetal growth restriction is a reduction in amniotic fluid. Placental *PHLDA2* expression was increased in participants with a low AFI (amniotic fluid index) during pregnancy, however this difference was not statistically significant (Figure 3.6). Finally, ultrasound assessment of umbilical artery blood flow can also used to ascertain the presence and severity of growth restriction. Only one participant exhibited

Doppler abnormalities (reversed end diastolic flow) and therefore differences in placental target gene expression could not be analysed.

3.3.2.3. Fetal growth restriction and *PHLDA2* expression

The Wales Cohort included seven participants with infants delivered preterm. These participants had a prenatal IUGR diagnosis and indications for early delivery included IUGR, placental abruption, reduced fetal movements and fetal distress. Given that there was no significant correlation between placental *PHLDA2* expression and gestational age (r = - 0.11, p = 0.16, n = 181) and no significant difference in expression between term and preterm placentas (p = 0.31, n = 181), participants with preterm deliveries were included in the overall cohort analysis. Any significant associations with target gene expression were confirmed with and without the addition of these participants.

With the exclusion of any participants with complications of pregnancy (PE or GDM, see Chapter 4), there was a significant inverse correlation between placental *PHLDA2* expression and birth weight (r = - 0.25, p = 0.001, n = 181) and custom birth weight centiles (r = - 0.28, p <0.001, n = 181), Figure 3.7. Linear regression analysis indicated that placental *PHLDA2* expression accounted for 6% of variance in birth weight (F = 12.3, p = 0.001, n = 181) and 8% of variance in custom birth weight centiles (F = 13.96, p <0.001, n = 181). For every 1 CT increase in *PHLDA2* expression (corresponding to a doubling of gene expression) there was a 180g reduction in birth weight and a 9 centile reduction in custom birth weight centiles.

Placental *PHLDA2* expression was on average 2 fold higher in participants with SGA compared with AGA infants (Figure 3.7). 18 SGA placentas demonstrated ≥ 2 fold higher expression compared with controls, suggesting aberrant *PHLDA2* expression in 27% of the SGA participants. Interestingly, *PHLDA2* expression was also 2 fold higher than the AGA mean for 7 AGA placentas; this included one participant with a normal birth weight but a custom birth weight centile of 10.5. There was no obvious distinguishing feature of the other high *PHLDA2* expressing AGA cases.

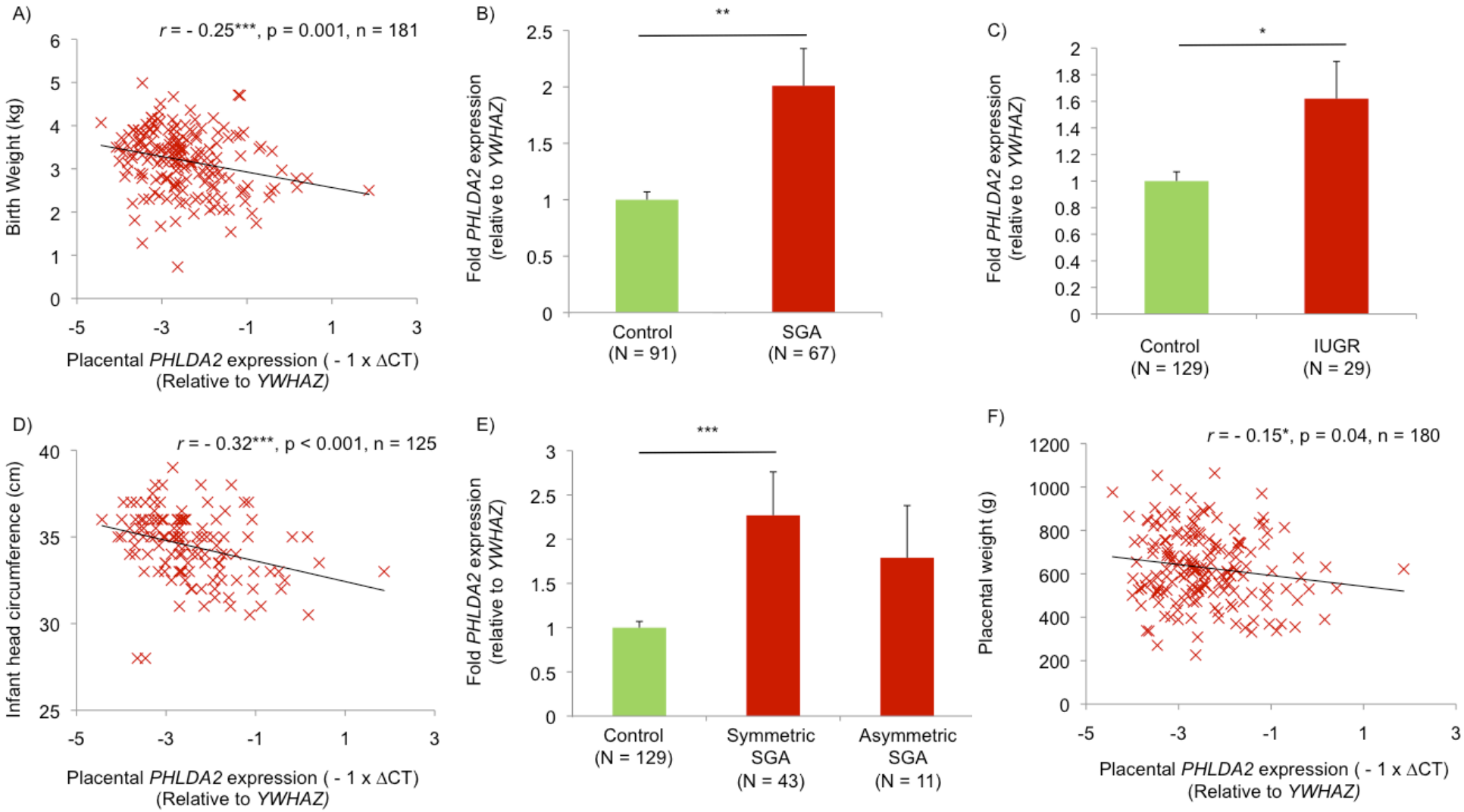


Figure 3.7: Placental *PHLDA2* expression and fetal growth. *PHLDA2* expression is shown in relation to birth weight (A), SGA (B) and IUGR (C) pregnancies, head circumference (D), asymmetric and symmetric growth restriction (E) and placental weight (F). Error bars represent SEM. * P <0.05, ** p < 0.01, *** p < 0.001.

PHLDA2 Expression was also significantly 62% higher in placentas of women delivering IUGR term infants (Figure 3.7) although there was no significant correlation between placental *PHLDA2* expression and week of IUGR diagnosis (r = - 0.08, p = 0.68,n = 29). In contrast, *PHLDA2* expression was not significantly altered in placentas of LBW infants (p = 52, n = 181), possibly as a result of the few number of participants (N=32) delivering LBW infants.

Head circumference at delivery was significantly inversely associated with placental *PHLDA2* expression (r = - 0.32, p < 0.001, n = 125), Figure 3.7. Linear regression analysis indicated that placental *PHLDA2* expression accounted for 1% of variance in head circumference (F = 13.83, p < 0.001, n = 125) with every 1 CT increase in *PHLDA2* expression associated with a 0.6cm reduction in head circumference.

There was also a significant inverse correlation between *PHLDA2* expression and placental weight (r = - 0.15, p = 0.04, n = 180). Linear regression analysis indicated that placental *PHLDA2* expression accounted for 2% of variance in placental weight (F = 4.34, p = 0.04, n = 180) with every 1 CT increase in *PHLDA2* expression associated with a 25g reduction in placental weight.

In order to determine whether there was any difference in placental gene expression between symmetric and asymmetrically growth restricted infants, prenatal ultrasound head (HC) and abdominal (AC) circumferences were analysed with an SGA abdominal circumference but not head circumference being indicative of brain sparing. A one-way ANOVA revealed a significant difference in *PHLDA2* expression between groups (F(2,142) = 6.73, p = 0.002) however expression was only significantly increased in SGA (symmetric) and not SGA (asymmetric) placentas compared with controls, Figure 3.7.

Finally, previous obstetric history was available for 46 of the 67 participants delivering SGA infants with 17% of participants having previously delivered a LBW infant. There was no significant difference in placental *PHLDA2* expression between these participants and those previously delivering a normal birth weight infant (p = 0.50, n = 46).

3.3.2.4. Poor perinatal outcomes and *PHLDA2* expression

In the overall cohort, 7 infants were admitted to NICU at delivery for respiratory distress. While placental *PHLDA2* expression was almost three fold higher in these placentas, there was a large variation in the NICU expression group and the difference was found not to be statistically significant (Figure 3.8). Similarly, *PHLDA2* expression was not significantly altered in infants exhibiting fetal distress during delivery (Figure 3.8).

There was no significant correlation between placental *PHLDA2* expression and arterial cord blood pH (r = 0.05, p = 0.62, n = 103). There was also no significant association between placental *PHLDA2* expression and apgar scores at 1 minute (r = - 0.06, p = 0.44, n = 163) or at 5 minutes (r = - 0.03, p = 0.74, n = 163).

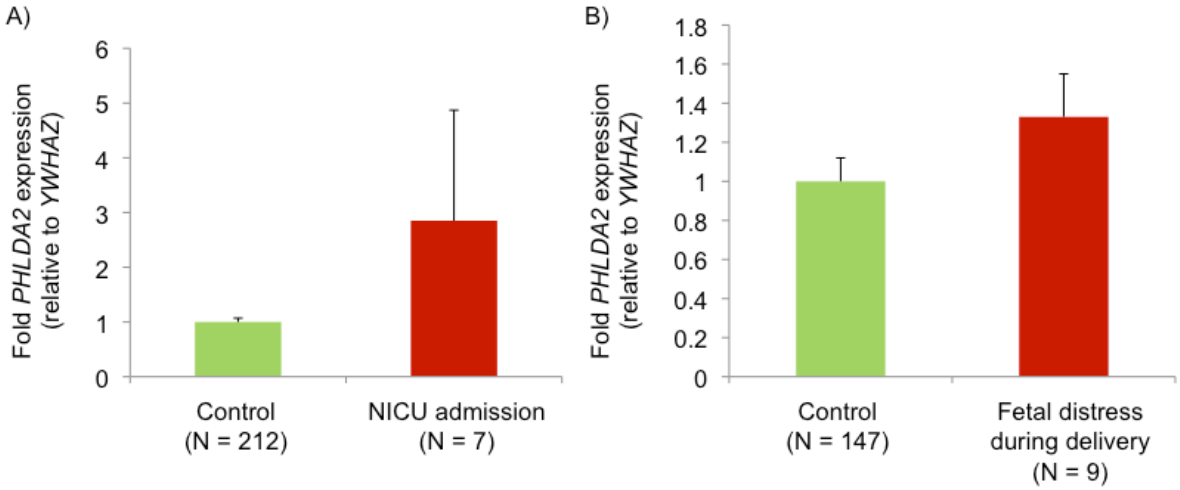


Figure 3.8: Placental *PHLDA2* expression and infant outcomes. Placental *PHLDA2* expression was not significantly altered in infants admitted to NICU following delivery (A) or those exhibiting fetal distress during delivery (B). Error bars represent SEM

3.3.2.5. Fetal growth restriction and *CDKN1C*

When analysed as a group, without accounting for mode of delivery, placental *CDKN1C* expression was significantly inversely correlated with birth weight (r = - 0.20, p = 0.01, n = 181) and custom birth weight centiles (r = - 0.25, p = 0.001, n = 181) but not placental weight (r = - 0.11, p = 0.16, n = 180), Figure

3.9. However, when the results were split according to labour status, there was no significant correlation between *CDKN1C* and birth weight in either labouring or non-labouring groups independently. Given the previous finding of increased *CDKN1C* expression in women delivering by EMCS or SVD (labouring placentas) (section 3.3.1.5), the correlation observed in the overall cohort was likely a result of differences in mode of delivery. Similarly, *CDKN1C* expression was not significantly altered in SGA placentas when results were split by labour status (Figure 3.9).

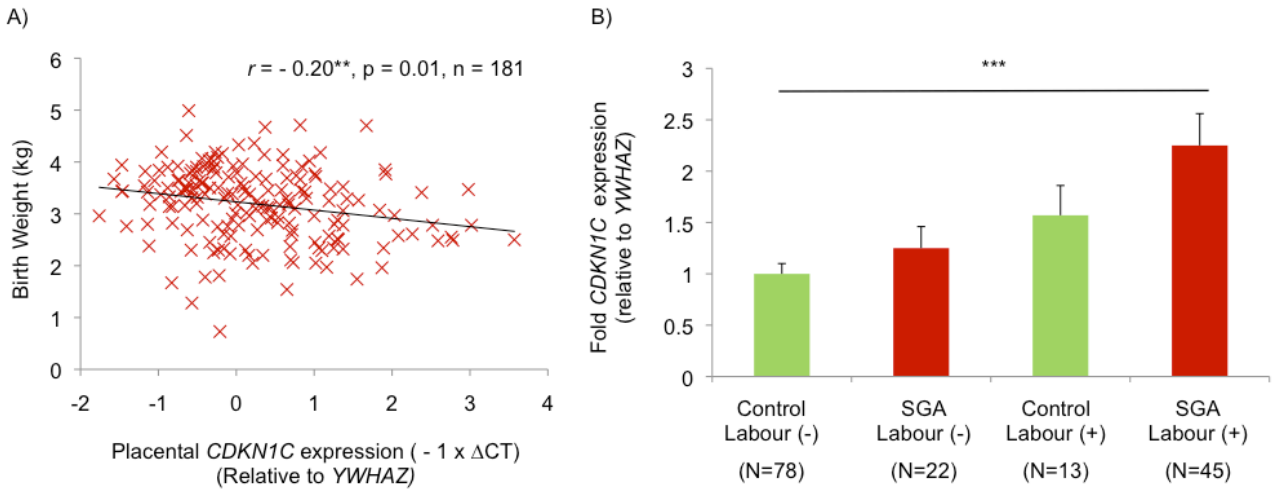


Figure 3.9: Placental *CDKN1C* expression and fetal growth. Although placental *CDKN1C* expression was significantly inversely correlated with birth weight (A), this association was not significant when results were analysed according to labour status (B). ** $p < 0.01$, *** $p < 0.001$. Error bars represent SEM.

Finally, placental *CDKN1C* expression was not significantly altered in placentas of infants admitted to NICU at delivery ($p = 0.43$, $n = 219$) and there was no significant correlation between *CDKN1C* expression and apgar scores or arterial cord blood pH (results not shown).

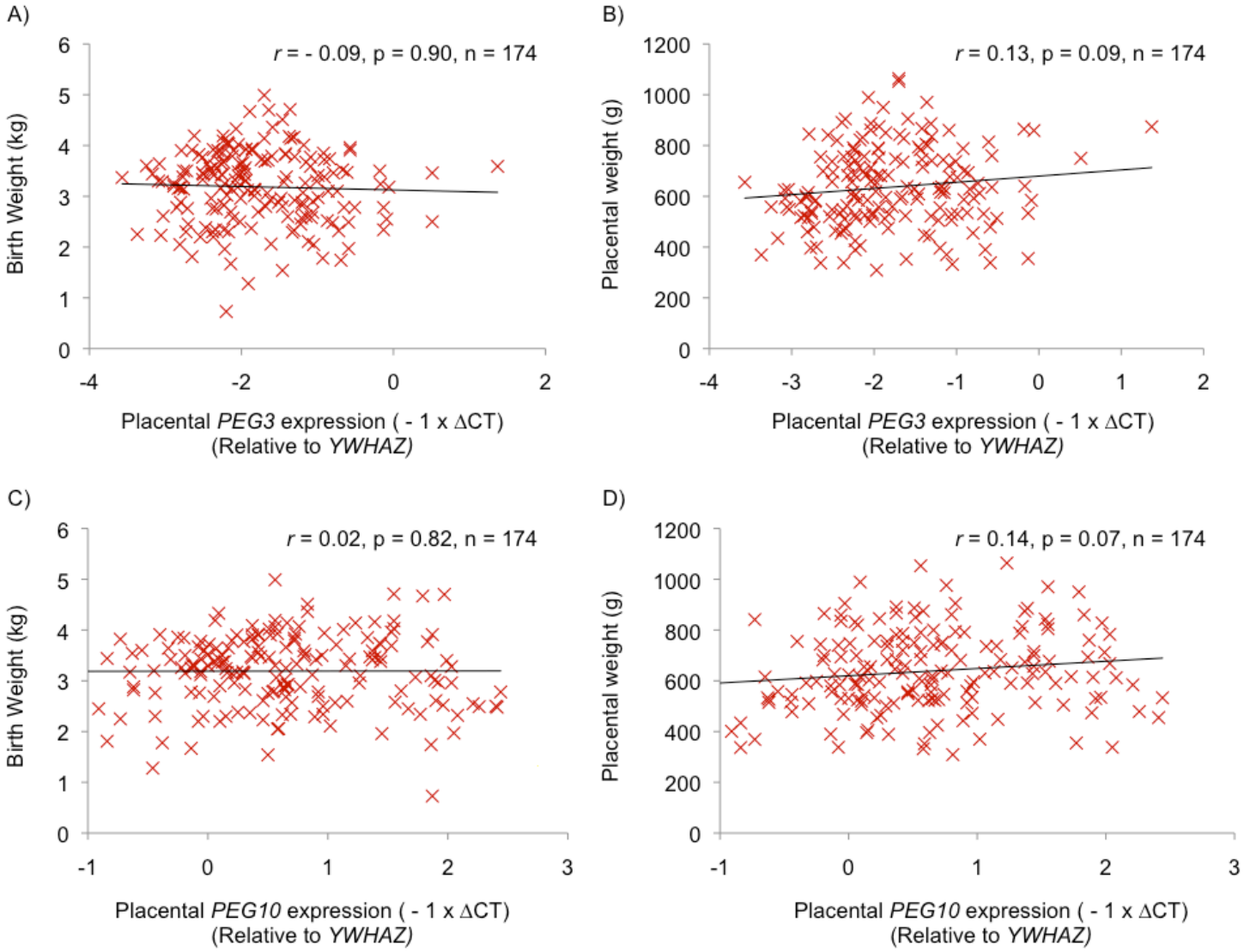


Figure 3.10: Fetal growth and placental *PEG3* and *PEG10* expression. There was no significant correlation between *PEG3* or *PEG10* and birth weight (A and C) although both genes showed a trend for a positive correlation with placental weight (B and D).

3.3.2.6. Fetal growth restriction and *PEG3* and *PEG10* expression

Placental *PEG3* (r = - 0.09, p = 0.90, n = 174) and *PEG10* expression (r = 0.02, p = 0.82, n = 174) was not significantly associated with birth weight (Figure 3.10). Similarly, placental *PEG3* (r = - 0.01, p = 0.99, n = 174) and *PEG10* expression (r = 0.01, p = 0.88, n = 174) was not significantly associated with custom birth weight centiles. Placental *PEG3* (p = 0.43, n = 158) and *PEG10* (p = 0.56, n = 158) expression was also not significantly altered in SGA placentas. Finally, there was no significant correlation between placental weight and *PEG3* (r = 0.13, p = 0.09, n = 174) and *PEG10* (r = 0.14, p = 0.07, n = 174) expression, Figure 3.10.

Placental *PEG3* (p = 0.86, n = 219) and *PEG10* (p = 0.85, n = 219) expression was not significantly altered in placentas of infants admitted to NICU at delivery and there was no significant correlation between expression and apgar scores (at 1 minute and 5 minutes) or arterial cord blood pH (results not shown).

3.3.2.7. Fetal growth restriction and *hPL* gene expression

Placental *hPL* expression was significantly decreased in participants with antenatal growth concerns (p = 0.02, n = 195), although there was no significant correlation with week of first documented concern over fetal growth (r = - 0.12, p = 0.41, n = 45). Expression was also significantly decreased in placentas of infants with a prenatal SGA abdominal and head circumference (results not shown).

Placental *hPL* expression was significantly positively correlated with birth weight (r = 0.17, p = 0.03, n = 174) and custom birth weight centiles (r = 0.17, p = 0.03, n = 174). There was also a significant positive correlation between *hPL* expression and placental weight (r = 0.16, p = 0.03, n = 180). However, *hPL* expression was not significantly altered in SGA (Figure 3.11), LBW (p = 0.39, n = 164) or IUGR placentas (p = 0.24, n = 158).

Placental *hPL* expression was not significantly altered (p = 0.38, n = 164) in placentas of term infants admitted to NICU at delivery (Figure 3.11). There was also no significant correlation between *hPL* expression and apgar scores at 1 minute (*r* = 0.08, p = 0.34, n = 163) or at 5 minutes (*r* = 0.10, p = 0.23, n = 163). Placental *hPL* expression was however significantly correlated with arterial cord blood pH (*r* = 0.20, p = 0.04, n = 103), Figure 3.11.

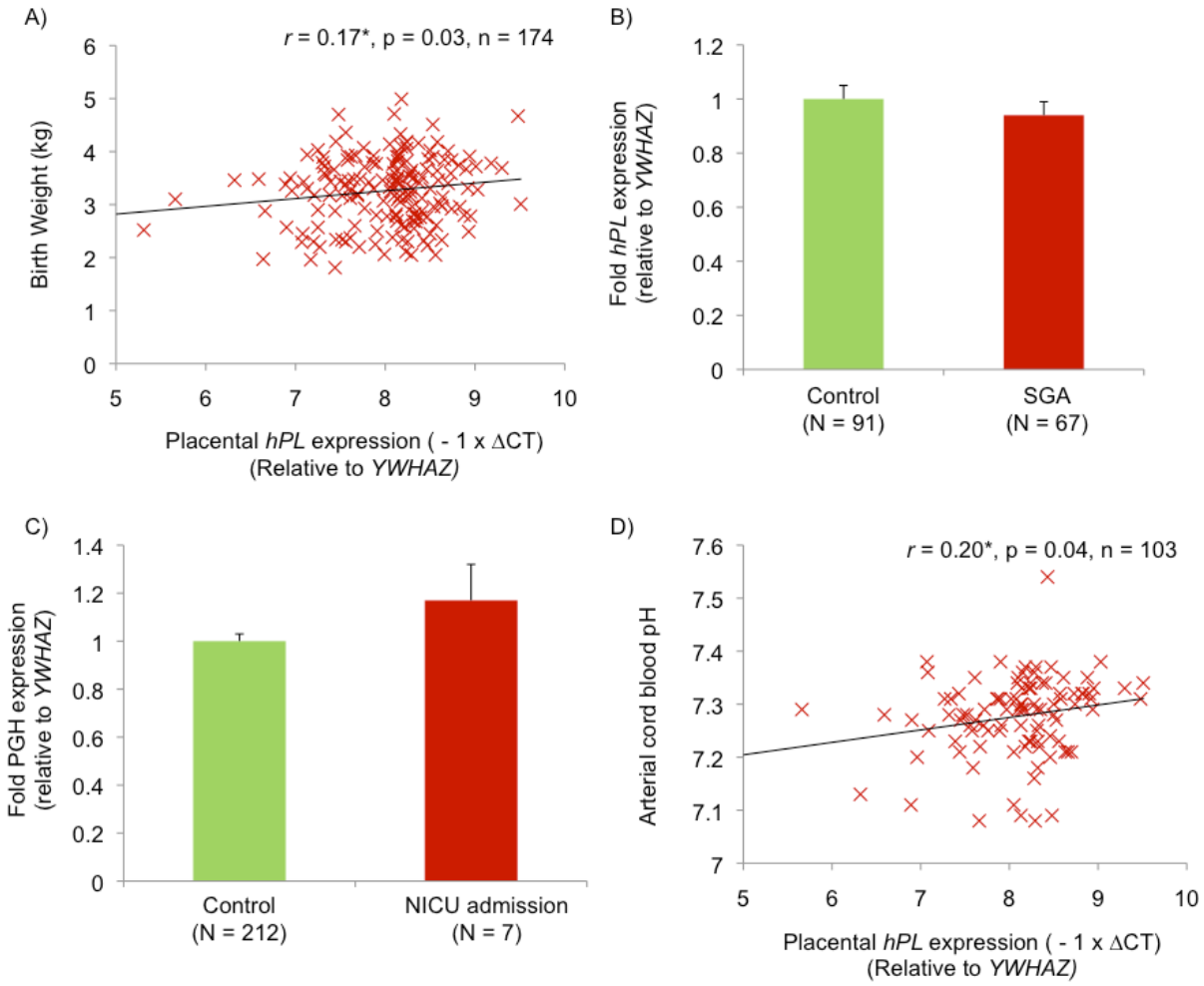


Figure 3.11: Placental *hPL* expression and fetal growth. Placental *hPL* expression was significantly positively associated with birth weight (A) but was not significantly altered in SGA placentas (B). There was no significant difference in *hPL* expression in placentas of infants admitted to NICU (C) although expression was positively associated with arterial cord blood pH (D). Error bars represent SEM. * P <0.05.

3.3.2.8. Fetal growth restriction and *PGH* expression

While initial analysis suggested placental *PGH* expression was significantly positively correlated with birth weight (r = 0.16, p = 0.03, n = 181), this association was not significant in a partial correlation controlling for gestational age (partial r = 0.06, p = 0.40, n = 181)(Figure 3.13). This likely reflects the significant association between *PGH* expression and gestational age observed in section 3.2.3. Similarly, there was no significant difference in *PGH* expression between AGA and SGA (term only) placentas, Figure 3.12.

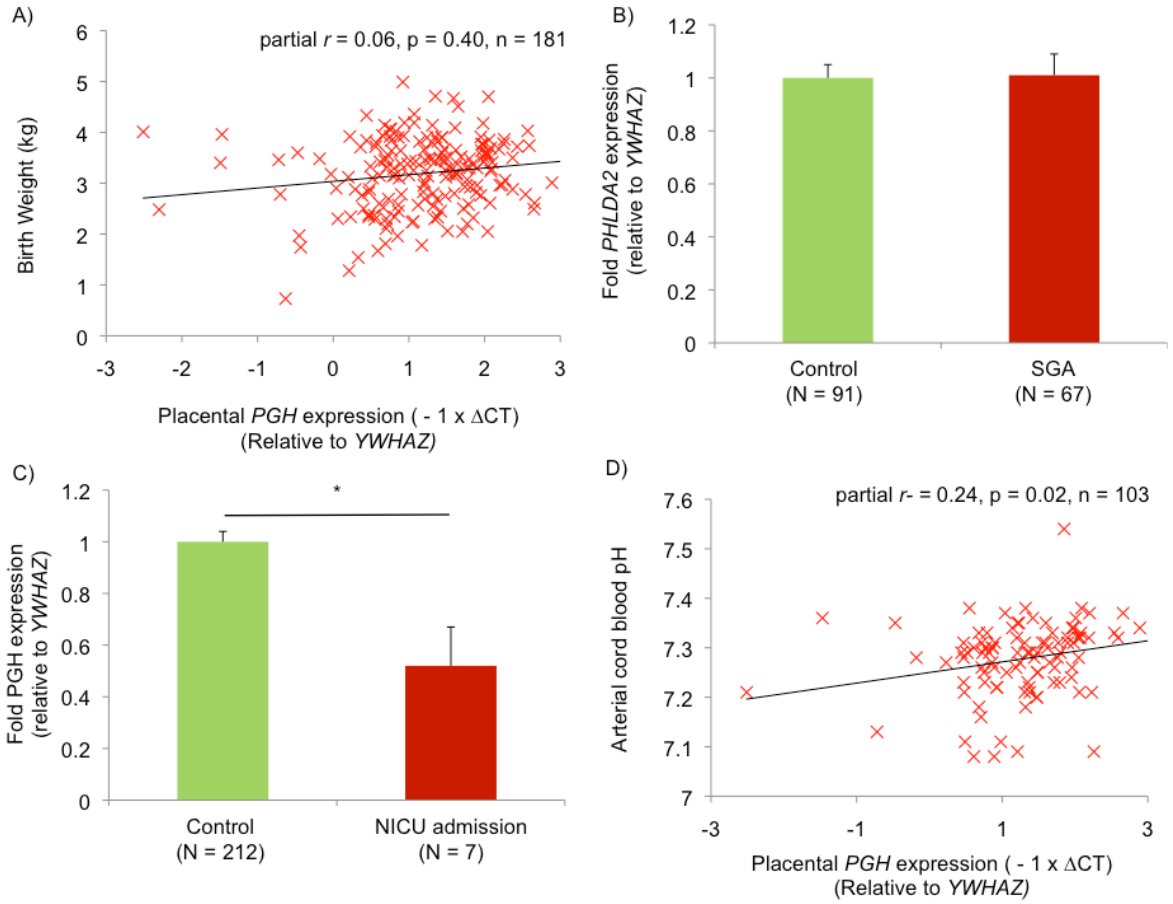


Figure 3.12: Placental *PGH* expression and fetal growth. Placental *PGH* expression was not significantly correlated with birth weight in a partial correlation controlling for gestational age (A) and was not significantly altered in SGA placentas (B). *PGH* expression was significantly decreased in placentas of infants admitted to NICU (C) and expression was positively associated with arterial cord blood pH (D). * P <0.05. Error bars represent SEM.

Placental *PGH* expression was significantly decreased (p = 0.04, n = 164) in placentas of term infants admitted to NICU (Figure 3.12). *PGH* expression was not significantly correlated with apgar scores at 1 minute (*r* = 0.02, p = 0.83, n = 163) or at 5 minutes (*r* = 0.04, p = 0.60, n = 163). Placental *PGH* expression was however significantly correlated with arterial cord blood pH (*r* = 0.24, p = 0.02, n = 103), Figure 3.12. Results remained significant in a partial correlation controlling for gestational age (partial *r* = 0.24, p = 0.02, n = 103).

Table 3.7: Relationship between imprinted gene expression and placental *hPL* and *PGH* expression. Results were analysed using Pearson correlation test. ** p < 0.01 *** p < 0.001

	Placental *hPL* expression	**Placental *PGH* expression**
Placental *PHLDA2* expression	***r* = - 0.27*** p < 0.001 n = 219**	*r* = 0.02 p = 0.84 n = 219
Placental *CDKN1C* expression	***r* = - 0.20** p = 0.002 n = 216**	*r* = 0.04 p = 0.58 n =219
Placental *PEG3* expression	*r* = - 0.01 p = 0.93 n = 219	*r* = - 0.09 p = 0.25 n = 219
Placental *PEG10* expression	*r* = 0.04 p = 0.62 n = 219	*r* = - 0.07 p = 0.38 n = 219

3.3.2.9. Placental hormone and imprinted gene expression

In the overall cohort, there was a significant inverse association between placental *PHLDA2* and *hPL* expression (*r* = - 0.27, p < 0.001, n = 219), Figure 3.13. There was also a significant inverse correlation between placental *CDKN1C* expression and *hPL* expression (Table 3.7), which remained significant after controlling for mode of delivery (*r* = - 0.20, p = 0.002, n = 216).

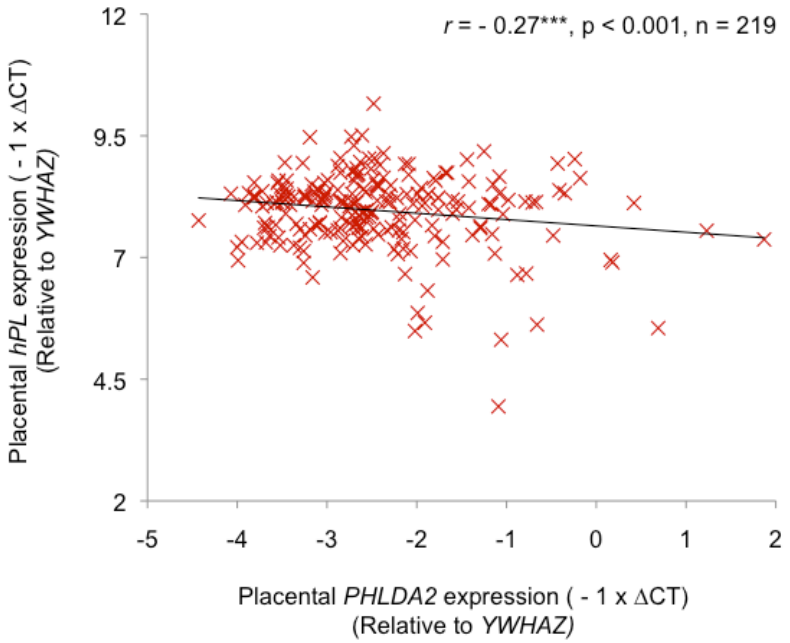


Figure 3.13: Placental *PHLDA2* and *hPL* expression. Placental gene expression was significantly correlated as determined by Pearson correlation analysis. *** p < 0.001

Table 3.8: Summary of target gene expression changes in SGA placentas. Fold gene expression is shown relative to AGA participants. Results highlighted in bold were statistically significant using an independent samples T test. ** p ≤ 0.01.

Gene	**AGA Participants** (N = 91)	**SGA participants** (N = 67)	P Value
PHLDA2	**1.00**	**2.01**	**P = 0.004****
CDKN1C	1.00	1.25	P = 0.88
PEG3	1.00	1.06	P = 0.43
PEG10	1.00	1.28	P = 0.56
hPL	1.00	0.94	P = 0.36
PGH	1.00	1.01	P = 0.89

3.3.2.10. Wales Cohort Summary

A summary of the changes in target gene expression in SGA compared with AGA placentas is shown in Table 3.8. Only placental *PHLDA2* expression

was significantly increased in SGA participants. The increase observed in placental *PHLDA2* expression remained significant after controlling for multiple comparisons using a Bonferroni correction (p = 0.01).

3.3.3. Fetal growth restriction: Manchester Cohort

In order to replicate the finding of a significant inverse correlation between placental *PHLDA2* expression and birth weight demonstrated in the Wales Cohort, target gene expression was analysed in a second independent cohort.

3.3.3.1. Participant demographics

The characteristics of the Manchester cohort (N=110) are shown in Table 3.9. All participants (N=110) experienced reduced fetal movements during pregnancy. The cohort included 21 participants delivering infants that were SGA by custom growth centiles of which ten infants were also born LBW.

3.3.3.2. Prenatal fetal growth restriction and placental *PHLDA2*

There was no significant correlation between placental *PHLDA2* expression and any prenatal measure of fetal growth (Table 3.10), although a trend for an inverse correlation with head circumference on scan was shown (*r*(s) = -0.16, p = 0.09, n = 110). 14 participants were identified during prenatal scanning to be carrying a growth restricted fetus (defined as estimated fetal weight <10th centile). Although placental *PHLDA2* expression was increased in pregnancies identified as growth restricted prenatally, this did not reach statistical significance (p = 0.16, n = 110), Figure 3.14.

Another prenatal indicator of fetal growth restriction is a reduction in amniotic fluid. In this cohort there was no significant correlation between placental *PHLDA2* expression and amniotic fluid volume (*r* (s) = - 0.02, p = 0.81, n = 105) and no significant difference in gene expression between participants with a low AFI during pregnancy and controls (p = 0.90, n = 108).

Table 3.9: Characteristics of Manchester Cohort Participants (N=110). Mean (SD)/Range or Number (%) are shown.

	Mean (SD)/Range or Number (%)		Mean (SD)/Range or Number (%)
Birth Outcomes		**Maternal characteristics**	
Fetal Sex		*Ethnicity*	
Male	55 (50%)	Caucasian	72 (65%)
Female	55 (50%)	African/Afro-Caribbean	10 (9%)
		Indian/Pakistani/Bangladeshi	22 (20%)
Gestational age *(weeks)*	39 (1.90) / 30 – 42	Middle Eastern	3 (3%)
		Other	3 (3%)
Birth weight *(g)*	3304 (595) / 850 - 4680		
Custom birth weight centile	40 (29) / 0 - 100	Age (years)	29 (5.81) / 17 – 46
Placental Weight *(g)*	587 (127) / 353 – 854	Maternal BMI	26.01 (5.34) / 17 – 46
Apgar Scores (1 min)	9 (1.44) / 0 - 10	Parity	1 (1.22) / 0 – 7
Apgar Scores (5 min)	10 (1.01) / 0 – 10		
		Smoking during Pregnancy	
Birth weight Classification		Yes	17 (15.5%)
AGA	81 (74%)	No	93 (84.5%)
SGA	21 (19%)		
LGA	8 (7%)	*Alcohol consumption*	
		None	109 (99%)
NICU Admission		1-5 units / week	1 (1%)
Yes	6 (5.5%)		
No	104 (94.5%)	*Obstetric complications of Pregnancy*	
Mode of Delivery		Preeclampsia/PIH	5 (4.5%)
SVD	60 (54%)	PV bleed	1 (1%)
ELCS	10 (9%)	Oligohydramnios	1 (1%)
EMCS	14 (13%)	Prenatal Suboptimal growth	3 (2.5%)
Instrumental	26 (24%)	Reduced Fetal Movements only	100 (91%)

Ultrasound assessment of the placenta and umbilical artery blood flow are also used to ascertain the presence and severity of growth restriction. There was no significant correlation between placental *PHLDA2* expression and mean placental diameter on scan (*r*(s) = - 0.02, p = 0.84, n = 104) or umbilical artery pulsatility index (*r* (s) = - 0.08, p = 0.40, n = 106).

Table 3.10: Relationship between placental *PHLDA2* expression and prenatal measures of fetal growth. No significant correlation was observed, as determined using Spearman rank order correlation test.

	Placental *PHLDA2* expression
Estimated fetal weight (g)	*r*(s) = - 0.03 p = 0.73 n = 110
Estimated growth centile	*r*(s) = - 0.07 p = 0.47 n = 110
Abdominal circumference (mm)	*r*(s) = - 0.07 p = 0.46 n = 110
Head circumference (mm)	*r*(s) = - 0.16 p = 0.09 n = 110
Femur length (mm)	*r*(s) = 0.01 p = 0.95 n = 110

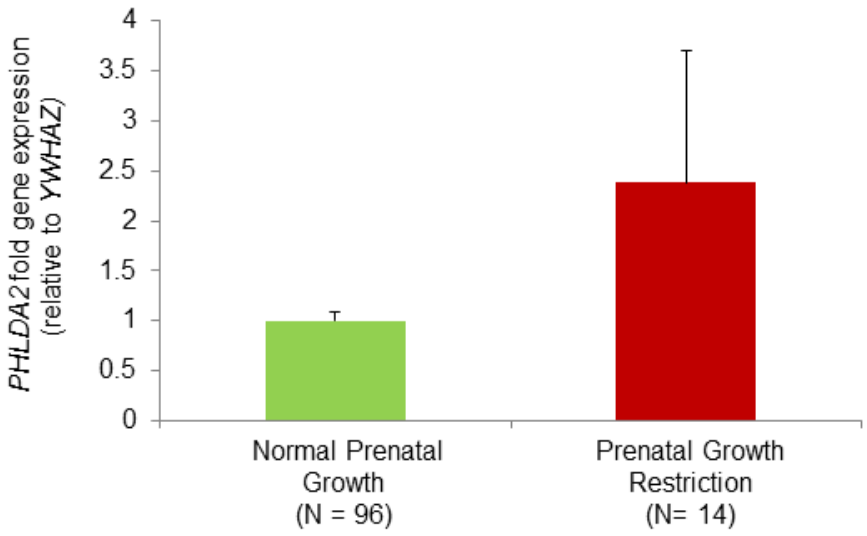


Figure 3.14: Placental *PHLDA2* expression and prenatal growth restriction. Prenatal growth restriction was determined by a custom growth centile <10th during prenatal scans. Differences in *PHLDA2* expression were not statistically significant using a Mann Whitney U test. Error bars represent SEM

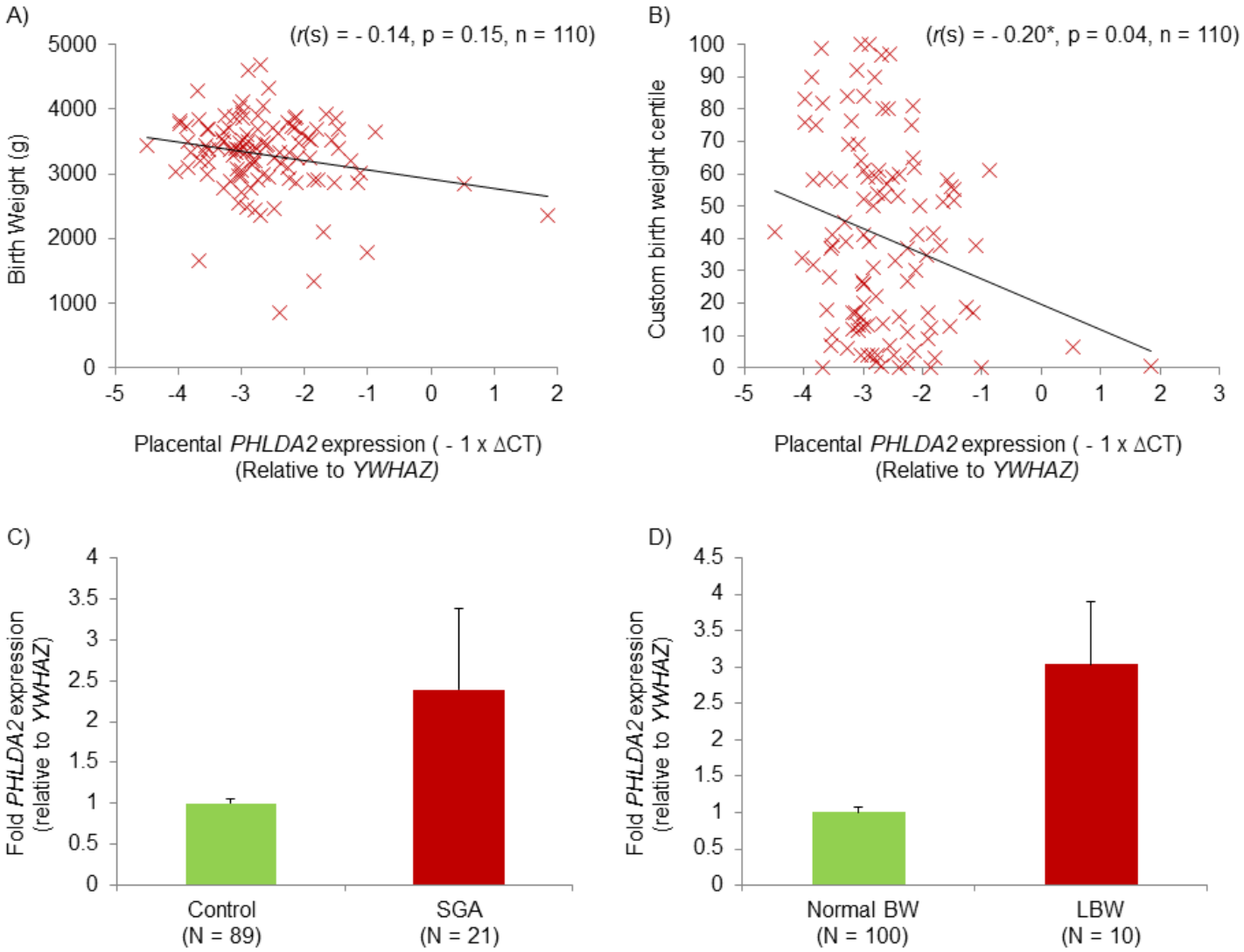


Figure 3.15: Placental *PHLDA2* expression and fetal growth. *PHLDA2* expression is shown in relation to birth weight (A) and custom birth weight centiles (B) and in SGA (C) and LBW (D) pregnancies. SGA = small for gestational age, LBW = Low birth weight. Error bars represent SEM. * P <0.05.

3.3.3.3. Fetal growth restriction and placental *PHLDA2*

In this cohort, 6 participants delivered an infant preterm and low birth weight. As there was no significant correlation between placental *PHLDA2* expression and gestational age (*r* (s) = 0.01, p = 0.89, n = 110) and no significant difference in gene expression between preterm and term pregnancies (p = 0.25, n = 110), these participants were included in the overall cohort for analysis of *PHLDA2* expression and measures of fetal growth.

Placental *PHLDA2* expression was not significantly correlated with birth weight (*r*(s) = - 0.14, p = 0.15, n = 110) in the overall cohort, Figure 3.15. However, there was a statistically significant inverse correlation between placental *PHLDA2* expression and custom birth weight centiles (*r* (s) = - 0.20, p = 0.04, n = 110), Figure 3.15. Interestingly, both birth weight (*r*(s) = - 0.21, p = 0.03, n = 104) and custom birth weight centiles (*r*(s) = - 0.24, p = 0.02, n = 104) were significantly inversely associated with *PHLDA2* expression when only term placentas were analysed. There was also a significant inverse correlation between placental *PHLDA2* expression and placental weight (*r* (s) = -0.4, p = 0.03, n = 31).

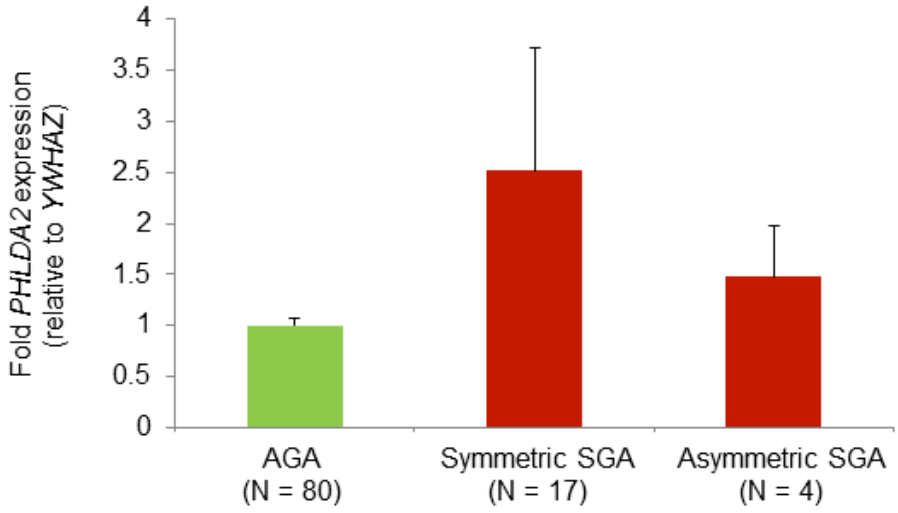


Figure 3.16: Placental *PHLDA2* expression and asymmetric versus symmetric fetal growth restriction. Fold *PHLDA2* expression is shown relative to AGA expression. Asymmetric or symmetric growth restriction was determined using the HC:AC ratio. AGA = average for gestational age, SGA = small for gestational age. Error bars represent SEM.

There was a trend for increased placental *PHLDA2* expression in placentas of SGA compared with control (either AGA or LGA) pregnancies (p = 0.06, n = 110), Figure 3.15, and in placentas of low birth weight compared with normal birth weight infants (p = 0.06, n = 110) Figure 3.15.

In order to determine whether there was any difference in placental gene expression between symmetric and asymmetrically growth restricted infants, prenatal ultrasound head (HC) and abdominal (AC) circumferences were analysed with an elevated HC:AC ratio being indicative of asymmetric growth restriction. There was no significant correlation between placental *PHLDA2* expression and HC:AC ratio (*r* (s) = 0.08, p = 0.47, n = 98) and no significant difference in gene expression between infants born AGA, SGA (symmetric) or SGA (asymmetric), Figure 3.16.

3.3.3.4. Poor perinatal outcomes and *PHLDA2* expression

There was a significant 2.27 fold increase in placental *PHLDA2* expression in participants with a poor perinatal outcome (including preterm labour, fetal growth restriction, NICU admission), as shown in Figure 3.17.

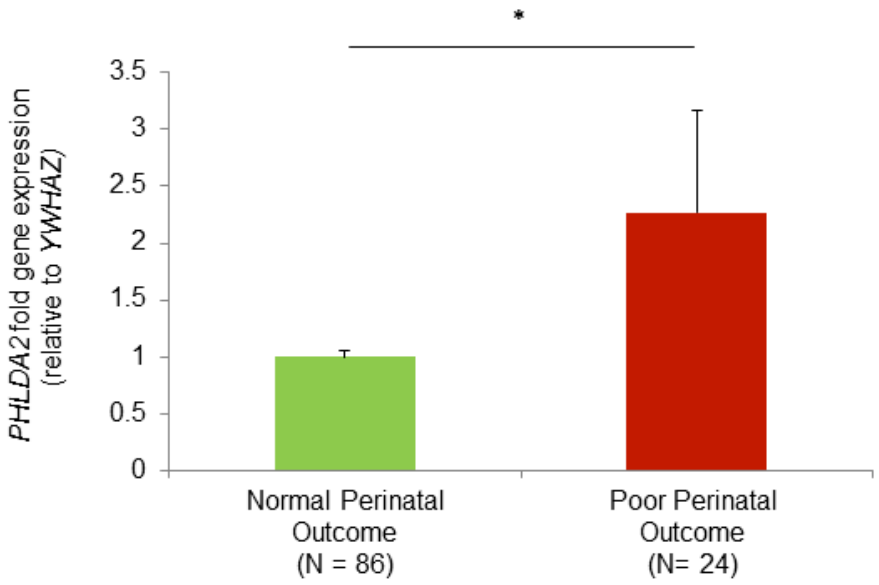


Figure 3.17: Placental *PHLDA2* expression and poor perinatal outcome. Poor Perinatal outcome included preterm birth (< 37 weeks), SGA (birth weight centile < 10th) or term NICU admission. *PHLDA2* expression was significantly reduced using a Mann Whitney U test. * p≤ 0.05. Error bars represent SEM.

The relationship between placental *PHLDA2* expression and birth outcomes in the overall cohort is shown in Table 3.11. Results did not differ when SGA cases were analysed independently. Finally, placental *PHLDA2* expression did not differ significantly in infants admitted to NICU at delivery (Figure 3.18).

Table 3.11: Relationship between placental *PHLDA2* expression and birth outcomes. Placental *PHLDA2* expression was not significantly correlated with any birth outcome as determined using Spearman rank order correlation test.

	Placental *PHLDA2* expression
Arterial Cord Blood pH	*r*(s) = - 0.07 p = 0.57 n = 78
Venous Cord Blood pH	*r*(s) = - 0.05 p = 0.69 n = 85
Apgar Score at 1min	*r*(s) = 0.14 p = 0.14 n = 106
Apgar Score at 5min	*r*(s) = 0.05 p = 0.61 n = 105

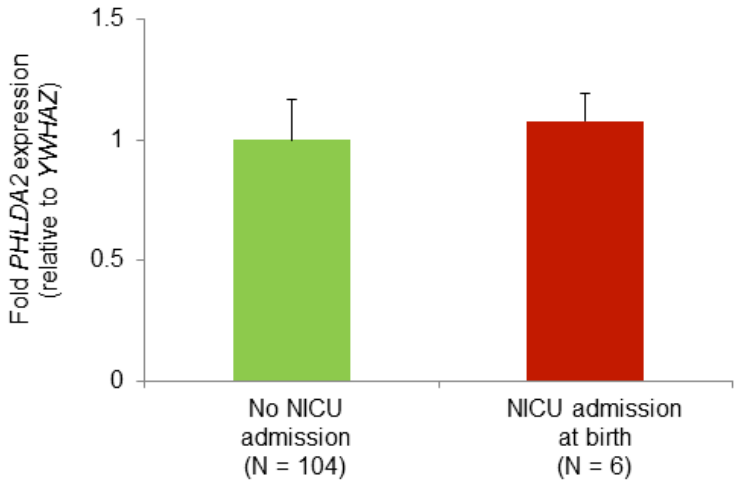


Figure 3.18: Placental *PHLDA2* expression and NICU admission at birth. *PHLDA2* expression was not significantly altered as determined by a Mann Whitney U test. Error bars represent SEM.

3.3.3.5. Fetal growth and *CDKN1C* expression

There was no significant correlation placental *CDKN1C* expression and any prenatal measure of fetal growth (results not shown). *CDKN1C* expression was also not significantly altered in placentas of participants identified during prenatal scanning to be carrying a growth-restricted fetus, see Figure 3.19.

Birth weight was not significantly correlated with placental *CDKN1C* expression (r = - 0.07, p = 0.48, n = 110) and expression did not differ significantly between SGA and AGA pregnancies, see Figure 3.19. Placental *CDKN1C* expression was also not significantly correlated with custom birth weight centiles (r = - 0.10, p = 0.30, n = 110) or placental weight (r = - 0.07, p = 0.72, n = 31).

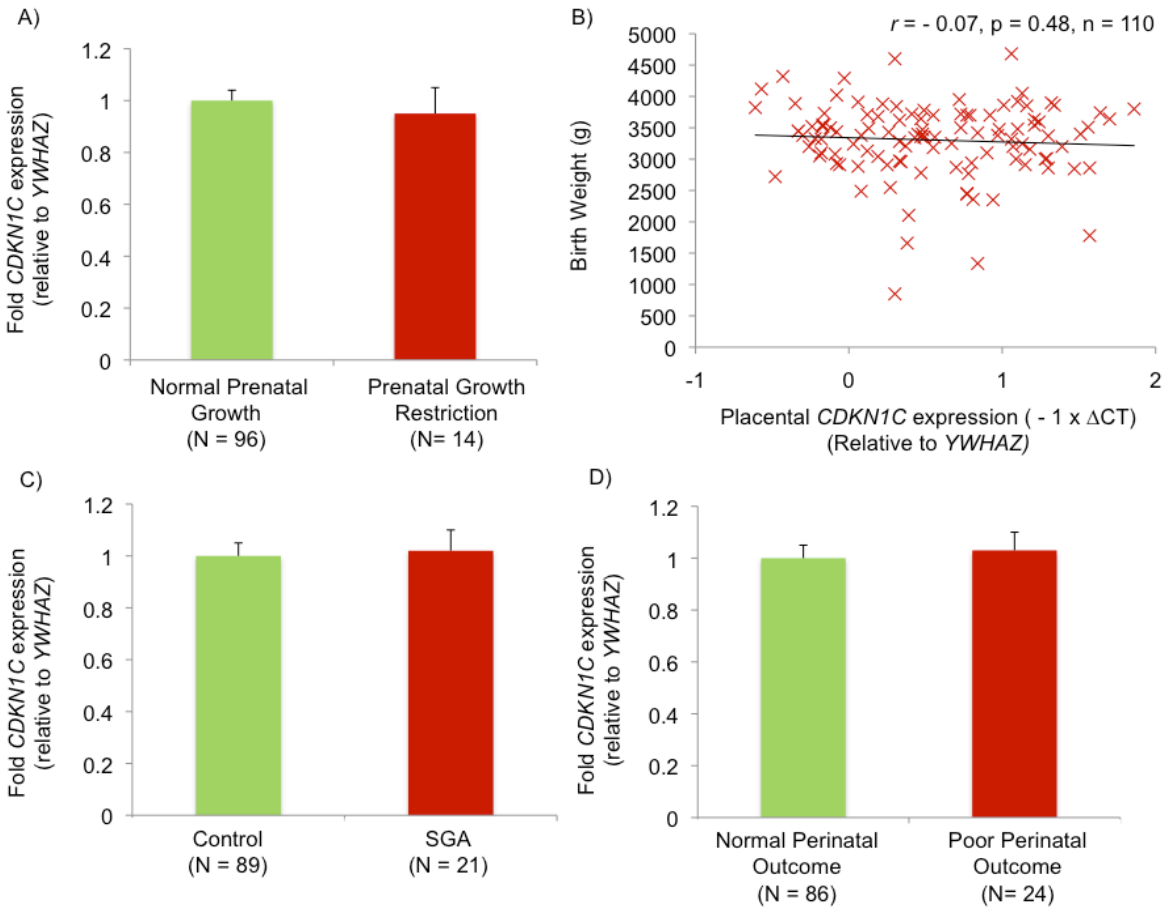


Figure 3.19: Placental *CDKN1C* expression and fetal growth. *CDKN1C* expression was not significantly altered in participants with prenatal growth restriction (A) and there was no correlation between expression and birth weight (B). *CDKN1C* expression was not significantly altered in SGA infants (C) or those with a poor perinatal outcome (D). Error bars represent SEM.

Finally, placental *CDKN1C* expression was not significantly altered in participants with a poor perinatal outcome, as shown in Figure 3.19. There was also no significant correlation between *CDKN1C* expression and any other birth outcome measure and expression was not significantly altered in placentas of infants admitted to NICU at delivery (p = 0.97, n = 110).

3.3.3.6. Fetal growth and *PEG3* expression

Placental *PEG3* expression was not significantly correlated with any prenatal measure of fetal growth (results not shown) and expression was not significantly altered in placentas of participants identified during prenatal scanning to be carrying a growth-restricted fetus (Figure 3.20).

There was no significant correlation between placental *PEG3* expression and birth weight (r = 0.05, p = 0.59, n = 110), custom birth weight centile (r = 0.08, p = 0.44, n = 110) or placental weight (r = - 0.14, p = 0.46, n = 31) and expression did not differ significantly between SGA and AGA pregnancies (Figure 3.20).

Finally, placental *PEG3* expression was not significantly altered in participants with a poor perinatal outcome, as shown in Figure 3.20. Similarly, *PEG3* expression was not significantly correlated with any birth outcome measure and was not significantly altered in placentas of infants admitted to NICU at delivery (p = 0.58, n = 110).

3.3.3.7. Fetal growth and *PEG10* expression

There was a significant positive correlation between placental *PEG10* expression and prenatal abdominal circumference (r = 0.26, p = 0.01, n = 110) and femur length (r = 0.21, p = 0.03, n = 110) as well as a trend for a positive correlation with estimated fetal weight on ultrasound scan (r = 0.17, p = 0.07, n = 110). However, placental *PEG10* expression was not significantly altered in participants with a prenatally identified growth-restricted fetus, see Figure 3.21.

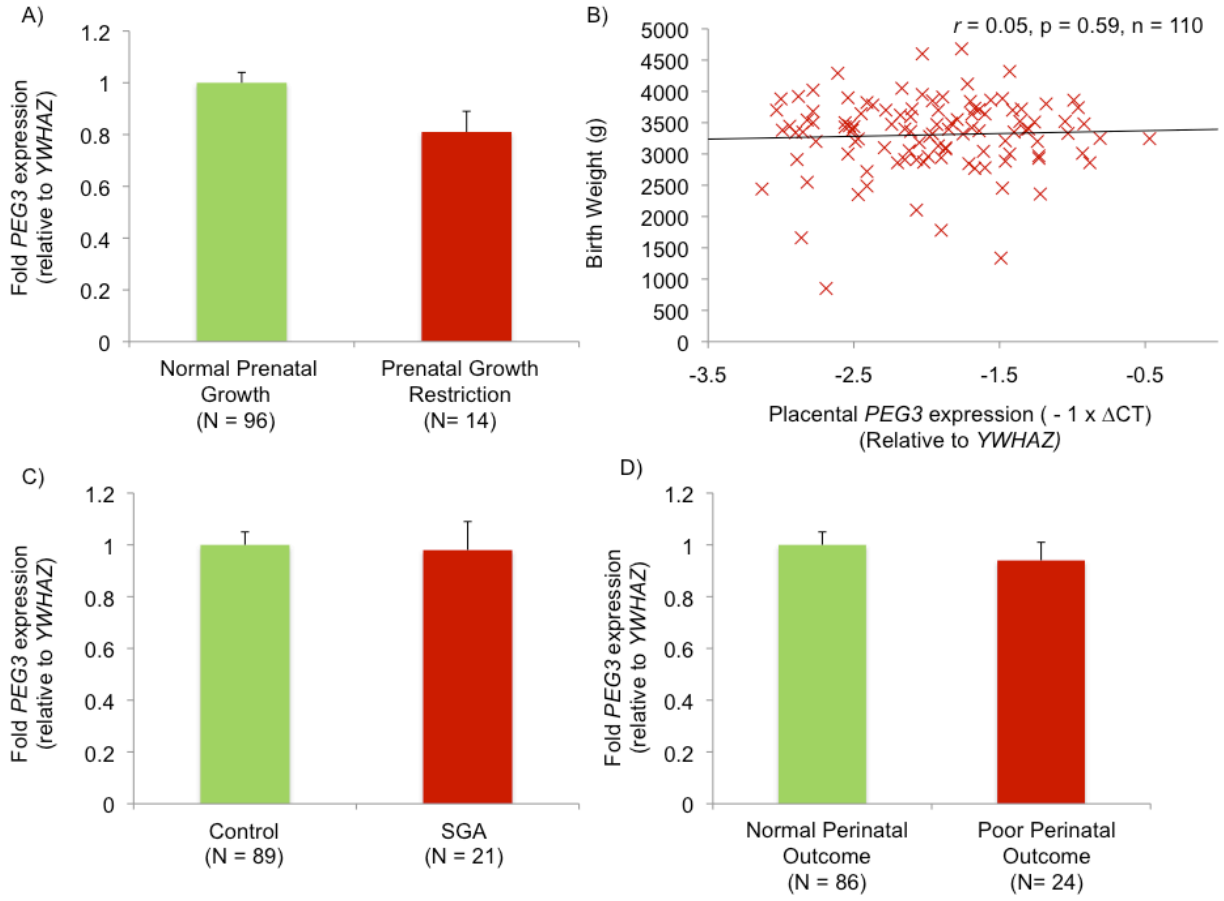


Figure 3.20: Placental *PEG3* expression and fetal growth. *PEG3* expression was not significantly altered in participants with prenatal growth restriction (A) and there was no correlation between expression and birth weight (B). *PEG3* expression was not significantly altered in SGA infants (C) or those with a poor perinatal outcome (D). Error bars represent SEM.

Placental *PEG10* expression was also significantly positively correlated with birth weight (r = 0.25, p = 0.01, n = 110) but not with custom birth weight centile (r = 0.15, p = 0.12, n = 110), Figure 3.21. *PEG10* expression was significantly decreased in placentas of SGA compared with AGA pregnancies (p = 0.046, n = 110) and in LBW compared with normal birth weight pregnancies (p = 0.03, n = 110), Figure 3.21. Analysis of the HC: AC ratio was used to distinguish between symmetric and asymmetric growth restriction. Interestingly, there was no significant difference in *PEG10* expression between AGA and SGA (symmetric) placentas (p = 0.26) however a trend was seen for a 60% reduction in expression in SGA (asymmetric) placentas (p = 0.09).

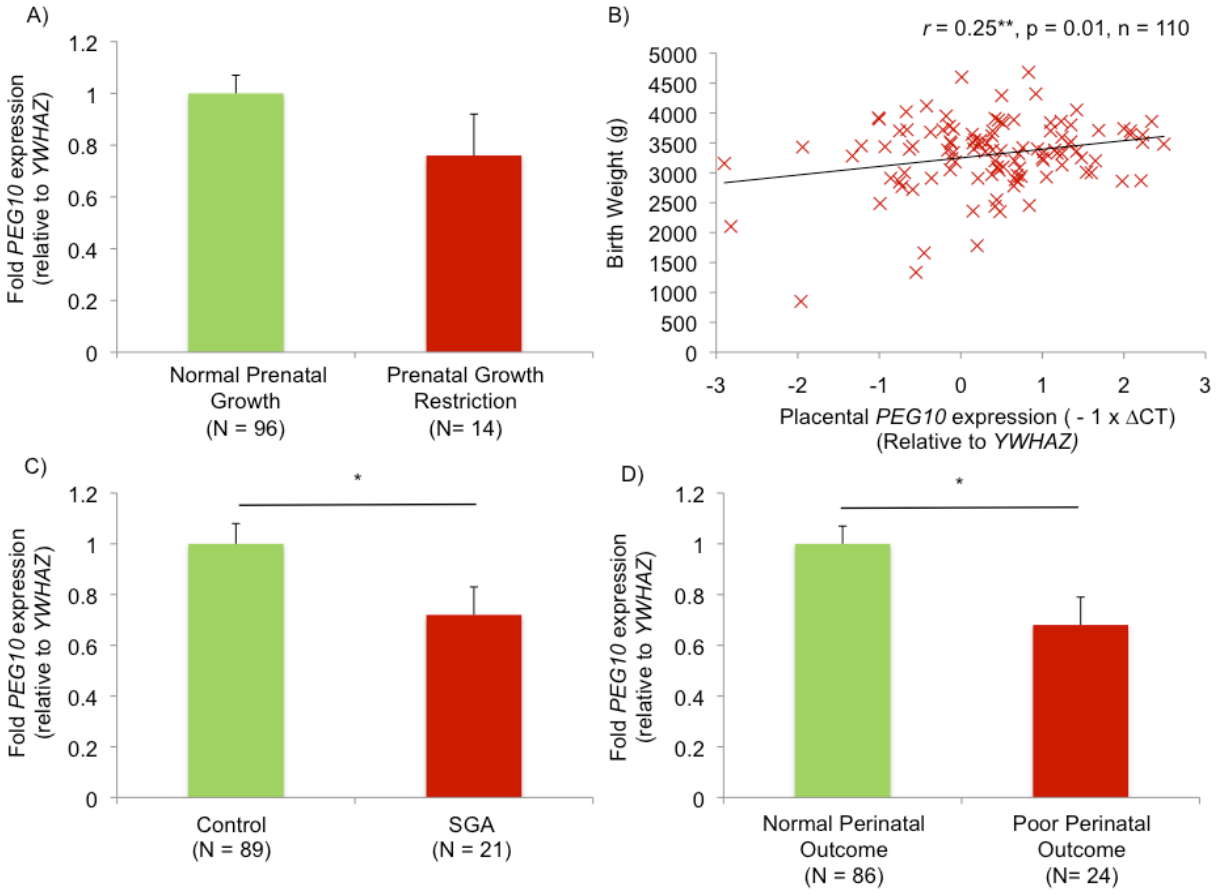


Figure 3.21: Placental *PEG10* expression and fetal growth. *PEG10* expression was not significantly altered in participants with prenatal growth restriction (A) although there was a significant positive correlation between expression and birth weight (B). *PEG10* expression was significantly reduced in SGA infants (C) and in those with a poor perinatal outcome (D). Error bars represent SEM. * P <0.05, ** p < 0.01.

Placental *PEG10* expression was significantly decreased by 32% in infants with a poor perinatal outcome (Figure 3.21) and expression was significantly positively correlated with Apgar scores at 1 minute (r = 0.28, p = 0.004, n = 106) and at 5 minutes (r = 0.30, p = 0.002, n = 105). Finally, *PEG10* expression was not significantly altered in infants admitted to NICU at delivery (p = 0.22, n = 110).

3.3.3.8. Fetal growth restriction and placental hormone gene expression

In terms of prenatal indicators of fetal growth restriction, there was a significant positive correlation between placental *hPL* expression and femur length (r = 0.23, p = 0.01, n = 109). There was also a significant positive correlation between placental *PGH* expression and head circumference (r = 0.25, p = 0.01, n = 98). Although placental *hPL* (p = 0.21, n = 110) and *PGH* expression (p = 0.21, n = 110) were decreased in pregnancies identified as growth restricted prenatally, these differences were not statistically significant.

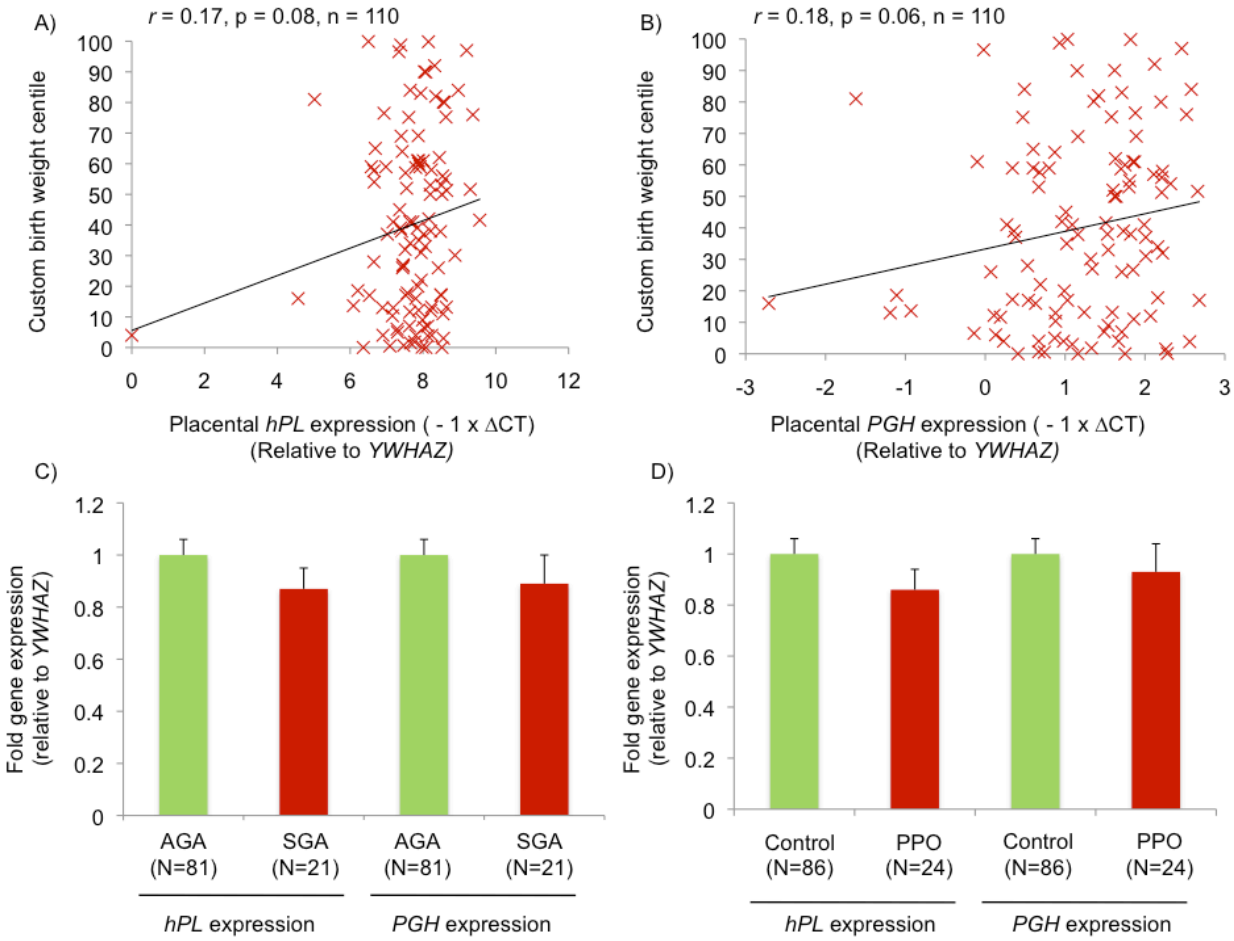


Figure 3.22: Placental *hPL* and *PGH* expression and fetal growth. There was a trend for a postive correlation between custom birth weight centiles and *hPL* (A) and *PGH* (B) expression. Gene expression was not significantly altered in SGA infants (C) or in those with a poor perinatal outcome (D). PPO = poor perinatal outcome. Error bars represent SEM.

There was a trend for a positive correlation between custom birth weight centile and placental *hPL* (r = 0.17, p = 0.08, n = 110), and *PGH* (r = 0.18, p = 0.06, n = 110) expression as shown in Figure 3.22. However, there was no significant correlation with birth weight or placental weight (results not shown). Finally, placental *hPL* and *PGH* expression was not significantly altered in placentas of SGA pregnancies (Figure 3.22).

In terms of birth outcomes, there was also no significant difference in placental *hPL* and *PGH* expression in participants with a poor perinatal outcome (Figure 3.22). Finally, there was no significant correlation between placental *hPL* or *PGH* expression and any other birth outcome examined (results not shown).

3.3.3.9. Imprinted gene and placental hormones

As can be seen in Table 3.12, there was no significant correlation between any of the imprinted genes examined and placental *hPL* and *PGH* expression. Only a trend was observed for a positive correlation between placental *PEG3* and *hPL* expression.

Table 3.12: Relationship between imprinted gene expression and placental *hPL* and *PGH* expression. No significant correlation was found using Spearman rank order or Pearson correlation test.

	Placental *hPL* expression	**Placental *PGH* expression**
Placental *PHLDA2* expression	r(s) = 0.09 p = 0.33 n = 110	r(s) = - 0.14 p = 0.16 n = 110
Placental *CDKN1C* expression	r(p) = - 0.10 p = 0.30 n = 110	r(p) = - 0.10 p = 0.29 n = 110
Placental *PEG3* expression	r(p) = 0.17 p = 0.07 n = 110	r(p) = 0.15 p = 0.12 n = 110
Placental *PEG10* expression	r(p) = 0.13 p = 0.17 n = 110	r(p) = 0.12 p = 0.21 n = 110

Table 3.13: Summary of target gene expression changes in SGA placentas. Fold gene expression is shown relative to AGA participants. Results highlighted in bold were statistically significant using an independent samples T test. * $p \leq 0.05$.

Gene	**AGA Participants** (N = 81)	**SGA participants** (N = 21)	P Value
PHLDA2	1.00	2.33	P = 0.09
CDKN1C	1.00	1.02	P = 0.86
PEG3	1.00	0.98	P = 0.84
PEG10	**1.00**	**0.72**	**P = 0.046***
hPL	1.00	0.87	P = 0.29
PGH	1.00	0.89	P = 0.42

3.3.3.10. Manchester cohort summary

A summary of the changes in target gene expression in SGA compared with AGA placentas is shown in Table 3.13. Only a trend for increased *PHLDA2* expression was observed in this cohort, although placental *PHLDA2* expression was significantly inversely correlated with birth weight and custom birth weight centiles. In addition, placental *PEG10* expression was significantly reduced in SGA placentas.

3.3.4. Fetal overgrowth

Given the well established role of imprinted genes in fetal growth and the associations observed in the Wales and Manchester Cohorts between birth weight and expression of a number of the target genes examined (section 3.3), it is possible that imprinted genes and placental hormones also play a role in fetal overgrowth resulting in delivery of an LGA (large for gestational age) or macrosomic (≥4,000g) infant.

Table 3.14: Comparison of birth outcomes between AGA and LGA participants. Mean (SD)/Range or Number (%) is shown. Differences were analysed using an independent samples T test or Chi – square test where appropriate. AGA = average for gestational age, LGA = large for gestational age. * P <0.05, *** p < 0.001.

	AGA Participants (N = 91)	**LGA participants** (N = 67)	**P Value**
Maternal characteristic			
Maternal BMI (kg/m2)	27 (6) / 17 - 42	29 (6) / 22 - 43	P = 0.07
Maternal age (years)	31 (5) / 19 - 41	32 (7) / 20 - 44	P = 0.75
Birth Outcome			
Mode of Delivery:			
Vaginal	7 (8%)	2 (9%)	
Elective C section	78 (86%)	17 (74%)	
Emergency C section	4 (4%)	2 (9%)	
Instrumental	2 (2%)	2 (9%)	P = 0.17
Birth weight (g)	3480 (277)/ 2830 - 4190	4206 (314) / 3820 - 4990	**P < 0.001*** **
Head circumference (cm)	35 (1) / 32 - 39	36 (1) / 33 - 38	**P = 0.03***
Gestational age (weeks)	39 (1) / 37 - 42	39 (1) / 38 - 42	P = 0.21
Placental weight (g)	703 (124) / 309 - 905	850 (113) / 637 - 1064	**P < 0.001*** **
Gender			
Male	44 (48%)	13 (57%)	
Female	47 (52%)	10 (43%)	P = 0.48
Apgar score (1 min)	9 (1) / 4 - 10	9 (1) / 5 - 10	P = 0.25
Apgar score (5 min)	10 (0.6) / 7 – 10	10 (1) / 7 - 10	P = 0.34
Arterial cord blood pH	7.3 (0.07) / 7.1 – 7.4	7.3 (0.05) / 7.2 – 7.3	P = 0.93

3.3.4.1. Fetal overgrowth: Wales cohort

The Wales Cohort included 23 participants delivering LGA infants of which 19 were also macrosomic (≥ 4,000g). A comparison of maternal characteristics and birth outcomes between AGA and LGA groups is shown in Table 3.14. Only birth weight, placental weight and head circumference were significantly different between AGA and LGA participants.

There was no significant difference in placental *PHLDA2*, *CDKN1C* or *PEG3* expression between LGA and SGA placentas, as shown in Figure 3.23.

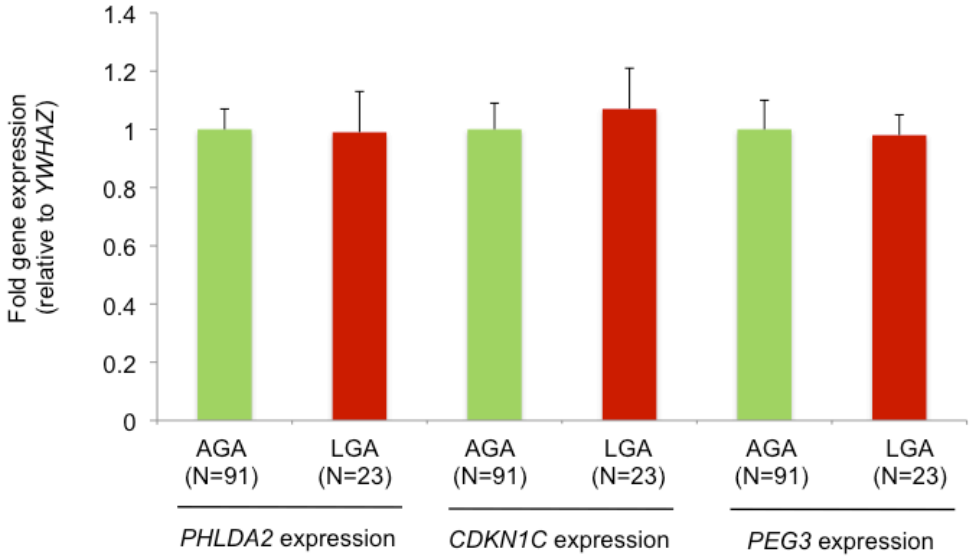


Figure 3.23: Imprinted gene expression and LGA. Placental *PHLDA2*, *CDKN1C* and *PEG3* expression was not significantly altered in placentas of LGA infants as determined by an independent samples T test. AGA = average for gestational age, LGA = large for gestational age. Error bars represent SEM.

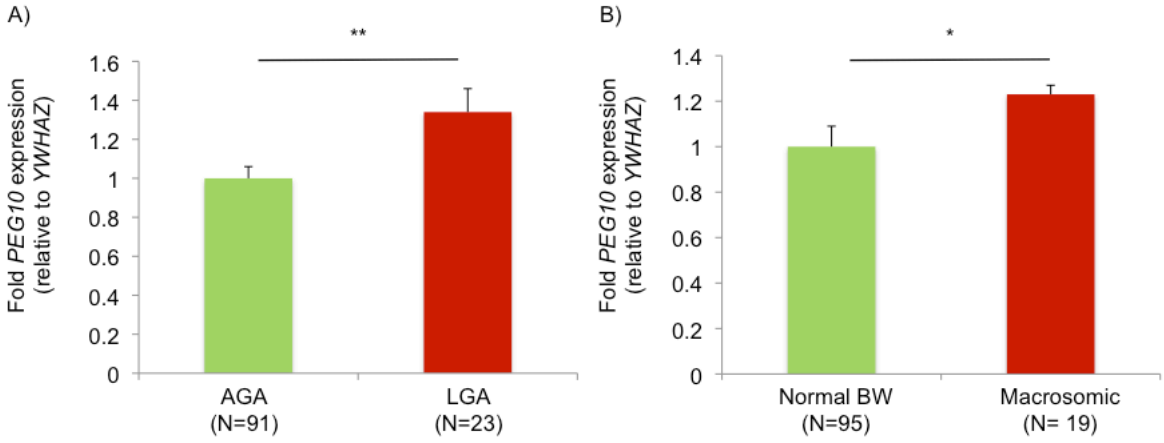


Figure 3.24: *PEG10* expression and fetal overgrowth. Placental *PEG10* expression was significantly increased in placentas of LGA (A) and macrosomic (B) infants as determined by an independent samples T test. AGA = average for gestational age, LGA = large for gestational age. * P <0.05, ** p < 0.01. Error bars represent SEM.

In contrast, placental *PEG10* expression was significantly increased by 34% in LGA placentas and by 23% in macrosomic placentas (Figure 3.24). Placental *PEG10* expression was also significantly increased in infants

measuring LGA prenatally by abdominal centiles (p = 0.02, n = 107) and head centiles (p = 0.04, n = 107). However, there was no significant correlation between *PEG10* expression and head circumference at delivery (*r* = 0.08, p = 0.49, n = 82). Interestingly, placental *PEG10* expression was not significantly altered in participants with a previous macrosomic delivery compared with those previously delivering normal birth weight infants (p = 0.58, n = 144). In terms of placental hormone gene expression, there was no significant difference in placental *hPL* or *PGH* expression between LGA and SGA placentas, as shown in Figure 3.25.

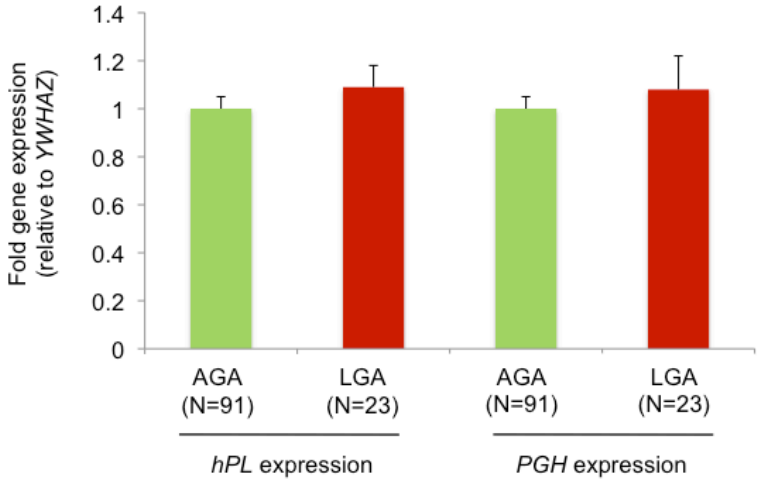


Figure 3.25: Fetal overgrowth and placental hormone gene expression. Placental *hPL* and *PGH* expression was not significantly altered in placentas of LGA infants as determined by an independent samples T test. AGA = average for gestational age, LGA = large for gestational age. Error bars represent SEM.

3.3.4.2. Fetal overgrowth: Manchester Cohort

The Manchester cohort included eight participants delivering an LGA infant. Only *PHLDA2* expression was significantly decreased in placentas of LGA infants (Figure 3.26). Placental *PHLDA2* expression was also decreased in placentas of macrosomic infants (p = 0.02, n = 90). No other imprinted gene examined showed significantly altered expression (Figure 3.26).

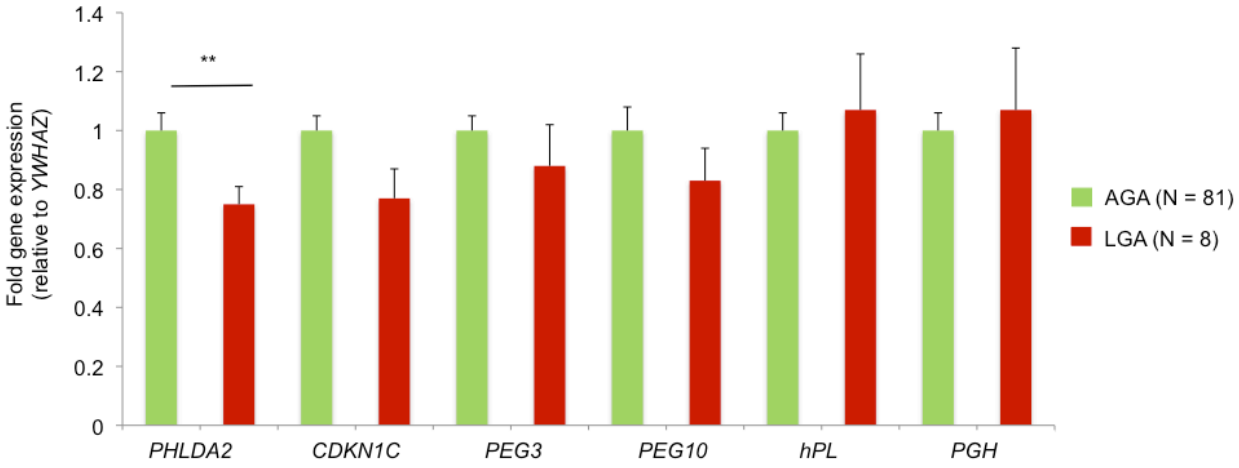


Figure 3.26: Target gene expression in LGA placentas. Only placental *PHLDA2* expression was significantly decreased in LGA placentas as determined by a Mann Whitney U test. AGA = average for gestational age, LGA = large for gestational age.

3.3.4.3. Fetal overgrowth summary

A summary of the changes in target gene expression in LGA compared with AGA placentas is shown in Table 3.15. In the Wales Cohort, only *PEG10* expression was significantly increased in LGA placentas whereas in the Manchester Cohort only *PHLDA2* expression was significantly decreased in LGA placentas.

Table 3.15: Summary of target gene expression changes in LGA placentas in Wales and Manchester Cohorts Percentage change in expression relative to controls is shown.

Gene	**LGA placentas Wales Cohort**	**LGA placentas Manchester Cohort**
PHLDA2	-	↓ 25%
CDKN1C	-	-
PEG3	-	-
PEG10	↑ 34%	-
hPL	-	-
PGH	-	-

3.4. Discussion

This chapter examined a role for aberrant placental imprinted gene expression in pregnancies complicated by fetal growth restriction and fetal overgrowth. A summary of imprinted gene expression alterations in SGA and LGA placentas is shown in Table 3.16. These results suggest a role for placental *PHLDA2* in the negative regulation of fetal growth and a role for placental *PEG10* in the positive regulation of fetal growth.

Table 3.16: Summary of target gene expression changes in SGA and LGA placentas from the Wales and Manchester Cohorts. Percentage change in expression is shown relative to AGA controls.

Gene	**SGA Wales Cohort**	**SGA Manchester Cohort**	**LGA Wales Cohort**	**LGA Manchester Cohort**
PHLDA2	↑ 101%	-	-	↓ 25%
CDKN1C	-	-	-	-
PEG3	-	-	-	-
PEG10	-	↓ 25%	↑ 34%	-
hPL	-	-	-	-
PGH	-	-	-	-

3.4.1. Protocol Optimisation

Time between delivery and placental dissection did not significantly affect placental RNA concentrations or expression of the housekeeping gene *YWHAZ. YWHAZ* gene expression can be used as a marker of RNA integrity with significantly decreased *YWHAZ* expression associated with increased RNA degradation (Huang *et al.* 2013). Thus, these results suggest that placental samples taken up to two hours after delivery are of sufficient quality for gene expression analysis. Similarly, time to sampling was not significantly associated with changes in placental target gene expression. These results are consistent with those of Fajardy *et al.* (2009) who reported stable placental RNA integrity and gene expression up to 96hrs after delivery,

provided samples were stored in RNA*later*. Similarly, although Avila *et al.* (2010) observed significant mRNA degradation of target genes with increasing processing time up to 24hrs, the greatest reduction in target gene expression was observed between 2 and 3 hrs post-delivery suggesting that samples taken within 2 hours of delivery were of sufficient quality for qPCR. Therefore, only samples obtained within 2 hours of delivery were used in the current study.

Placental *PHLDA2* expression differed significantly with sampling site, with increased expression at distal sampling sites compared with sites close to the umbilical cord. Although no previous study has examined placental *PHLDA2* expression in relation to sampling site, a number of studies have similarly reported significant intraplacental variation in gene expression (Hempstock *et al*. 2003; Pidoux *et al*. 2004; Wyatt *et al*. 2005; Sood *et al*. 2006; Tzschoppe *et al.* 2010). Given that *PHLDA2* expression is altered in response to hypoxia (Roh *et al*. 2005; Kim *et al.* 2007; Tomlinson *et al.* 2010), it is possible that differences in perfusion between distal sites and sites close to the umbilical cord (Hempstock *et al.* 2003; Wyatt *et al.* 2005) could underlie the differences observed in placental *PHLDA2* expression. No significant difference was observed in placental *CDKN1C*, *PEG3*, *PEG10*, *PGH* or *hPL* expression between sampling sites. The observation of intraplacental variation in *PHLDA2* expression is novel and has implications for future studies with consistency in placental sampling of key importance; based on these results all placental samples for the current study were consistently taken from site 2B (middle layer, mid way between cord insertion and distal edge).

Only placental *PGH* expression was significantly positively correlated with gestational age, with 2.5 fold increased expression between early term and late term placentas. This result is consistent with observations of increasing maternal serum *PGH* as gestation progresses (Lacroix *et al.* 2002). There was no significant correlation between gestational age and expression of any other target gene. However, it should be noted that placental *PHLDA2* and *PEG3* expression was greatly increased in late term placentas but due to large standard deviations and a small sample size this

was not statistically significant. Thus, gestational age was controlled for in all analyses of placental *PGH*, *PHLDA2* and *PEG3* expression.

There was no significant difference in target gene expression between male and female control placentas. Moore *et al.* (2015) similarly report no significant effect of fetal sex on placental *PHLDA2*, *CDKN1C*, *PEG3* or *PEG10* expression. Mannik *et al.* (2010) also observed no significant differences in *hPL* or *PGH* expression between male and female placentas despite reports of increased maternal serum *hPL* and *PGH* in mothers of female fetuses (Fuglsang and Ovesen 2006). However, it should be noted that sexual dimorphism exists in the fetal response to an adverse environment (Clifton 2010) and therefore although no difference in placental target gene expression was observed in the control placentas analysed, it is possible that gene expression may be altered to different extents in male and female placentas by complications of pregnancy such as FGR.

Placental *CDKN1C* expression was significantly increased by almost three fold in labouring compared with non-labouring placentas. Although no previous study has examined placental *CDKN1C* expression in response to labour, significant differential gene expression has previously been reported between labouring and non-labouring placentas (Lee *et al.* 2010; Peng *et al.* 2011; Cindrova-Davies *et al.* 2007). This result is novel and has implications for interpretation of results in future studies. In addition, it could explain the conflicting results reported regarding *CDKN1C* and human fetal growth restriction. For example, Rajamaran *et al.* 2010 analysed *CDKN1C* expression in placentas from both vaginal and elective C-section deliveries, which could confound the results reported. Therefore, in the current study mode of delivery was controlled for in any analysis of *CDKN1C* expression.

In conclusion, placental samples taken up to two hours after delivery are of sufficient quality for gene expression analysis. In addition, consistency in placental sampling is necessary to ensure comparable gene expression data. Finally, both gestational age and mode of delivery must be fully described in all studies and should be accounted for in analyses of placental gene expression.

3.4.2. *PHLDA2* and FGR

In the Wales Cohort, placental *PHLDA2* expression was significantly increased by 60% in participants with prenatal concern over fetal growth including those with fetus' measuring SGA by head or abdominal circumference on ultrasound. Placental *PHLDA2* expression was also increased (by 150%) in participants with prenatal growth restriction in the Manchester cohort, although this difference was not statistically significant due to the large variation in *PHLDA2* expression in infants with prenatal growth concern. There was no significant association between placental *PHLDA2* expression and time of fetal growth restriction onset, amniotic fluid levels, umbilical artery pulsatility index or placental diameter on scan. No previous study has reported on placental *PHLDA2* expression and prenatal growth restriction or time of onset (McMinn *et al*. 2006; Diplas *et al*. 2009; Kumar *et al*. 2012). This is therefore the first evidence that increased placental *PHLDA2* expression is associated with fetal growth restriction that becomes evident during pregnancy, although gene expression was not significantly associated with time of onset or other factors used to ascertain the presence and severity of growth restriction.

In both the Manchester and Wales Cohorts, placental *PHLDA2* expression was significantly inversely associated with birth weight and custom birth weight centiles. In the Wales Cohort *PHLDA2* expression accounted for 6% of variance in birth weight and 8% of variance in custom birth weight centiles. Apostolidou *et al.* (2007) and Lim *et al.* (2012) similarly reported an inverse association between *PHLDA2* expression and birth weight. However, these results contrast those of Lambertini *et al. (*2012), Lewis *et al.* (2012) and Demetriou *et al.* (2014) which observed no significant association between placental *PHLDA2* expression and birth weight. These conflicting results could be due to differences in study methodology and population. For example, Demetriou *et al*. (2014) analysed placental *PHLDA2* expression from first trimester chorionic villus samples, suggesting that the role of *PHLDA2* in the control of fetal growth is specific to late pregnancy. It is also possible that no association between placental *PHLDA2* expression and birth weight was detected in the studies by Lambertini *et al.*

(2012) and Lewis *et al.* (2012) because of the relatively small number of placentas analysed (106 and 102 respectively) compared with the current study (181 and 110 in the Wales and Manchester Cohorts).

A two fold increase in *PHLDA2* expression was observed in SGA placentas of the Wales Cohort. A similar two fold increase in *PHLDA2* expression was observed in the Manchester cohort although this difference did not reach statistical significance ($p = 0.09$) possibly due to the smaller number of SGA placentas in this cohort (21 compared with 67). A two fold increase in placental *PHLDA2* expression is slightly less than the three fold increase in gene expression observed in IUGR placentas by Diplas *et al.* (2009). This may result from differences in the definition of growth restriction; while the current study defined SGA as birth weight < 10^{th} centile, Diplas *et al.* (2009) used a more stringent cut off, with IUGR defined as birth weight <3^{rd} centile.

In the Wales Cohort, 27% of SGA placentas displayed aberrant placental *PHLDA2* expression (defined as ≥ 2 fold expression compared with controls). This is consistent with 25% of placentas examined by McMinn *et al.* (2006) demonstrating increased *PHLDA2* expression. Interestingly, aberrant *PHLDA2* expression was also observed in a number of AGA placentas in the Wales Cohort, including one participant with a custom birth weight centile close to the SGA cut off. This result is novel and suggests it is possible that *PHLDA2* expression could be used to identify growth restriction *in utero* not resulting in LBW or SGA. Although no obvious clinical feature linked these cases, further studies are needed to fully characterise these infants and their growth during pregnancy.

In the Manchester Cohort, no significant difference was observed in placental *PHLDA2* expression between AGA infants and either symmetric or asymmetric SGA infants possibly due to the small number of each (17 and 4 respectively). However, in the Wales Cohort, placental *PHLDA2* expression was increased in both symmetric and asymmetric SGA pregnancies relative to AGA controls, although this difference was only statistically significant for symmetric SGA infants. Tunster *et al.* (2014) observed asymmetric growth

restriction, with evidence of head sparing, in the mouse model of *Phlda2* over expression. No previous studies have analysed human placental *PHLDA2* expression in symmetric and asymmetric SGA cases independently. The results of the current study therefore provide the first evidence that *PHLDA2* may also play a role in symmetric growth restriction. It should also be noted that given the non-significant, almost 2 fold increase in *PHLDA2* expression observed in asymmetric SGA placentas, this study does not discount a role for *PHLDA2* in asymmetric growth restriction in human pregnancies which may become apparent in further studies with larger sample sizes.

In terms of other definitions of growth restriction, there was no significant difference in *PHLDA2* expression in LBW placentas in either cohort, likely reflecting the heterogeneous causes of being born LBW including pathological growth restriction and constitutional smallness. Only 43% of SGA pregnancies in the Wales Cohort were diagnosed as IUGR prenatally. Placental *PHLDA2* expression was significantly increased in IUGR pregnancies although there was no significant correlation between gene expression and time of onset. Diplas *et al.* (2009) and McMinn *et al.* (2006) similarly demonstrated increased *PHLDA2* expression in cases of clinically diagnosed IUGR although neither study reported on time of onset.

Placental *PHLDA2* expression was also significantly inversely associated with head circumference at birth in the Wales Cohort, although *PHLDA2* expression only accounted for 1% of variance in head circumference. Previous studies (Apostolidou *et al.* 2007; Lewis *et al.* 2012; Lambertini *et al.* 2012) reported no significant association between placental *PHLDA2* expression and head circumference. These conflicting results may be due to the relatively small number of pregnancies analysed (Lambertini *et al.* 2012) and/or that only normal birth weight infants were examined (Apostolidou *et al.* 2007; Lewis *et al.* 2012). Therefore, these results provide the first evidence that human placental *PHLDA2* expression is negatively associated with head circumference. Importantly, this is consistent with the increased placental *PHLDA2* expression observed in symmetric SGA cases in the current study, suggesting that in cases of fetal growth restriction characterised by aberrant placental *PHLDA2* expression, head sparing (the

typical fetal adaptation to an adverse intrauterine environment), may be impaired.

Finally, *PHLDA2* expression was inversely correlated with placental weight in both the Wales and Manchester Cohorts with *PHLDA2* expression accounting for 2% of variance in placental weight. Although human studies have previously reported no significant association between placental *PHLDA2* expression and placental weight (Apostolidou *et al*. 2006; Lewis *et al*. 2011), as with head circumference, this may be due to the cohorts examined including only normal birth weight infants. However, the results of the current study are consistent with the impaired placental growth observed in the mouse model of *Phlda2* over expression (Tunster *et al*. 2010). The novel results of the current study therefore suggest a role for *PHLDA2* in the control of placental growth in human pregnancies.

3.4.3. *PHLDA2* and poor perinatal outcomes

In the Manchester Cohort, placental *PHLDA2* expression was significantly increased (by 130%) in infants with a poor perinatal outcome, defined as preterm birth, SGA or term NICU admission. However, there was no significant difference in placental *PHLDA2* expression in infants admitted to NICU in this cohort and no significant correlation between gene expression and gestational age. This suggests that the difference observed in infants with poor perinatal outcome is due to the relationship between *PHLDA2* expression and fetal growth restriction alone. Similarly, in the Wales Cohort, although placental *PHLDA2* expression was three fold higher in infants admitted to NICU this difference was not statistically significant. This is likely due to the large variation seen in *PHLDA2* expression in the NICU group reflecting the heterogeneous group of infants admitted to NICU.

Finally, placental *PHLDA2* expression was not significantly associated with measures of adverse infant outcomes such as fetal distress during delivery, umbilical cord blood pH or apgar scores. No previous study has examined placental *PHLDA2* expression in relation to poor perinatal outcomes. Therefore, this study is the first to suggest that aberrant placental

PHLDA2 expression does not identify infants at high risk of poor perinatal outcomes at birth. However, it should be noted that few infants in either cohort suffered extreme adverse outcomes at delivery and therefore larger studies are needed to fully determine the role of *PHLDA2* expression in predicting adverse outcomes.

3.4.4. *CDKN1C* and FGR

Although initial analysis of the Wales Cohort suggested an inverse correlation between placental *CKN1C* expression, birth weight and custom birth weight centiles, this association was subsequently demonstrated not to be significant when results were analysed according to labour status. Similarly, placental *CDKN1C* expression was not significantly associated with any measure of fetal growth in the Manchester Cohort. There was also no significant difference in *CDKN1C* expression between SGA and AGA placentas in either cohort. Few previous studies, with conflicting results, have examined placental *CDKN1C* expression in relation to growth restriction. Our results are consistent with those of Diplas *et al*. (2009) demonstrating no significant difference in *CDKN1C* expression in IUGR placentas. In contrast, McMinn *et al*. (2006) reported significantly increased and Rajaraman *et al*. (2010) significantly decreased placental *CDKN1C* expression in growth restricted pregnancies. These conflicting results may have arisen from a failure to control for mode of delivery, for example Rajaraman *et al*. (2010) analysed *CDKN1C* expression in vaginal and elective c-section placentas combined whereas the labour status of the placentas examined by McMinn *et al*. (2006) was not determined. Thus, the results of the current study do not support a role for placental *CDKN1C* expression in human FGR and highlight the need to thoroughly control for mode of delivery in future studies.

Finally, there was no significant difference in placental *CDKN1C* expression in infants admitted to NICU at delivery and no significant correlation between *CDKN1C* expression and measures of adverse infant outcomes such as umbilical cord blood pH or apgar scores in either cohort.

These results suggest that aberrant placental *CDKN1C* expression does not identify infants at high risk of poor perinatal outcomes at birth.

3.4.5. *PEG3* and FGR

Placental *PEG3* expression was not significantly correlated with birth weight or custom birth weight centiles in either cohort. Similarly, *PEG3* expression was not significantly altered in SGA placentas. This is consistent with results from four previous studies (McMinn *et al*. 2006; Diplas *et al*. 2009; Kumar *et al*. 2011 and Moore *et al*. 2015) reporting no association between placental *PEG3* expression and measures of fetal growth. Similarly, placental *PEG3* expression was not significantly associated with placental weight in the Wales or Manchester Cohort. Moore *et al*. (2015) also reported no significant association between *PEG3* expression and placental weight. Thus, consistent with previous studies, the findings of the current study do not support a role for *PEG3* in human fetal growth restriction. It is possible that the 10% decrease in weight of *Peg3* mutants at birth (Li *et al*. 1999; Kim *et al*. 2013) represents a mouse-specific response or that the relatively small effect of *Peg3* loss of function on fetal growth cannot be detected in a cohort of this size. Finally, there was no significant difference in placental *PEG3* expression in infants admitted to NICU at delivery and no significant correlation between *PEG3* expression and umbilical cord blood pH or apgar scores in either cohort, suggesting that aberrant placental *PEG3* expression does not identify infants at high risk of poor perinatal outcomes at birth.

3.4.6. *PEG10* and FGR

In the Wales Cohort, placental *PEG10* expression was not significantly associated with birth weight or custom birth weight centiles and expression did not differ significantly between AGA and SGA pregnancies. In contrast, a significant positive association was observed between placental *PEG10* expression and prenatal abdominal circumference and femur length on scan, as well as birth weight, in the Manchester Cohort. Placental *PEG10*

expression was also significantly decreased in SGA compared with AGA placentas. The greatest reduction in placental *PEG10* expression was observed in the asymmetric SGA placentas, although this difference did not reach statistical significance ($p = 0.09$). This is consistent with the positive correlation between placental *PEG10* expression, abdominal circumference and femur length, (but not head circumference), suggesting a role for *PEG10* in asymmetric fetal growth restriction characterised by reduced length and abdominal circumferences but relative sparing of the head. Similarly, placental *PEG10* expression was not significantly associated with head circumference at delivery. These results contrast those of Diplas *et al*. (2009) demonstrating increased placental *PEG10* expression in IUGR placentas and those of Moore *et al*. (2015) demonstrating no significant association with birth weight. Lim *et al*. (2012) however, have previously demonstrated a significant positive association between birth weight and umbilical cord *PEG10* expression. This is therefore the first evidence of an association between human placental *PEG10* expression and fetal growth, with a specific correlation with abdominal and femur growth during pregnancy.

Furthermore, placental *PEG10* expression was significantly reduced in infants with poor perinatal outcome. *PEG10* expression was not altered in infants admitted to NICU after delivery, suggesting that the association with poor perinatal outcome was due to fetal growth restriction cases alone. Placental *PEG10* expression was significantly positively associated with apgar scores at 1 minute and 5 minutes after delivery. No previous study has examined placental *PEG10* expression in relation to infant outcomes at delivery. Therefore this is the first evidence that placental *PEG10* expression may predict apgar scores at delivery. Further research is required to determine whether placental *PEG10* expression can be used to identify infants at increased risk of adverse outcomes at delivery.

3.4.7. *hPL* and FGR

In the Wales Cohort, placental *hPL* expression was significantly decreased in pregnancies with prenatal growth concerns, including those with SGA

abdominal and head circumferences on scan. A significant positive correlation between placental *hPL* expression and femur length was also observed in the Manchester Cohort, which may underlie prenatal growth restriction.

Placental *hPL* expression was significantly positively correlated with birth weight, custom birth weight centiles and placental weight in the Wales Cohort. In contrast, only a trend was observed for a positive correlation between placental *hPL* expression and custom birth weight centiles in the Manchester Cohort ($p = 0.08$), possibly as a result of the smaller number of placentas examined. Despite the association observed between placental *hPL* expression and birth weight in the Wales Cohort, expression was not significantly altered in SGA, LBW or IUGR pregnancies in either cohort. These findings are consistent with those reported by Mannik *et al*. (2010) of no significant difference in *hPL* expression between SGA and AGA placentas. It is possible that placental *hPL* expression plays a role in the control of fetal growth but that perturbations in this pathway do not underlie FGR. Further research is required to fully determine the role of *hPL* in human growth restricted pregnancies.

Finally, as reported by Dutton *et al*. (2012), placental *hPL* expression was not significantly altered in infants with poor perinatal outcomes, NICU admission at delivery or low apgar scores. However, placental *hPL* expression was significantly associated with arterial cord blood pH in the Wales Cohort. It is possible that this association reflects the inverse association reported between placental *hPL* expression and hypoxia (Roh *et al*. 2005), suggesting placental *hPL* expression as a potential biomarker of hypoxia during delivery.

3.4.8. *PGH* and FGR

There was no significant correlation between placental *PGH* expression and birth weight, custom birth weight centiles or placental weight in either cohort, after controlling for gestational age. Similarly, placental *PGH* expression was not significantly altered in SGA compared with AGA pregnancies. These

results contrast those of Mannik *et al*. (2010) reporting a significant association between placental *PGH* expression and birth weight. However previous studies have noted only a modest reduction in placental *PGH* expression (approximately 10%) in SGA compared with AGA infants (Mannik *et al*. 2010; Koutsaki *et al*. 2011), which may not have been detectable in our cohorts. Furthermore, given the significant correlation between placental *PGH* expression and gestational age observed in the current study, conflicting result may be explained by failure of previous studies to control for gestational age (Koutsaki *et al*. 2011). The findings of the current study therefore do not support a role for aberrant placental *PGH* expression in fetal growth restriction.

Finally, placental *PGH* expression was not significantly altered in infants with poor perinatal outcomes in the Manchester Cohort. However, in the Wales Cohort placental *PGH* expression was significantly reduced in infants admitted to NICU at delivery. In addition, placental *PGH* expression was significantly positively correlated with arterial cord blood pH after controlling for gestational age. This contrasts results by Trollmann *et al.* (2007) of no significant difference in placental *PGH* expression in response to acute birth asphyxia characterised by reduced umbilical artery pH. Thus, this is the first report of aberrant placental *PGH* expression in infants admitted to NICU and in association with reduced umbilical artery pH. Larger studies of infant birth outcomes are needed to determine the significance of these findings.

3.4.9. Imprinted genes and placental hormones

Placental *PHLDA2* expression was significantly inversely associated with *hPL* but not *PGH* expression in the Wales Cohort. A similar correlation was not observed in the Manchester Cohort, possible as a result of the fewer numbers of placentas examined (110 compared with 219). An inverse association between placental *PHLDA2* and *hPL* expression in the Wales Cohort is consistent with findings of decreased placental lactogen expression and loss of spongiotrophoblast cells (the endocrine lineage of the placenta)

in the mouse model of *Phlda2* over expression (Tunster *et al.* 2010). No previous study has examined the relationship between imprinted gene expression and hormone production in the human placenta. Therefore, the results of the current study provide the first evidence for an association between human placental *PHLDA2* and *hPL* expression with the mouse model of *Phlda2* over expression suggesting that this relationship is causal, with *PHLDA2* expression negatively regulating *hPL* expression.

An inverse correlation between placental *CDKN1C* and *hPL* but not *PGH* expression was also observed in the Wales Cohort, after controlling for mode of delivery. An inverse association between placental *CDKN1C* and *hPL* expression in the Wales Cohort is consistent with findings of decreased placental lactogen expression and loss of spongiotrophoblast cells (the endocrine lineage of the placenta) in the mouse model of *Cdkn1c* over expression (Tunster *et al*. 2011). No previous study has examined the relationship between imprinted gene expression and hormone production in the human placenta. Therefore, the results of the current study provide the first evidence for an association between human placental *CDKN1C* and *hPL* expression with the mouse model of *Cdkn1c* over expression suggesting that this relationship is causal with *CDKN1C* expression negatively regulating *hPL* expression.

Neither placental *PEG3* nor *PEG10* expression was significantly associated with *hPL* or *PGH* expression in the Wales or Manchester Cohorts. A trend was observed for a positive correlation between placental *PEG3* and *hPL* expression ($p = 0.07$) in the Manchester Cohort. Given the effect of loss of function of *Peg3* on placental lactogen expression in a mouse model (Broad and Keverne 2011; Kim *et al*. 2013), it is possible that *PEG3* positively regulates placental *hPL* expression in the human placenta however this needs to be confirmed in future studies.

3.4.10. Fetal overgrowth

In the Wales Cohort, placental *PHLDA2*, *CDKN1C* and *PEG3* expression was not significantly altered in LGA or macrosomic pregnancies. However,

placental *PHLDA2* was significantly decreased in LGA and macrosomic pregnancies in the Manchester Cohort. Given the significant correlation between placental *PHLDA2* and birth weight in both cohorts, it is possible that *PHLDA2* expression is altered in cases of fetal overgrowth. However, given the discrepancies between the two cohorts, this remains to be confirmed in future studies.

Placental *PEG10* expression was significantly increased by 34% in LGA pregnancies and by 23% in macrosomic pregnancies of the Wales Cohort. There was no significant difference in *PEG10* expression in LGA placentas of the Manchester Cohort, possibly due to the smaller number of LGA placentas examined. However, results are consistent with the positive correlation observed between placental *PEG10* expression and birth weight in the Manchester Cohort. Although no study has previously examined placental *PEG10* in relation to LGA, these results are consistent with the role of *PEG10* in the positive regulation of fetal growth as suggested by both human (Lim *et al*. 2012) and animal studies (Ono *et al*. 2006). In addition, the results of the current study uniquely suggest a role for aberrant placental *PEG10* expression in pregnancies complicated by fetal overgrowth.

Finally, there was no significant change in placental *hPL* or *PGH* expression in LGA or macrosomic pregnancies of either cohort.

3.4.11. Summary

In summary, the results presented in this chapter support a role for placental *PHLDA2* in the negative regulation of fetal growth with abnormally increased expression observed in pregnancies complicated by fetal growth restriction. A role for placental *PEG10* expression in the positive regulation of fetal growth was also suggested. These results therefore support the first and second study hypotheses of aberrant placental imprinted gene expression in pregnancies complicated by growth restriction and fetal overgrowth. Further research will be required to elucidate the mechanisms by which aberrant placental imprinted gene expression drives growth restriction.

CHAPTER 4: IMPRINTED GENES AND OTHER COMPLICATIONS OF PREGNANCY

4.1. Introduction

Results from Chapter 3 support a role for placental imprinted gene expression, particularly *PHLDA2* and *PEG10*, in the control of fetal growth. Abnormal fetal growth is known to occur in a number of pregnancy complications, such as preeclampsia, gestational diabetes and maternal depression and/or anxiety, prompting investigation of placental imprinted gene expression in these pregnancies. In addition, results from chapter 3 provide evidence for an association between imprinted gene expression and expression of the placental hormone *hPL,* which has an established role in maternal adaptation to pregnancy (Newbern and Freemark 2011). Pregnancy complications such as gestational diabetes are suggested to result from inadequate maternal adaptation to pregnancy (Newbern and Freemark 2011), further supporting investigation of placental imprinted gene and placental hormone gene expression in study participants suffering from these complications. Thus, the main aim of this chapter was to explore a possible association between aberrant placental imprinted gene expression and development of the pregnancy complications preeclampsia, gestational diabetes and maternal mood disorders.

Preeclampsia (PE) is defined as new onset hypertension and proteinuria during pregnancy (NICE 2010), differing from pregnancy induced hypertension (PIH) in which proteinuria is absent. PE is associated with an estimated four fold increased risk of FGR (Villar *et al.* 2006; Hutcheon *et al.* 2011), highlighting the abnormal fetal growth phenotype associated with this pregnancy complication. Given the role of imprinted genes in the control of fetal growth, it is possible aberrant placental expression of these genes underlies the co-morbidity of PE and FGR. In addition, the mouse model of *Cdkn1c* loss of function supports a role for imprinted genes in the pathogenesis of preeclampsia. Preeclampsia-like symptoms develop in wild type mice carrying mutant *Cdkn1c* pups an effect that was attributed to shallow trophoblast invasion and increased spongiotrophoblast proliferation in the placenta (Kanayama *et al.* 2002). As the imprinted genes *Phlda2*, *Peg3* and *Peg10* are also known to regulate the spongiotrophoblast cell

lineage of the mouse placenta (reviewed in John 2013), it is possible that these genes also play a role in preeclampsia. However, previous studies of imprinted gene expression in human PE placentas have yielded conflicting results. A severe form of preeclampsia (HELLP) has been reported to occur in pregnancies in which the infant suffers from BWS as a result of a *CDKN1C* mutation (Romanelli *et al.* 2009) whereas another study demonstrated increased *CDKN1C* expression PE placentas (Enquobahrie *et al.* 2008). In contrast, placental *PHLDA2* expression has previously been demonstrated not to be significantly altered in a small number of PE placentas (McMinn *et al.* 2006). While no previous study has examined placental *PEG3* expression in PE pregnancies, Lambertini *et al*. (2008) demonstrated LOI of *PEG3* in 50% of the PE placentas examined. Finally, both increased (Chen *et al*. 2012) and decreased (Liang *et al*. 2014) placental *PEG10* expression has been reported in PE pregnancies.

Gestational diabetes (GDM) is defined as new onset glucose intolerance during pregnancy (Buchanan 2007; Hartling *et al*. 2013; Mitanchez *et al*. 2014). Pregnancies complicated by GDM are twice as likely to result in delivery of an LGA or macrosomic infant (Hartlingt *et al*. 2013), possibly as a result of increased neonatal body fat (Catalano *et al*. 2003). Given the role of imprinted genes in the control of fetal growth, it is possible aberrant placental expression of these genes underlies the co-morbidity of GDM and fetal overgrowth. The association between placental imprinted gene expression and *hPL* expression demonstrated in Chapter 3 further suggests a role for imprinted genes in the pathogenesis of GDM. The placental hormone hPL is known to prevent maternal glucose intolerance as a consequence of metabolic adaptation to pregnancy (reviewed in Fuglsang and Ovesen 2006; Newbern and Freemark 2011). This is supported by reports of altered maternal hPL serum levels in diabetic pregnancies (Ursell *et al*. 1973; McIntyre *et al*. 2000; Mannik *et al*. 2012). No previous study has examined placental *PHLDA2*, *CDKN1C*, *PEG3* or *PEG10* expression in GDM pregnancies, although an increased risk of GDM has been reported in women inheriting a genetic variant of *KCNQ1* (the imprinting cluster in which *PHLDA2* and *CDKN1C* are located) (Mao *et al*. 2012).

It is thought that women are particularly prone to developing symptoms of depression and anxiety during and after pregnancy, which may be due to the numerous physiological and hormonal changes that occur during pregnancy (Tan and Tan 2013). The placenta is a key source of hormones that act on the maternal brain, priming the mother for pregnancy and postnatal care (Glynn and Sandman 2011) and thereby inducing maternal psychological adaptation to pregnancy. In particular, hPL produced by the placenta has been demonstrated to induce maternal behaviour in animal studies (Bridges *et al*. 1985; Bridges *et al*. 1990; Bridges and Freeman 1995; Bridges *et al*. 1997) and to be reduced in the serum of human mothers with postnatal depression (Abou-Saleh *et al*. 1998; Ingram *et al*. 2003; Groer and Morgan 2007). It has therefore been suggested that aberrant placental function may influence maternal mood (Glynn and Sandman 2011). Based on the association between placental imprinted gene expression and *hPL* expression reported in Chapter 3, it was proposed that imprinted genes could contribute to maternal psychological adaptation to pregnancy via control of placental hormone production, with inadequate adaptation manifesting as symptoms of anxiety and/or depression during pregnancy. In support of this hypothesis, *Peg3* has previously been demonstrated to be necessary for the induction of nurturing and nest building behaviour in rodents (Li *et al*. 1999; Curley *et al*. 2004; Champagne *et al*. 2009; Chiavegatto *et al*. 2012). Investigation of placental imprinted gene expression in pregnancies complicated by maternal mood disorders is also warranted based on the established association between maternal anxiety and depression during pregnancy and fetal growth restriction (Khashan *et al*. 2008; Berkowitz *et al*. 2003; Pritchard and Teo 1994; Paarlberg *et al*. 1999; Steer *et al*. 1992; Henrichs *et al*. 2010; Uguz *et al*. 2013). Given the role of placental imprinted genes in the control of fetal growth, it is possible that aberrant gene expression could explain the co-occurrence of maternal mood disorders and fetal growth restriction.

Thus, this chapter aimed to examine placental imprinted gene and placental hormone gene expression in pregnancies complicated by preeclampsia, gestational diabetes and maternal mood disorders.

4.2. Chapter specific methods

Methods were as described in Chapter 2. For ease of interpretation of the results presented in this chapter, methods related to the study of placental target gene expression in pregnancies complicated by PE, GDM and maternal mood disorders are summarised below.

4.2.1. Methods: preeclampsia

Target gene expression was analysed in 22 placentas from PE or PIH pregnancies in the Wales Cohort. In this study, preeclampsia was defined as diastolic blood pressure ≥ 90 – 99 mm Hg and systolic blood pressure ≥ 140 – 149 mm Hg in association with ≥ 300mg protein in a 24hr urine test. A diagnosis of PE and the gestational age at diagnosis, was recorded from the participant's medical notes. Gestational age at first occurrence of hypertension and proteinurea were recorded to distinguish between pregnancy induced hypertension (hypertension without proteinurea) and preeclampsia (presence of both hypertension and proteinurea). Finally, the treatment for preeclampsia was noted as well as the gestation age at which treatment began.

4.2.2. Methods: gestational diabetes

Target gene expression was analysed in 16 placentas from GDM pregnancies in the Wales Cohort. In this study, GDM was defined as fasting glucose ≥ 6.1mmol/L or ≥ 7.8mmol/L at 2 hours following a 75g oral GTT. Gestational age at first abnormal GTT and GDM diagnosis was recorded from the participants' medical notes. A previous GDM pregnancy was also noted. Finally, the form of GDM management (diet, exercise and/or medication) was recorded. The Wales Cohort also included a number of women with a confirmed normal GTT (at ≥ 28 weeks) who had been tested for GDM because of risk factors such as previous history, family history of diabetes, LGA infant, and/or BMI ≥ 30.

4.2.3. Methods: Maternal mood disorders

Target gene expression was analysed with respect to maternal depression in the Imperial and Manchester Cohorts. In the Imperial Cohort, symptoms of depression were assessed using the Edinburgh Postnatal Depression Scale (EPDS) with higher scores indicating higher levels of depression. A median split of the cohort by maternal EPDS scores was carried out for the purpose of analysing the effects of maternal depression on placental gene expression. In addition, placental target expression was compared between participants with an EPDS score ≥13 (indicative of clinical depression) and those with EPDS scores < 13. In the Manchester Cohort, a diagnosis of depression during pregnancy (including any treatment prescribed) was recorded from the participant's medical notes.

Target gene expression was analysed with respect to maternal anxiety in the Imperial Cohort. Maternal anxiety was measured using the Spielberger Trait Anxiety Inventory (STAI), a self-administered psychometric questionnaire with higher scores indicating higher levels of anxiety. A median split of the cohort by maternal STAI scores was carried out for the purpose of analysing the effects of maternal depression on placental gene expression. This median split also divided participants into those with a STAI score ≥ 40 (indicative of clinical anxiety) and those with a STAI score < 40.

4.2.4. Statistical analysis

All statistical analysis was carried out using IBM SPSS statistics for Windows (version 20.0, 2011) with a p value <0.05 considered statistically significant.

Normal distribution was assessed using P-P plots and a Shapiro-Wilk test. All data was normally distributed and therefore parametric statistical tests used. Associations between placental gene expression and measures of maternal mood (e.g. EPDS scores) were assessed using a Pearson correlation test. Differences in placental gene expression between control

and complicated pregnancies were analysed using an independent samples T tests or one-way ANOVA with a Tukey post hoc test where appropriate.

Given the differences in placental *CDKN1C* expression according to mode of delivery as demonstrated in Chapter 3, multiple linear regression analysis was also carried out to determine whether labour status and preeclampsia independently predicted placental *CDKN1C* expression.

4.3. Results

4.3.1. Maternal preeclampsia

4.3.1.1. Participant demographics

22 Caucasian participants were diagnosed with preeclampsia or pregnancy induced hypertension during pregnancy. Of these 22 participants, five were diagnosed with PIH and 17 with PE. There was no significant difference in any maternal lifestyle factor examined between PE/PIH and control participants as shown in Figure 4.1.

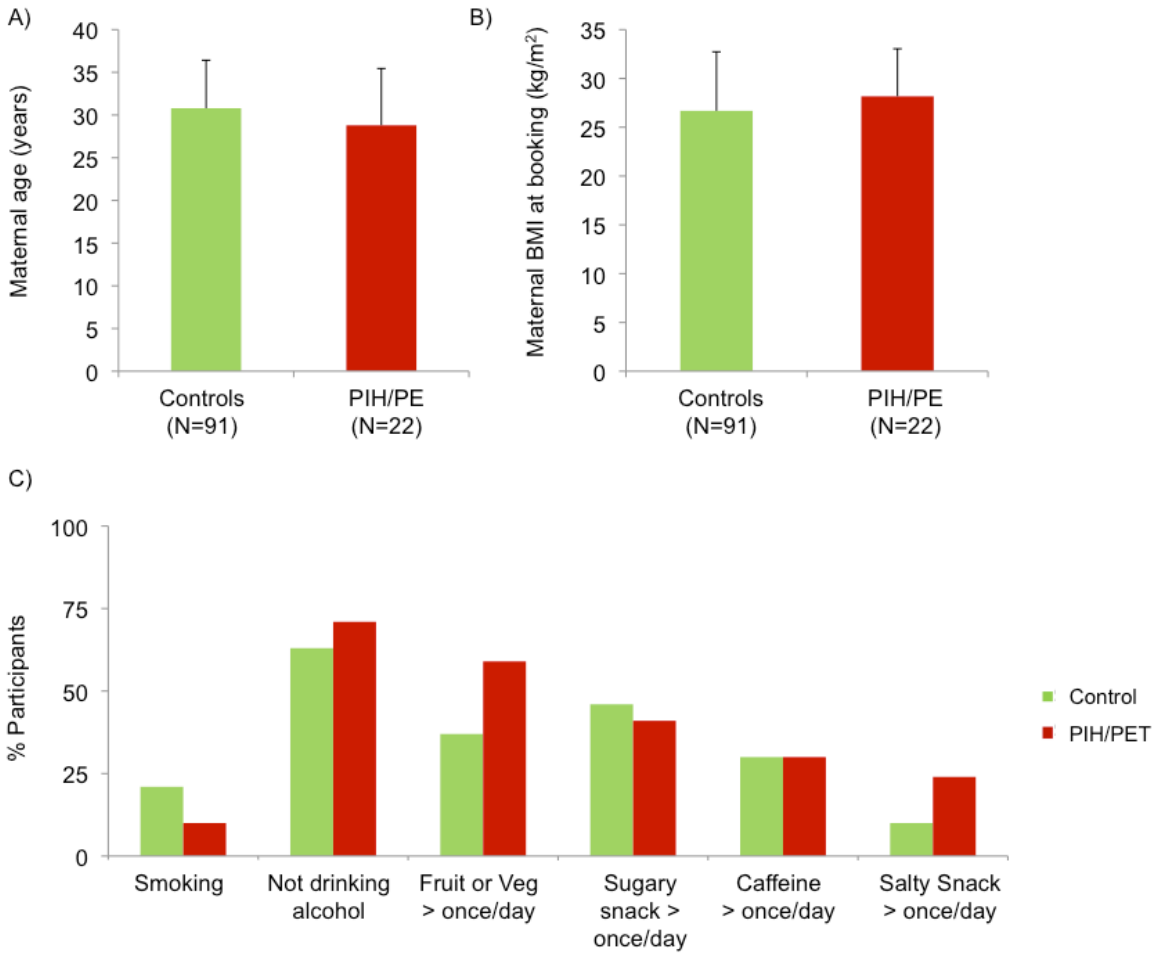


Figure 4.1: Maternal lifestyle and PIH/PE. There was no significant difference in maternal age (A), BMI at booking (B) or lifestyle factors (C) between PIH/PE and control participants as determined by an independent samples t-test or chi-squared test where appropriate. PIH = pregnancy induced hypertension, PE = preeclampsia. Error bars represent SEM.

4.3.1.2. Preeclampsia and birth weight

Birth outcomes for PE/PIH and control participants are shown in Table 4.1. A significantly smaller number of PIH/PET participants delivered by elective c-section highlighting the need to control for mode of delivery in target gene expression analysis.

Table 4.1: Comparison of birth outcomes between control and PIH/PE participants. Mean (SD)/Range or Number (%) is shown. Differences were analysed using an independent samples T test or Chi – square test where appropriate. PE = preeclampsia, PIH = pregnancy induced hypertension.

	Control Participants (N = 91)	**PE/PIH participants** (N = 22)	**P Value**
Birth Outcome			
Mode of Delivery:			
Vaginal	7 (8%)	6 (27%)	
Elective C section	78 (86%)	6 (27%)	
Emergency C section	4 (4%)	6 (27%)	
Instrumental	2 (2%)	4 (18%)	**P < 0.001*** **
Birth weight (g)	3480 (277)/ 2830 - 4190	3123 (560)/ 1830 - 3910	**P = 0.007** **
Custom birth weight centile	53 (22) / 11 - 89	33 (26) / 0 - 86	**P = 0.001*** **
Head circumference (cm)	35 (1) / 32 - 39	35 (1) / 33 - 38	**P = 0.04***
Gestational age (weeks)	39 (1) / 37 - 42	39 (1) / 35 - 42	P = 0.24
Placental weight (g)	703 (124) / 309 – 905	591(120) / 367– 820	P = 0.10
Gender			
Male	44 (48%)	15 (68%)	
Female	47 (52%)	7 (32%)	P = 0.08

Birth weight was significantly reduced by 360g in infants born to PE/PIH participants compared with controls ($p = 0.01$, $n = 113$, Table 4.1). A one-way ANOVA revealed significant differences in birth weight when participants were divided into those with PIH or PE ($p < 0.001$, $n = 113$) Figure 4.2. A Tukey post hoc test further revealed decreased birth weights for PE ($p < 0.001$, $n = 108$) but not PIH participants ($p = 0.44$, $n = 96$). Due to the differing effects on birth weight, placental target gene expression was examined in the PE and PIH participants combined and independently.

As with birth weight, custom growth centiles were significantly reduced in PIH/PE participants compared with controls ($p = 0.001$, $n = 113$). Further

analysis revealed that custom growth centiles were significantly reduced only in PE (p = 0.001, n = 108) but not PIH participants (p = 0.58, n = 96), Figure 4.2. Finally, a trend was observed for decreased placental weight in PE/PIH participants compared with controls (p = 0.10, n = 113).

Only one participant was diagnosed with early onset preeclampsia (≤ 34 weeks) and therefore fetal growth and placental gene expression could not be compared between severity groups.

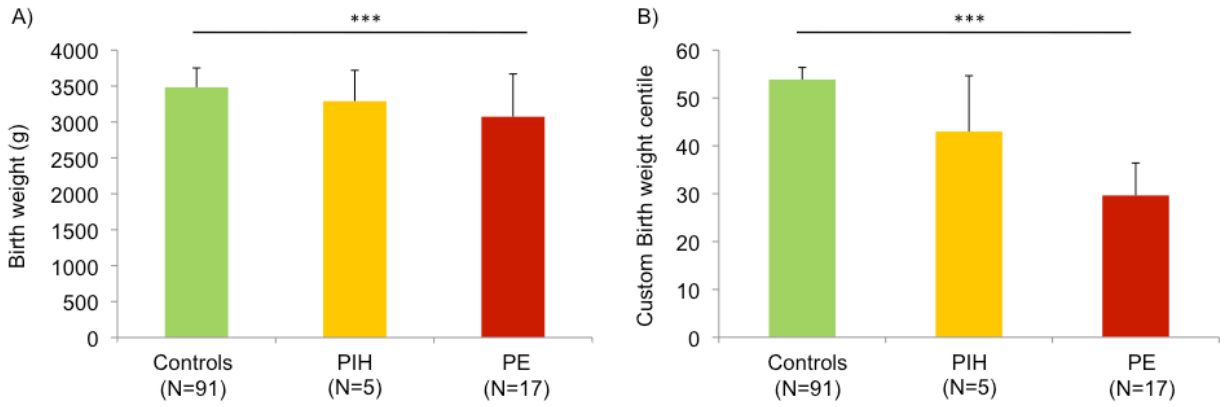


Figure 4.2: Birth weight and preeclampsia. Birth weight (A) and custom birth weight centiles (B) were significantly reduced in PE but not PIH participants compared with controls, as determined by a one-way ANOVA and Tukey post-hoc test. PIH = pregnancy induced hypertension, PE = preeclampsia. *** p < 0.001. Error bars represent SD.

4.3.1.3. Preeclampsia and placental *PHLDA2* expression

Placental *PHLDA2* expression was significantly increased by 63% in PIH/PE placentas compared with controls, Figure 4.3. A one-way ANOVA revealed significant differences in placental *PHLDA2* expression when participants were divided into those with PIH or PE (p = 0.02, n = 113, Figure 4.3). A Tukey post hoc test further demonstrated increased placental *PHLDA2* expression for PE (p = 0.01, n = 108) but not PIH participants (p = 0.96, n = 96). Results remained significant with the exclusion of one preterm participant delivered at 36 weeks (+5 days) (results not shown).

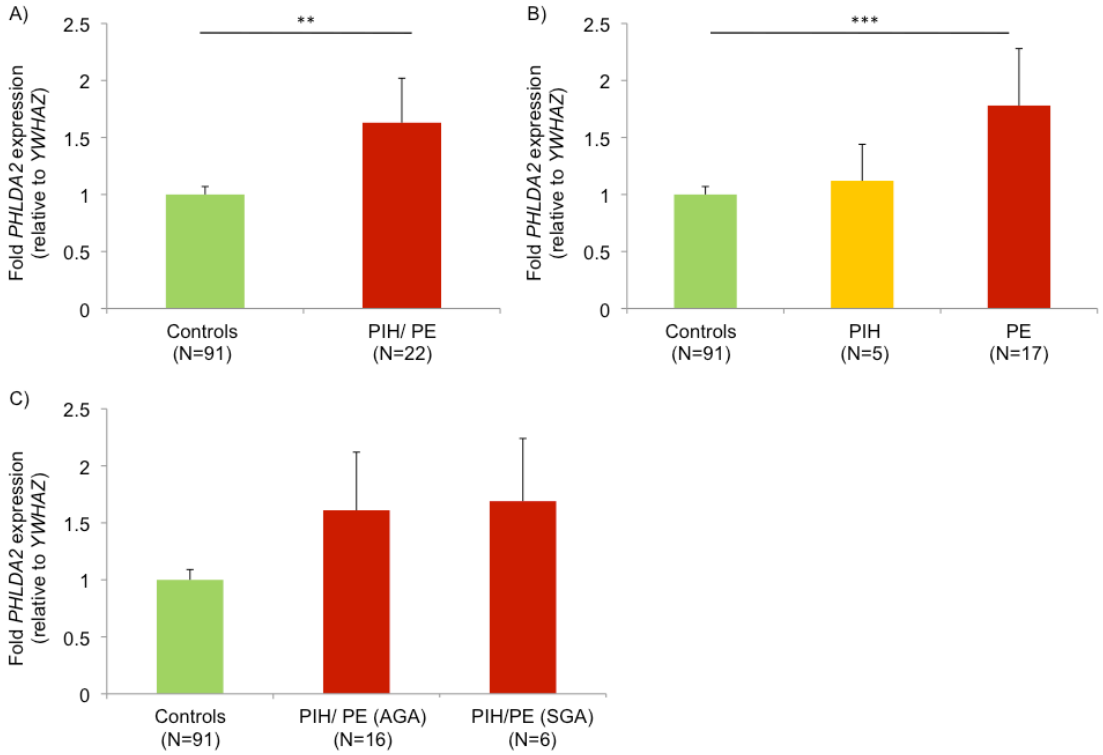


Figure 4.3: Placental *PHLDA2* expression and preeclampsia. *PHLDA2* expression was significantly increased in PIH/PE placentas (A) as determined by an independent samples t-test. Increased expression was significant only in PE placentas (B). Differences in expression were not significant when participants were divided according to AGA/SGA status (C) as determined by one-way ANOVA and Tukey post-hoc test. PIH = pregnancy induced hypertension, PE = preeclampsia.

To determine whether the increase observed in placental *PHLDA2* expression was associated with the growth restriction reported in 38% of the PIH/PE participants, expression was compared between controls and PIH/PE participants with or without the presence of SGA. There was a significant difference in placental *PHLDA2* expression between groups as determined by a one-way ANOVA ($p = 0.04$, $n = 113$). However, although increased expression compared with controls was observed for both PIH/PE (SGA) and PIH/PE (AGA) groups respectively these differences were not statistically significant (Figure 4.3).

4.3.1.4. Preeclampsia and *CDKN1C, PEG3* and *PEG10* expression

Placental *CDKN1C* expression was significantly increased by 45% in participants with PIH/PE compared with controls, Figure 4.4. A one-way

ANOVA revealed a trend for differences in placental *CDKN1C* expression when participants were divided into those with PIH or PE ($p = 0.07$, $n = 113$) with increased *CDKN1C* expression in the PE group only (Figure 4.4). However, there was no significant difference in placental *CDKN1C* expression when PIH/PE participants were divided into those with or without SGA ($p = 0.12$, $n = 113$) although expression was increased in both groups compared with controls (results not shown).

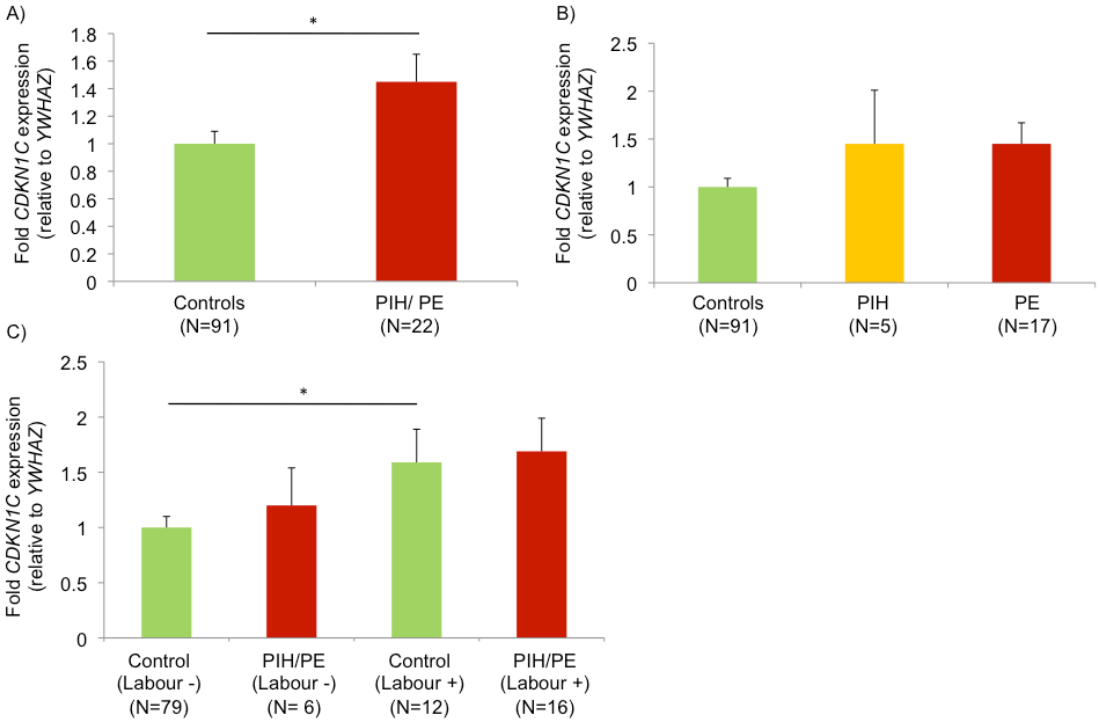


Figure 4.4: Placental *CDKN1C* expression and preeclampsia. *CDKN1C* expression was significantly increased in PIH/PE placentas (A) as determined by an independent samples t-test. Expression was increased in both PIH and PE placentas (B) although differences were not significant as determined by one-way ANOVA. When divided according to labour status, *CDKN1C* expression was only significantly increased in response to labour (C). PIH = pregnancy induced hypertension, PE = preeclampsia.

Given the significant increase in *CDKN1C* expression in labouring placentas (section 3.3.1.5) and the significant difference in mode of delivery between PIH/PE and control participants (Table 4.1), *CDKN1C* expression in PIH/PE placentas was further analysed according to labour status. As shown in Figure 4.4, *CDKN1C* expression was only increased between control labouring and non-labouring placentas, as determined by a one-way ANOVA

and Tukey post hoc test. Multiple linear regression analysis was also carried out to determine whether labour status and preeclampsia independently predicted placental *CDKN1C* expression. This model significantly predicted placental *CDKN1C* expression ($F(2, 110) = 3.71$, $p = 0.03$, $R^2 = 0.06$), however only labour status ($p = 0.02$) and not preeclampsia ($p = 0.37$) significantly predicted placental *CDKN1C* expression.

There was no significant difference in placental *PEG3* or *PEG10* expression between control and PIH/PE participants (Figure 4.5). Results remained non-significant when participants were divided into those with PIH or PE, and into those with or without SGA (results not shown).

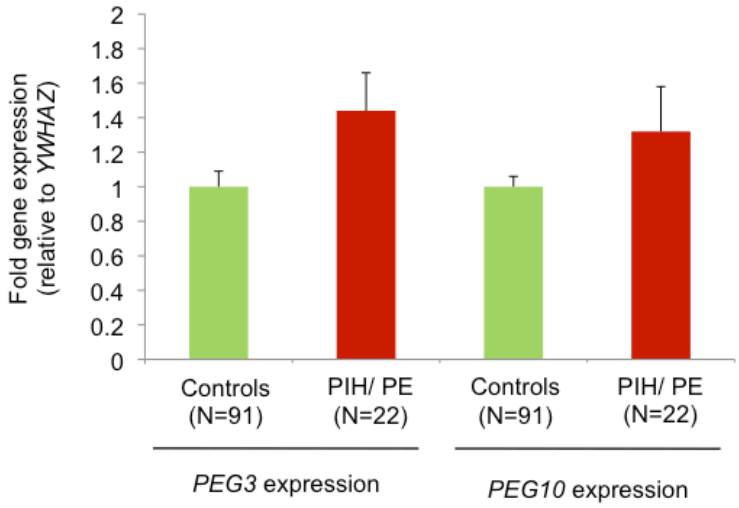


Figure 4.5: Placental *PEG3* and *PEG10* expression and preeclampsia. There was no significant difference in expression between PIH/PE placentas and controls as determined by an independent samples T-test. PIH = pregnancy induced hypertension, PE = preeclampsia. Error bars represent SEM.

4.3.1.5. Preeclampsia and placental hormone gene expression

Placental *hPL* and *PGH* expression was not significantly altered in PIH/PE participants compared with controls (Figure 4.6). Results remained non-significant when participants were divided into those with PIH or PE and into those with or without SGA (results not shown).

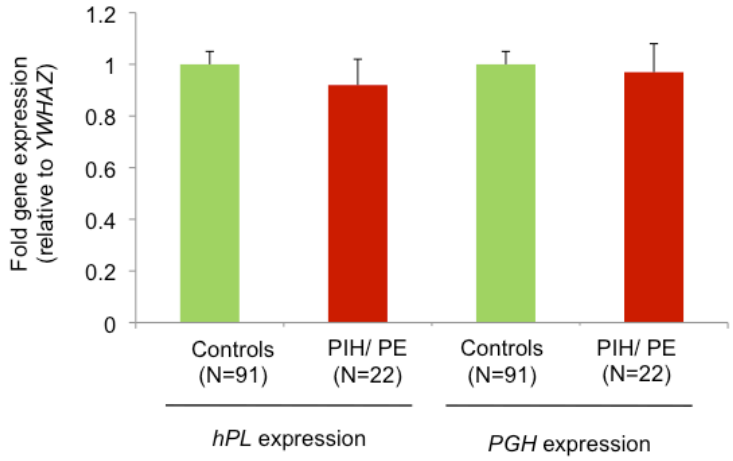


Figure 4.6: Placental *hPL* and *PGH* expression and preeclampsia. There was no significant difference in expression between PIH/PE placentas and controls as determined by an independent samples T-test. PIH = pregnancy induced hypertension, PE = preeclampsia. Error bars represent SEM.

4.3.1.6. Preeclampsia Summary

A summary of the changes in target gene expression in PIH/PE placentas is shown in Table 4.2. Placental *PHLDA2* expression was significantly increased in placentas of PIH/PE participants. While *CDKN1C* expression was also altered in these placentas, this was demonstrated to be due to differences in labour status.

Table 4.2. Summary of placental gene expression changes associated with preeclampsia. Fold gene expression is shown relative to control participants. Results highlighted were statistically significant using an independent samples T test. ** $p \leq 0.01$. [(a)] p value after controlling for labour using multilinear regression.

Gene	Control Participants (N=91)	PE/PIH participants (N=22)	P Value
PHLDA2	**1.00**	**1.63**	**P = 0.01 ****
CDKN1C	1.00	1.45	P = 0.37 [(a)]
PEG3	1.00	1.44	P = 0.24
PEG10	1.00	1.32	P = 0.24
hPL	1.00	0.92	P = 0.44
PGH	1.00	0.97	P = 0.82

4.3.2. Maternal Gestational Diabetes Mellitus (GDM)

4.3.2.1. Participant demographics

16 Caucasian participants were diagnosed with GDM during their pregnancy. A comparison of maternal lifestyle factors between GDM and control participants is shown in Figure 4.7.

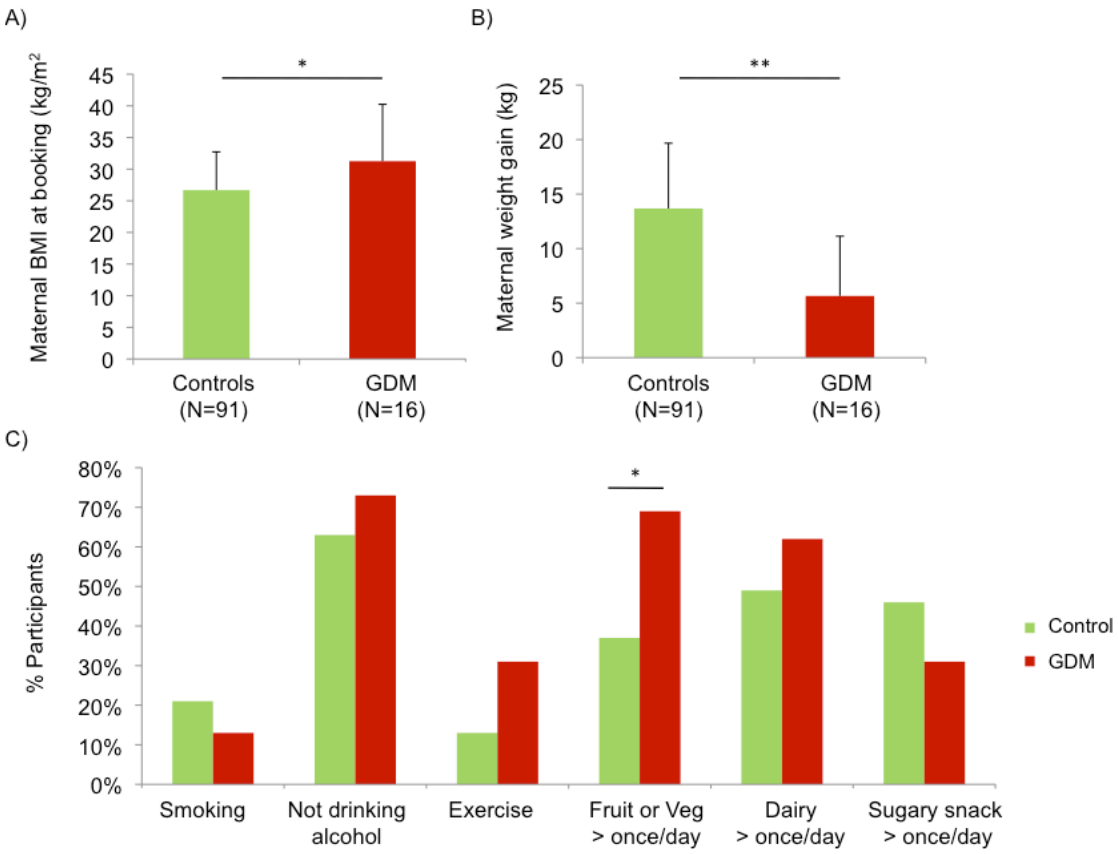


Figure 4.7: Maternal lifestyle and GDM. Maternal BMI was significantly higher in GDM compared with control participants (A) although weight gain during pregnancy was significantly less (B). Fruit or vegetable consumption was significantly higher in GDM participants (C) but there was no significant difference in any other lifestyle factors determined by an independent samples t-test or chi-squared test where appropriate. GDM = gestational diabetes mellitus. Error bars represent SEM.

Maternal BMI was significantly higher in GDM compared with control participants ($p = 0.04$, $n = 107$), Figure 4.7. Therefore, for the purposes of analysing the association between target gene expression and GDM, placental expression was compared with all controls (no known GTT status) and with normal or high BMI controls (known negative GTT test) respectively.

In contrast, maternal weight gain was significantly lower in GDM compared with control participants (Figure 4.7) possibly as a result of 75% of the GDM participants receiving weekly nutrition advice and weight management. Similarly, maternal fruit and vegetable consumption was significantly higher in GDM participants (Figure 4.7). There was no significant difference in any other maternal lifestyle factor examined between GDM and control participants as shown in Figure 4.7.

4.3.2.2. Maternal GDM and birth weight

Birth outcomes for the gestational diabetes (GDM) and control participants are compared in Table 4.3.

Table 4.3: Comparison of birth outcomes between control and GDM participants. Mean (SD)/Range or Number (%) is shown. Differences were analysed using an independent samples T test or Chi – square test where appropriate. GDM = Gestational Diabetes Mellitus.

	Control Participants (N = 91)	**GDM participants** (N = 16)	**P Value**
Birth Outcome			
Mode of Delivery:			
Vaginal	7 (8%)	2 (13%)	
Elective C section	78 (86%)	11 (69%)	
Emergency C section	4 (4%)	3 (19%)	
Instrumental	2 (2%)	0 (0%)	P = 0.18
Birth weight (g)	3480 (277)/ 2830 - 4190	3543 (426)/ 2710 - 4160	P = 0.15
Custom birth weight centile	53 (22) / 11 - 89	64 (26) / 13 - 95	P = 0.11
Head circumference (cm)	35 (1) / 32 - 39	35 (1) / 33 - 38	P = 0.80
Gestational age (weeks)	39 (1) / 37 - 42	39 (1) / 37 - 40	P = 0.22
Placental weight (g)	703 (124) / 309 – 905	718 (144) / 451– 1050	P = 0.37
Gender			
Male	44 (48%)	11 (69%)	
Female	47 (52%)	5 (31%)	P = 0.22

There was no significant difference in birth weight (p = 0.15, n = 107) or placental weight (p = 0.37) between control and GDM participants. Similarly, differences remained non significant when participants were divided into those with a normal BMI and high BMI (overweight/obese), Figure 4.8. There

was also no significant correlation between time of onset and placental (r = 0.11, p = 0.73, n = 16) or birth weight (r = 0.18, p = 0.57, n = 16). Finally, there was no significant difference in birth weight or placental weight between participants requiring metformin or insulin treatment for GDM and those with diet and/or exercise management, Figure 4.8.

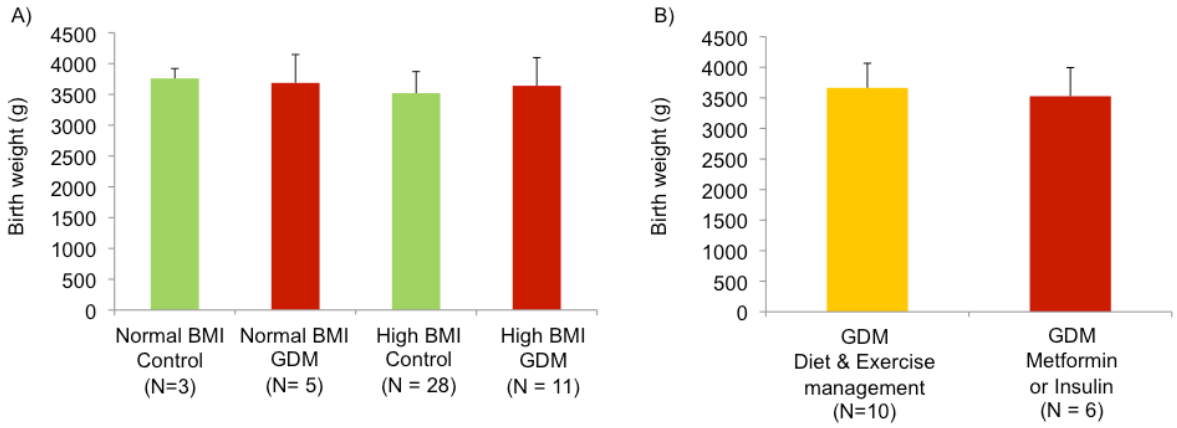


Figure 4.8: Birth weight and GDM. Birth weight was not significantly different when GDM participants were compared with BMI matched controls (A) or when divided according to management type (B) as determined by a one-way ANOVA and independent samples t-test respectively. GDM = gestational diabetes mellitus.

Of the 16 GDM participants, 3 delivered LGA infants and 13 AGA infants (determined by custom birth weight centiles). Therefore, placental gene expression was also compared between control (AGA) and GDM (AGA) participants and control (LGA) and GDM (LGA) participants respectively.

4.3.2.3. Maternal GDM and placental *PHLDA2* expression

Placental *PHLDA2* expression was approximately two fold higher in GDM pregnancies (Figure 4.9), however this difference was not statistically significant due to the large variation in *PHLDA2* expression in the GDM placentas. When results were analysed according to BMI category, placental *PHLDA2* expression was decreased in placentas of normal BMI GDM participants in comparison to normal BMI controls whereas expression was increased in high BMI GDM participants in comparison to high BMI controls

(Figure 4.9) suggesting an effect of BMI on the association between GDM and placental *PHLDA2* expression. However, these differences were not statistically significant. Finally, there was no significant difference in placental *PHLDA2* expression when participants were divided into those delivering AGA or LGA infants, Figure 4.9.

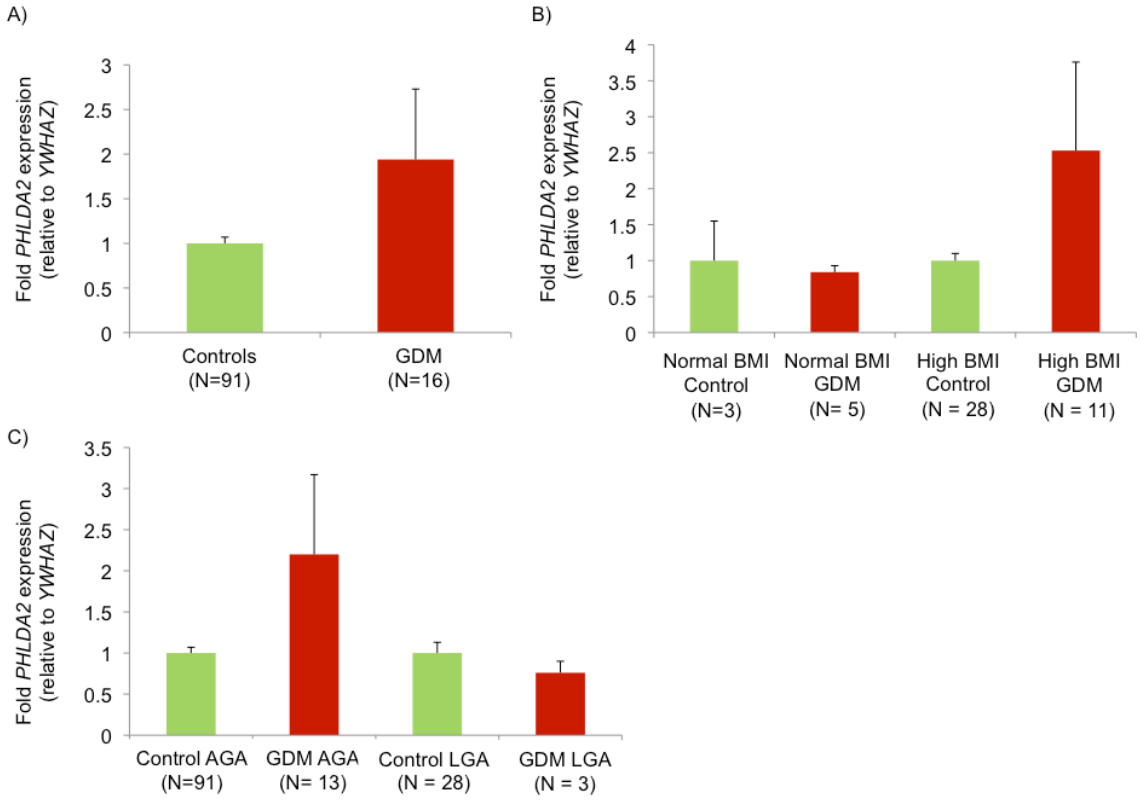


Figure 4.9: Placental *PHLDA2* expression and GDM. *PHLDA2* expression was not significantly altered in GDM placentas (A). Results remained non-significant when participants were compared with BMI matched controls (B) and according to AGA or LGA status (C). GDM = gestational diabetes mellitus.

4.3.2.4. Maternal GDM and *CDKN1C*, *PEG3* and *PEG10* expression

There was no significant difference in placental *CDKN1C*, *PEG3* or *PEG10* expression between control and GDM participants, Figure 4.10. Results remained non-significant when gene expression was compared with BMI matched controls and when participants were divided into those delivering AGA or LGA infants (results not shown).

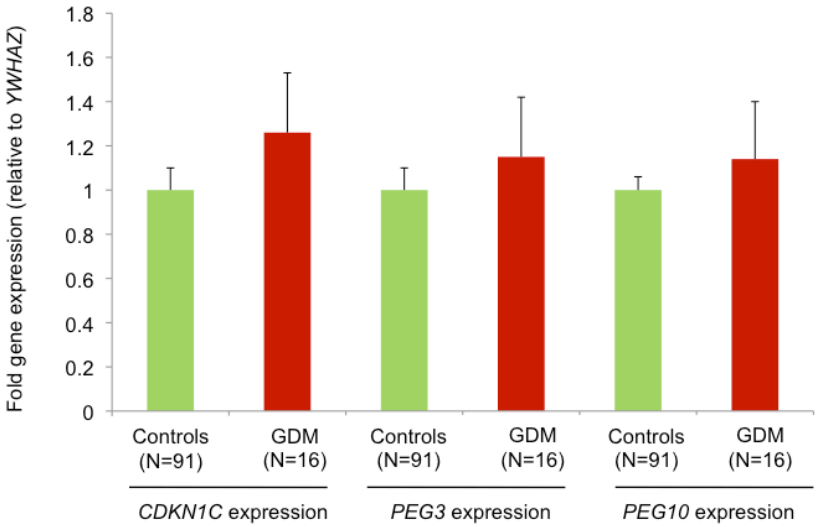


Figure 4.10: Placental *CDKN1C, PEG3* and *PEG10* expression and GDM. There was no significant difference in expression between GDM placentas and controls as determined by an independent samples T-test. GDM = gestational diabetes mellitus.

4.3.2.5. Maternal GDM and placental hormone gene expression

Placental *hPL* and *PGH* expression was not significantly altered in GDM participants (Figure 4.11). Results remained non significant when *hPL* expression was compared with BMI matched controls, see (Figure 4.11). There was however, a significant 36% increase in *PGH* expression in high BMI GDM placentas compared with matched controls (Figure 4.11). This was in contrast to a non-significant 19% decrease in normal BMI GDM placentas. Finally, there was no significant difference in placental *hPL* or *PGH* expression when participants were divided into those delivering AGA or LGA infants (results not shown).

4.3.2.6. Gestational diabetes summary

A summary of the changes in target gene expression in GDM placentas is shown in Table 4.4. Target gene expression was not significantly altered in placentas of GDM participants compared with controls.

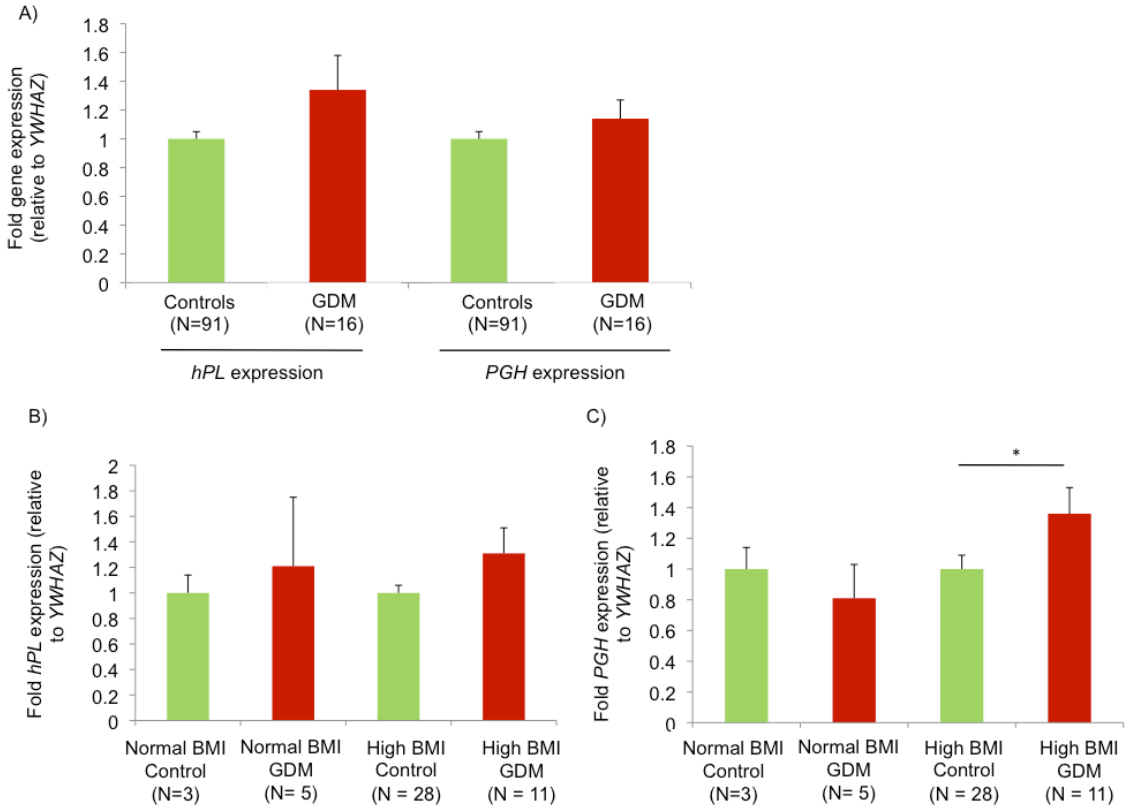


Figure 4.11: Placental *hPL* and *PGH* expression and GDM. Gene expression was not significantly altered in GDM placentas (A). There was also no significant difference in *hPL* expression (B) when GDM participants were compared to BMI matched controls. In contrast, placental *PGH* expression was significantly increased in high BMI GDM participants compared with high BMI controls (C). Error bars represent SEM.

Table 4.4. Summary of placental gene expression changes associated with maternal gestational diabetes. Fold gene expression is shown relative to control participants. Differences were not statistically significant using an independent samples T test.

Gene	Control Participants (N=91)	GDM participants (N=16)	P Value
PHLDA2	1.00	1.94	P = 0.25
CDKN1C	1.00	1.26	P = 0.32
PEG3	1.00	1.15	P = 0.58
PEG10	1.00	1.14	P = 0.43
hPL	1.00	1.34	P = 0.18
PGH	1.00	1.14	P = 0.31

4.3.3. Maternal prenatal depression: Imperial cohort

4.3.3.1. Participant demographics

Placentas from the Imperial Cohort (N=30) were sampled following delivery by elective caesarean section and all infants were normal birth weight. A median split of the cohort by maternal EPDS scores was carried out for the purpose of analysing association between maternal depression and placental gene expression. Gene expression was compared between participants with low EPDS scores (mean EPDS score 4.5) and high EPDS scores (mean EPDS score 14.5). Importantly, participants did not differ significantly in terms of any other maternal characteristic or birth outcome (Table 4.5).

Table 4.5: Comparison of maternal characteristics and birth outcomes between high and low EPDS scorers. Mean (SD)/Range or Number (%) is shown. Differences were analysed using an independent samples T test or Chi – square test where appropriate. *** $p < 0.001$.

	Low EPDS scorers (N = 15)	**High EPDS scorers** (N = 15)	**P Value**
Maternal Characteristic			
Total EPDS score	4.5 (2) / 1 - 8	14.5 (4) / 11 - 25	**P < 0.001*** **
Maternal age	36 (4) / 29 - 41	35 (5) / 27 - 41	P = 0.83
Maternal BMI (kg / m^2)	25 (4) / 20 - 35	25 (5) / 20 - 38	P = 0.78
Parity	2 (2) / 0 -11	2 (2) / 0 - 5	P = 0.87
Currently Smoking:			
No	14 (93%)	13 (87%)	
Yes	1 (7%)	2 (13%)	P = 0.60
Drinking Alcohol:			
No	14 (93%)	11 (73%)	
Yes	1 (7%)	4 (27%)	P = 0.14
Birth Outcome			
Birth weight (g)	3501 (525) / 2930 - 4700	3441 (402) / 2640 - 4140	P = 0.73
Gestational age (weeks)	39 (0.5) / 38 - 40	39 (0.7) / 38 - 41	P = 0.75
Placental weight (g)	622 (163) / 387 - 932	644 (125) / 460 - 851	P = 0.68
Gender			
Male	6 (40%)	9 (60%)	
Female	9 (60%)	6 (40%)	P = 0.27

Placental gene expression was also compared between participants with an EPDS score ≥ 13 (N = 9), (participants at risk of developing a depressive

disorder), and participants with an EPDS score <13 (N = 21). Notably, no participant had a prenatal diagnosis of depression and none were currently taking antidepressants.

4.3.3.2. Maternal depression and birth weight

There was no significant correlation between maternal prenatal EPDS scores and birth weight (r = - 0.24, p = 0.21, n = 30). Although birth weight was reduced in participants with the highest EPDS scores compared with the lowest scores (3501g v. 3441g, p = 0.73 n = 30) this difference was not statistically significant (Figure 4.12). Similarly there was no significant difference in birth weight when participants were divided into those with EPDS scores < 13 and ≥ 13 (cut off for clinical depression), Figure 4.12.

As with birth weight, no significant association was observed between placental weight and maternal EPDS or when participants were divided into low and high EPDS scorers (results not shown).

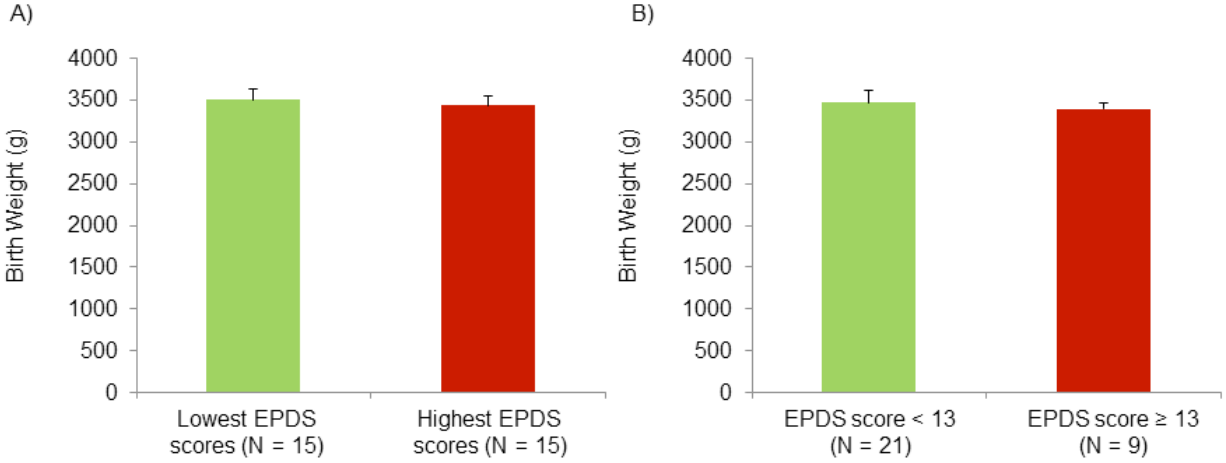


Figure 4.12. Maternal depression and birth weight. (A) No significant difference in mean birth weight between 15 participants with lowest and highest EPDS scores. (B) No significant difference in mean birth weights between participants with EPDS scores < or ≥ 13. Error bars represent SEM.

4.3.3.3. Maternal depression and *PHLDA2* and *CDKN1C* expression

There was no significant association between placental *PHLDA2* expression and maternal EPDS scores (r = 0.15, p = 0.42, n = 30) in the overall cohort.

Although placental *PHLDA2* expression was increased in the highest EPDS scorers, this difference was not statistically significant (p = 0.33, n = 30), see Figure 4.13. Similarly, there was no significant difference in *PHLDA2* expression between participants with EPDS scores < 13 and ≥ 13 (p = 0.44, n = 30).

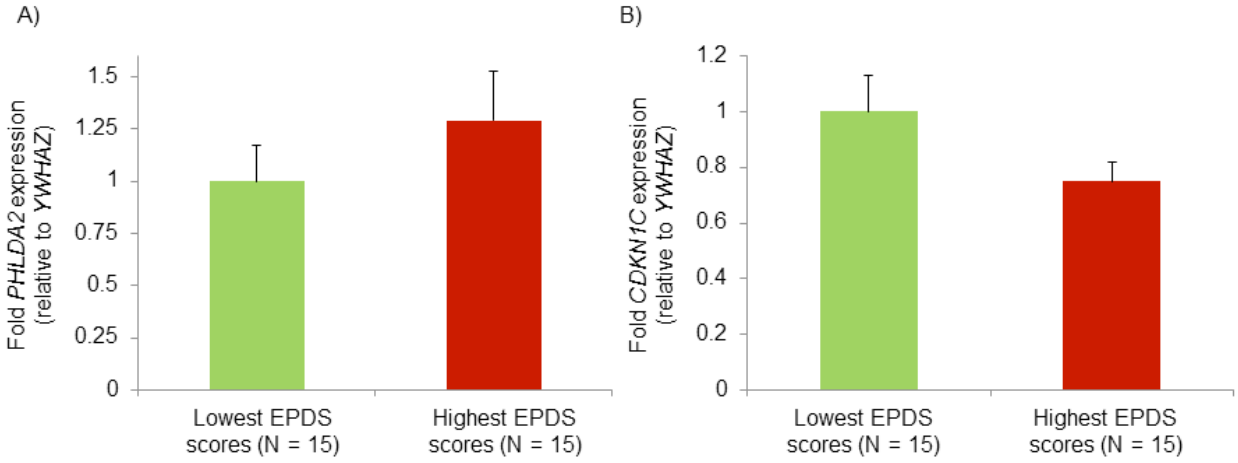


Figure 4.13: Maternal depression and placental *PHLDA2* (A) and *CDKN1C* (B) expression. (A) No significant difference in *PHLDA2* expression between 15 participants with lowest and highest EPDS scores. (B) No significant difference in *CDKN1C* expression between 15 participants with lowest and highest EPDS scores.

There was no significant correlation between placental *CDKN1C* expression and maternal EPDS scores (r = - 0.20, p = 0.31, n = 30). Similarly, no significant difference was seen in *CDKN1C* expression between the low and high EPDS scorers (Figure 4.13) or when comparing those participants with EPDS scores < 13 and ≥ 13 (p = 0.30, n = 30).

4.3.3.4. Maternal depression and *PEG3* expression

There was a significant inverse association between placental *PEG3* expression and maternal EPDS scores (r = - 0.36, p < 0.05, n = 30), Figure 4.14. There was also a significant 15% decrease in placental *PEG3* expression in the high EPDS scorers (Figure 4.14).

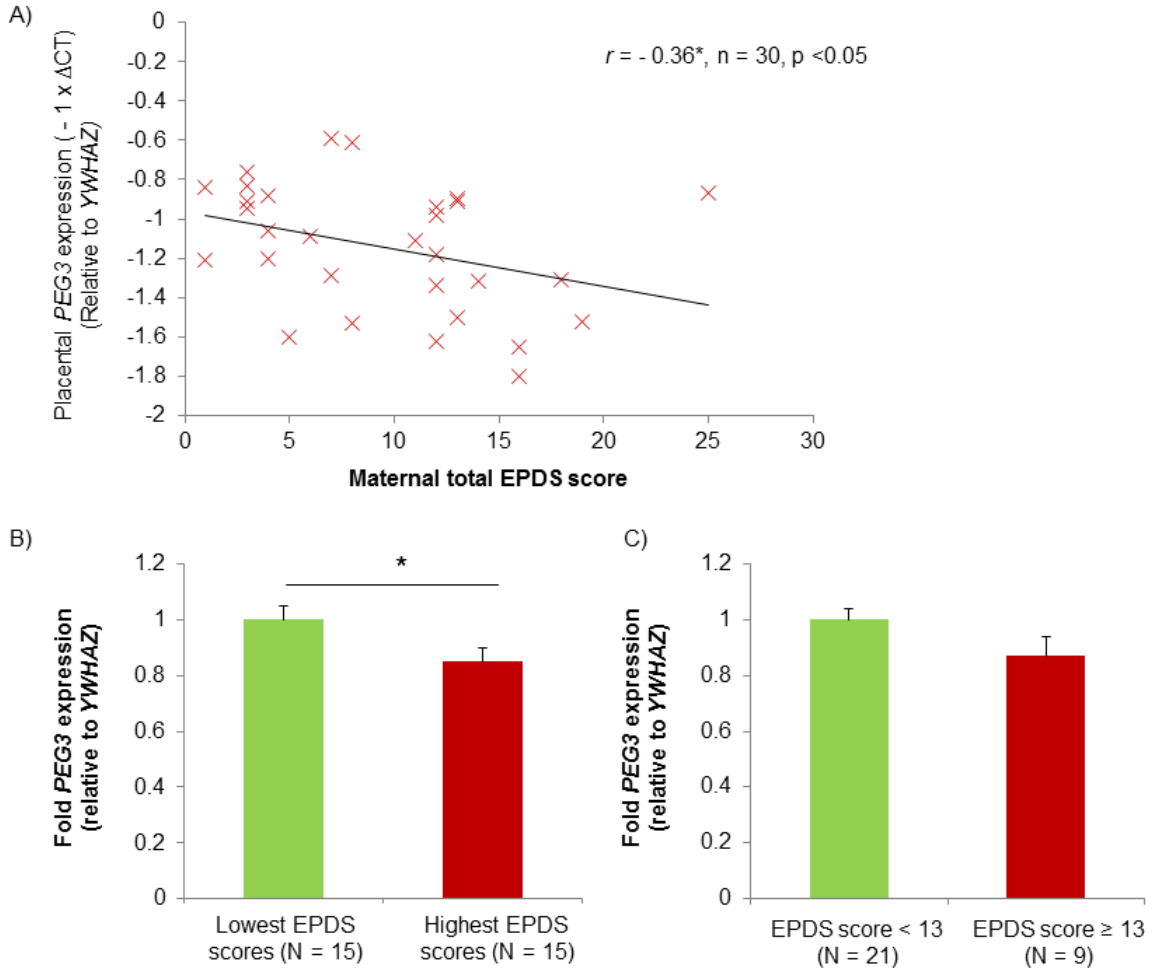


Figure 4.14: Maternal depression and placental *PEG3* expression. (A) A significant inverse correlation between placental *PEG3* expression and maternal EPDS scores. B) A significant decrease in *PEG3* expression between 15 participants with lowest and highest EPDS scores. (B) A trend for decreased *PEG3* expression between participants with EPDS scores <13 and ≥ 13. Error bars represent SEM. * P <0.05.

Placental *PEG3* expression was also decreased in participants with EPDS scores ≥ 13 compared with those scoring <13, although this difference did not reach statistical significance (p = 0.08, n = 30), Figure 4.14.

4.3.3.5. Maternal depression and *hPL* expression

There was a trend for an inverse correlation between placental *hPL* expression and maternal EPDS scores (*r* = - 0.33, p = 0.08, n = 30), Figure 4.15. A significant 31% decrease in placental *hPL* expression was observed in the 15 participants with the highest EPDS scores compared with the 15 participants with the lowest EPDS scores (Figure 4.15).

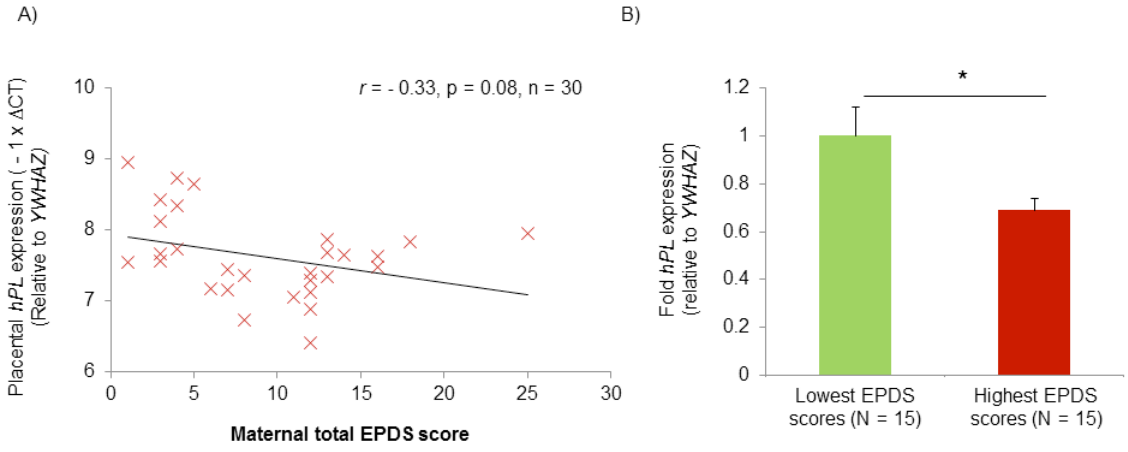


Figure 4.15: Maternal depression and placental *hPL* expression. (A) A trend for an inverse association between *hPL* expression and maternal EPDS scores. (B) A significant decrease in *hPL* expression between 15 participants with lowest and highest EPDS scores. Error bars represent SEM. * P <0.05.

However when participants were divided into those with EPDS score < 13 and ≥ 13 expression was not significantly different, perhaps as a result of the few number of participants with an EPDS score ≥13 (p = 0.77, n = 30).

There was no significant correlation between placental *PEG3* and *hPL* expression (*r* = 0.04 p = 0.85, n = 30). Similarly, placental *hPL* expression was not significantly correlated with *PHLDA2* (*r* = 0.12, p = 0.53, n = 30) or *CDKN1C* expression (*r* = 0.28, p = 0.14, n = 30).

4.3.3.6. Maternal depression and *PGH* expression

Placental *PGH* expression was significantly inversely correlated with maternal EPDS scores (r = -0.38, p = 0.04, n = 30). However, no significant difference in *PGH* expression was seen between the 15 participants with the lowest and highest EPDS scores (p = 0.10, n = 30) or when comparing those participants with EPDS scores < 13 and ≥ 13 (p = 0.14, n = 30).

There was no significant correlation between placental *PGH* expression and *PHLDA2* (r = -0.07, p = 0.72, n = 30), *CDKN1C* (r = 0.14, p = 0.45, n = 30) or *PEG3* (r = 0.20, p = 0.30, n = 30) gene expression.

Table 4.6. Summary of placental gene expression changes associated with maternal prenatal depression in the Imperial depression cohort. Fold gene expression is shown relative to low depression scorers. Results highlighted in bold were statistically significant using an independent samples T test. * p ≤ 0.05.

Gene	Low Depression scores (N=15)	High Depression scores (N=15)	P Value
PHLDA2	1.00	1.29	P = 0.33
CDKN1C	1.00	0.75	P = 0.10
PEG3	**1.00**	**0.85**	**P = 0.03***
hPL	**1.00**	**0.69**	**P = 0.03***
PGH	1.00	0.68	P = 0.10

4.3.3.7. Maternal depression summary

A summary of the changes in placental gene expression in association with maternal prenatal depression in the Imperial Cohort is shown in Table 4.6. Only placental *PEG3* and *hPL* expression were significantly reduced in placentas of participants with high total EPDS scores.

4.3.4. Maternal depression: Manchester cohort

In order to replicate the findings of a significant inverse association between placental *PEG3* and *hPL* expression and maternal depression, gene expression was analysed in a second independent cohort.

4.3.4.1. Participant demographics

Characteristics of the overall Manchester cohort are shown in Table 3.9 (Section 3.3.3). This cohort did not have EPDS scores, but included a subset of women (N = 7) with diagnosed depression during pregnancy. Placental gene expression was compared with control participants (N=70) with no previous history of mood disorders or other maternal complication (e.g. asthma, epilepsy). Maternal characteristics and birth outcomes did not differ significantly between depressed participants and controls (Table 4.7).

4.3.4.2. Maternal depression and birth weight

As with the Imperial Cohort, infants of mothers with depression during pregnancy were lighter than control infants (3296g v. 3092g, p = 0.41 n = 77) although this difference was not statistically significant (Table 4.7). For this cohort, custom growth centiles were available; there was a non-significant decrease in mean growth centile of infants born to depressed mothers compared with control infants (Table 4.7).

4.3.4.3. Maternal depression and imprinted gene expression

For this cohort, placental *PEG10* expression was also analysed due to the larger amounts of cDNA available for gene expression analysis. There was no significant difference in placental *PHLDA2*, *CDKN1C* or *PEG10* expression between depressed and control participants (Figure 4.16). However, as with the Imperial Cohort, maternal prenatal depression was associated with a significant decrease in placental *PEG3* expression (Figure

4.16). Compared with the 15% decrease in *PEG3* expression observed with maternal depression in the Imperial Cohort, in the Manchester cohort maternal depression was associated with a 40% decrease in gene expression ($p = 0.02$, $n = 77$).

Table 4.7: Comparison of maternal characteristics and birth outcomes between control and depressed participants. Mean (SD)/Range or Number (%) is shown. Differences were analysed using an independent samples T test or Chi – square test where appropriate.

	Control Participants (N = 70)	**Depressed participants** (N = 7)	**P Value**
Maternal Characteristic			
Maternal age	28 (5) / 17 - 42	29 (7) / 19 - 35	P = 0.86
Maternal BMI (kg / m^2)	26 (4) / 19 - 38	24 (4) / 20 – 30	P = 0.53
Parity	3 (2) / 1 - 9	4 (3) / 1 - 9	P = 0.11
Currently Smoking:			
No	64 (86%)	5 (83%)	
Yes	10 (14%)	1 (17%)	P = 1.00
Drinking Alcohol:			
No	73 (99%)	7 (100%)	
Yes	1 (1%)	0 (0%)	P = 0.75
Birth Outcome			
Mode of Delivery:			
Vaginal	38 (51%)	4 (67%)	
Elective C section	4 (5%)	1 (17%)	
Emergency C section	9 (12%)	0 (0%)	
Instrumental	23 (31%)	1 (17%)	P = 0.11
Birth weight (g)	3331 (619) / 850 - 4680	3092 (507) / 2440 - 3720	P = 0.41
Custom birth weight centile	42 (30) / 0 – 99	28 (25) / 2 - 61	P = 0.30
Gestational age (weeks)	39 (2) / 30 - 42	39 (2)/ 36 - 41	P = 0.49
Gender			
Male	35 (47%)	3 (50%)	
Female	39 (53%)	3 (50%)	P = 0.72

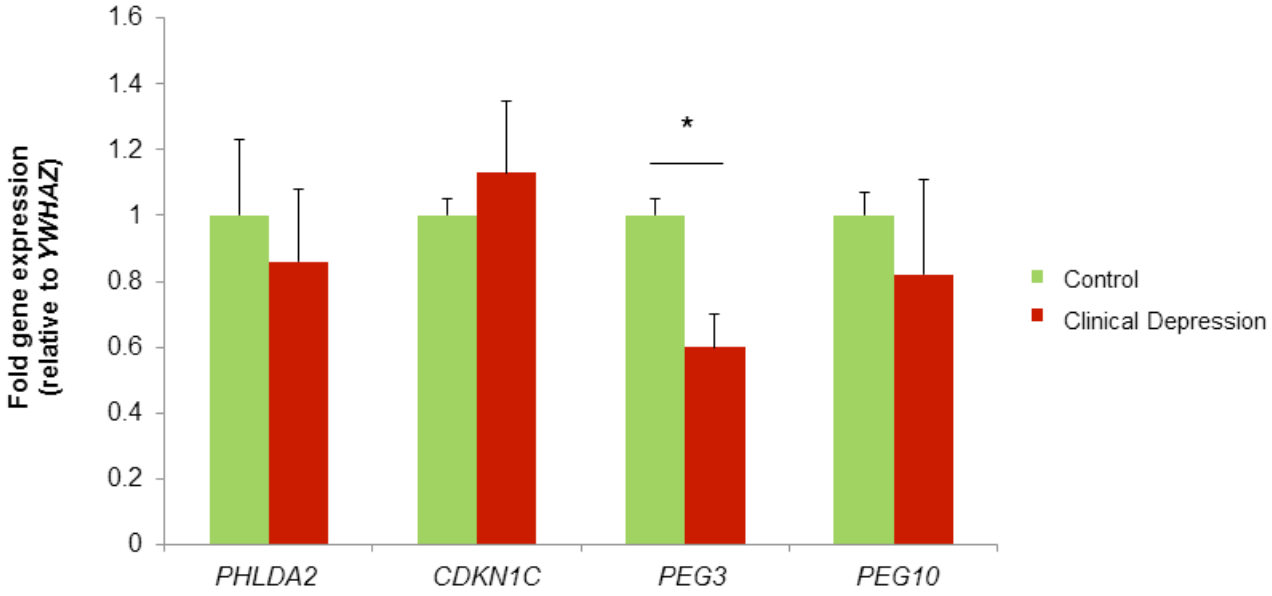


Figure 4.16: Maternal clinical depression and placental imprinted gene expression. Only placental *PEG3* expression was significantly altered in mothers with clinical depression during pregnancy. * p ≤ 0.05. Error bars represent SEM.

4.3.4.4. Maternal depression and placental hormones

Placental *hPL* expression was significantly decreased by 44% in mothers with diagnosed depression in pregnancy compared with controls (p = 0.02, n = 77)(Figure 4.17), replicating results from the Imperial cohort (section 4.3.3).

Maternal serum hPL levels were measured for 54 participants in the Manchester Cohort. Serum hPL was reduced in mothers with prenatal diagnosed depression (Figure 4.17), however due to the small number of depressed participants (N=2) with serum hPL measures this difference could not be statistically analysed.

In the Manchester Cohort, placental *PEG3* and *hPL* expression were significantly correlated (r = 0.28, p = 0.01, n = 77) as shown in Figure 4.17. However, there was no significant correlation between maternal serum hPL levels and placental *PEG3* (r = - 0.05, p = 0.75, n = 54) or *hPL* expression (r = -0.07, p = 0.61, n = 54), as shown in Figure 4.17.

In addition, placental *PGH* expression was significantly decreased by 43% in mothers with diagnosed depression (p = 0.03, n = 77) (Figure 4.17).

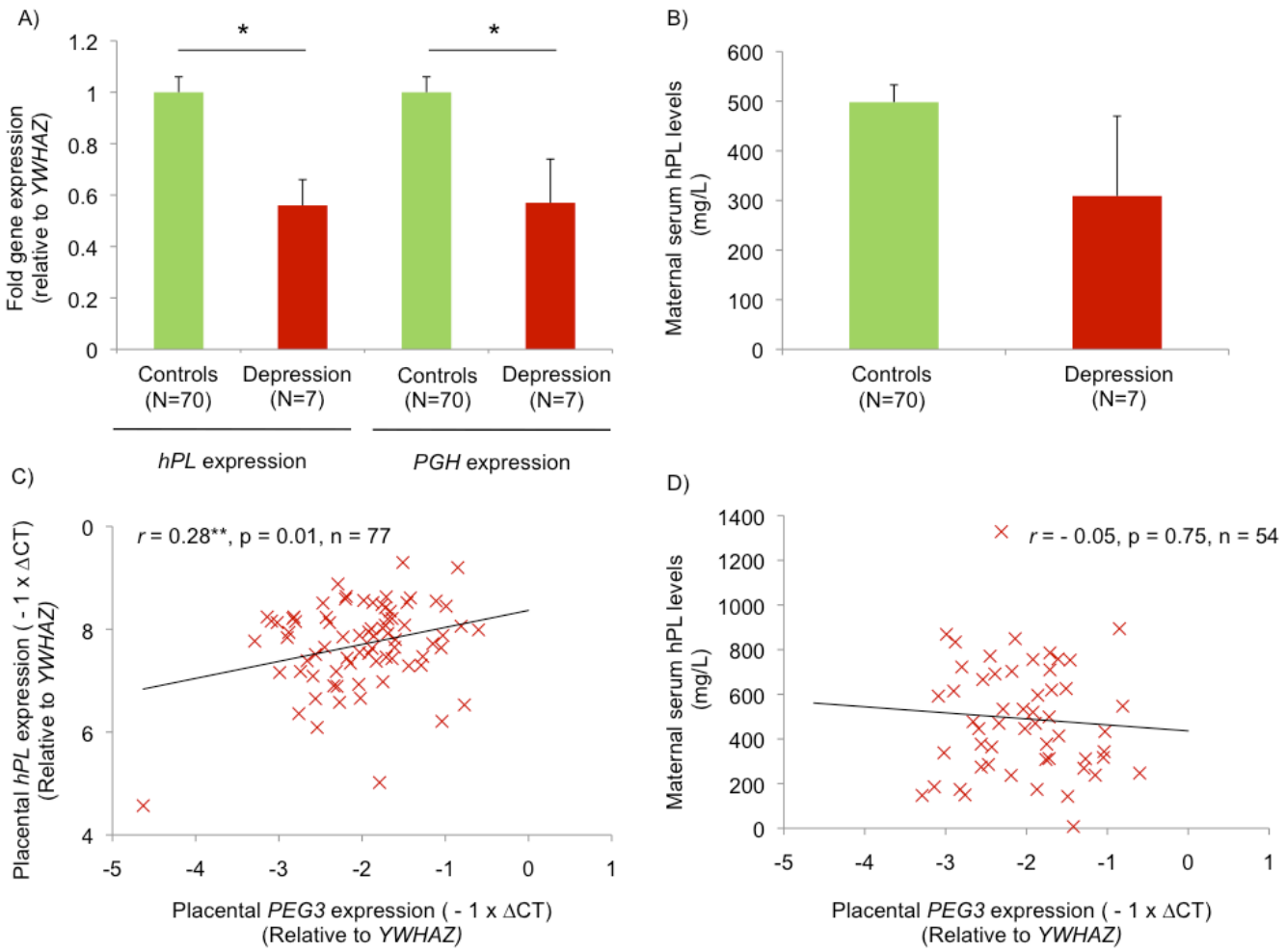


Figure 4.17: Maternal depression and placental hormones. (A) Placental *hPL* and *PGH* expression were significantly reduced in depressed participants. (B) Serum hPL levels were also decreased although this could not be analysed statistically due to the small sample size. Placental *PEG3* expression was significantly positively associated with *hPL* expression (C) but not serum hPL levels (D).

4.3.4.5. Maternal depression medication and placental gene expression

Of the seven participants with diagnosed prenatal depression, four participants were taking prescribed medication for the treatment of depression (most commonly selective serotonin reuptake inhibitors, SSRIs). There was no significant difference in placental target gene expression between depressed participants on prescribed SSRIs during pregnancy and those not on prescribed medication for depression (Figure 4.18). Given the small numbers of participants in this analysis, these results must be interpreted with caution. However, they do suggest that the difference in placental *PEG3*, *hPL* and *PGH* expression in clinically depressed participants may not be a result of medication differences.

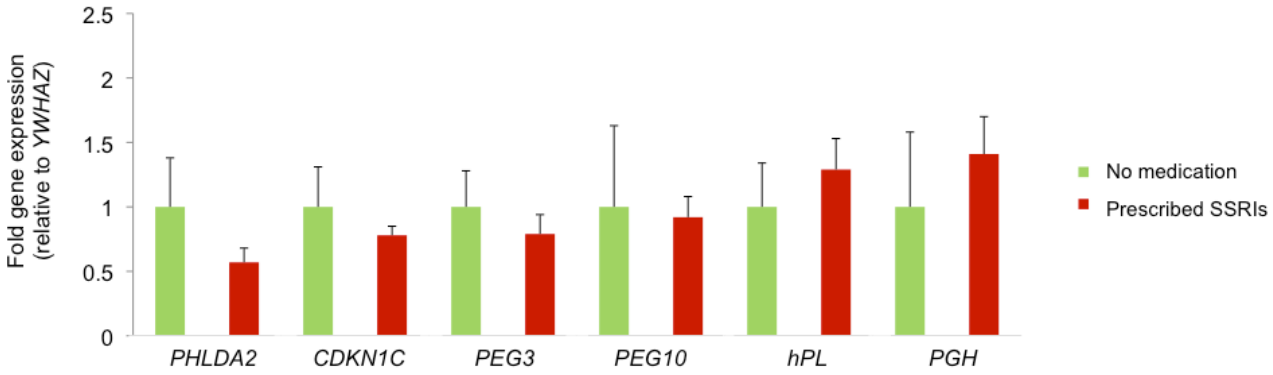


Figure 4.18: Maternal prescribed medication for clinical depression and placental target gene expression. There was no significant difference in target gene expression between depressed participants prescribed SSRIs and those not prescribed medication during pregnancy as determined by an independent samples T test. SSRI = selective serotonin reuptake inhibitor. Error bars represent SEM.

4.3.4.6. Manchester depression cohort summary

A summary of the changes in placental gene expression in association with maternal clinical depression in the Manchester Cohort is shown in Table 4.6. Replicating the results from the Imperial Cohort (see section 4.3.3) placental *PEG3* and *hPL* expression were significantly reduced in placentas of participants with clinical depression. In addition, placental *PGH* was also significantly reduced in depression participants.

Table 4.8. Summary of placental gene expression changes associated with maternal clinical depression in the Manchester cohort. Fold gene expression is shown relative to controls. Results highlighted in bold were statistically significant using an independent samples T test. * $p \leq 0.05$.

Gene	Controls (N = 70)	Clinical Depression (N = 7)	P Value
PHLDA2	1.00	0.86	P = 0.87
CDKN1C	1.00	1.13	P = 0.49
PEG3	**1.00**	**0.60**	**P = 0.02***
PEG10	1.00	0.82	P = 0.49
hPL	**1.00**	**0.56**	**P = 0.02***
PGH	**1.00**	**0.57**	**P = 0.03***

4.3.5. Maternal prenatal anxiety

Maternal anxiety and depression are often co-morbid and indeed within the Imperial Cohort maternal EPDS and STAI scores were significantly correlated (r = 0.81**, $p<0.001$, n= 30), Figure 4.19. However, it has also previously been demonstrated that anxiety and depression are independently associated with altered placental gene expression (O'Donnell *et al.* 2012; Blakeley *et al.* 2013). Therefore, placental imprinted and hormone gene expression was also analysed in relation to maternal prenatal trait anxiety in the Imperial Anxiety Cohort.

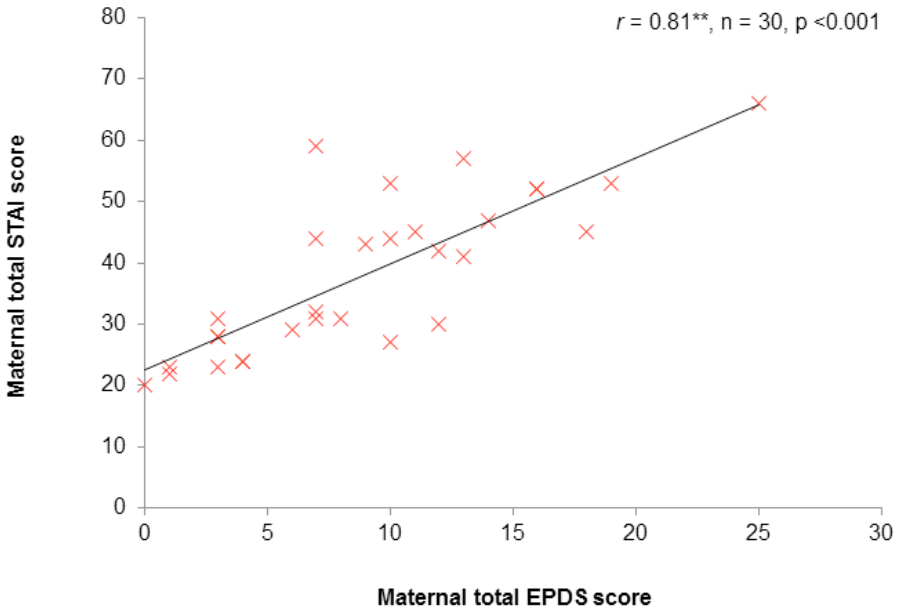


Figure 4.19: Maternal depression and trait anxiety scores. Maternal trait anxiety was measured using the Spielberger Trait Anxiety Inventory (STAI). Maternal depression was measured using the Edinburgh Postnatal Depression Scale. ** p < 0.001.

Table 4.9: Comparison of maternal characteristics and birth outcomes between high and low STAI scorers. Mean (SD)/Range or Number (%) is shown. Differences were analysed using an independent samples T test or Chi – square test where appropriate.

	STAI score < 40 (N = 15)	**STAI score ≥40** (N = 15)	**P Value**
Maternal Characteristic			
Total STAI score	27 (4) / 20 - 32	50 (7) / 41 - 66	**P < 0.001*** **
Maternal age	36 (4) / 27 - 42	36 (5) / 26 - 41	P = 0.85
Maternal BMI (kg / m^2)	25 (5) / 19 - 35	24 (5) / 20 - 38	P = 0.69
Parity	2 (3) / 0 -11	2 (2) / 1 - 6	P = 0.87
Currently Smoking:			
No	14 (93%)	14 (93%)	
Yes	1 (7%)	1 (7%)	P = 0.37
Drinking Alcohol:			
No	14 (93%)	8 (53%)	
Yes	1 (7%)	7 (47%)	**P = 0.01****
Birth Outcome			
Birth weight (g)	3448 (426) / 2930 – 4700	3360 (382) / 2640 - 4140	P = 0.56
Gestational age (weeks)	39 (0.5) / 38 - 39	39 (0.6) / 38 - 41	P = 0.75
Placental weight (g)	629 (143) / 422 - 932	585 (93) / 422 - 762	P = 0.33
Gender			P = 1.00
Male	8 (53%)	8 (53%)	
Female	7 (47%)	7 (47%)	

4.3.5.1. Participant demographics

All placentas (N=30) were sampled following delivery by elective caesarean section and all infants were normal birth weight.

The mean maternal STAI score was 38.2, with 15 women scoring ≥ 40, highlighting them as at risk of developing an anxiety disorder. Placental gene expression was compared between participants with a low STAI score <40 (N=15) and those with a high STAI score ≥ 40 (N=15). This also represented a median split of the Imperial Anxiety Cohort. Notably, no participant had a prenatal diagnosis of or was receiving treatment for an anxiety disorder. Participant characteristics and birth outcomes are compared in Table 4.9. Maternal alcohol consumption during pregnancy was significantly more frequent in participants with an STAI score ≥ 40 (table 4.9). Participants did not differ significantly in any other maternal characteristic of birth outcome examined.

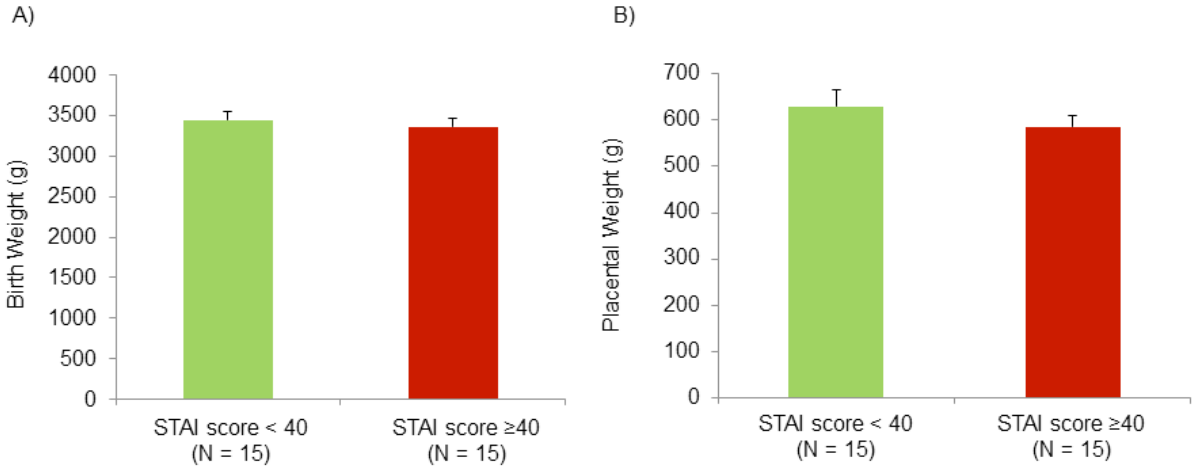


Figure 4.20. Effects of maternal anxiety on birth weight (A) and placental weight (B). No significant difference in mean birth or placental weight between participants with STAI scores < or ≥ 40. Error bars represent SD.

4.3.5.2. Maternal anxiety and birth weight

No significant correlation was observed between birth weight and maternal prenatal STAI scores (r = - 0.13, p = 0.50, n = 30). Similarly, placenta and

birth weight were not significantly altered in participants with STAI scores ≥40 (Figure 4.20)

4.3.5.3. Maternal anxiety and imprinted gene expression

There was no significant association between maternal STAI scores and placental *PHLDA2* (r = 0.12, p = 0.58, n = 30), *CDKN1C* (r = - 0.10, p = 0.60, n = 30) or *PEG3* (r = - 0.24, p = 0.20, n = 30) gene expression. Furthermore, no significant difference was seen in placental imprinted gene expression when participants were divided into those with STAI scores < 40 or ≥40 (Figure 4.21).

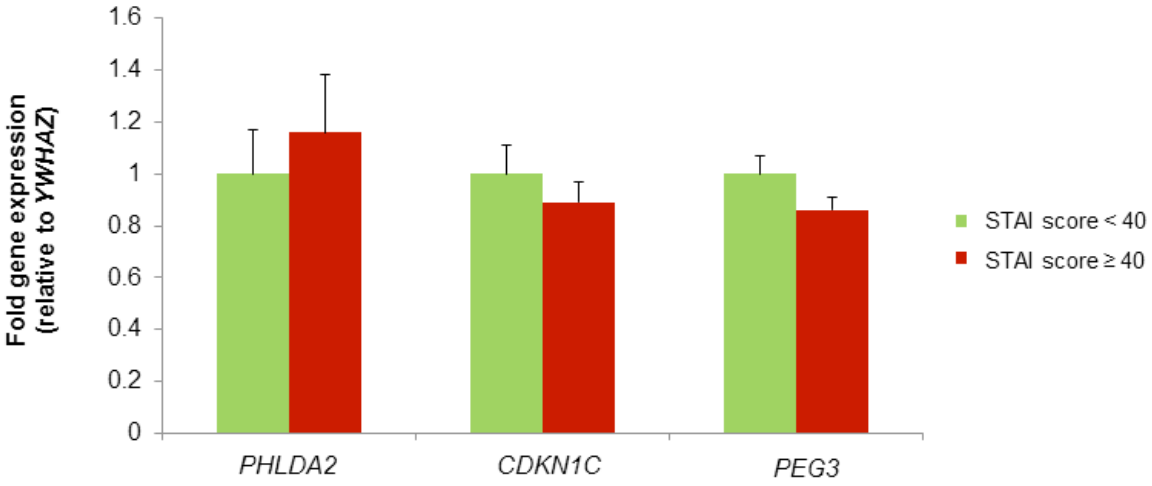


Figure 4.21. Maternal Anxiety and placental imprinted gene expression. There was no significant difference in *PHLDA2*, *CDKN1C* or *PEG3* expression between participants with STAI scores < or ≥ 40. Error bars represent SEM.

In contrast to the significant decrease in placental *PEG3* expression with maternal depression, only a trend was observed for maternal STAI scores ≥ 40 (p = 0.09, n = 30).

4.3.5.4. Maternal anxiety and placental hormone gene expression

A trend was observed for an inverse association between maternal STAI scores and placental *hPL* expression (r = - 0.35, p = 0.07, n = 30). Similarly, although a decrease was seen in *hPL* expression in placentas of mothers

with an STAI score ≥40, this did not reach statistical significance (p = 0.09, n = 30), Figure 4.22. Placental *PGH* expression was significantly inversely associated with maternal STAI scores (*r* = - 0.39, p = 0.04, n = 30). However, there was no significant difference in *PGH* expression between mothers with STAI score ≥40 and <40 (p = 0.09, n = 30), Figure 4.22.

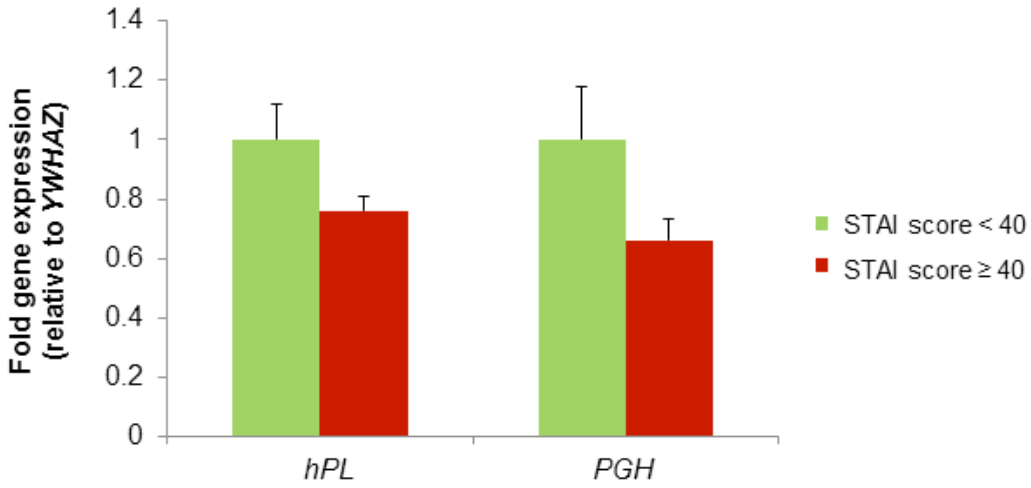


Figure 4.22. Maternal Anxiety and placental hormone gene expression. No significant difference in *hPL* or *PGH* expression between participants with STAI scores < or ≥ 40. Error bars represent SEM.

Table 4.10. Summary of placental gene expression changes associated with maternal prenatal trait anxiety (STAI). Fold gene expression is shown relative to participants with STAI score < 40. Results were not statistically significant using an independent samples T test.

Gene	Low Anxiety, STAI < 40 (N=15)	High Anxiety, STAI ≥ 40 (N=15)	P Value
PHLDA2	1.00	1.16	P = 0.56
CDKN1C	1.00	0.89	P = 0.29
PEG3	1.00	0.86	P = 0.09
hPL	1.00	0.76	P = 0.09
PGH	1.00	0.66	P = 0.09

A summary of the changes in placental gene expression in association with maternal prenatal trait anxiety is shown in Table 4.10. Trait anxiety is a measure of how the participant “generally feels”. Maternal SSAI scores, measuring how the participant felt “at that moment” (day before elective Caesarean section) were also available for the Imperial Anxiety Cohort. There was no significant association between maternal SSAI scores and placental *PHLDA2*, *CDKN1C*, *PEG3*, *hPL* or *PGH* expression (results not shown), suggesting a specific effect of trait anxiety.

4.4. Discussion

This chapter examined a role for placental imprinted gene expression in the pregnancy complications preeclampsia, gestational diabetes and maternal mood disorders. A summary of imprinted gene expression alterations in these pregnancy complications is shown in Table 4.11. These results suggest a role for placental *PHLDA2* in the pathogenesis of preeclampsia and a role for *PEG3* and placental hormones in maternal mood disorders during pregnancy.

Table 4.11. Summary of target gene expression changes following complications of pregnancy. Percentage change in expression relative to controls is shown. PIH = pregnancy induced hypertension, PE = preeclampsia, GDM = gestational diabetes mellitus.

Gene	PIH/PE	GDM	Depression (Imperial Cohort)	Depression (Manchester cohort)	Trait Anxiety
PHLDA2	↑ 63%	-	-	-	-
CDKN1C	-	-	-	-	-
PEG3	-	-	↓ 15%	↓ 40%	-
PEG10	-	-	-	-	-
hPL	-	-	↓ 31%	↓ 44%	-
PGH	-	-	-	↓ 43%	-

4.4.1. Preeclampsia

Birth weight and custom birth weight centiles were significantly reduced in PE but not PIH pregnancies compared with controls. Head circumference was similarly reduced in PIH/PE pregnancies. Although placental weight was also decreased in PIH/PE pregnancies, this difference did not reach statistical

significance. This is consistent with previous studies reporting increased prevalence of FGR in PIH/PE pregnancies (Villar *et al*. 2006; Hutcheon *et al*. 2011). Villar *et al*. (2006) estimated that IUGR occurred in 22% of women with PE and in 13% of women with PIH; indeed SGA was observed in 38% of the PIH/PE pregnancies in the current study.

Placental *PHLDA2* expression was significantly increased by 63% in PIH/PE pregnancies. Furthermore, this increase was found to be specific to PE pregnancies. Given the increased prevalence of SGA in these pregnancies, it is possible the increased *PHLDA2* expression observed in PIH/PE placentas reflects the association between placental *PHLDA2* and fetal growth observed in the current study (Chapter 3), rather than a specific association with preeclampsia. However, placental *PHLDA2* expression was increased by over 50% in PIH/PE pregnancies with or without the presence of SGA. Unfortunately, these differences were not statistically significant, possibly as a result of the relatively small number of PIH/PE pregnancies in each group (16 AGA and 6 SGA with PIH/PE). These results are consistent with those of McMinn *et al*. (2006) reporting a non-significant increase in *PHLDA2* expression in PE placentas. Uniquely, the results of the current study also suggest a role for increased placental *PHLDA2* expression in the pathogenesis of preeclampsia, independent of any associated effect on fetal growth.

PIH/PE was also associated with a 45% increase in placental *CDKN1C* expression. However, multiple linear regression analysis revealed that preeclampsia did not independently predict placental *CDKN1C* expression after controlling for labour status. Previous studies examining placental *CDKN1C* expression in human preeclamptic pregnancies have yielded conflicting results. Enquobahrie *et al*. (2008) and Unek *et al.* (2014) reported increased *CDKN1C* expression in preeclamptic placentas while Kawasaki *et al.* (2015) reported significantly reduced placental *CDKN1C* expression. Given the association between labour and placental *CDKN1C* expression demonstrated in chapter 3, it is possible that these conflicting results result from a failure to control for labour status. For example, the caesarean delivery rate differed between control and PE pregnancies in the

study by Enquobahrie *et al*. (2008) whereas in the study by Unek *et al.* (2014), labour status was not described.

Placental *PEG3* and *PEG10* expression was not significantly altered in PIH/PE pregnancies. Lambertini *et al*. (2008) reported LOI of *PEG3* in 50% of PE placentas examined although gene expression was not analysed. Studies of placental *PEG10* expression in relation to PE have yielded conflicting results; Chen *et al*. (2012) reported increased expression and Liang *et al*. (2014) decreased expression of *PEG10* in PE placentas. In comparison, it is possible that the current study did not detect any differences in *PEG10* expression due to cohort differences. For example, Chen *et al*. (2012) analysed severe cases of preeclampsia (onset before 34 weeks) and Liang *et al*. (2014) excluded cases of PIH. In summary the results of the current study do not support a role for placental *PEG3* or *PEG10* in the pathogenesis of preeclampsia.

Finally, placental *hPL* and *PGH* expression was not significantly altered in PIH/PE pregnancies. Although Mannik *et al*. (2012) demonstrated significantly reduced *hPL* and *PGH* expression in PE placentas, the majority of pregnancies were complicated by severe preeclampsia. It is therefore possible that the current study did not detect any association preeclampsia and *hPL* or *PGH* expression due to differences in disease severity. Thus, the results of the current study do not support a role for placental *hPL* or *PGH* in preeclampsia.

4.4.2. Gestational diabetes

Maternal BMI was significantly higher in GDM compared with control pregnancies. Therefore, placental gene expression analysis was split by BMI category. Results are consistent with reports of raised BMI increasing GDM risk (Teh *et al*. 2011; Mitanchez *et al*. 2014). In contrast, maternal weight gain during pregnancy was significantly lower in GDM participants, possible as a result of diet and exercise management of GDM. Similarly, Hartlington *et al*.

(2013) observed significantly reduced maternal weight gain in GDM pregnancies.

Birth weight, custom birth weight centiles and placental weight were not significantly altered in GDM pregnancies. This contrasts reports by Hartlington *et al*. (2013) of an almost two fold increased risk of LGA in GDM pregnancies. However, this meta-analysis included studies of up to 34,000 participants. It is therefore possible, that an effect of GDM on birth weight was not observed in the current study due to the relatively small number of GDM participants recruited.

Placental *PHLDA2* expression was almost two fold higher in GDM pregnancies, although this difference failed to reach statistical significance due to the large variation in *PHLDA2* expression in the GDM group and small sample sizes. Aberrant placental *PHLDA2* expression (≥ 2 fold) was demonstrated in 19% of GDM participants. When results were split by maternal BMI, placental *PHLDA2* expression was only increased in high BMI GDM participants compared with high BMI controls (although again this result was not statistically significant). This suggests an effect of BMI on the association between GDM and placental *PHLDA2* expression. No previous study has examined placental *PHLDA2* expression in relation to GDM. Therefore, these novel preliminary results, although not significant, prompt additional research as to the role of placental *PHLDA2* in GDM pregnancies.

In contrast, placental *CDKN1C*, *PEG3* and *PEG10* expression were not significantly altered in GDM pregnancies. No previous study has examined placental *CDKN1C*, *PEG3* or *PEG10* expression in relation to GDM. The results of the current study do not support a role for *CDKN1C*, *PEG3* or *PEG10* in the pathogenesis of GDM.

Placental *hPL* expression was not significantly altered in GDM pregnancies (independent of maternal BMI). Mannik *et al*. (2010) similarly reported no significant difference in *hPL* expression in GDM placentas. This is surprising given the key role of hPL in preventing glucose intolerance during pregnancy (Newbern and Freemark 2011) and the increase in placental *PHLDA2* expression observed in GDM placentas (which was

demonstrated to be inversely correlated with placental *hPL* expression). Studies of larger GDM cohorts are required to fully determine the role of placental *PHLDA2* in GDM and the possible role of *hPL* in mediating this association.

Finally, placental *PGH* expression was significantly increased in high BMI GDM pregnancies compared with high BMI controls. This was in contrast to a non-significant decrease in normal BMI GDM placentas. Mannik *et al*. (2012) similarly reported increased *PGH* expression in placentas of GDM women delivering LGA infants. The results of the current study are also consistent with the known role of PGH in promoting fetal growth through induction of peripheral insulin resistance during pregnancy (Newbern and Freemark 2011). The effect of maternal BMI on the association between placental *PGH* expression and GDM is intriguing. A similar effect of maternal obesity on the association between GDM and adverse infant outcomes has been noted (Makgoba *et al*. 2012; Mitanchez *et al*. 2014). Thus, the findings of the current study support a role for placental *PGH* in GDM and uniquely suggest that maternal BMI mediates this association.

4.4.3. Maternal depression

Maternal prenatal depression was associated with decreased placental *PEG3* expression in both the Imperial and Manchester Cohorts. In the Imperial Cohort, placental *PEG3* expression was significantly decreased by 15% in mothers with the highest EPDS scores compared with those with the lowest EPDS scores. While the change in gene expression was modest, this finding was replicated in a second independent cohort. In the Manchester Cohort, placental *PEG3* expression was decreased by 40% in participants with diagnosed depression during pregnancy. The role of *Peg3* in regulating rodent maternal behaviour via a direct action in the brain is well established (Li *et al*. 1999; Curley *et al*. 2004; Champagne *et al*. 2009; Chiavegatto *et al*. 2012), however this is the first report that placental *PEG3* is associated with maternal mood in humans.

Peg3 in the rodent placenta is known to be responsive to environmental stimuli, such as maternal diet (Broad and Keverne 2011; Radford *et al*. 2012). It is therefore possible that human placental *PEG3* is responsive to maternal prenatal stress, or alterations in maternal lifestyles associated with prenatal stress. However, the association between maternal mood and placental expression of *PEG3* also raises the possibility that this gene may modulate maternal psychological adaptation to pregnancy indirectly via the placenta.

Maternal prenatal depression was also associated with decreased placental *hPL* expression in both the Imperial and Manchester Cohorts. In the Imperial Cohort, placental *hPL* expression was significantly decreased by 31% in mothers with the highest EPDS scores compared with those with the lowest EPDS scores. Similarly, maternal diagnosed depression during pregnancy was associated with a 44% decrease in placental *hPL* expression in the Manchester Cohort. Maternal serum hPL levels were only available for two participants with diagnosed depression in the Manchester Cohort; although hPL levels were decreased compared with controls this could not be statistically analysed.

hPL is a key placental lactogenic hormone, closely related to the pituitary hormone prolactin, which has been demonstrated to induce maternal behaviour in animal studies (Bridges *et al*. 1985; Bridges *et al*. 1990; Bridges and Freemark 1995; Bridges *et al*. 1997). Previous studies have demonstrated decreased serum prolactin levels in mothers with postnatal depression symptoms (Abou-Saleh *et al*. 1998; Ingram *et al*. 2003; Groer and Morgan 2007). This study is the first to report that perturbed *hPL* expression in the human placenta is associated with abnormal maternal mood. Further research is required both in a larger study cohort and one in which maternal serum levels of *hPL* can be measured to determine whether reduced placental *hPL* expression in the term placenta manifests as reduced hormone serum level during pregnancy. This is of clinical relevance since it may be possible to use maternal serum *hPL* levels as a biomarker in combination with self-report questionnaires to identify mothers at high risk of maternal depression.

There was no significant association between *PEG3* and *hPL* expression (p = 0.59) in the Imperial cohort. However, placental *PEG3* expression was significantly positively associated with *hPL* expression in the Manchester cohort (p = 0.03), perhaps reflecting the larger sample size. A relationship between *PEG3* and *hPL* expression is consistent with the finding that loss of function of *Peg3* in the mouse placenta results in a marked alteration in the expression of several mouse placental lactogens as gestation proceeds (Broad and Keverne 2011; Kim *et al*. 2013a).

There was no significant association between maternal prenatal depression and placental *PHLDA2* expression in either cohort. In an animal model, female mice carrying *Phlda2* mutant pups display altered maternal behaviours (RMJ lab, unpublished data). It is therefore possible that placental *PHLDA2* expression is associated with other aspects of maternal psychological adaptation to pregnancy, such as mother-infant bonding which requires investigation in future studies.

Finally, placental *PGH* expression was significantly decreased by 43% in mothers with diagnosed depression during pregnancy. PGH promotes fetal growth through induction of peripheral insulin resistance in the mother resulting in preferential transfer of glucose to the fetus (Newbern and Freemark 2011). The novel finding of decreased placental *PGH* expression in mothers with diagnosed depression may underlie the reported association between depression during pregnancy and fetal growth restriction (Steer *et al*. 1992; Henrichs *et al*. 2010; Uguz *et al*. 2013). This remains to be confirmed in a larger study where maternal serum PGH levels can be measured.

4.4.4. Maternal anxiety

Although anxiety and depression are co-morbid, placental *PEG3* and *hPL* expression was not significantly altered in mothers with the highest STAI scores compared with those with the lowest STAI scores. However, a trend for reduced placental expression of *PEG3* and *hPL* in mothers with higher symptoms of trait anxiety (p = 0.09) was observed. Blakeley *et al*. (2013) and

O'Donnell *et al*. (2012) similarly reported different effects of maternal prenatal anxiety and depression on placental gene expression. The differential associations, if any, of placental *PEG3* and *hPL* expression with depression and anxiety, remain to be determined in a larger cohort.

4.4.5. Summary

In summary, the results presented in this chapter suggest a role for aberrant placental *PHLDA2* in preeclampsia. Placental *PHLDA2* expression was significantly increased in PE pregnancies, independent of any associated effect on fetal growth. In contrast, the results of the current study do not support a role for aberrant imprinted gene expression in gestational diabetes, although further studies on larger GDM cohorts are needed to confirm this result. Finally, this study demonstrated decreased placental *PEG3* and *hPL* expression in pregnancies complicated by maternal mood disorders. These novel results provide the first evidence to support the hypothesis that aberrant placental function may influence maternal mood during pregnancy. In conclusion, this chapter supports the third study hypothesis of aberrant placental imprinted gene expression in pregnancies complicated by PE and maternal mood disorders and therefore highlights a role for placental imprinted genes in maternal adaptation to pregnancy, an observation which warrants further research.

CHAPTER 5: HUMAN PLACENTAL IMPRINTED GENE EXPRESSION AND MATERNAL LIFESTYLE

5.1. Introduction

Results from Chapters 3 and 4 support a role for aberrant imprinted gene expression in fetal growth restriction and other complications of pregnancy such as preeclampsia and maternal mood disorders. Therefore, identifying factors responsible for altered imprinted gene expression is of paramount importance. Of particular interest are maternal lifestyle factors, as these may be most amenable to intervention.

A number of studies have examined the relationship between maternal lifestyle factors and fetal growth. Smoking during pregnancy in particular has been associated with a two fold increased risk of LBW (Pollack *et al*. 2000; Figueras *et al*. 2008; Rasmussen and Irgens 2006) and has been estimated to account for 11% of LBW pregnancies (Pollack *et al*. 2000). Although current NICE guidelines recommend cessation of smoking during pregnancy (NICE 2010), an estimated 16% of mothers in Wales continue smoking during pregnancy (Health and Social Care information Centre 2012). Despite the well established association between smoking and growth restriction, the mechanisms underlying this relationship are unclear. Proposed mechanisms include restriction of placental oxygen supply, transfer of toxic compounds to the fetus and/or direct effects on placental development and function (Pollack *et al*. 2000; Zdravkovi *et al*. 2006).

Although light, recreational exercise is considered safe during pregnancy (in the absence of any medical complications) (RCOG 2006), a number of studies have reported significantly decreased birth weight in women carrying out strenuous exercise during pregnancy (Clapp 2003; Bell *et al*. 1995). This has been proposed to be due to an exercise-induced reduction in placental blood flow and growth (Clapp 2003; Juhl *et al*. 2010).

Heavy alcohol consumption during pregnancy (> one drink/day) has also been demonstrated to negatively affect birth weight, although it is unclear whether light to moderate drinking has any effect on fetal growth (Patra *et al*. 2011; Henderson *et al*. 2007). NICE guidelines recommend that pregnant women should not consume alcohol in the first trimester and those choosing to drink alcohol during pregnancy should consume no more than 1 to 2 units a week (NICE 2008). However, approximately 39% of women in

Wales are estimated to continue drinking alcohol during pregnancy (Infant Feeding Survey 2010). In addition, illegal drug use during pregnancy has been associated with a significantly increased risk of growth restriction (Pinto *et al*. 2010).

Both extremes of maternal BMI have been associated with fetal growth restriction. Low pre-pregnancy BMI, short stature and poor weight gain during pregnancy significantly increase the risk of delivering a LBW infant (Valero de Bernabe *et al*. 2004). In contrast, although maternal obesity is generally thought to be protective against growth restriction, Gardosi (2009) demonstrated that in fact the risk of SGA is also increased in obese mothers. NICE guidelines recommend weight loss in obese women before pregnancy in order to minimise associated risks for mother and fetus during pregnancy (NICE 2010b). Given that 58% of people in Wales are overweight and 23% obese (Health and Social Care information Centre 2012), it is likely that a substantial number of women are obese during pregnancy.

Finally, a number of studies have analysed maternal diet with respect to effects on fetal growth. Indeed, the importance of a healthy diet during pregnancy is recognised in clinical practice with current NICE guidelines recommending that pregnant women receive information on diet and nutrition at their booking appointment (NICE 2008). Fruit and vegetable consumption in particular have been demonstrated to be positively associated with birth weight (Mikkelsen *et al*. 2006; Rao *et al*. 2001; Thompson *et al*. 2010; Matthews *et al*. 1999). However, it is estimated that only 33% of people in Wales consume the minimum five portions of fruits or vegetables a day (Health and Social Care information Centre 2012) that is recommended (FSA 2007). Dairy consumption during pregnancy is similarly positively associated with birth weight (Ford 2011; Xue *et al*. 2008; Olsen *et al*. 2007; Ludvigsson and Ludvigsson 2004). It is recommended that a healthy daily diet include moderate amounts of dairy (FSA 2007) and that this should be increased during pregnancy (Ortega 2001).

Studies have yielded conflicting results regarding the relationship between maternal carbohydrate consumption and fetal growth with some reporting a positive association (Mitchell *et al*. 2004; Thompson *et al*. 2010) and others a negative association (Godfrey *et al*. 1996; Moore *et al*. 2004). It

is currently recommended that as part of a healthy diet, 50% of food energy should come from total carbohydrates (FSA 2007) but that this should not be increased during pregnancy (Williamson *et al*. 2006). Conflicting results have also been reported regarding the relationship between maternal protein consumption during pregnancy and fetal growth with some studies reporting a positive association (Godfrey *et al*. 1996; Ford 2011; Thompson *et al*. 2010; Moore *et al*. 2004) and others a negative association (Knudsen *et al*. 2008; Campbell *et al*. 1996; Ricci *et al*. 2010). It is recommended that a healthy daily diet contain protein-rich foods (such as meat, fish or meat alternatives) (FSA 2007) with some studies suggesting that this should be increased during pregnancy (Ortega 2001; Williamson 2006). Fish is one source of protein in particular, which has been positively associated with birth weight (Makrides *et al*. 2009; Ricci *et al*. 2010; Rogers *et al*. 2004). Indeed, it is recommended that at least two portions of fish are consumed a week as part of a healthy diet (FSA 2007).

Maternal caffeine consumption has also been demonstrated to be negatively associated with fetal growth (Santos *et al*. 1998; Xue *et al*. 2008; Vik *et al*. 2003) and as such it is recommended that pregnant women consume no more than 200mg of caffeine a day, which equates to approximately two mugs of instant coffee (FSA 2007). Finally, micronutrients in the form of maternal folate and iron supplementation are positively associated with birth weight (Godfrey *et al*. 1996; Mitchell *et al*. 2004), although only daily folate supplementation is routinely recommended to all pregnant women (NICE 2008).

Importantly, with respect to analysing the effect of maternal diet on fetal growth, it has been suggested that analysis of diet patterns (rather than individual food items or groups as described above) provides more information on the interacting effects of food items and is of greater relevance in the design of future healthy eating interventions (Crozier *et al.* 2008). In particular, analysis of diet patterns during pregnancy has previously revealed two contrasting diets; the “Western Diet” or “Junk Food Diet” pattern characterised by frequent consumption of high fat and high sugar food items and the “Prudent Diet” or “Healthy Diet” pattern characterised by frequent consumption of fruit, vegetables, fish and dairy products (Hu 2002; Crozier *et*

al. 2006; Thompson *et al*. 2010). A prudent or healthy diet has been demonstrated to be associated with a significantly reduced risk of delivering an SGA infant (Thompson *et al*. 2010).

Thus, a number of studies highlight the effect of maternal lifestyle on fetal growth. However, the mechanisms underlying these associations are not well understood. The growth regulating functions of the placenta are vulnerable to changes in the maternal environment, providing a possible mechanism by which an adverse maternal lifestyle could result in fetal growth restriction (Fowden *et al*. 2011). For example, imprinted gene expression in the placenta is known to be responsive to environmental signals (Fowden *et al*. 2011), although the majority of these studies have focussed on maternal diet alterations in an animal model. It is therefore possible that aberrant placental imprinted gene expression may underlie the association between adverse maternal lifestyles and FGR, although this requires further investigation in human pregnancies.

Human placental *PHLDA2* expression has been demonstrated to be significantly increased in mothers carrying out strenuous exercise during pregnancy (Lewis *et al*. 2011). Bruchova *et al*. (2010) also reported increased placental *PHLDA2* expression in women smoking during pregnancy, although a subsequent study by Moore *et al*. (2015) failed to replicate this finding. While aberrant placental *Phlda2* expression has been demonstrated in response to ethanol consumption and calorie restriction in pregnant rat dams (Shukla *et al*. 2011), the effects of maternal diet and alcohol consumption on human placental *PHLDA2* expression remain to be determined. Similarly, placental *CDKN1C* expression was not significantly altered in mothers who continued smoking during pregnancy (Moore *et al*. 2015). In an animal model, placental *Cdkn1c* expression was increased in response to maternal ethanol consumption and calorie restriction (Shukla *et al*. 2011), but not significantly altered by a high fat – high sugar diet (Sferruzi-Perri *et al*. 2013). *PEG3* expression was not significantly altered in placentas of smokers (Moore *et al*. 2015) or in the umbilical cord of obese mothers (Keshari *et al*. 2014). However, placental *Peg3* expression was significantly decreased as a result of transient maternal starvation (Broad and Keverne 2011) and increased due to calorie restriction in a mouse model (Radford *et al*. 2012). In

contrast, no significant effect of maternal high fat or high fat- high sugar diet was observed on mouse placental *Peg3* expression (Gallou-Kabani *et al*. 2010; Sferruzzi-Perri *et al*. 2013). Finally, placental *Peg10* expression has been demonstrated not to be significantly altered in response to a maternal high fat diet in a mouse model (Gallou-Kabani *et al*. 2010) or in response to human maternal obesity (Soubry *et al*. 2013) or antibiotic use during pregnancy (Vidal *et al*. 2014).

In summary, few studies have examined human placental expression of the imprinted genes *PHLDA2*, *CDKN1C*, *PEG3* and *PEG10* in response to maternal lifestyle during pregnancy. Given the evidence presented in Chapters 3 and 4 supporting a role for aberrant imprinted gene expression in fetal growth restriction and other complications of pregnancy such as preeclampsia and maternal mood disorders, identifying maternal lifestyle factors associated with aberrant placental imprinted gene expression was a key aim of this chapter. In addition, in light of the association reported between placental imprinted gene (*PHLDA2* and *CDKN1C*) and hormone gene expression (*hPL*) in Chapter 3, the effects of maternal lifestyle on placental hormone gene expression were also analysed.

5.2. Chapter specific methods

5.2.1. Study participants

The effects of maternal lifestyle on fetal growth and placental imprinted gene expression were examined in Caucasian control participants and (where relevant) in SGA participants of the Wales Cohort. Control participants were defined as those delivering AGA infants and with no documented complication of pregnancy or medical disorder. Otherwise healthy participants, with singleton pregnancies, were recruited antenatally at two research sites: UHW (Cardiff) and RGH (Newport). Only control placentas were used to determine maternal lifestyle effects on placental imprinted gene expression such that any effect observed was not due to the underlying association between imprinted gene expression and fetal growth.

5.2.2. Maternal lifestyle measures

A self-administered participant questionnaire was used to assess maternal BMI, smoking, alcohol consumption and illegal drug use before and during pregnancy. This data was compared with that recorded in the participant's medical notes to ensure consistency in self-report measures. In addition, information on the number of cigarettes smoked and alcohol units consumed were recorded from the medical notes.

The participant questionnaire also included a food frequency questionnaire (FFQ), with which the participant indicated how frequently they consumed each food item during their pregnancy. This FFQ (Appendix 3) was designed and trialled by A Janssen and J Crovini (Cardiff based nutritionist) as previous FFQs used in the study of diet during pregnancy were not suitable due to length, target population and/or diet components of interest. Although a basic, non-quantitative method for the assessment of diet, the FFQ was chosen in order to assess habitual diet during pregnancy in a format easily self-administered as part of the recruitment process.

5.2.3. Statistical analysis

All statistical analysis was carried out using IBM SPSS statistics for Windows (version 20.0, 2011) with a p value <0.05 considered statistically significant. P-P plots were generated and a Shapiro-Wilk test carried out to test for normal distribution of the data. All variables in this chapter, including placental target gene expression, were normally distributed and therefore parametric tests were used in data analysis. Pearson correlation tests or partial correlations (when controlling for a potential confounding variable) were used to analyse associations between variables. Differences between two groups or more were analysed using a T-test or one-way ANOVA (with a Tukey post-hoc test) respectively.

In addition, specific to this chapter, principle component analysis (PCA) was used to analyse maternal diet patterns. PCA is a multivariate statistical method used in dietary research to group food items based on how strongly the frequency of their consumption is correlated, with the aim of identifying food patterns that explain the greatest proportion of variance in FFQ data (Hu 2002). In this study, PCA of reported consumption of 17 food items was carried out in SPSS. Firstly, a scree plot was generated to determine the number of components (or diet patterns) present in the data as well as the proportion of variance in FFQ data explained by each component. A correlation matrix was further used to determine the degree of correlation between each food item within a component or diet pattern. Within each diet pattern, positive and negative coefficients were generated for each food item. PCA coefficients explain the degree to which each food item is consumed more or less frequently as part of the diet pattern, with coefficients ≤ - 0.15 or ≥ 0.15 considered a characteristic food item. The PCA coefficients for each food item were then multiplied by the frequency of consumption reported by the participant to generate a food item score. A diet pattern score was subsequently calculated (the sum of the food item scores) with higher scores indicating a diet more closely matching the diet pattern described.

5.3. Results

The Wales Cohort included 91 control participants (delivering AGA infants with no known complications of pregnancy) of which 96% were Caucasian. Furthermore, 78% of control participants were Welsh and 70% described their baby's father as Caucasian-Welsh. Due to the known differences in birth weight between ethnic groups and differences in culture, which may impact on maternal lifestyle during pregnancy, only Caucasian control participants (N = 87) were included in the analysis of lifestyle effects on birth weight and target gene expression. Maternal characteristics and birth outcomes for these participants are summarised in table 5.1.

One factor of relevance when analysing maternal lifestyle during pregnancy is that of maternal education and family income. Low maternal educational attainment and/or low family income have been demonstrated to be associated with adverse lifestyle during pregnancy and low birth weight (Collingwood Bakeo and Clarke 2006). In the overall cohort 49% of participants reported GCSE/O levels as their highest level of education and 17% reported a combined family income (before deductions) of < £18,000. There was a trend for an increased proportion of participants reporting a family income < £18,000 at the RGH site (20%) ($p = 0.09$, $n = 216$) but there was no significant difference in maternal education between sites ($p = 0.37$, $n = 216$).

5.3.1. Pre-pregnancy smoking

In the overall cohort ($n = 219$), 31% of participants reported smoking in the three months before pregnancy. The majority of smokers reported daily smoking (83%) in contrast to occasional smoking (17%) with a mean of 7 cigarettes smoked per day. The proportion of pre-pregnancy smokers was highest at the RGH site (38%) compared with UHW (27%) although this difference did not reach statistical significance ($p = 0.08$). The participants' reported smoking in the study questionnaire and that recorded in the medical notes was significantly positively correlated ($r = 0.79$, $p < 0.001$, $n = 188$).

Table 5.1: Maternal characteristics and birth outcomes for control participants included in analysis of lifestyle effects on placental gene expression. Mean (SD)/Range or Number (%) is shown.

	Control Caucasian Participants (N = 87)
Maternal Lifestyle	
Maternal age	30 (5) / 19 – 41
Parity	2 (1) / 0 – 7
Smoking during pregnancy:	
No	63 (72%)
Yes	17 (20%)
Not known	7 (8%)
Strenuous exercise:	
No	57 (66%)
Yes	9 (10%)
Not known	21 (24%)
Drinking Alcohol during pregnancy:	
No	53 (61%)
Yes	32 (37%)
Not known	2 (2%)
Maternal BMI (kg / m^2)	27 (6) / 19 – 42
Underweight	0 (0%)
Normal weight	41 (47%)
Overweight	17 (20%)
Obese	25 (29%)
Not known	4 (4%)
Birth Outcome	
Mode of Delivery:	
Vaginal	6 (7%)
Elective C section	76 (87%)
Emergency C section	4 (5%)
Instrumental	1 (1%)
Birth weight (g)	3485 (280) / 2830 – 4190
Custom birth weight centile	53 (22) / 11 – 89
Gestational age (weeks)	39 (1) / 37 - 42
Gender	
Male	42 (48%)
Female	45 (52%)

Pre-pregnancy smoking information was available for 85 control (Caucasian) participants with 28% reporting pre-pregnancy smoking. There was no significant difference in birth weight or custom birth weight centiles between pre-pregnancy smokers and non-smokers (Figure 5.1). Similarly, placental weight was not significantly altered in smokers (p = 0.29, n = 85).

As shown in Figure 5.1, there was no significant difference in placental expression of the imprinted genes and placental hormones examined. Differences in birth weight and gene expression remained non-significant when participants were divided into those reporting daily or occasional smoking (results not shown). Finally, there was no significant association between the number of cigarettes smoked and birth weight (r = 0.01, p = 0.96, n = 74) or placental *PHLDA2* (r = 0.16, p = 0.19, n = 74), *CDKN1C* (r = 0.15, p = 0.19, n = 74), *PEG3* (r = - 0.07, p = 0.54, n = 74), *PEG10* (r = - 0.04, p = 0.72, n = 74), *hPL* (r = - 0.13, p = 0.27, n = 74) or *PGH* (r = 0.08, p = 0.53, n = 74) expression.

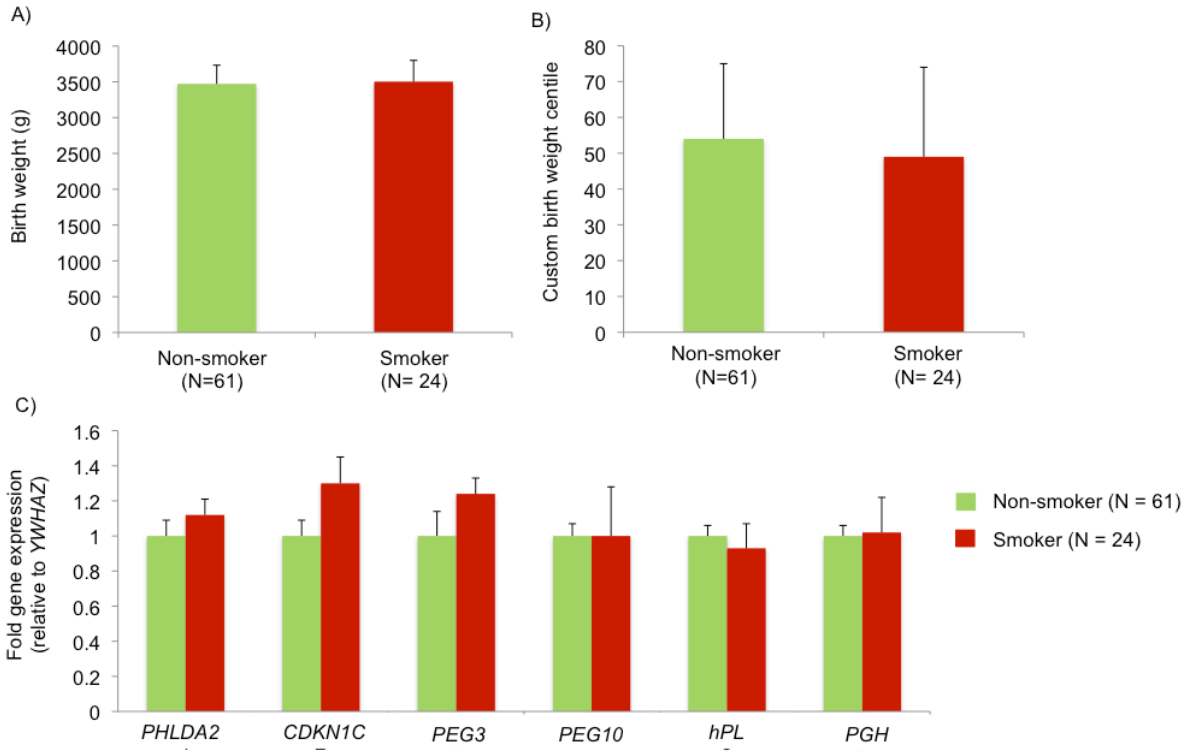


Figure 5.1: Effects of pre-pregnancy smoking on birth weight and placental target gene expression. There was no significant effect of maternal pre-pregnancy smoking on birth weight (A), custom birth weight centiles (B) or placental target gene expression (C). Error bars represent SEM.

5.3.2. Smoking during pregnancy

In the overall cohort (n = 219), 25% of participants reported smoking during pregnancy. 25% of participants reported smoking during the first trimester and 22% in the second and third trimesters combined, with a mean of 10 cigarettes smoked per day. As with pre-pregnancy smoking, the proportion of participants smoking during pregnancy was highest at the RGH site (33%) compared with UHW (22%) although this difference did not reach statistical significance (p = 0.06).

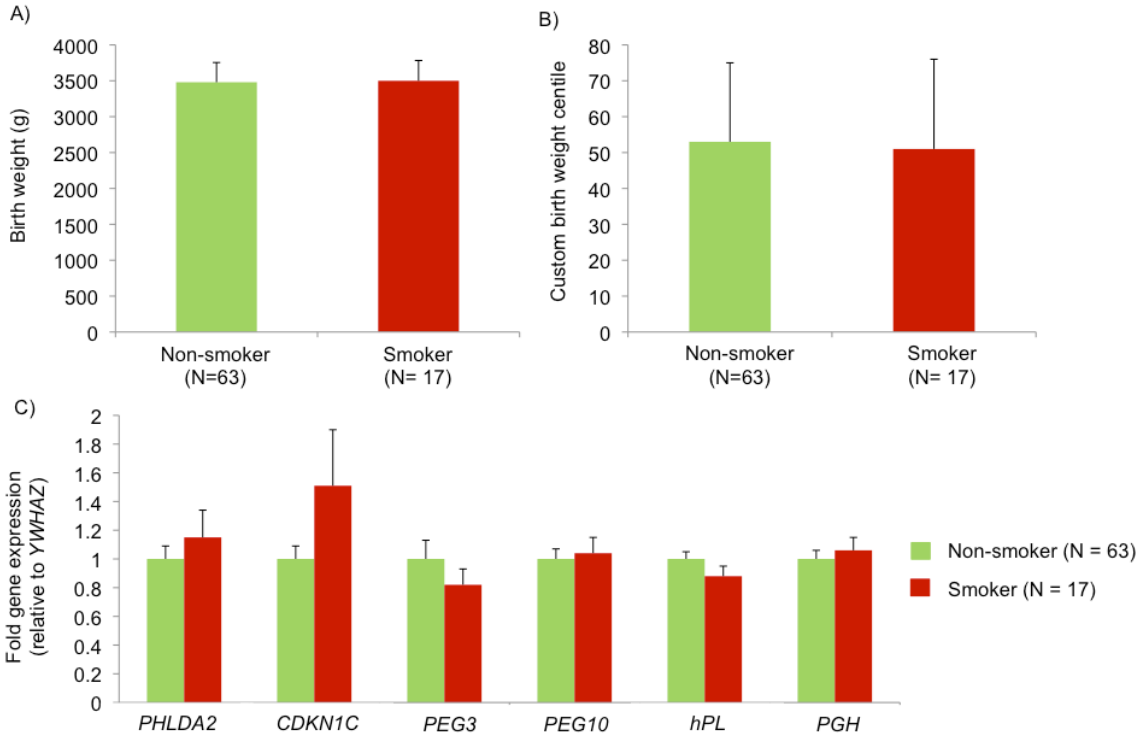


Figure 5.2: Effects of smoking during pregnancy on birth weight and placental target gene expression. There was no significant effect of maternal smoking during pregnancy on birth weight (A), custom birth weight centiles (B) or placental target gene expression (C). Error bars represent SEM.

Pregnancy smoking information was available for 80 control (Caucasian) participants with 21% reporting smoking during pregnancy. There was no significant difference in birth weight or custom birth weight centiles between smokers and non-smokers (Figure 5.2). Similarly, placental weight was not significantly altered in smokers (p = 0.19, n = 80). However, the proportion of smokers was significantly higher in SGA compared with

AGA participants (p = 0.01). In addition, smokers were 2 times more likely to deliver an SGA infant than non-smokers as determined by logistic regression analysis (OR = 2.26 (1.09, 4.67); p = 0.03; n = 158), further suggesting a role for smoking in fetal growth.

As shown in Figure 5.2, there was no significant difference in placental expression of the imprinted genes and placental hormones examined. Differences in birth weight and gene expression remained non-significant when participants were divided into those reporting daily or occasional smoking (results not shown). Similarly, there was no significant association between the number of cigarettes smoked and birth weight (r = 0.03, p = 0.79, n = 79) or placental *PHLDA2* (r = 0.07, p = 0.57, n = 79), *CDKN1C* (r = 0.06, p = 0.58, n = 79), *PEG3* (r = - 0.14, p = 0.21, n = 79), *PEG10* (r = - 0.07, p = 0.53, n = 79), *hPL* (r = 0.06, p = 0.62, n = 79) or *PGH* (r = - 0.12, p = 0.30, n = 79) expression.

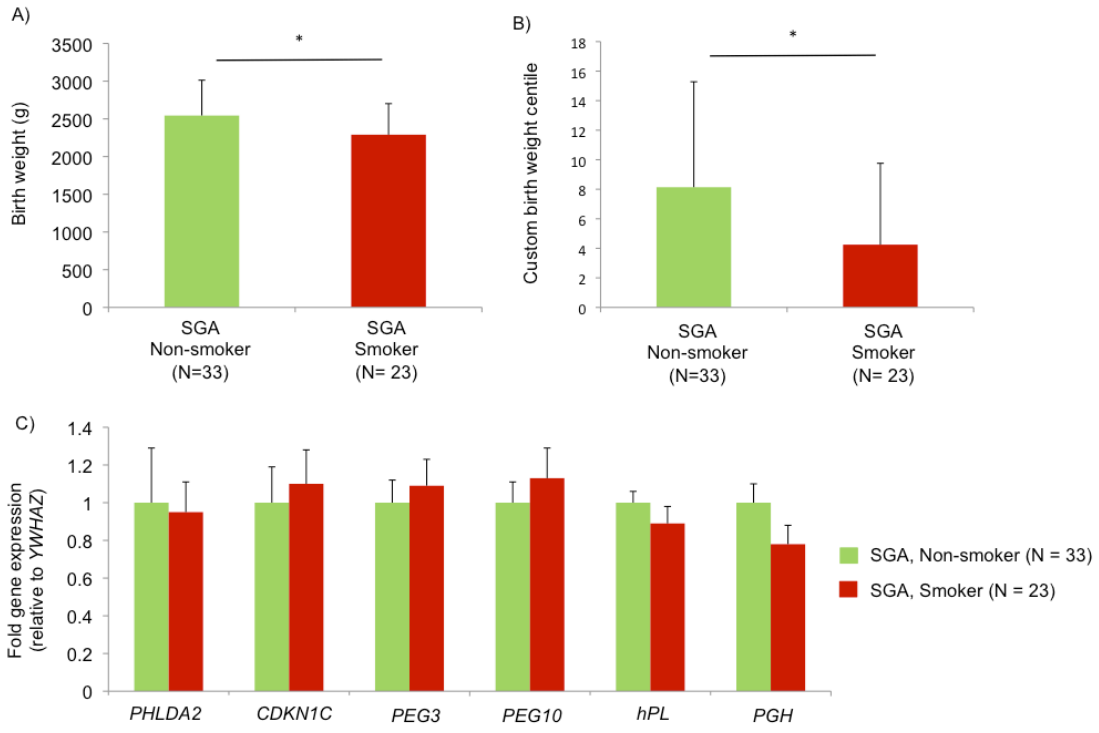


Figure 5.3: Effects of smoking during pregnancy on SGA placentas. Birth weight (A) and custom birth weight centiles (B) were significantly reduced in SGA participants smoking during pregnancy but there was no change in placental target gene expression (C). Error bars represent SEM. * P <0.05.

Birth weight and custom birth weight centiles were significantly lower in SGA participants reporting smoking during pregnancy compared with non-smokers (Figure 5.3). However, there was no significant difference in placental imprinted gene or placental hormone gene expression between smokers and non-smokers delivering SGA infants (Figure 5.3).

5.3.3. Strenuous exercise

15% of participants in the overall cohort (n = 216) reported undertaking strenuous exercise (defined as exercise at least 30 minutes in duration, at least once a week). There was no significant difference in the proportion of participants undertaking strenuous exercise between the RGH (10%) and UHW (16%) sites (p = 0.81).

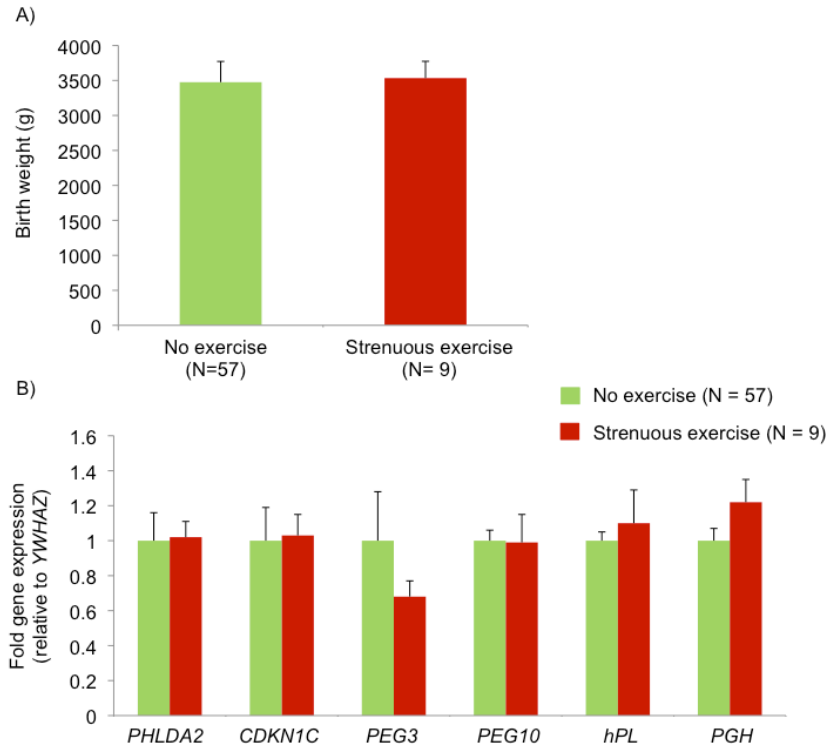


Figure 5.4: Effects of strenuous exercise during pregnancy. There was no significant effect of strenuous exercise on birth weight (A) or placental target gene expression (B). Error bars represent SEM.

Exercise information was available for 66 of the Caucasian control participants of which 14% were exercising during pregnancy. There was no significant difference in birth weight (Figure 5.4), custom birth weight centiles

(p = 0.12, n = 66) or placental weight (p = 0.63, n = 66) between exercisers and non-exercisers. Similarly, there was no significant difference in the proportion of participants undertaking strenuous exercise between AGA, SGA or LGA groups (p = 0.81, n = 66). There was no significant difference in placental expression of any of the imprinted genes or placental hormones examined (Figure 5.4). Results remained non-significant when the overall cohort was analysed.

5.3.4. Pre-pregnancy alcohol consumption

Alcohol consumption in the three months before pregnancy was reported in 59% of the overall cohort with a mean of 1.7 units per week consumed. Maternal pre-pregnancy alcohol consumption was significantly increased at UHW (65%) compared with RGH (47%) sites (p = 0.02). Participant self-reported alcohol consumption in the study questionnaire was significantly positively correlated with that recorded in the medical notes (*r* = 0.31, p <0.001, n = 149).

Pre-pregnancy alcohol consumption information was available for 85 of the Caucasian control participants of which 62% were consuming alcohol during the three months before their pregnancy. Birth weight, custom birth weight centiles (Figure 5.5) and placental weight (p = 0.84, n = 85) were not significantly reduced in participants consuming alcohol before pregnancy. Similarly, there was no significant difference in the proportion of participants consuming alcohol before pregnancy between AGA and SGA groups (p = 0.62, n = 85).

There was no significant difference in placental expression of any of the imprinted genes or placental hormones examined (Figure 5.5), although a trend was shown for increased placental *hPL* (p = 0.06, n = 85) and *PGH* (p = 0.07, n = 85) expression in participants consuming alcohol. Results remained non-significant when the overall cohort was analysed. Finally, there was no significant correlation between the number of units consumed per week and birth weight (*r* = 0.10, p = 0.40, n = 73) or placental *PHLDA2* (*r* = 0.05, p = 0.65, n = 73), *CDKN1C* (*r* = 0.06, p = 0.60, n = 73), *PEG3* (*r* = -

0.02, p = 0.98, n = 73), *PEG10* (*r* = - 0.06, p = 0.61, n = 73), *hPL* (*r* = 0.14, p = 0.23, n = 73) or *PGH* (*r* = 0.09, p = 0.47, n = 73) expression.

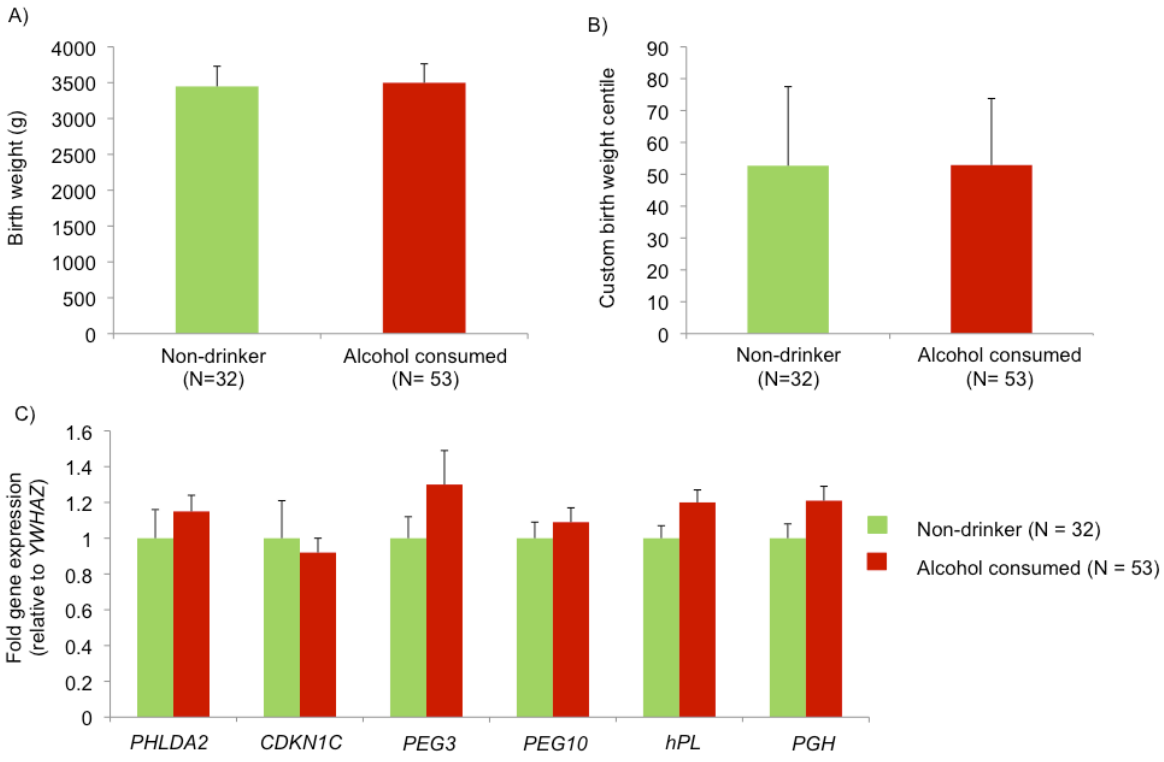


Figure 5.5: Effects of pre-pregnancy alcohol consumption. There was no significant effect of alcohol consumption on birth weight (A), custom birth weight centiles (B) or placental target gene expression (C). Error bars represent SEM.

5.3.5. Alcohol consumption during pregnancy

In the overall cohort 33% of participants reported consuming alcohol during their pregnancy with a mean of 1.1 units consumed per week. In contrast to pre-pregnancy alcohol consumption, there was no significant difference in the proportion of participants consuming alcohol during pregnancy between RGH (27%) and UHW (36%) sites. Interestingly, alcohol consumption was less frequent in the first trimester (24%) compared with the second and third trimesters (27%). There was also a significant positive correlation between alcohol consumption before and during pregnancy (*r* = 0.55, p <0.001, n = 197).

Information on alcohol consumption during pregnancy was available for 85 of the Caucasian control participants of which 38% were consuming alcohol during pregnancy. Birth weight, custom birth weight centiles (Figure 5.6) and placental weight (p = 0.21, n = 85) were not significantly reduced in participants consuming alcohol during pregnancy. Similarly, there was no significant difference in the proportion of participants consuming alcohol during pregnancy between AGA and SGA groups (p = 0.55, n = 85).

There was no significant difference in placental expression of the imprinted genes or placental hormones examined (Figure 5.6), although as with pre-pregnancy alcohol consumption, a trend was observed for increased *hPL* expression in mothers consuming alcohol during pregnancy (p = 0.06).

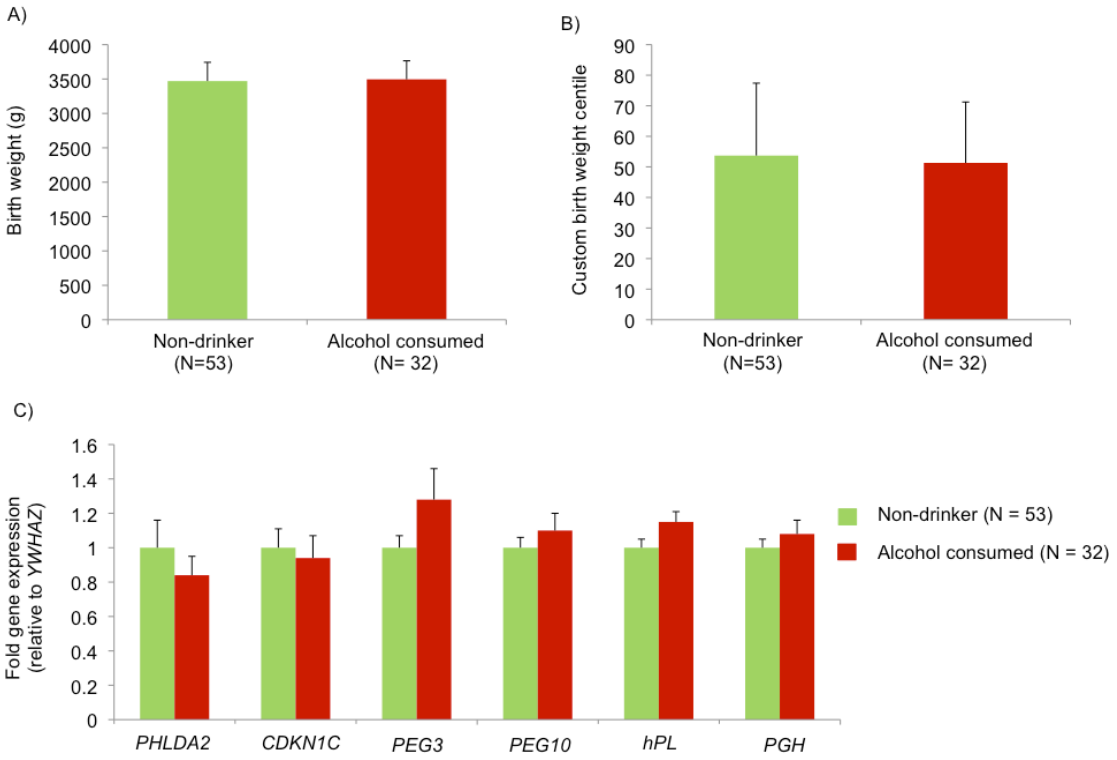


Figure 5.6: Effects of alcohol consumption during pregnancy. There was no significant effect of alcohol consumption on birth weight (A), custom birth weight centiles (B) or placental target gene expression (C). Error bars represent SEM.

There was also no significant correlation between the number of units consumed per week and birth weight (r = 0.08, p = 0.41, n = 73) or placental *PHLDA2* (r = 0.05, p = 0.65, n = 73), *CDKN1C* (r = 0.06, p = 0.60, n = 73), *PEG3* (r = - 0.02, p = 0.98, n = 73), *PEG10* (r = - 0.06, p = 0.61, n = 73), *hPL*

(r = 0.14, p = 0.23, n = 73) or *PGH* (r = 0.09, p = 0.47, n = 73) expression. Finally, there was no significant difference in birth weight (p = 0.66, n =55) or custom birth weight centiles (p = 0.19, n = 55) in SGA participants reporting consuming alcohol during pregnancy. Similarly, placental imprinted gene or placental hormone gene expression was not significantly altered (results not shown).

5.3.6. Illegal drug use

In the overall cohort (n = 219), only 3 participants (1 AGA and 2 SGA participants) had a record of pre-pregnancy illegal drug use with none continuing throughout pregnancy.

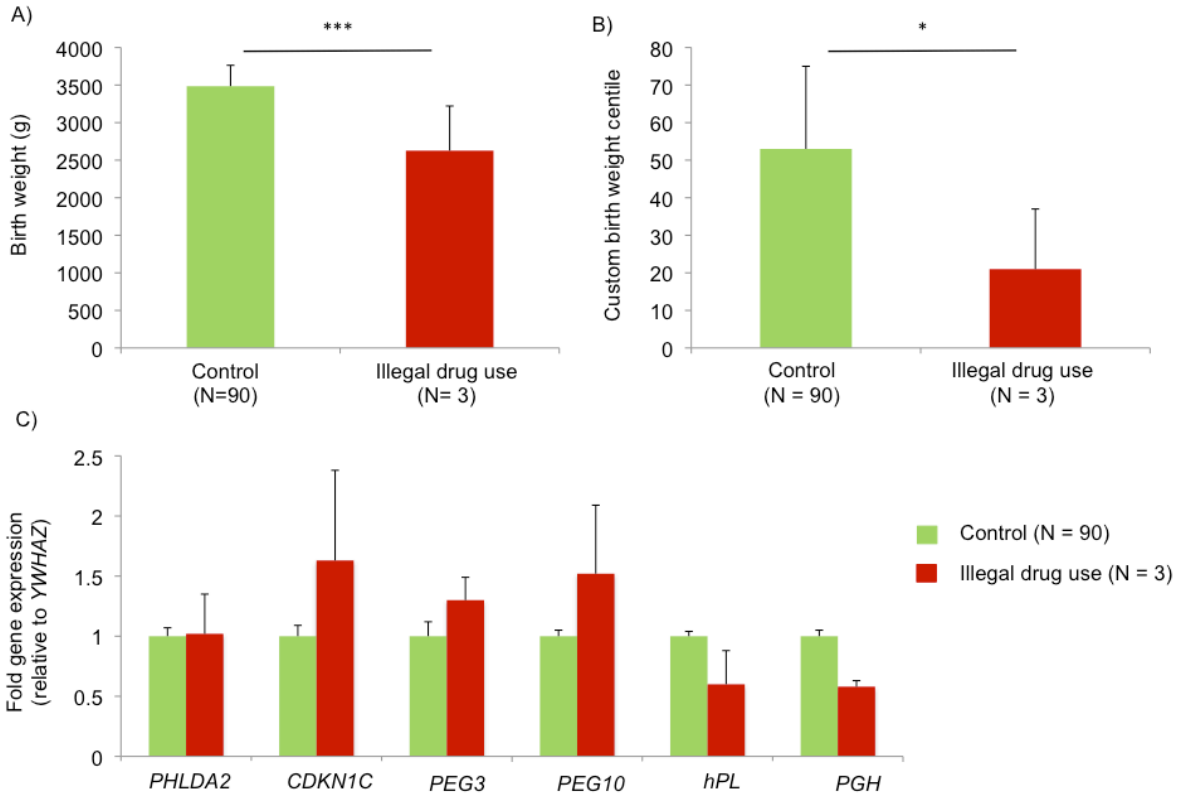


Figure 5.7: Effects of pre-pregnancy illegal drug use. Birth weight (A) and custom birth weight centiles (B) were significantly reduced in participants with pre-pregnancy illegal drug use, however there was no significant effect on placental target gene expression (C). Error bars represent SEM. * P <0.05, *** p < 0.001.

Birth weight and custom birth weight centiles were significantly reduced in these participants compared with controls with no documented

illegal drug use (Figure 5.7). Although placental weight was also reduced by 89g, this difference was not statistically significant (p = 0.91, n = 93). There was no significant difference in placental expression of the imprinted genes or placental hormones examined (Figure 5.7).

5.3.7. Maternal BMI

In the overall cohort (n = 219), 1% of participants were underweight, 47% were a healthy BMI, 24% were overweight and 28% were obese at booking. There was no significant difference in the proportion of BMI categories between RGH and UHW sites (p = 0.51, n = 219). Importantly, maternal self-report of BMI was significantly positively correlated with that recorded in the medical notes (r = 0.94, p < 0.001, n = 147).

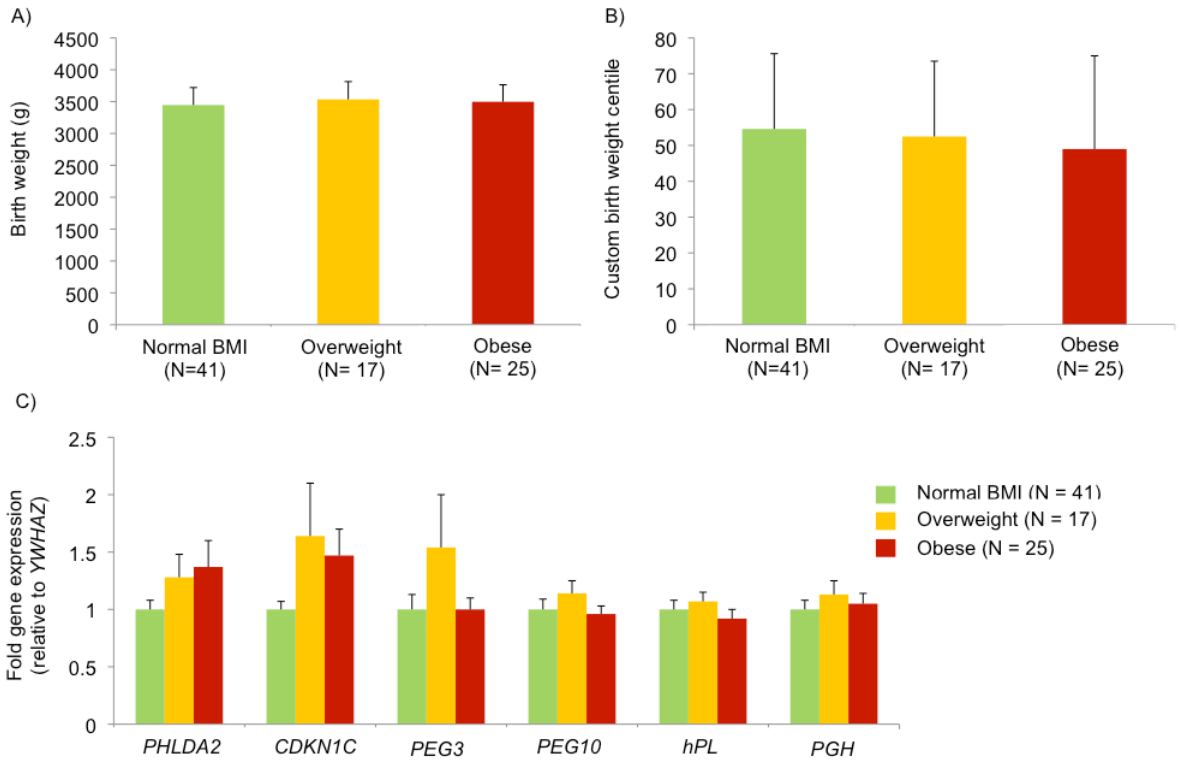


Figure 5.8: Effects of maternal BMI. There was no significant effect of maternal BMI at booking on birth weight (A), custom birth weight centiles (B) or placental target gene expression (C). Error bars represent SEM.

Maternal BMI information was available for 83 of the Caucasian control participants of which 51% were overweight or obese at booking. Of the obese participants, 36% were classified as Class 1 Obese (BMI 30.0 – 34.9), 52% as Class 2 Obese (BMI 35 – 39.9) and 12% as Class 3 Obese (BMI ≥ 40). Maternal BMI was not significantly correlated with birth weight (r = 0.06, p = 0.59, n = 83), custom birth weight centiles (r = - 0.15, p = 0.21, n = 83) or placental weight (r = 0.06, p = 0.62, n = 83). Similarly, there was no significant difference in birth weight (p = 0.49, n = 83), custom birth weight centiles (p = 0.64, n = 83) or placental weight (p = 0.88, n = 83) between normal BMI, overweight and obese participants as determined by a one-way ANOVA (Figure 5.8).

As shown in Figure 5.8, there was no significant difference in placental expression of the imprinted genes or placental hormones examined, although a trend was observed for increased *CDKN1C* (p = 0.08) expression in obese participants. When participants were divided into those with a healthy BMI compared with overweight or obese participants, placental *CDKN1C* expression was significantly increased by 54% in overweight or obese participants (p = 0.03, n = 83). Given that there was no significant difference in mode of delivery between participants of different BMI categories (p = 0.15, n = 83), this suggests that the observed increase in *CDKN1C* expression in overweight/obese participant placentas is independent of any effects of labour on *CDKN1C* expression. Placental *PHLDA2* expression was also increased by 33% in overweight/obese participants, although this difference failed to reach statistical significance (p = 0.06, n = 83). Finally, there was no significant correlation between maternal BMI at booking and placental *PHLDA2* (r = 0.17, p = 0.14, n = 83), *CDKN1C* (r = 0.17, p = 0.12, n = 83), *PEG3* (r = 0.11, p = 0.34, n = 83), *PEG10* (r = - 0.01, p = 0.93, n = 83), *hPL* (r = - 0.04, p = 0.71, n = 83) or *PGH* expression (r = 0.08, p = 0.45, n = 83). Results remained non-significant when maternal height and weight data were analysed independently (results not shown).

In the overall cohort the mean maternal weight gain during pregnancy was 12kg with no significant correlation between maternal BMI at booking and weight gain (r = - 0.17, p = 0.17, n = 68). As maternal weight gain during

pregnancy is not routinely assessed at either research site, maternal weight gain information was only available for 25 Control Caucasian participants. There was no significant correlation between maternal weight gain and placental *PHLDA2* (r = 0.10, p = 0.65, n = 25), *CDKN1C* (r = - 0.10, p = 0.65, n = 25), *PEG3* (r = 0.20, p = 0.20, n = 25), *PEG10* (r = 0.02, p = 0.91, n = 25), *hPL* (r = 0.14, p = 0.50, n = 25) or *PGH* expression (r = - 0.14, p = 0.51, n = 25).

5.3.8. Fruit and vegetable consumption

In the overall cohort, 50% of participants reported consuming fresh fruit, fresh vegetables or dried fruit more than once a day during pregnancy, with 30% of participants reporting increased consumption compared with before pregnancy. There was no significant difference in fruit and vegetable consumption between UHW (51%) and RGH (50%) sites (p = 0.93).

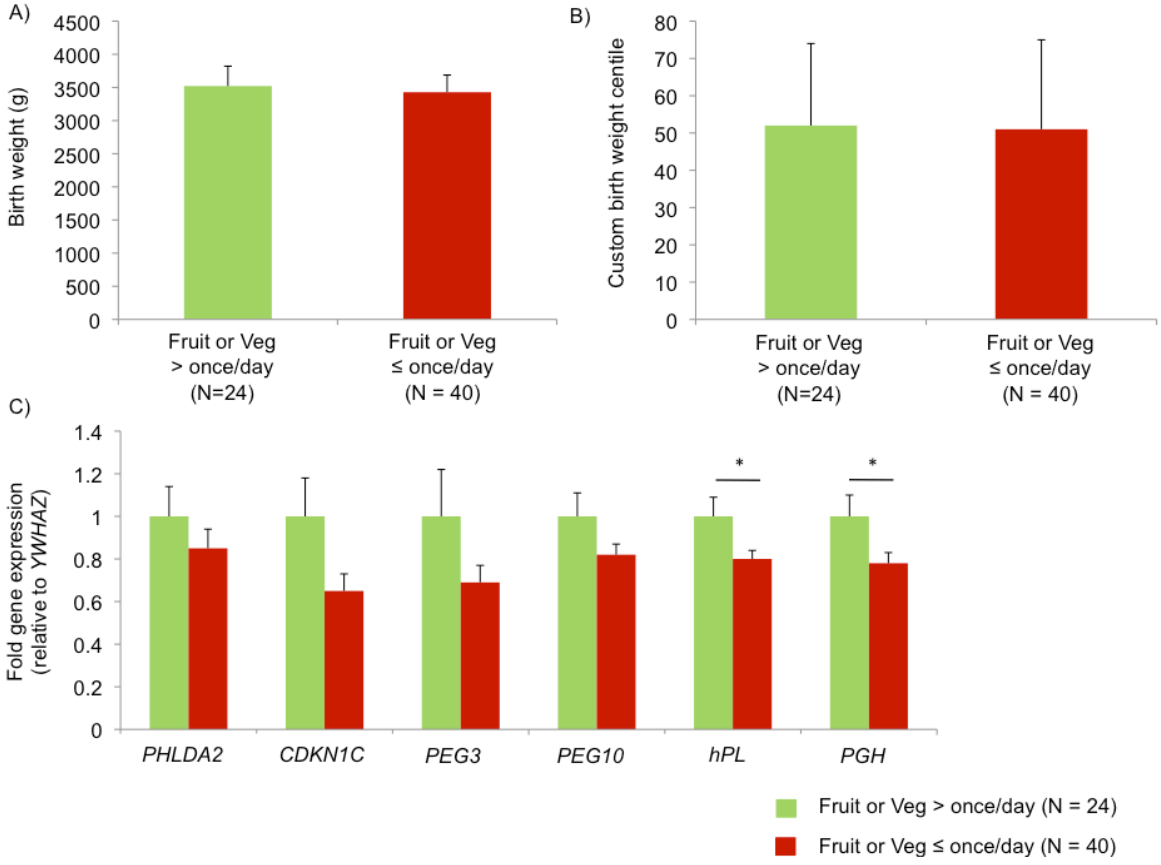


Figure 5.9: Effects of maternal fruit and vegetable consumption during pregnancy. There was no significant effect of fruit and veg consumption on birth weight (A) or custom birth weight centiles (B). Placental *hPL* and *PGH* expression

was significantly decreased in participants with low fruit and veg consumption (C). Error bars represent SEM. * P <0.05.

Information on maternal fruit and vegetable consumption was available for 64 Caucasian control participants of which 38% reported consuming fresh fruit, fresh vegetables or dried fruit more than once a day during pregnancy. Birth weight ($p = 0.22$, $n = 64$), custom birth weight centiles ($p = 0.89$, $n = 64$) and placental weight ($p = 0.95$, $n = 64$) were not significantly altered in participants consuming fruit and vegetables once a day or less (Figure 5.9), i.e. less than government recommendations.

Placental *hPL* and *PGH* expression was significantly reduced in participants reporting consuming fruit or vegetables ≤ once a day during pregnancy (Figure 5.9). There was also a significant positive correlation between placental *hPL* expression and frequency of vegetable consumption during pregnancy ($r = 0.30$, $p = 0.02$, $n = 64$) and between placental *PGH* expression and dried fruit consumption ($r = 0.28$, $p = 0.03$, $n = 63$) respectively. Placental *PHLDA2*, *CDKN1C*, *PEG3* and *PEG10* expression was not significantly altered in participants reporting consuming fruit or vegetables ≤ once a day during pregnancy (Figure 5.9). Results remained non-significant when gene expression was analysed in relation to fruit and vegetable consumption in the overall cohort.

5.3.9. Dairy consumption

In the overall cohort 58% of participants reported consuming dairy products (such as milk, cheese or yoghurt) at least once a day during pregnancy, with 26% of participants reporting increased consumption compared with before pregnancy. There was no significant difference in dairy consumption between UHW (54%) and RGH (67%) sites ($p = 0.09$).

Information on maternal dairy consumption was available for 64 Caucasian control participants of which 66% reported consuming dairy products at least once a day during pregnancy. Birth weight ($p = 0.65$, $n = 64$), custom birth weight centiles ($p = 0.56$, $n = 64$) and placental weight ($p = 0.08$, $n = 64$) was not significantly altered in participants consuming dairy less than once a day (Figure 5.10).

There was a trend for decreased placental *PEG3* (p = 0.08, n = 64) and *PEG10* (p = 0.06, n = 64) expression in participants consuming dairy products less than once a day (Figure 5.10). However, there was no significant difference in placental *PHLDA2*, *CDKN1C*, *hPL* or *PGH* expression (Figure 5.10).

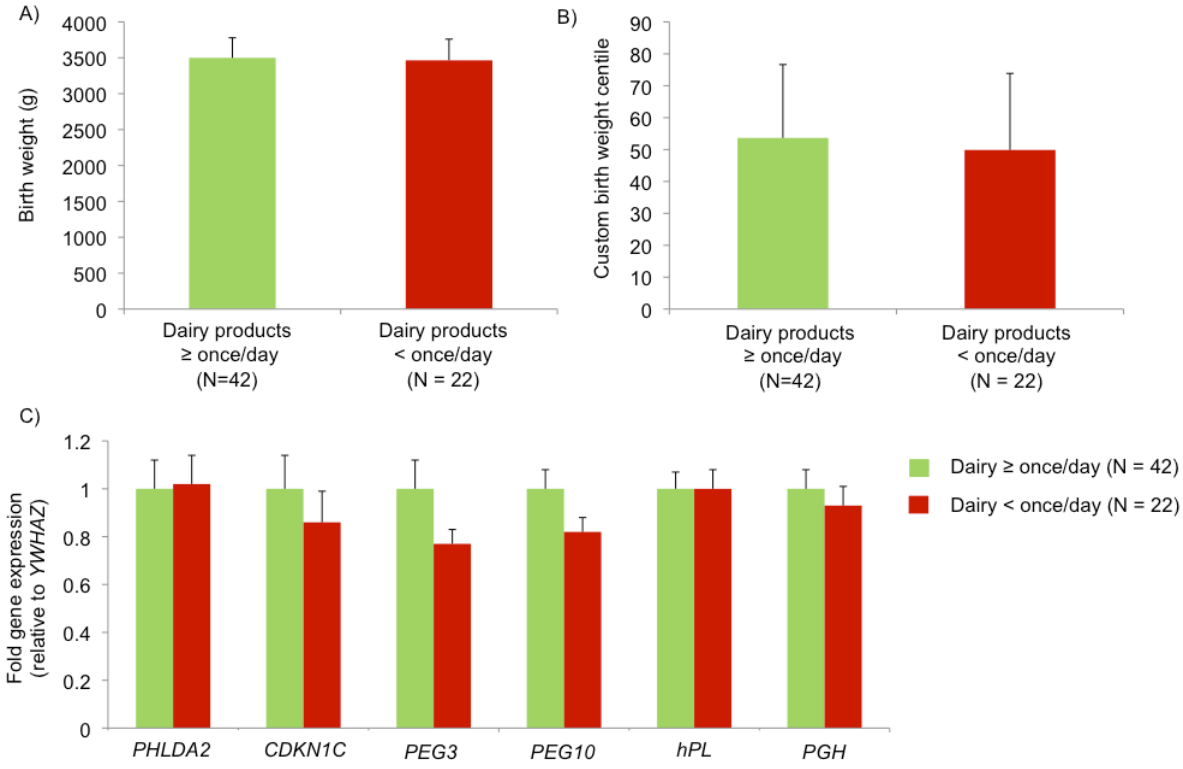


Figure 5.10: Effects of maternal dairy consumption during pregnancy. There was no significant effect of dairy consumption on birth weight (A), custom birth weight centiles (B) or placental target gene expression (C). Error bar represent SEM.

5.3.10 Carbohydrate consumption

In the overall cohort 52% of participants reported consuming carbohydrate rich foods (such as bread, potatoes and pasta) more than once a day during pregnancy, with 23% of participants reporting increased consumption compared with before pregnancy. There was no significant difference in

carbohydrate consumption between UHW (51%) and RGH (54%) sites (p = 0.52).

Information on maternal carbohydrate consumption was available for 63 Caucasian control participants of which 56% reported consuming carbohydrate rich foods more than once a day during pregnancy. There was no significant difference in birth weight (p = 0.28, n = 63) or placental weight (p = 0.96, n = 63) in these participants (Figure 5.11). However, there was a trend for higher custom birth weight centiles in these participants (p = 0.09, n = 63), and frequency of maternal carbohydrate consumption was significantly positively associated with custom birth weight centiles (*r* = 0.27, p = 0.04, n = 63).

Placental *PEG10* (p = 0.04, n = 63) and *hPL* (p = 0.04, n = 63) expression was significantly increased in participants consuming carbohydrate rich foods more than once a day (Figure 5.11). There was no significant difference in placental *PHLDA2*, *CDKN1C*, *PEG3* or *PGH* expression (Figure 5.11).

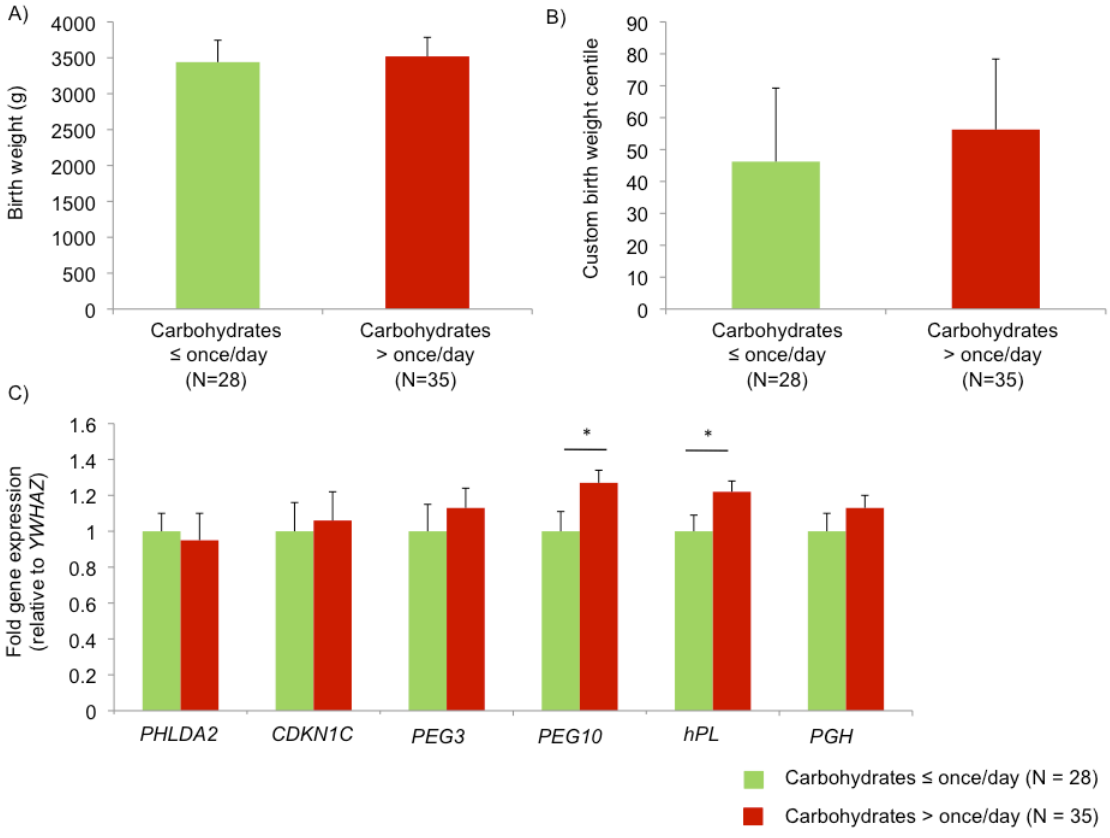


Figure 5.11: Effects of maternal carbohydrate consumption during pregnancy. There was no significant effect of dairy consumption on birth weight (A) or custom

birth weight centiles (B). Placental *PEG10* and *hPL* expression was significantly increased in participants with a carbohydrate rich diet (C). Error bars represent SEM. * P <0.05

5.3.11. Protein consumption

In the overall cohort 52% of participants reported consuming protein rich foods (meat, fish and meat alternatives) less than once a day during pregnancy. There was no significant difference in protein consumption between UHW (52%) and RGH (39%) sites (p = 0.11).

Information on maternal protein consumption was available for 65 Caucasian control participants of which 55% reported consuming protein rich foods less than once a day during pregnancy. Birth weight (p = 0.90, n = 65), custom birth weight centiles (p = 0.64, n = 65) and placental weight (p = 0.88, n = 65) was not significantly reduced in these participants (Figure 5.12). There was also no significant difference in placental expression of the imprinted genes or placental hormones examined (Figure 5.12). Results remained non-significant when gene expression was analysed in relation to protein consumption in the overall cohort.

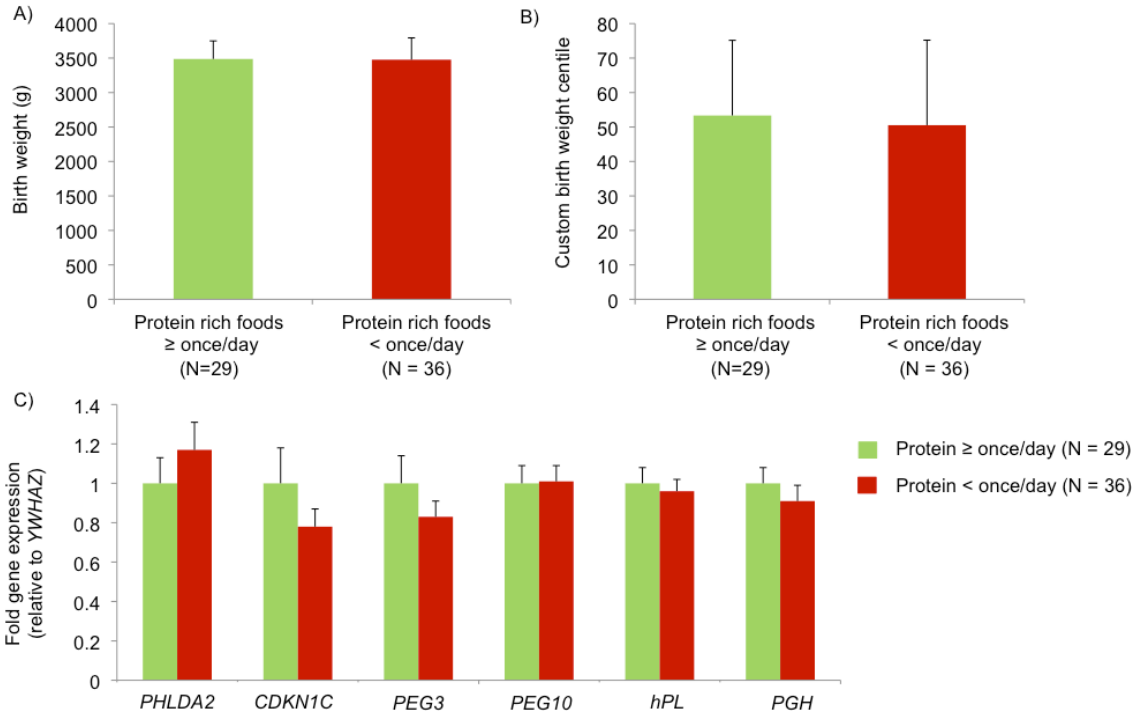


Figure 5.12: Effects of maternal protein consumption during pregnancy. There was no significant effect of protein consumption on birth weight (A), custom birth

weight centiles (B) or placental target gene expression (C). Error bars represent SEM.

Maternal fish consumption in particular has previously been positively associated with birth weight. However, in this cohort there was no significant difference in birth weight (p= 0.91, n = 62), custom birth weight centiles (p = 0.51, n = 62) or placental weight (p = 0.19) in participants consuming fish less than the recommended 2 times a week. Similarly, placental target gene expression was not significantly altered in these participants (Figure 5.13).

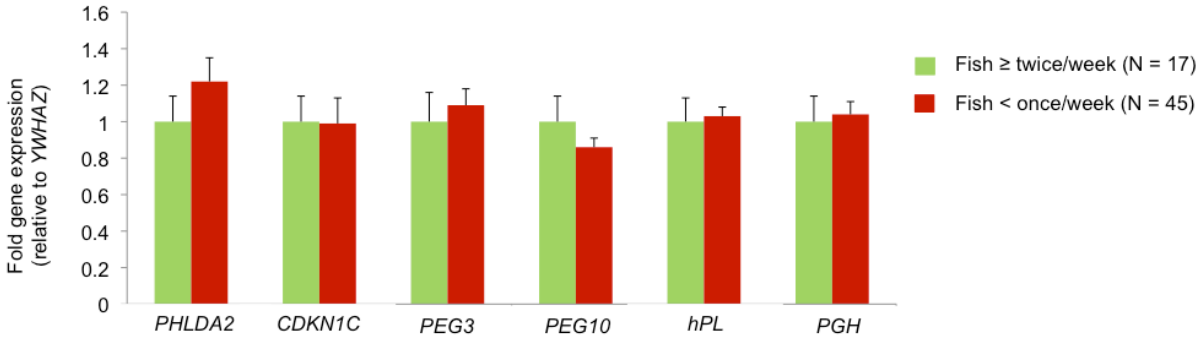


Figure 5.13: Effects of maternal fish consumption during pregnancy. There was no significant effect of fish consumption on placental target gene expression.

5.3.12. High sugar snacks

In the overall cohort 45% of participants reported consuming a high sugar snack (chocolate, cake, biscuits and soft drinks) more than once a day during pregnancy. This did not differ significantly between UHW (43%) and RGH (49%) sites (p = 0.41).

Information on high sugar snack consumption was available for 65 Caucasian control participants of which 46% reported consuming a sugary snack more than once a day during pregnancy. Birth weight (p = 0.32, n = 65), custom birth weight centiles (p = 0.19, n = 65) and placental weight (p = 0.98, n = 65) were not significantly altered in these participants (Figure 5.14). Similarly, placental target gene expression was not significantly altered (Figure 5.14). In terms of individual sugar rich snacks, only maternal soft drink consumption was significantly positively associated with placental *PEG3* expression (r = 0.26, p = 0.04, n = 64).

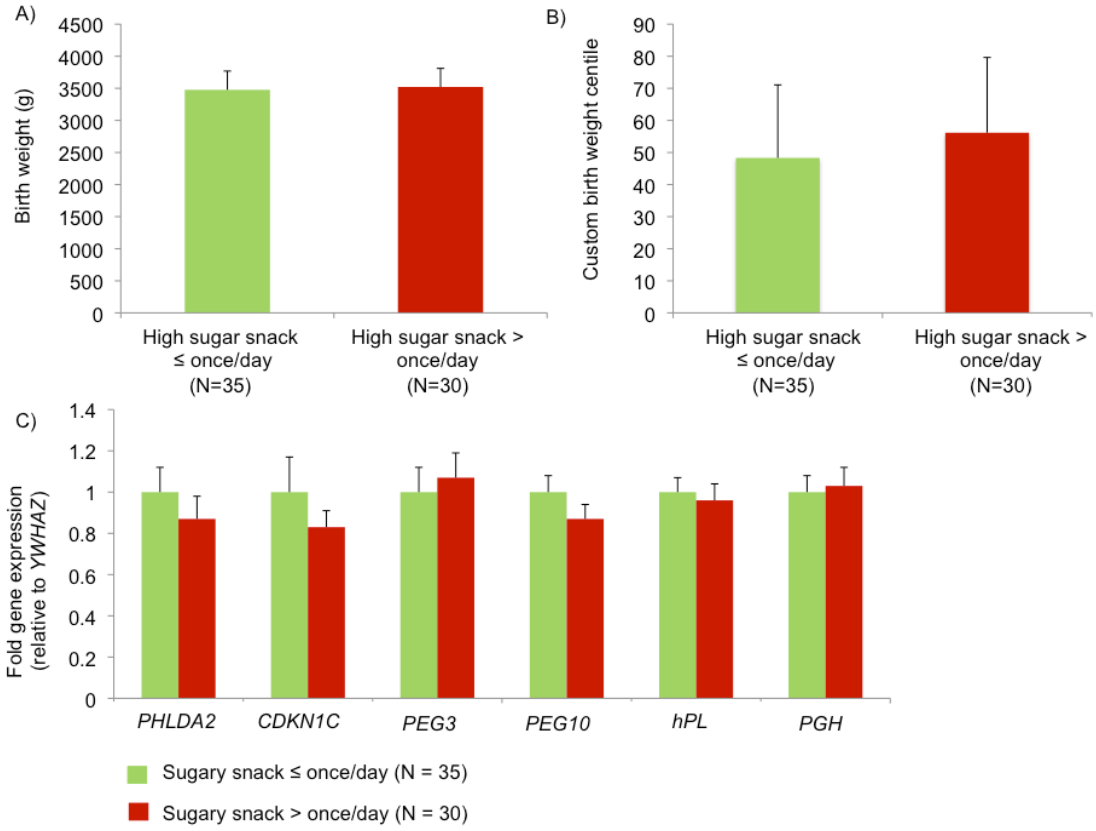


Figure 5.14: Effects of maternal high sugar snack consumption during pregnancy. There was no significant effect of high sugar snack consumption on birth weight (A), custom birth weight centiles (B) or placental target gene expression (C). Error bars represent SEM.

5.3.13. Caffeine consumption

30% of participants in the overall cohort reported consuming caffeinated drinks (tea, coffee, power drinks) more than once a day during pregnancy with 27% of participants reporting decreased consumption compared with before pregnancy. This did not differ significantly between UHW (26%) and RGH (38%) sites ($p = 0.09$).

Information on caffeine consumption was available for 64 Caucasian control participants of which 30% reported consuming a caffeinated drink more than once a day during pregnancy. Although birth weights and custom growth centiles were reduced for these participants, these differences were not statistically significant (Figure 5.15). Similarly, there was no significant difference in placental weight ($p = 0.45$, $n = 64$). There was also no

significant difference in placental target gene expression in mothers consuming caffeinated drinks more than once a day during pregnancy, although placental *PGH* expression was significantly inversely correlated with frequency of caffeine consumption (r = - 0.24, $p < 0.05$, n = 64).

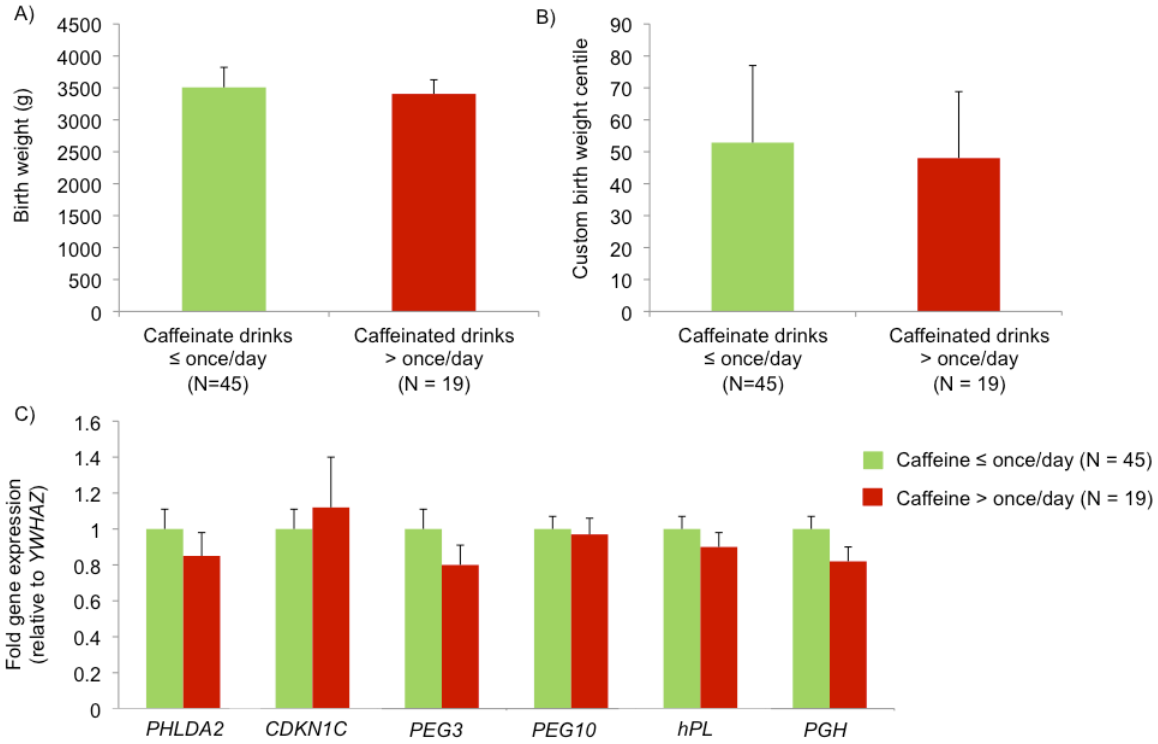


Figure 5.15: Effects of maternal caffeinated drink consumption during pregnancy. There was no significant effect of caffeine consumption on birth weight (A), custom birth weight centiles (B) or placental target gene expression (C).

Interestingly, for participants delivering SGA infants placental *PHLDA2* expression was significantly increased in participants consuming caffeinated drinks more than once a day (Figure 5.16). These participants also demonstrated decreased birth weights, custom birth weight centiles and placental weights, although these differences did not reach statistical significance (Figure 5.16).

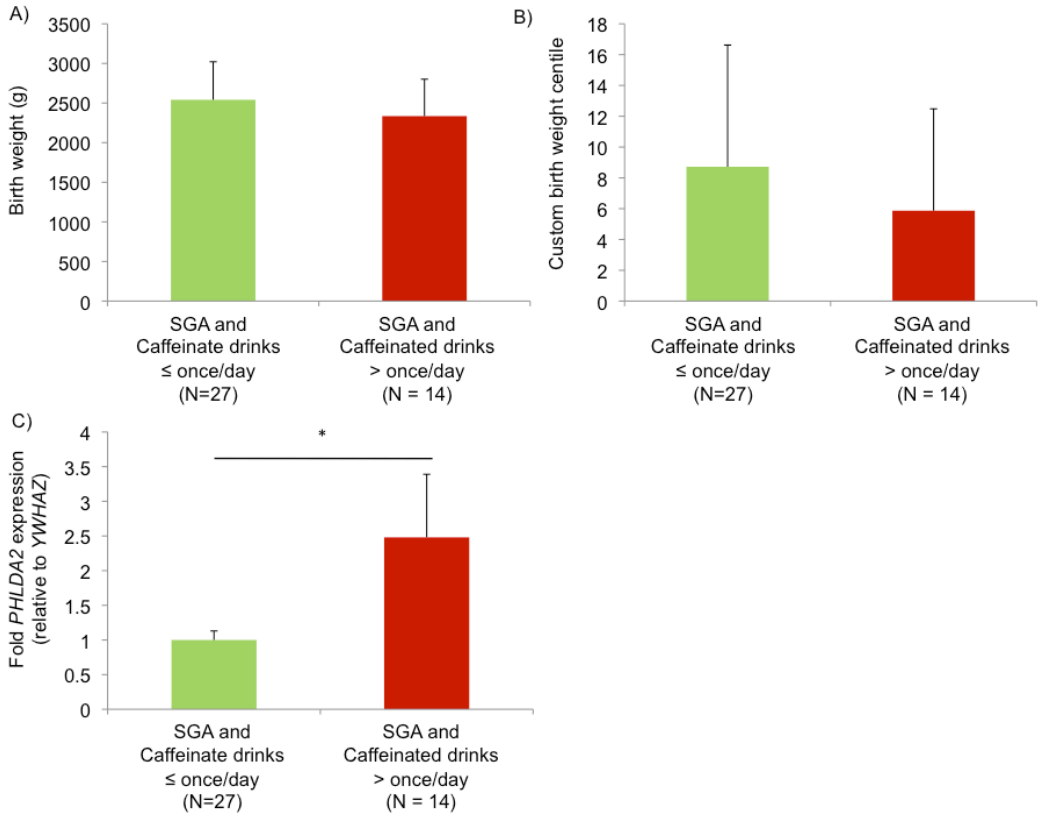


Figure 5.16: Effects of maternal caffeinated drink consumption during pregnancy on SGA placentas. Birth weight (A) and custom birth weight centiles (B) were not significantly altered in SGA participants with high caffeine consumption but placental *PHLDA2* expression was significantly increased (C). Error bars represent SEM. * P <0.05.

5.3.14. Supplement use

59% of participants in the overall cohort reported taking supplements (iron, folate and/or vitamin) at least once a day during pregnancy with 28% of participants reporting increased use compared with before pregnancy. This did not differ significantly between UHW (52%) and RGH (39%) sites (p = 0.17).

Information on maternal supplement use was available for 65 Caucasian control participants of which 59% reported taking supplements at least once a day during pregnancy. There was no significant difference in birth weight (p = 0.58, n = 65) or custom birth weight centiles (p = 0.41, n =

65) between these participants and those taking supplements less than once a day (Figure 5.17). Similarly, there was no significant in placental expression of the imprinted genes and placental hormones examined (Figure 5.17).

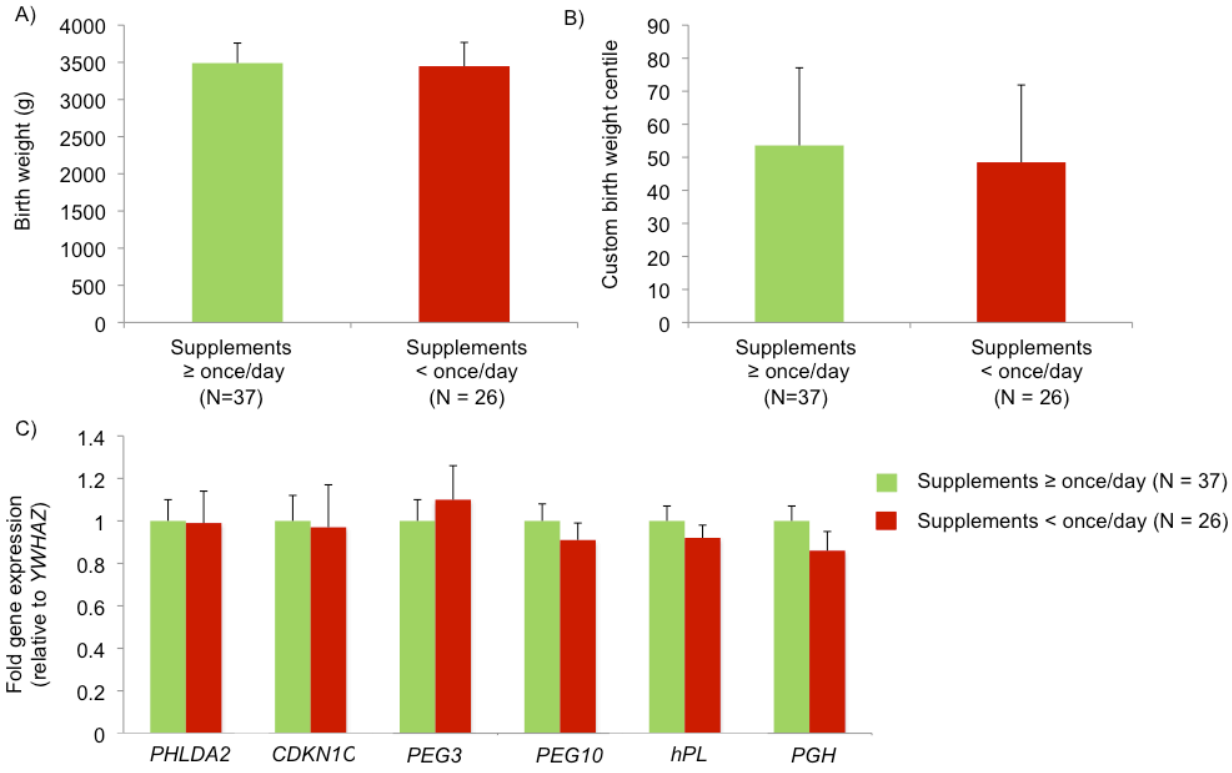


Figure 5.17: Effects of maternal supplement use during pregnancy. There was no significant effect of supplement use on birth weight (A), custom birth weight centiles (B) or placental target gene expression (C). Error bars represent SEM.

5.3.15. Maternal diet patterns

Principle component analysis of reported consumption of 17 food items was carried out to examine participant diet patterns and associated effects on fetal growth and placental target gene expression. Examining diet patterns, in contrast to individual diet components may provide more information on the interacting effects of food items and has been suggested to be of greater relevance for designing healthy eating interventions (Crozier *et al.* 2008).

PCA identified two diet components accounting for 19% and 9% of variation in maternal diet information respectively. Component 1 was characterised by frequent consumption of high fat and high sugar snacks

(such as chips, crisps, chocolate and cake), processed meat and soft drinks, and a low intake of fruit, vegetables and supplements (Table 5.2). Component 1 therefore displayed many similarities with the "Western diet" pattern observed in similar studies (Crozier *et al.* 2006; Thompson *et al.* 2010; Hu 2002). A western diet score was calculated for each participant based on the component coefficients and reported consumption of each food item, with higher scores indicating a more Western diet.

Table 5.2: Principle component analysis coefficients. Positive and negative coefficients are shown for food items that are consumed more or less frequently as part of the diet pattern. The Western diet and Healthy diet patterns explained 19% and 9% of variance in maternal diet data. Coefficients ≥ 0.15 are highlighted in bold.

Food item or group	Component 1 (Western Diet)	Component 2 (Healthy Diet)
Chips and Crisps	**0.80**	**- 0.15**
Chocolate	**0.65**	0.04
Takeaway meals	**0.64**	0.06
Cakes, biscuits, ice cream	**0.60**	0.12
Processed meat	**0.59**	**0.35**
Dried Fruit	**- 0.53**	0.004
Soft drinks	**0.49**	- 0.01
Cheese and Yoghurt	- 0.003	**- 0.30**
Bread, cereals, rice and pasta	- 0.05	**- 0.27**
Vegetables or Salad	**- 0.39**	**0.27**
Milk	0.09	**- 0.42**
Fruit	**- 0.39**	**0.17**
Unprocessed meat	0.03	**0.56**
Fish and shellfish	- 0.06	**0.42**
Supplements	**- 0.30**	**- 0.20**
Caffeine	0.09	**- 0.42**
Meat alternatives	0.02	**0.40**

In the Caucasian control participants, there was no significant correlation between maternal western diet scores and birth weight (r = 0.14, p = 0.27, n = 65), custom birth weight centiles (r = 0.06, p = 0.65, n = 65) or placental weight (r = - 0.13, p = 0.32, n = 65). Similarly, there was no significant correlation between maternal western diet scores and placental *PHLDA2* (r = 0.04, p = 0.75, n = 65), *CDKN1C* (r = - 0.07, p = 0.54, n = 65), *PEG3* (r = 0.04, p = 0.72, n = 65), *PEG10* (r = 0.20, p = 0.09, n = 65), *PGH* (r = - 0.01, p = 0.90, n = 65) or *hPL* expression (r = 0.05, p = 0.69,n = 65).

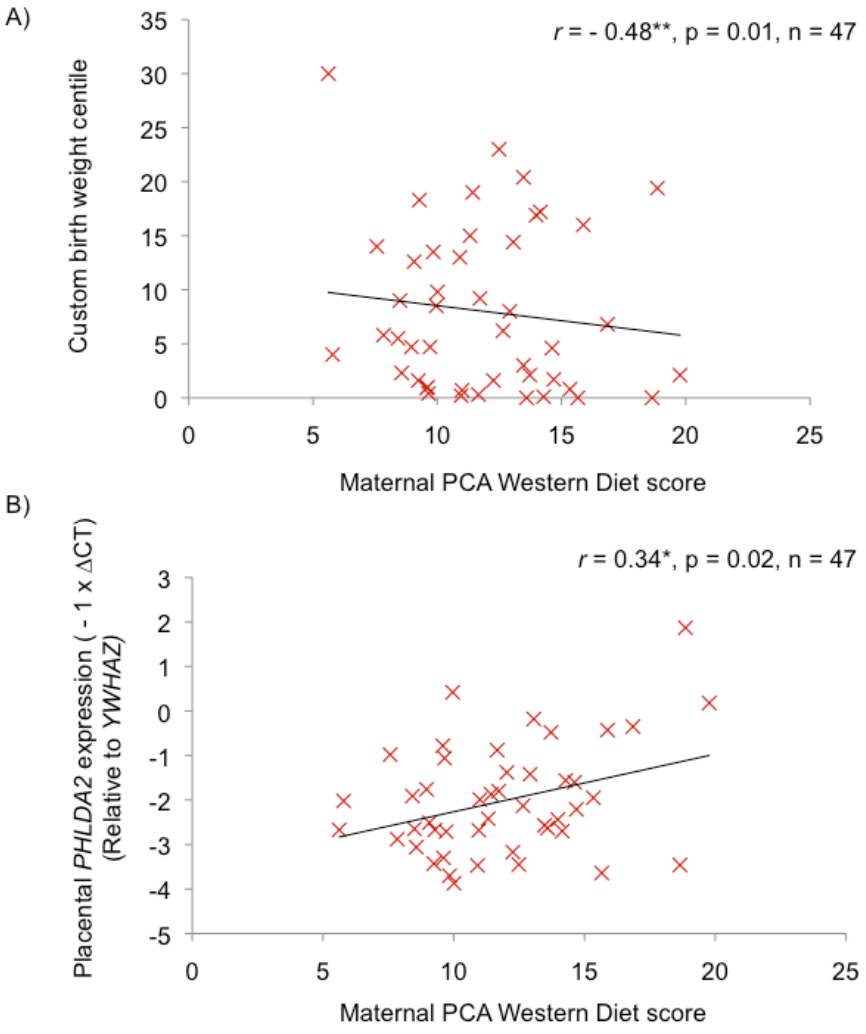


Figure 5.18: Effects of a maternal western diet during pregnancy in SGA participants. In SGA participants, maternal western diet scores were significantly inversely correlated with custom birth weight centiles (A) and positively correlated with placental *PHLDA2* expression. * P <0.05

However, in participants delivering SGA infants there was a significant inverse correlation between maternal western diet scores and birth weight (r = - 0.29, p = 0.04, n = 47) and custom birth weight centiles (r = - 0.48, p = 0.01, n = 47), Figure 5.18. Maternal western diet scores were also significantly positively associated with placental *PHLDA2* expression (r = 0.34, p = 0.02, n = 47), Figure 5.18, but not with any other imprinted gene or placental hormone examined.

PCA Component 2 was characterised by high intake of fruit and vegetables, unprocessed meat, fish and meat alternatives and a low intake of caffeine, dairy products and carbohydrates (Table 5.2). Component 2 therefore displayed similarities with the "Prudent" or "healthy diet" pattern observed in similar studies (Crozier *et al.* 2006; Thompson *et al.* 2010; Hu 2002). A healthy diet score was calculated for each participant based on the component coefficients and reported consumption of each food item, with higher scores indicating a more healthy diet.

In the Caucasian control participants, there was no significant correlation between maternal healthy diet scores and birth weight (r = - 0.03, p = 0.83, n = 65), custom birth weight centiles (r = 0.12, p = 0.36, n = 65) or placental weight (r = - 0.08, p = 0.52, n = 65). Similarly, there was no significant correlation between maternal healthy diet scores and placental *PHLDA2* (r = 0.07, p = 0.58, n = 65), *CDKN1C* (r = 0.17, p = 0.19, n = 65), *PEG3* (r = - 0.02, p = 0.86, n = 65), *PEG10* (r = 0.03, p = 0.81, n = 65), *hPL* (r = 0.20, p = 0.12, n = 65) or *PGH* expression (r = 0.09, p = 0.51, n = 65). Results remained non-significant when analysed in SGA placentas independently.

5.4. Discussion

This chapter examined the possible influence of maternal lifestyle on placental imprinted gene and placental hormone gene expression. A summary of target gene expression changes is shown in Table 5.3. These results suggest that imprinted gene expression in the human placenta may be responsive to maternal lifestyle factors during pregnancy. Similarly, expression of placental hormones *hPL* and *PGH* was also altered in response to maternal lifestyle.

Table 5.3. Summary of significant changes in target gene expression in association with maternal lifestyle factors. Percentage change in expression relative to controls is shown.

Maternal lifestyle factor	*PHLDA2*	*CDKN1C*	*PEG3*	*PEG10*	*hPL*	*PGH*
Smoking	-	-	-	-	-	-
Exercise	-	-	-	-	-	-
Alcohol	-	-	-	-	-	-
Illegal drug use	-	-	-	-	-	-
Overweight or Obese	-	↑ 54%	-	-	-	-
Low fruit intake	-	-	-	-	↓ 20%	↓ 22%
Dairy intake	-	-	-	-	-	-
High carb intake	-	-	-	↑ 27%	↑ 22%	-
Protein intake	-	-	-	-	-	-
Sugary snacks	-	-	-	-	-	-
Caffeine	↑ 150% (SGA only)	-	-	-	-	-
Supplement use	-	-	-	-	-	-
Western diet pattern	+ ve association (SGA only)	-	-	-	-	-
Healthy diet pattern	-	-	-	-	-	-

5.4.1. Maternal smoking

28% of control participants reported smoking before pregnancy. Pre-pregnancy smoking was not significantly associated with any measure of fetal growth and target gene expression was not significantly altered between smokers and non-smokers. Smoking during pregnancy (21% prevalence), was also not significantly associated with birth weight, custom birth weight centiles or target gene expression in control participants. However, women smoking during pregnancy were twice as likely as non-smokers to deliver an SGA infant. This is consistent with previous reports of a two fold increased risk of LBW in smokers (Pollack *et al*. 2000; Figueras *et al*. 2008; Rasmussen and Irgens 2006). Although birth weight was significantly lower in SGA infants born to smokers compared to SGA infants born to non-smokers, there was no associated effect on placental target gene expression. Bruchova *et al*. (2010) reported increased placental *PHLDA2* expression in pregnant smokers in a small microarray study, with results not subsequently validated by qPCR. However, results of the current study are consistent with those of Moore *et al*. (2015) demonstrating no significant association between maternal smoking and placental *PHLDA2*, *CDKN1C*, *PEG3* or *PEG10* expression. Thus, these results do not provide evidence for an effect of maternal smoking on placental imprinted gene expression.

5.4.2. Maternal exercise

Only 15% of participants reported carrying out strenuous exercise during pregnancy, defined as exercise at least 30 minutes in duration at least once a week (Statistics for Wales 2013). Maternal exercise was not significantly associated with birth weight, custom birth weight centiles or placental weight. A number of studies have previously demonstrated decreased birth weight in response to maternal exercise (Juhl *et al*. 2010; Clapp 2003; Bell *et al*. 1995; Hopkins *et al*. 2010). However, the study of effects of exercise on fetal growth are complicated by issues of pre-pregnancy exercise levels, time of exposure, duration of exercise as well as effects of interacting maternal lifestyle factors. The results of the current study are consistent with those of

Hegaard *et al.* (2010) and Kramer and McDonald (2006) reporting no significant association between exercise and measures of fetal growth. Finally, there was no significant effect of maternal exercise during pregnancy on placental target gene expression. Only placental *PHLDA2* expression has previously been suggested to be altered in response to strenuous exercise (Lewis *et al*. 2012). As with birth weight, it is possible that no difference in placental *PHLDA2* expression was observed in the current study due to differences in exercise intensity or duration.

5.4.3. Alcohol consumption

62% of control participants reported consuming alcohol in the three months before pregnancy. There was no effect of maternal pre-pregnancy alcohol consumption on any measure of fetal growth or placental target gene expression. 38% of participants reported consuming alcohol (of any quantity) during pregnancy, with a mean of 1 unit consumed per week. As with pre-pregnancy alcohol consumption, drinking alcohol during pregnancy had no significant effect on birth weight or placental target gene expression. Although maternal alcohol consumption has previously been reported to be associated with fetal growth restriction (Patra *et al*. 2011; Henderson *et al*. 2007), the risk of LBW was demonstrated to increase only above 1 – 1.5 drinks / day. It is unclear whether light to moderate drinking has any effect on fetal growth (Patra *et al*. 2011; Henderson *et al*. 2007). It is therefore possible that no effect of alcohol consumption was observed in the current study in which a mean of 1 units per week was consumed. Similarly, the study by Shukla *et al*. (2011) demonstrating increased placental *Phlda2* and *Cdkn1c* expression in response to an ethanol-containing diet was proposed to model effects of moderate alcohol consumption. In conclusion, the results of the current study do not provide evidence for an effect of maternal alcohol consumption on placental imprinted gene expression.

5.4.4. Pre-pregnancy illegal drug use

Three participants (1 AGA and 2 SGA participants) had a record of pre-pregnancy illegal drug use with none continuing throughout pregnancy. Birth weight was significantly reduced by 858g in these participants compared with controls. Pinto *et al*. (2010) similarly estimated an almost four fold increased risk of LBW and IUGR in UK women using drugs during pregnancy. Furthermore, placental target gene expression was not significantly altered in response to maternal pre-pregnancy illegal drug use. However, given the small number of participants reporting illegal drug use, this study also does not discount a role for placental imprinted genes in mediating the association between illegal drug use and fetal growth restriction.

5.4.5. Maternal BMI

51% of control participants were overweight or obese at booking. There was no significant correlation between maternal BMI and birth weight, custom birth weight centiles or placental weight. Although birth weight was similar between normal BMI, overweight and obese participants, there was a non-significant gradual reduction in custom birth weight centiles consistent with the observation that the use of custom birth weight centiles identifies a significantly higher number of SGA infants in obese mothers compared with controls (Gardosi 2009). Placental *CDKN1C* expression was significantly increased by 54% in overweight or obese participants compared with controls. Moore *et al*. (2015) previously reported no significant association between maternal pre-pregnancy weight and placental *CDKN1C* expression in a similar sample size, although placental *CDKN1C* expression was not analysed in relation to maternal BMI or maternal BMI categories. Thus, the results of the current study provide the first evidence of an effect of maternal high BMI (overweight or obese) on placental *CDKN1C* expression.

Placental *PHLDA2* expression was also increased by 33% in overweight or obese participants, however this failed to reach statistical significance ($p = 0.06$). Similarly, Moore *et al*. (2015) observed a trend

(p=0.099) for a positive association between maternal weight and placental *PHLDA2* expression. Thus, these findings suggest a possible effect of maternal BMI on placental *PHLDA2* expression, which must be confirmed in a larger cohort of obese participants.

5.4.6. Fruit and vegetables consumption

38% of control participants reported consuming fruit or vegetables more than once a day. Fruit and vegetable consumption was not significantly associated with birth weight, custom birth weight centiles or placental weight. Previous studies have reported a positive association between maternal fruit and vegetable consumption and birth weight (Mikkelsen *et al.* 2006; Rao *et al.* 2001; Thompson *et al.* 2010). However, these studies have generally involved analysis of large cohorts, for example Mikkelsen *et al*. (2006) analysed maternal diet in 43,000 women. In addition, it is possible that maternal fruit and vegetable consumption as part of a diet pattern, rather than the individual components alone, affect fetal growth as suggested by Thompson *et al*. (2010). Placental *hPL* and *PGH* expression was significantly reduced by 20% and 22% respectively in participants consuming fruit and vegetables once a day or less. In particular, placental *hPL* expression was positively associated with frequency of vegetable consumption and *PGH* expression with frequency of dried fruit consumption. These novel results require further investigation in a larger study using a FFQ specifically designed to analyse maternal fruit and vegetable consumption. There was no significant effect of maternal fruit and vegetable consumption on placental *PHLDA2*, *CDKN1C*, *PEG3* or *PEG10* expression. Thus, the findings of the current study do not support an effect of maternal fruit and vegetable consumption on placental imprinted gene expression.

5.4.7. Dairy consumption

66% of participants consumed dairy products at least once a day during pregnancy. There was no significant association between maternal dairy

consumption and birth weight, custom birth weight centiles or placental weight. Previous studies reporting a significant positive correlation between dairy consumption during pregnancy and birth weight were of large national cohorts with diet analysed in up to 50,000 women (Ludvigsson and Ludvigsson 2004; Olsen *et al*. 2007; Xue *et al*. 2008). It is therefore possible that no effect of maternal dairy consumption was shown in the current study due to the comparatively smaller sample size. In terms of placental gene expression, decreased *PEG3* ($p = 0.08$) and *PEG10* ($p = 0.06$) were observed in participants consuming dairy products less than once a day, however these differences did not reach statistical significance. These novel results, although not significant, could underlie the positive correlation between dairy consumption and birth weight reported in previous studies (Ludvigsson and Ludvigsson 2004; Olsen *et al*. 2007; Xue *et al*. 2008; Ford 2011). Examining placental imprinted gene expression in a larger cohort using a FFQ specifically designed to analyse consumption of dairy products, will determine the relevance of these findings.

5.4.8. Carbohydrate consumption

56% of participants consumed carbohydrate rich foods more than once a day. Birth weight and placental weight were not significantly altered in these participants although there was a trend ($p = 0.09$) for increased custom birth weight centiles. Similarly, frequency of maternal carbohydrate consumption was significantly associated with custom birth weight centiles. This is consistent with the previously reported positive association between a diet rich in carbohydrates and birth weight (Mitchell *et al*. 2004; Thompson *et al*. 2010). Placental *hPL* expression was increased by 22% in participants consuming carbohydrate rich foods more than once a day during pregnancy. Given the established role of hPL in the control of maternal insulin production, these results are consistent with the positive effect of carbohydrate consumption on blood glucose levels and insulin production (Sheard *et al.* 2004). Placental *PEG10* expression was also significantly increased by 27% in participants consuming carbohydrate rich foods more

than once a day. As placental *PEG10* plays a role in the positive control of fetal growth (chapter 3), it is possible that the association between maternal carbohydrate consumption and birth weight is mediated by an increase in placental *PEG10* expression. This novel result requires replication in a larger cohort using a FFQ specifically designed to assess maternal consumption of carbohydrate rich foods including quantity and glycemic load.

5.4.9. Protein consumption

55% of participants reported consuming protein rich foods less than once a day. Maternal protein consumption during pregnancy was not significantly associated with birth weight, custom birth weight centiles or placental weight. Similarly, placental target gene expression was not significantly altered in women consuming protein rich foods less than once a day. Results remained non-significant when maternal fish consumption was analysed independently. Previous studies have reported conflicting results with respect to the relationship between maternal protein consumption and birth weight (Godfrey *et al*. 1996; Ford 2011; Thompson *et al*. 2010; Moore *et al*. 2004; Knudsen *et al*. 2008; Campbell *et al*. 1996; Ricci *et al*. 2010). This may be due to differences in study methodology or population, sources of protein and/or the interacting effects of other diet components. For example, in the study by Thompson *et al*. (2010), diet patterns were assessed, with a diet rich in protein and fruit and vegetables being positively associated with birth weight. Therefore, the effects of protein consumption alone were not analysed. Although studies in animal models suggest an effect of low protein diet or calorie restriction on imprinted gene expression, this is the first study to analyse human placental imprinted expression in response to maternal diet. The results of the current study do not provide evidence for an effect of maternal low protein diet on placental imprinted gene expression although further studies are needed to confirm these findings.

5.4.10. Maternal high sugar consumption

46% of control participants reported consuming a high sugar snack more than once a day. Consumption of high sugar snacks during pregnancy was not significantly associated with birth weight, custom birth weight centiles or placental weight. Similarly, placental target gene expression was not significantly altered in women consuming a high sugar snack more than once a day during pregnancy. This is consistent with findings in animal studies, which demonstrated no significant effect of a high fat- high sugar diet on placental *Phlda2*, *Cdkn1c* or *Peg3* expression (Sferruzzi-Perri *et al*. 2013). However, this study is the first to examine human placental imprinted gene expression in response to increased sugar consumption. Although these results do not provide evidence for an effect of maternal high sugar snack consumption on placental imprinted gene expression, they do not discount a role for a maternal high fat – high sugar diet pattern with interacting effects of other diet components.

5.4.11. Caffeine consumption

30% of control participants consumed caffeinated drinks more than once per day during pregnancy. Frequency of maternal caffeine consumption was not significantly associated with birth weight, custom birth weight centiles or placental weight in control participants. Previous studies have demonstrated a significant negative effect of maternal caffeine consumption on fetal growth (Santos *et al*. 1998; Xue *et al*. 2008; Vik *et al*. 2003). It is possible that no effect of caffeine consumption was detected in the current study because of differences in methodology; for example, Vik *et al*. 2003 used food records to quantitatively assess caffeine intake. In addition, previous studies have highlighted differential effects of distinct sources of caffeine, with e.g. coffee having the greatest effect. Given the non-quantitative FFQ used in the current study, as well as grouping of caffeinated drinks, it is possible that more subtle effects on birth weight could not be detected in this relatively small population. In control participants, placental target gene expression

was not significantly altered in women consuming caffeinated drinks more than once a day.

Interestingly, placental *PHLDA2* expression was significantly 2.5 fold higher in SGA participants consuming caffeinated drinks more than once a day. Birth weight was also reduced by 206g in these participants, although this difference was not statistically significant ($p = 0.17$). Two possible explanations could underlie these novel findings. Firstly, *PHLDA2* may drive fetal growth restriction caused by environmental factors (such as increased caffeine consumption). In contrast, it is possible that increased caffeine consumption exacerbates fetal growth restriction in already compromised pregnancies, through alterations in *PHLDA2* expression. Animal models will aid in determining the mechanisms underlying the association between maternal caffeine consumption, fetal growth and *PHLDA2* expression. For example, fetal growth restriction in response to maternal caffeine consumption in a mouse model, associated with increased *PHLDA2* expression, would lend support to the first hypothesis. Alternatively, if fetal growth restriction is observed only in compromised pregnancies (such as in the mouse model of *Phlda2* over expression), this would support the latter hypothesis.

5.4.12. Supplement use

59% of control participants reported taking supplements (e.g. folate, iron or vitamins) at least once a day during pregnancy. Maternal supplement use was not significantly associated with birth weight, custom birth weight centiles or placental weight. Similarly, placental target gene expression was not significantly altered in women taking supplements less than once a day during pregnancy. Importantly, the current study did not quantitatively assess maternal levels of folate, iron or vitamins. Therefore, although these findings do provide evidence of an effect of maternal supplement use on fetal growth or target gene expression, this remains to be confirmed in larger quantitative studies of maternal supplement use during pregnancy.

5.4.13. Maternal diet patterns

Principle component analysis revealed 2 diet patterns accounting for 19% and 9% of variation in maternal diet data respectively. This diet pattern was characterised by increased consumption of high fat and high sugar snacks (chips, crisps, chocolate and cake), processed meat and soft drinks and a low intake of fruit, vegetables and supplements. The first diet pattern therefore displayed many similarities with the "western diet pattern" observed in similar studies (Crozier *et al*. 2006; Crozier *et al*. 2008; Crozier *et al*. 2009; Thompson *et al*. 2010). Maternal western diet scores were not significantly associated with birth weight, custom birth weight centiles or placental target gene expression in control participants.

However, as with caffeine consumption, in participants delivering SGA infants maternal western diet scores were significantly inversely correlated with birth weight and custom birth weight centiles. In addition, maternal western diet scores were positively correlated with placental *PHLDA2* expression, i.e. a more western diet was associated with increased placental *PHLDA2* expression. As increased caffeine consumption was not characteristic of the western diet, this suggests that the effect of maternal western diet on birth weight and placental *PHLDA2* expression was independent of caffeine consumption.

No previous study has examined human placental *PHLDA2* expression in relation to maternal diet. Sferruzzi-Perri *et al*. (2013) demonstrated no significant effect of a maternal high fat – high sugar diet on placental *Phlda2* expression in a mouse model. However, the western diet described in the current study involves other aspects of diet in addition to high fat – high sugar foods (such as fruit and vegetable consumption and supplement use); it is therefore possible that it is the interaction between the individual food items of the maternal western diet rather than the isolated effect of maternal high fat – high sugar snack consumption which are associated with decreased fetal growth and increased placental *PHLDA2* expression. As described previously, these novel findings may be explained by *PHLDA2* expression driving fetal growth restriction caused by environmental factors (such as maternal western diet) or maternal western

diet exacerbating fetal growth restriction in already compromised pregnancies, through alterations in *PHLDA2* expression.

Finally, the second diet pattern identified by PCA was characterised by a high intake of fruit and vegetables, unprocessed meat, fish and meat alternatives and a low intake of caffeine, dairy products and carbohydrates. This diet pattern therefore displayed similarities with the “Prudent” or “healthy diet” pattern observed in similar studies (Crozier *et al*. 2006; Crozier *et al*. 2008; Crozier *et al*. 2010; Thompson *et al*. 2010). Maternal health diet scores were not significantly associated with birth weight, custom birth weight centiles or placental target gene expression in control participants. Results remained non-significant in SGA pregnancies. These novel results therefore do not provide evidence of a protective effect of a healthy maternal diet on aberrant placental imprinted gene expression.

5.4.14. Summary

In summary, the results presented in this chapter suggest an effect of maternal lifestyle on imprinted gene and placental hormone gene expression in the human placenta. Placental *PHLDA2* expression was significantly increased in response to maternal caffeine consumption and a western diet pattern, an effect that was specific to SGA pregnancies. Placental *CDKN1C* expression was increased in overweight or obese participants. In contrast, placental *PEG3* expression however was not significantly altered in response to maternal lifestyle. Placental *PEG10* expression was significantly increased in response to a carbohydrate rich diet. In terms of placental hormone gene expression, placental *hPL* expression was increased in response to a carbohydrate rich diet and both *hPL* and *PGH* expression was decreased in association with low fruit intake. These novel results provide the first evidence to suggest an effect of maternal lifestyle, particularly maternal diet, on human placental imprinted gene expression and therefore support the fourth study hypothesis of aberrant placental imprinted gene expression in response to an adverse maternal lifestyle. Given the role of aberrant placental imprinted gene expression in fetal growth restriction and other

complications of pregnancy as demonstrated in chapters 3 and 4 respectively, these results could be of relevance in the design of future maternal lifestyle interventions.

CHAPTER 6: IMPRINTED GENES AND MATERNAL DIET IN AN ANIMAL MODEL

6.1. Introduction

In this chapter, a mouse model was used to further explore whether placental imprinted gene expression is responsive to environmental stimuli, in particular maternal diet during pregnancy.

Animal models of maternal low protein diet during pregnancy are commonly used in the study of fetal programming as offspring are born growth restricted and develop symptoms of hypertension, obesity and glucose intolerance in later life (reviewed in (Bertram and Hanson 2001; Martin-Gronert and Ozanne 2007)). Given that a two fold change in imprinted gene expression in the placenta can have a knock-on effect on fetal growth, such as in the case of *Phlda2* (Tunster *et al*. 2010), it is possible that alterations in placental imprinted gene expression underlie the growth restriction observed as a result of a maternal low protein diet. No previous study, however, has examined placental *Phlda2*, *Cdkn1c*, *Peg3* or *Peg10* expression in response to a low protein diet. Lillycrop *et al*. (2010) demonstrated no significant effect of low protein diet during pregnancy on *Phlda2* expression in the offspring liver but placental expression was not analysed. Similarly, while *Cdkn1c* expression in the offspring brain was shown to be significantly increased in response to a maternal low protein diet, expression was not analysed in the placenta (Vucetic *et al.* 2010). Placental *Peg3* and *Peg10* expression have not been examined in a mouse model of low protein diet during pregnancy. Related models of under - nutrition during pregnancy (such as calorie restriction or transient starvation) have however demonstrated perturbed placental *Phlda2*, *Cdkn1c* and *Peg3* expression (Shukla *et al*. 2011; Broad and Keverne 2011; Radford *et al*. 2012).

No significant association was observed between human placental imprinted gene expression and maternal protein consumption in Chapter 5. However, this may be due to only a small number of participants exhibiting sufficient severity of protein restriction to result in FGR and aberrant target gene expression, or that the presence of an association is obscured by confounding maternal lifestyle factors. Therefore, placental *Phlda2*, *Cdkn1c*,

Peg3 and *Peg10* expression was analysed in a mouse model of low protein diet during pregnancy to fully determine whether protein restriction results in altered placental imprinted gene expression.

It could be argued that animal models of over-nutrition are more relevant to western society. Effects of a maternal high fat diet during pregnancy have yielded conflicting results in mice, with some studies reporting growth restricted offspring and others increased fetal weight (reviewed in (Williams *et al.* 2014)), likely reflecting differences in study methodology. A number of studies have also demonstrated that offspring exposed to a high fat diet during pregnancy develop symptoms of hypertension, obesity and glucose intolerance in adulthood (Williams *et al*. 2014).

In Chapter 5, placental *PHLDA2* expression was demonstrated to be significantly associated with a maternal diet pattern characterised by increased consumption of high-fat and high-sugar food items, processed meat and soft drinks and a low intake of fruit, vegetables and supplements. Thus, placental *Phlda2*, *Cdkn1c*, *Peg3* and *Peg10* expression was also analysed in two mouse models of over-nutrition: a high fat diet during pregnancy and a high fat – high sugar diet before and/or during pregnancy. Placental *Phlda2*, *Cdkn1c* and *Peg3* have previously been suggested not to be significantly altered in response to a high fat – high sugar diet during pregnancy (Sferruzi-Perri *et al*. 2013), although the effect of pre-pregnancy diet was not examined. Similarly, placental *Peg3* and *Peg10* expression were not significantly altered in a mouse model of a maternal high fat diet during pregnancy (Gallou-Kabani *et al*. 2012). However, this study examined placental gene expression at an earlier time point (E15.5) suggesting that the period of exposure to a high fat diet may not have been sufficiently long to result in aberrant imprinted gene expression.

No significant sex differences were observed in human placental target gene expression in control pregnancies or in response to different maternal lifestyles (Chapter 5). However, previous studies in the mouse have demonstrated differential imprinted gene expression between male and

female placentas both under normal conditions (Faisal *et al.* 2014) and in response to maternal diet alteration (Radford *et al*. 2012) in the mouse. Thus results were further analysed for sex-specific responses to maternal low protein, high fat or high fat-high sugar diet in the mouse model.

In Chapter 3, an association was observed between imprinted gene expression and placental hormone gene expression in the human placenta. Similarly, a decrease in mouse placental spongiotrophoblast cells (and expression of placental prolactins) in response to aberrant *Phlda2*, *Cdkn1c*, *Peg3* and *Peg10* expression has been demonstrated in previous animal studies. Therefore, in the current study, placental expression of the spongiotrophoblast markers *Prl3b1* and *Prl8a8* was also analysed in response to maternal diet alteration to determine whether any aberrant placental imprinted gene expression observed was associated with a concurrent effect on placental spongiotrophoblast cells.

6.2. Chapter specific methods

Methods were as described in Chapter 2. For ease of interpretation of the results presented in this chapter, methods related to the study of placental target gene expression in a mouse model are summarised below.

6.2.1. Animals and maternal diet

Mus mus domesticus C57BL/6 mice carrying the *Mus spretus* region of distal chromosome 7 were used, as described in section 2.4. All females in the first experiment were fed a basal diet before pregnancy (diet energy from; protein 18.3%, fat 22.1%, carbohydrate 59.6%) (Test Diet) before pregnancy. On the day at which a vaginal plug was first detected (designated as E0.5), females were either fed a basal diet (control mice), a low protein diet (diet energy from; protein 8.1%, fat 21.8%, carbohydrate 70.1%) (Test Diet) or a high fat diet (diet energy from; protein 18.1%, fat 46.1%, carbohydrate 35.8%) (Test Diet) until E18.5.

Females in the second experiment were fed either a basal diet (15% sugar content and diet energy from; protein 17.7%, fat 10%, carbohydrate 61%) or a high fat – high sugar (HFHS) diet (34% sugar content and diet energy from; protein 17.8%, fat 20.0%, Carbohydrate 49.9%) for 12 weeks before mating. Mice were weighed every week to assess pre-pregnancy weight gain. At E0.5 females were placed on a control or HFHS diet for the remainder of pregnancy. Thus the following groups of mice were generated: Control-Control mice (fed basal diet before and during pregnancy), Control-HFHS mice (fed a HFHS diet in pregnancy only), HFHS-Control (fed a HFHS diet before pregnancy only) and HFHS-HFHS mice (fed a HFHS diet both before and during pregnancy). Control-HFHS females were used to model effects of poor diet specific to pregnancy. In addition, comparison of the HFHS-Control with the HFHS-HFHS females was used to model effectiveness of an intervention to improve maternal diet during pregnancy. Mice were weighed at E0.5 and E18.5 to determine maternal pregnancy weight gain.

Dissection was carried out at E13.5 and E18.5 for the low protein diet experiment in order to examine time specific effects of maternal protein restriction. For all other mice, dissection was carried out at E18.5. Processing of placentas for qPCR analysis of gene expression was as described in Chapter 2. Embryos and placentas were weighed at dissection.

6.2.2. *Phlda2* RFLP analysis

Phlda2 RFLP analysis was carried out as detailed in section 2.4.3. Briefly, cDNA samples amplified by PCR with the *Phlda2* RFLP primers were digested with the *TaqI* restriction enzyme and the products analysed by gel electrophoresis to show the presence of the different alleles. The *TaqI* restriction enzyme recognises a sequence on the C57BL/6 *Phlda2* allele and cuts the cDNA, resulting in the presence of two bands on the gel approximately 100bp and 50bp in length respectively. In contrast, a polymorphism in the *Spretus* sequence means that the *TaqI* enzyme cannot recognise the TaqI cut sites, resulting in the presence of a larger (undigested) band of 150bp in length. In this way, it is possible to identify the parental origin of the *Phlda2* allele; because *Phlda2* is a maternally expressed imprinted gene, inheritance of the maternal *Spretus* allele by the F1 offspring will result in the presence of one larger undigested band of 150bp in length. In contrast, inheritance of the paternal *Spretus* allele by the F1 offspring (which when imprinted is not expressed) will result in the presence of two (digested) bands of 100bp and 50bp in length. Thus, loss of imprinting (resulting in both maternal and paternal expression of *Phlda2*) will be indicated by the presence of two bands at 150bp and 100bp in length.

6.2.3. Statistical analysis

Embryo and placental weight was adjusted to account for differences in litter size using the following formula:

$$\frac{\text{Mean weight for diet}}{\text{Mean weight for litter}} \times \text{individual weight}$$

All statistical analysis was carried out using IBM SPSS statistics for Windows (version 20.0, 2011) with a p value <0.05 considered statistically significant.

Normal distribution was assessed using P-P plots and a Shapiro-Wilk test. All data was normally distributed and therefore parametric statistical tests used. Differences in placental gene expression between diet groups was analysed using an independent samples T tests or one-way ANOVA with a Tukey post hoc test where appropriate. A Bonferroni correction was also used to control for multiple comparisons in placental gene expression between maternal diet groups ($\alpha/6 = 0.008$). Finally, in order to examine sexual dimorphism in the placental response to maternal diet changes results were analysed in the overall diet group and split by sex.

6.3. Results

6.3.1. Sex differences in basal gene expression

Previous studies have suggested sex differences in expression of some imprinted genes expression under normal conditions (Faisal *et al.* 2014). As shown in Figure 6.1, there was no significant difference in placental *Phlda2* ($p = 0.51$, $n = 6$), *Cdkn1c* ($p = 0.41$, $n = 6$), *Peg3* ($p = 0.90$) or *Peg10* ($p = 0.54$, $n = 6$) imprinted gene expression between male and female control placentas. Similarly, there was no significant sex difference in placental gene expression of the spongiotrophoblast markers *Prl3b1* ($p = 0.63$, $n = 6$) or *Prl8a8* ($p = 0.72$, $n = 6$), Figure 6.1.

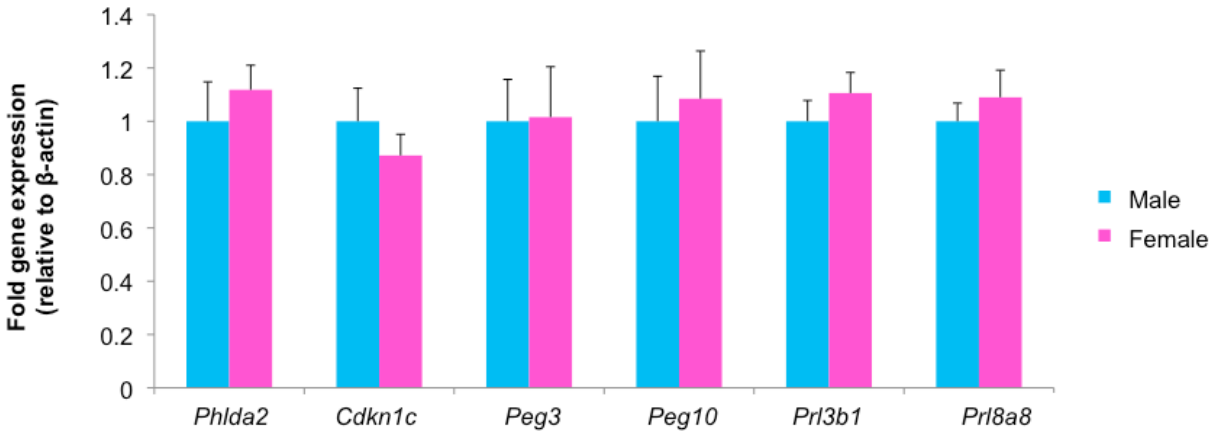


Figure 6.1: Sex differences in placental target gene expression at E18.5. Gene expression is shown relative to expression in male placentas. Error bars represent Standard Error. Differences in gene expression were not statistically significant using a t-test.

6.3.2. Maternal low protein diet

There was no significant effect of maternal low protein diet on fetal (1.06 g ± 0.11 v. 1.06 g ± 0.97, $n = 61$, $p = 0.88$) or placental weights (83.67 mg ± 7.66 v. 85.71 mg ± 9.08, $n = 61$, $p = 0.52$) at E18.5, as shown in Figure 6.2 A and B. Similarly, no significant differences were seen in fetal weight when results were split by embryo sex (Figure 6.2 C and D).

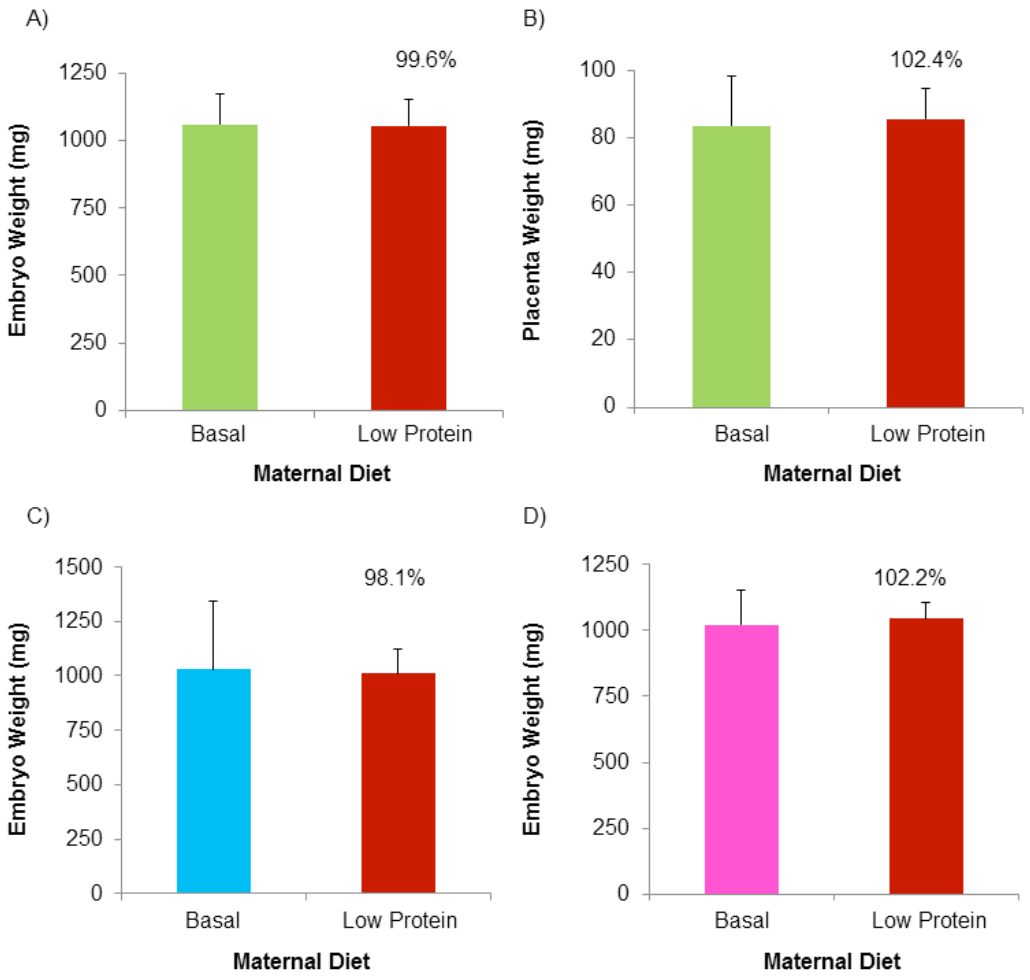


Figure 6.2: Effects of maternal low protein diet on fetal and placental weights. Mean embryonic (A) and placental (B) weights are shown at E18.5. Mean embryonic weights are also shown split by sex; male (C) and female (D). Error bars represent standard deviation. The relative embryonic and placental weights of low protein diet offspring are shown as a percentage of the control values. Differences in weight were not statistically significant using an independent samples t-test.

There was a significant 2.09 fold higher *Phlda2* expression in placentas from low protein diet pregnancies compared with controls ($p = 0.003$, $n = 12$), Figure 6.3 A. This difference was sex specific with increased *Phlda2* expression statistically significant in female placentas ($p = 0.02$, $n = 6$). In the male placentas, the elevated expression did not reach statistical significance ($p=0.09$, $n = 6$), Figure 6.3 B.

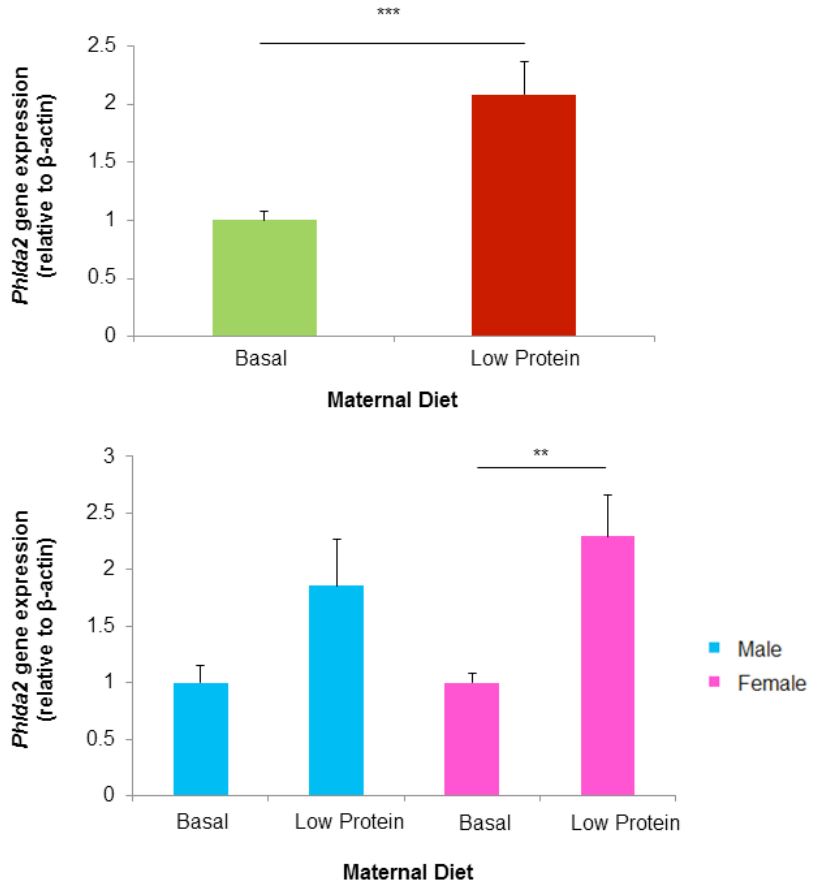


Figure 6.3: Effects of maternal low protein diet on placental *Phlda2* expression. *Phlda2* expression was significantly increased following low protein diet using an independent samples T test (A); this difference was significant in female but not male placentas (B). *** $p < 0.001$. Error bars represent SEM.

In contrast there was no significant effect of low protein diet on placental *Cdkn1c* expression ($p = 0.21$, $n = 12$). Results remained non-significant when split by fetal sex.

In terms of the paternally expressed imprinted genes, placental *Peg3* expression was significantly increased by 24% in low protein compared with control pregnancies ($p = 0.01$, $n = 12$), Figure 6.4. This difference was sex specific with increased expression statistically significant only in male ($p = 0.02$, $n = 6$) but not female ($p = 0.19$, $n = 6$) placentas, Figure 6.4.

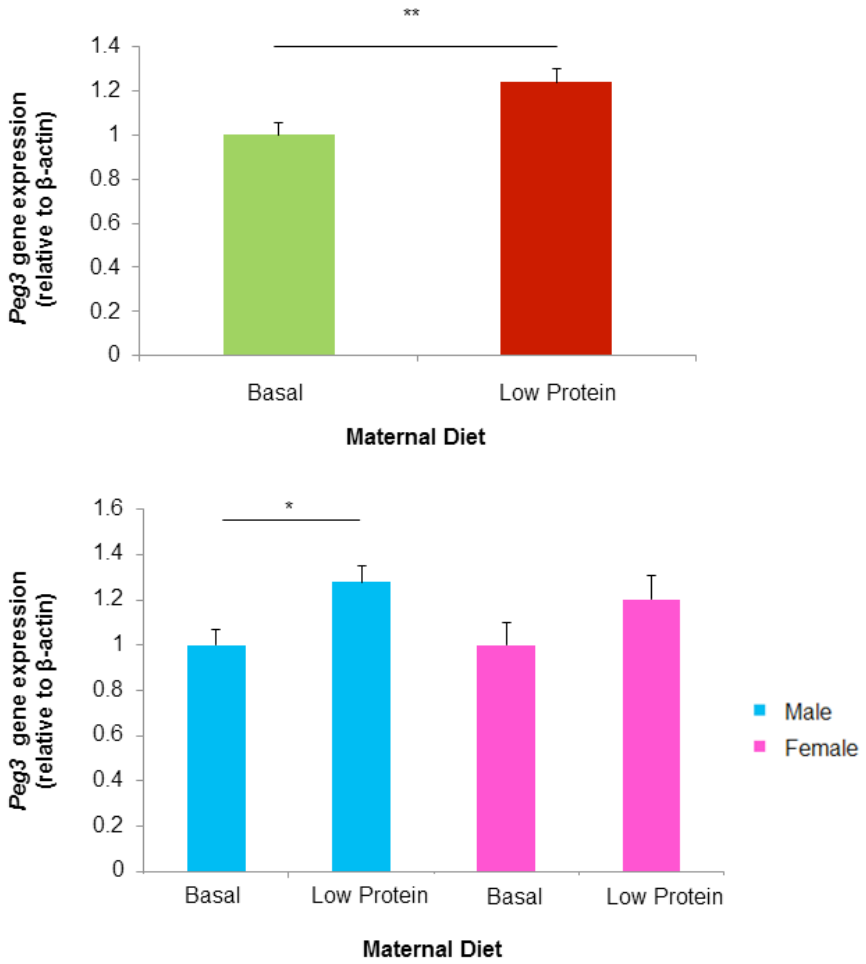


Figure 6.4: Effects of maternal low protein diet on placental *Peg3* expression. *Peg3* expression was significantly increased following low protein diet (A); this difference was significant in male but not female placentas (B). * $p \leq 0.05$, ** $p \leq 0.01$. Error bars represent SEM.

In contrast, placental *Peg10* expression was significantly decreased by 33% in low protein compared with control pregnancies ($p = 0.02$, $n = 12$), Figure 6.5. Although a decrease in expression was observed in both male and female placentas, this difference was only significant in female ($p = 0.02$, $n = 6$) and not male placentas ($p = 0.41$, $n = 6$).

A summary of the changes in placental imprinted gene expression following maternal low protein diet is shown in Table 6.1. Note only the increase in *Phlda2* expression and *Peg3* expression remained significant after controlling for multiple comparisons using a Bonferroni correction ($P = 0.01$).

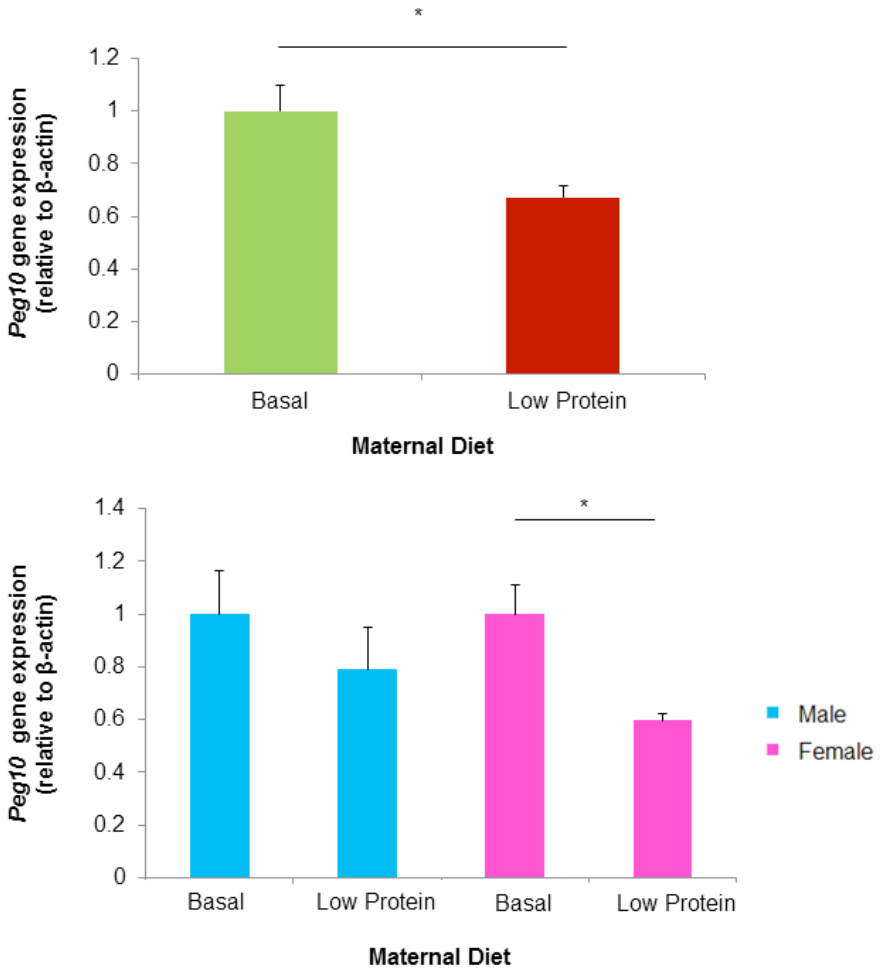


Figure 6.5: Effects of maternal low protein diet on placental *Peg10* expression. *Peg*10 expression was significantly increased following low protein diet (A); this difference was significant in female but not male placentas (B). * p ≤ 0.05. Error bars represent SEM.

Table 6.1. Summary of placental imprinted gene expression changes following maternal low protein diet at E18.5. Fold gene expression is shown relative to basal gene expression. Results highlighted in bold were statistically significant using an independent samples T test. * p ≤ 0.05, ** p ≤ 0.01. LPD = Low protein Diet.

Gene	Active Allele	Basal fold expression	LPD fold expression	P Value
Phlda2	**M**	**1.00**	**2.09**	**P = 0.003****
Cdkn1c	M	1.00	1.21	P = 0.21
Peg3	**P**	**1.00**	**1.24**	**P = 0.01****
Peg10	**P**	**1.00**	**0.67**	**P = 0.02***

Phlda2 is known to negatively regulate the spongiotrophoblast lineage of the mouse placenta (reviewed in John 2013). Increased *Phlda2* expression following maternal low protein diet might therefore result in a reduced spongiotrophoblast lineage in this model. To explore this possibility, expression of the spongiotrophoblast markers *Prl3b1* and *Prl8a8* was examined. As shown in Figure 6.6, there was no significant effect of maternal low protein diet on placental *Prl3b1* ($p = 0.86$, $n = 12$) or *Prl8a8* ($p = 0.31$, $n = 12$) expression. Results remained non-significant when analysed according to fetal sex.

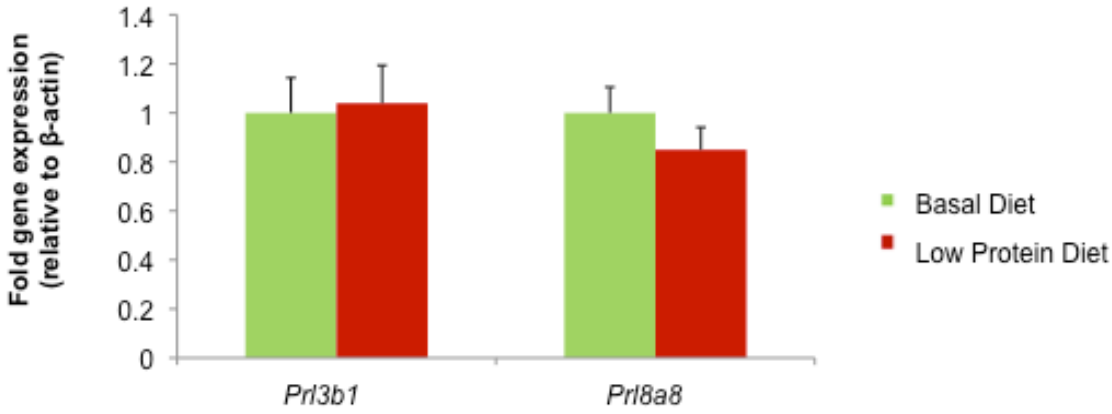


Figure 6.6: Effect of maternal low protein diet on spongiotrophoblast marker gene expression. There was no significant effect of low protein diet on placental *Prl3b1* or *Prl8a8* gene expression. Error bars represent SEM.

Furthermore, to determine whether the increase observed in placental *Phlda2* expression following low protein diet was organ specific, *Phlda2* expression was also examined in the kidney at E18.5. As can be seen in Figure 6.7, *Phlda2* expression in the kidney was not significantly altered following low protein diet in contrast to the increased placental expression observed (section 6.4.2), suggesting a placental specific response to maternal diet. There was no significant difference in *Phlda2* ($p = 0.42$, $n = 6$) expression between male and female control kidneys and no sex effect on the association between low protein diet and *Phlda2* expression (results not shown).

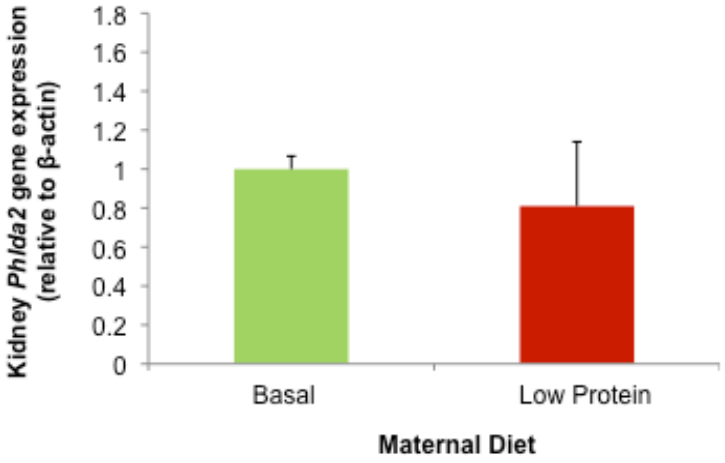


Figure 6.7: Effects of maternal low protein diet on kidney *Phlda2* gene expression. There was no significant effect of low protein diet on *Phlda2* gene expression in the kidney. Error bars represent standard error.

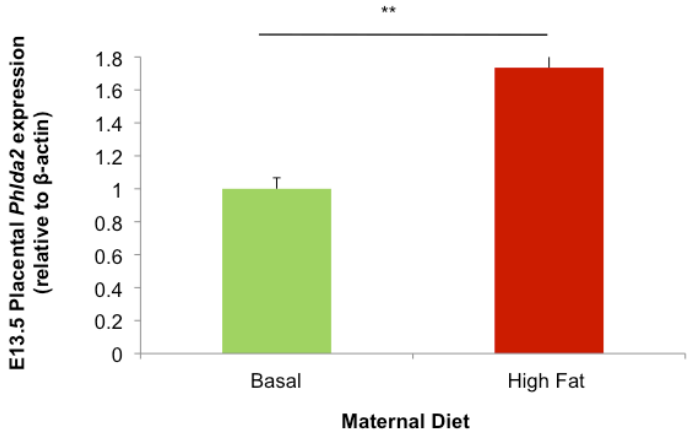


Figure 6.8: Effects of maternal low protein diet on E13.5 placental *Phlda2* gene expression. *Phlda2* expression was significantly increased using an independent samples T-test. Error bars represent standard error. ** $p \leq 0.01$.

Finally, to determine whether the changes observed in placental *Phlda2* gene expression in response to maternal low protein diet were time specific, imprinted gene expression was also analysed at the earlier time point of E13.5. *Phlda2* expression was significantly increased at E13.5 following low protein diet ($p=0.01$, $n = 8$), Figure 6.8. However, the increase in *Phlda2* expression at E13.5 (74%) was less than that seen at E18.5 (109%). There was no significant effect of maternal low protein diet on E13.5 placental expression of any other imprinted gene examined (results not shown).

Unaltered placental *Peg3* expression at E13.5 (p=0.48, n = 12) suggested that the increase observed in E18.5 *Peg3* placental expression was time specific.

6.3.3. Maternal high fat diet

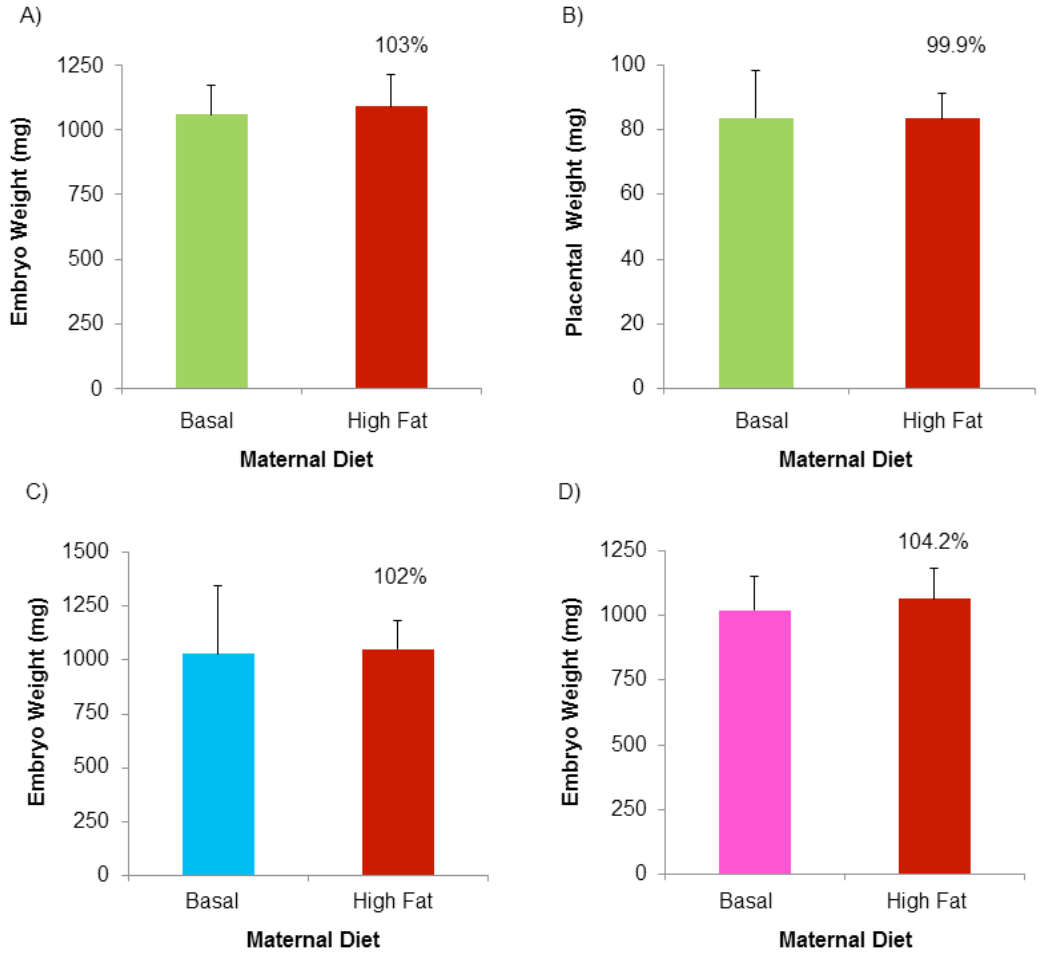


Figure 6.9: Effects of maternal high fat diet on fetal and placental weights. Mean embryonic (A) and placental (B) weights are shown at E18.5. Mean embryonic weights are also shown split by sex; male (C) and female (D). Error bars represent Standard Deviation. The relative embryonic and placental weights of high fat diet offspring are shown as a percentage of the control values. Differences in weight were not statistically significant using a t-test.

Given the responsiveness of a number of the imprinted genes examined (particularly *Phlda2*) to maternal low protein diet, other dietary alterations were explored. A maternal high fat diet was chosen as it has been suggested that animal models of over nutrition are currently more relevant to western

society. There was no significant effect of maternal high fat diet on fetal (1.06 g ± 0.11 v. 1.06 g ± 0.97, n = 61, p = 0.88) or placental weights (83.67 mg ± 7.66 v. 85.71 mg ± 9.08, n = 61, p = 0.52), Figure 6.9 A and B. Results remained non-significant when split by fetal sex (Figure 6.9 C and D).

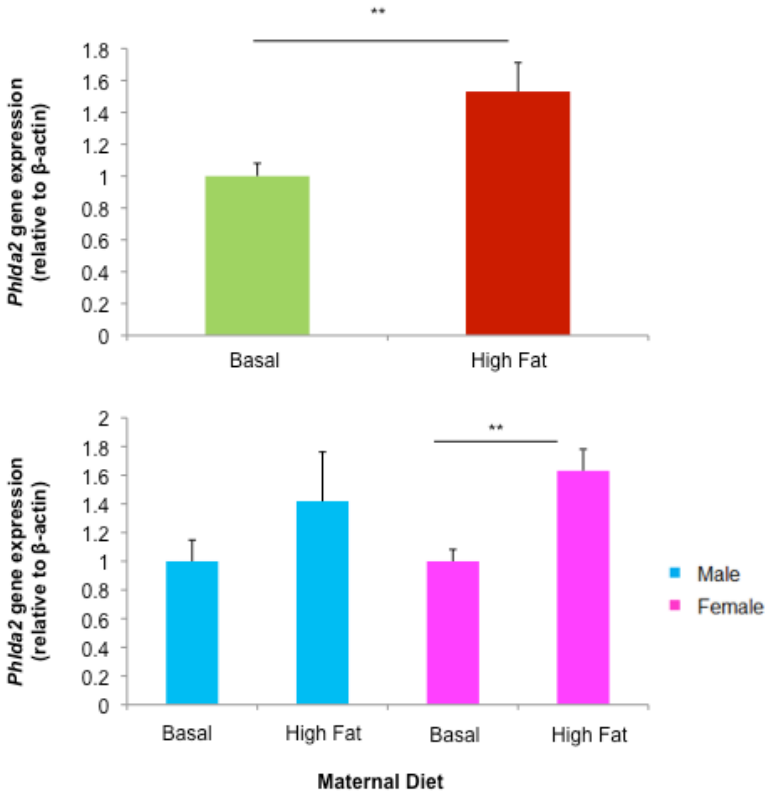


Figure 6.10: Effects of maternal high fat diet on placental *Phlda2* expression. *Phlda2* expression was significantly increased following high fat diet using an independent samples T test (A); this difference was significant in female but not male placentas (B). ** p ≤ 0.01.

There was a significant 53% increase in placental *Phlda2* expression in the maternal high fat compared with control pregnancies (p = 0.01, n = 12), Figure 6.10. This difference was sex specific with increased *Phlda2* expression significant in only female (p = 0.004, n = 6) and not male placentas (p=0.29, n = 6), Figure 6.10.

As shown in Figure 6.11, there was no significant effect of high fat diet on placental *Cdkn1c* (p = 0.20, n = 12) or *Peg3* (p = 0.98, n = 12) gene expression. Results remained non-significant when split by embryo sex. A

trend was seen for decreased placental *Peg10* expression in response to maternal high fat diet (p=0.07) (Figure 6.11), however this failed to reach statistical significance in either male (p = 0.48, n = 12) or female placentas (p = 0.07, n = 12).

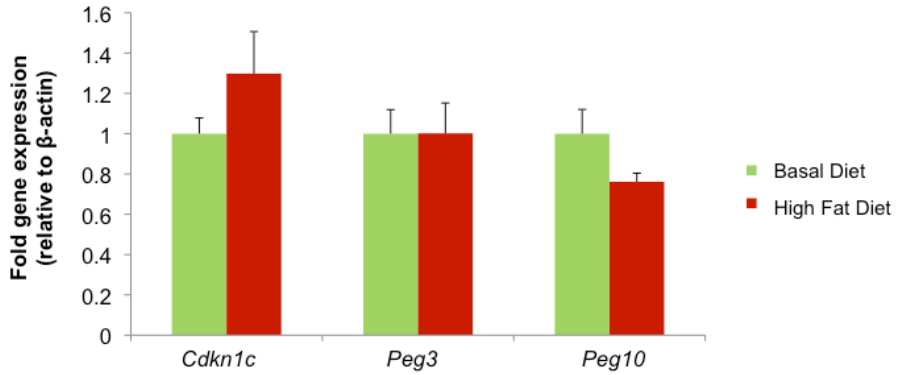


Figure 6.11: Effects of maternal high fat diet on placental imprinted gene expression. Gene expression was not significantly altered as a result of maternal high fat diet using an independent samples T test. Error bars represent SEM.

Table 6.2. Summary of placental imprinted gene expression changes following maternal high fat diet at E18.5. Fold gene expression is shown relative to basal gene expression. Results highlighted in bold were statistically significant using an independent samples T test. ** p ≤ 0.01. HFD = high fat diet.

Gene	Active Allele	Basal fold expression	HFD fold expression	P Value
Phlda2	**M**	**1.00**	**1.53**	**P = 0.01****
Cdkn1c	M	1.00	1.30	P = 0.20
Peg3	P	1.00	1.00	P = 0.98
Peg10	P	1.00	0.87	P = 0.07

A summary of the changes in placental imprinted gene expression following maternal high fat diet is shown in Table 6.2. Only placental *Phlda2* expression was significantly increased in response to a maternal high fat diet.

This increase in *Phlda2* expression remained significant after controlling for multiple comparisons using a Bonferroni correction (P = 0.01).

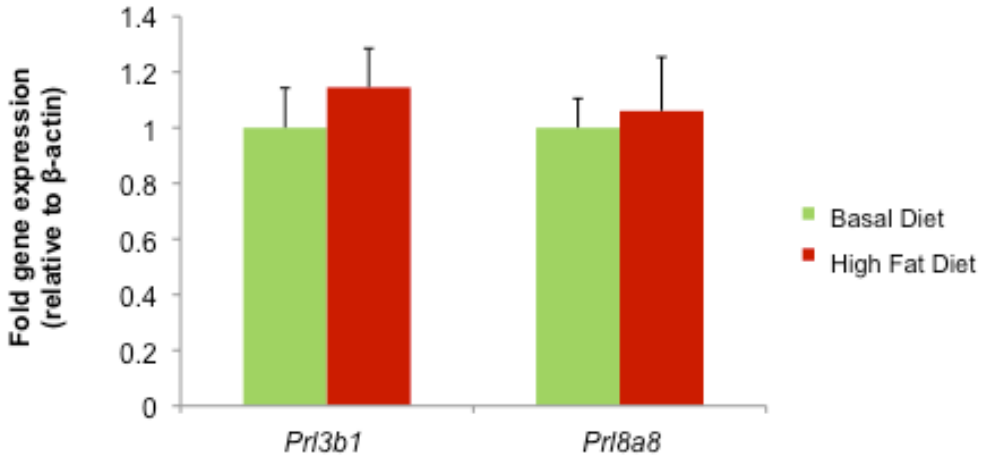


Figure 6.12: Effects of maternal high fat diet on spongiotrophoblast marker gene expression. There was no significant effect of high fat diet on placental *Prl3b1* or *Prl8a8* gene expression. Error bars represent standard error.

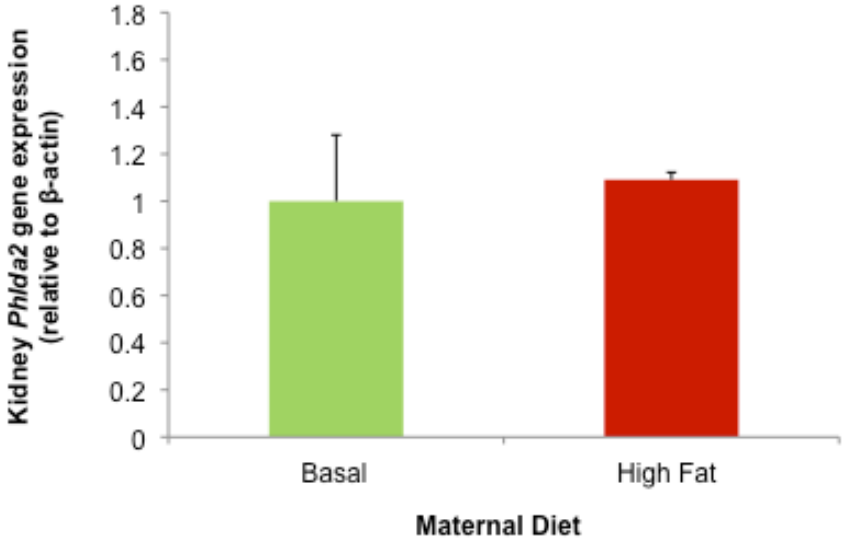


Figure 6.13: Effects of maternal high fat diet on kidney *Phlda2* gene expression. There was no significant effect of high fat diet on *Phlda2* gene expression in the kidney. Error bars represent standard error.

Given the significant increase in placental *Phlda2* gene expression resulting from a maternal high fat diet, expression of the spongiotrophoblast markers *Prl8a8* and *Prl3b1* was also examined. There was no significant effect of

maternal high fat diet on placental *Prl3b1* (p = 0.48, n = 12) or *Prl8a8* (p = 0.79, n = 12) expression, as shown in Figure 6.12.

Finally, to determine whether the effect of maternal high fat diet on placental *Phlda2* expression was organ specific, expression of *Phlda2* in the kidney was also analysed. As can be seen in Figure 6.13, maternal high fat diet did not significantly alter *Phlda2* (p = 0.84, n = 11) expression in the kidney. Differences remained non-significant for both male and female kidney.

6.3.4. Maternal high fat – high sugar diet

Females were on a control or high fat – high sugar (HFHS) diet 12 weeks before pregnancy and were weighed every week to examine pre-pregnancy weight gain. As can be seen in Figure 6.14, females on a HFHS diet were significantly heavier than control females after 2 weeks and on average 42% heavier by week 12 (at mating).

In addition, maternal weight gain during pregnancy was assessed between E0.5 and E18.5 (Figure 6.15). Females on a HFHS-HFHS diet gained significantly more weight than Control-Control females (1.33 g v. 2.44 g, n = 5, p = 0.02). However, there was no significant difference in weight gain during pregnancy between Control-Control females and Control-HFHS (1.33 g v. 1.78 g, n = 7, p = 0.42) or HFHS-Control females (1.33 g v. 1.07 g, n = 5, p = 0.28).

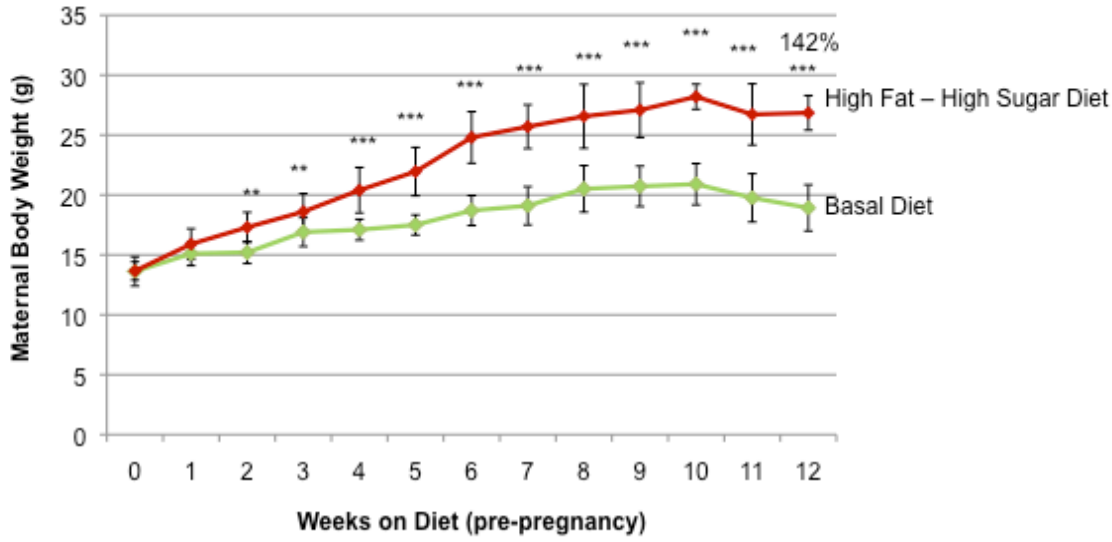


Figure 6.14: Effects of maternal pre-pregnancy diet on maternal body weight. Mean body weights are shown, error bars represent Standard Deviation. The relative body weight of HFHS mice compared with controls (basal diet) are shown in percentages at week 12. HFHS mice were significantly heavier than basal mice from week 2. ** $p \leq 0.01$, *** $p \leq 0.001$.

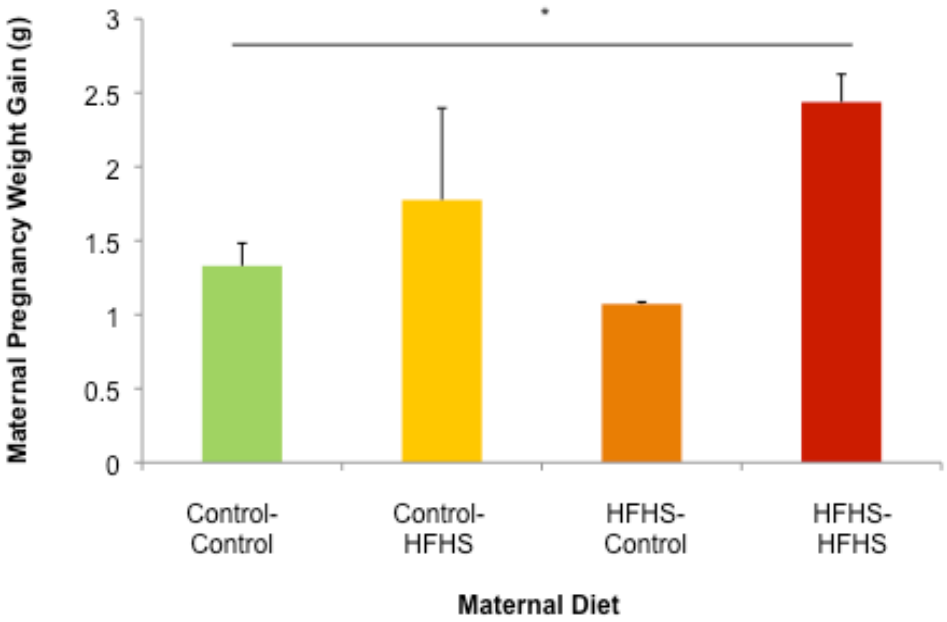


Figure 6.15: Effects of maternal diet on pregnancy weight gain. Mean pregnancy weight gain between E0.5 and E18.5 is shown for each diet condition. Error bars represent Standard Deviation. * $p \leq 0.05$. Maternal Weight gain was normalised to litter size.

There was a significant difference in fetal weight between maternal diet groups as determined by one-way ANOVA ($F(3,48) = 10.46$, $p \leq 0.001$), see Figure 6.16 A. A Tukey post-hoc test revealed significantly increased embryo weight in offspring of mothers fed a HFHS-Control diet compared with a Control-Control diet (1.11 ± 0.17 v. 1.35 g ± 0.05, n = 27, p = 0.001) and a Control-HFHS diet (1.03 ± 0.12 v. 1.35 g ± 0.05, n = 27, p < 0.001).

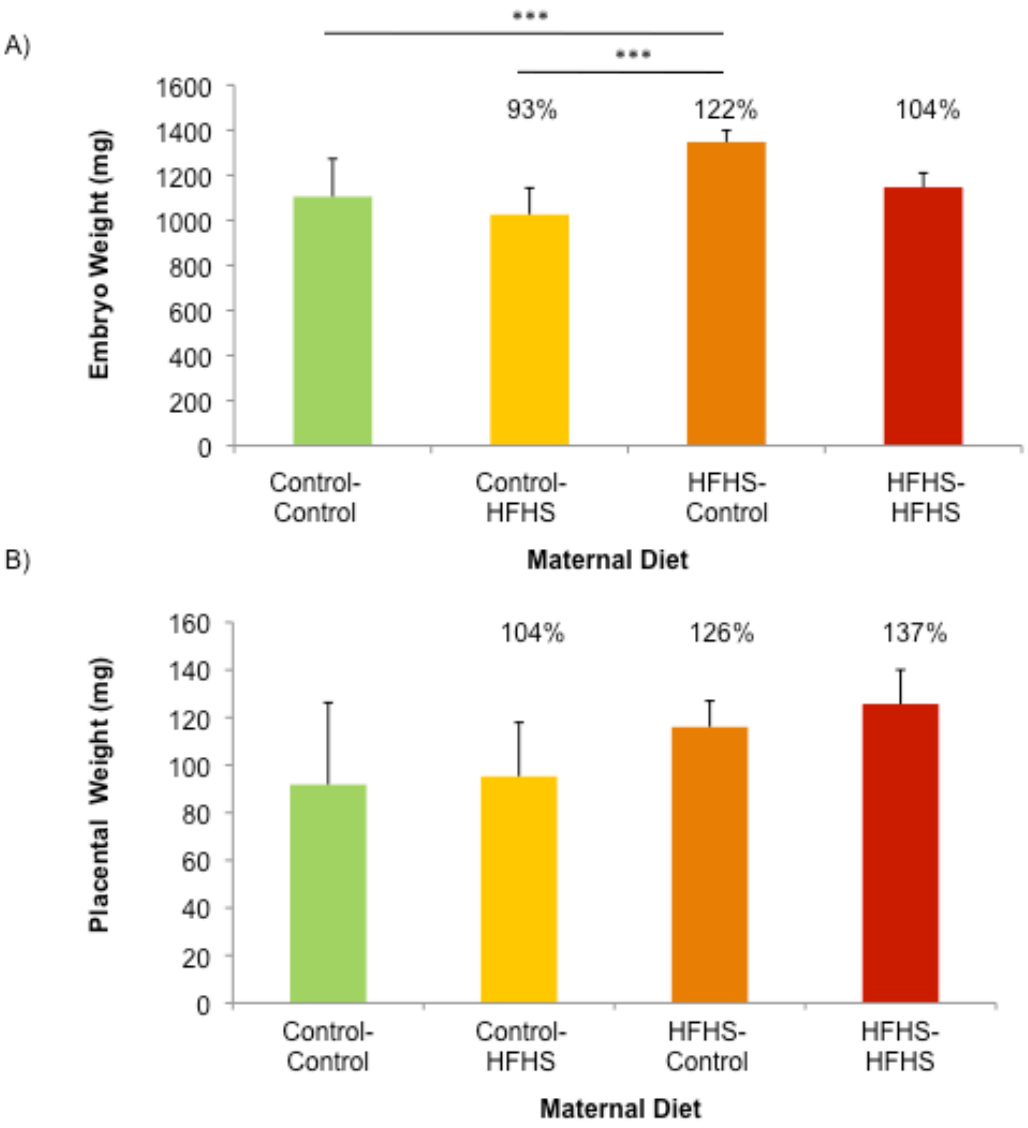


Figure 6.16: Effects of maternal diet on embryonic and placental weights. Mean embryonic (A) and placental (B) weights are shown at E18.5. Error bars represent Standard Deviation. Relative embryonic and placental weights compared with control-control are shown in percentages. HFHS = high fat – high sugar. *** $p \leq 0.001$.

There was also a significant difference in mean placental weights between maternal diet groups as determined by one-way ANOVA ($F(3,48) = 2.9$, $p = 0.04$), see Figure 6.16 B. However, a Tukey post-hoc test revealed only a trend for increased placental weight for mothers fed a HFHS-HFSH diet compared with a Control-Control diet (91.83 mg ± 3.44 v. 125.71 mg ± 1.44, $n = 24$, $p = 0.10$).

There was no significant difference in placental *Phlda2* expression between maternal diet groups as determined by a one-way ANOVA ($F(3,18) = 1.90$, $p = 0.17$). Results remained non-significant when split by embryo sex. Similarly, there was no significant difference in placental *Cdkn1c* ($F(3,18) = 1.67$, $p = 0.21$) expression between maternal diet groups (Figure 6.17).

There was however a significant difference in placental *Peg3* expression between maternal diet groups as determined by one-way ANOVA ($F(3,18) = 5.01$, $p = 0.01$), as shown in Figure 6.17. A Tukey post-hoc test revealed a significant 50% increase in *Peg3* expression in placentas of mothers fed a HFHS-Control diet as compared with a Control-Control diet ($n = 12$, $p = 0.01$). There was no significant difference in placental *Peg3* expression between any other diet groups as determined by a Tukey post-hoc test.

There was also a significant difference in placental *Peg10* expression between maternal diet groups as determined by one-way ANOVA ($F(3,18) = 3.5$, $p = 0.04$) (Figure 6.17). Although placental *Peg10* expression was increased in all diet groups compared with the Control-Control offspring, A Tukey post-hoc test revealed only a significant 54% increase in *Peg10* expression in placentas of mothers fed a Control-HFHS diet as compared with a Control-Control diet ($n = 12$, $p = 0.03$).

A summary of the changes in placental imprinted gene expression in response to maternal HFHS diet is shown in Table 6.3. The increase in placental *Peg3* expression remained significant after controlling for multiple comparisons using a Bonferroni correction ($P = 0.01$).

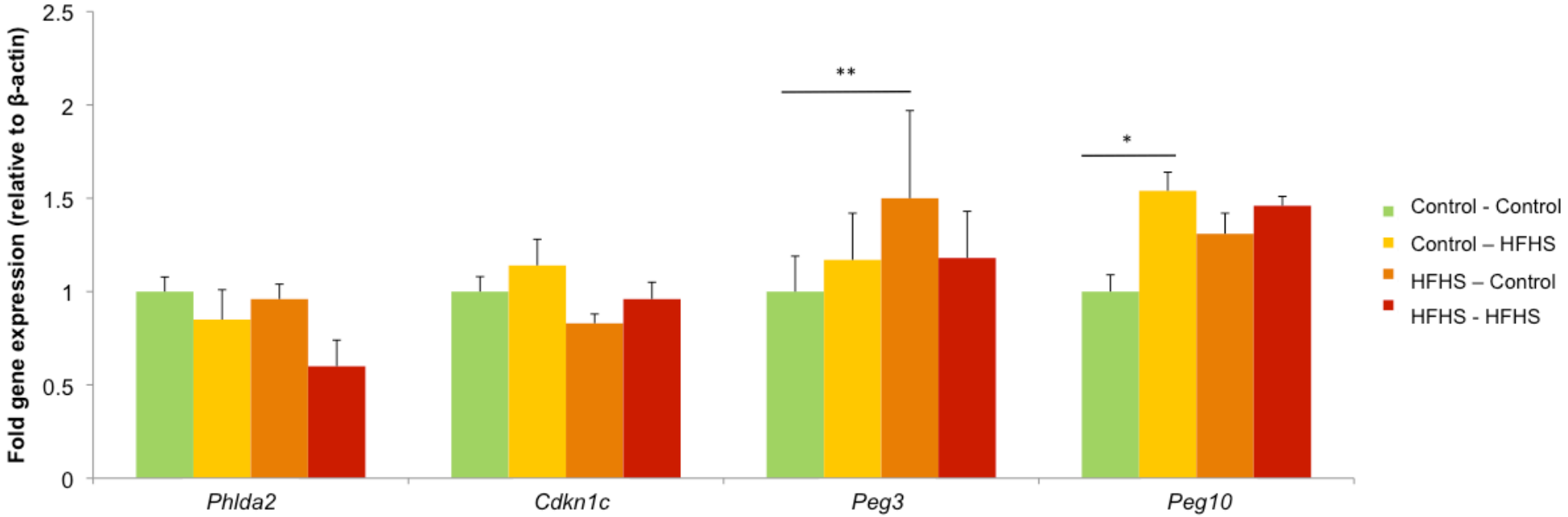


Figure 6.17: Effects of maternal high fat – high sugar (HFHS) diet on placental imprinted gene expression Error bars represent standard error. * $p \leq 0.05$, ** $p \leq 0.01$.

Table 6.3. Summary of placental imprinted gene expression changes following maternal high fat – high sugar diet. Fold gene expression is shown relative to controls. Results highlighted in bold were statistically significant using an independent samples T test. * p ≤ 0.05, ** p ≤ 0.01. C = control diet, F = High fat – high sugar diet.

Gene	Active Allele	C-C fold expression	C-F fold expression	F-C fold expression	F-F fold expression
Phlda2	M	1.00	0.85 (P = 0.80)	0.96 (P = 0.99)	0.6 (P = 0.15)
Cdkn1c	M	1.00	1.14 (P = 0.76)	0.83 (P = 0.61)	0.96 (P = 0.99)
Peg3	**P**	1.00	1.17 (P = 0.60)	**1.5 (P = 0.01)****	1.18 (P = 0.61)
Peg10	**P**	1.00	**1.54 (P = 0.03)***	1.31 (P = 0.32)	1.46 (P = 0.13)

In line with the absence of an effect of maternal HFHS diet on placental *Phlda2* expression, placental expression of the spongiotrophoblast markers *Prl3b1* and *Prl8a8* was not significantly altered, Figure 6.18.

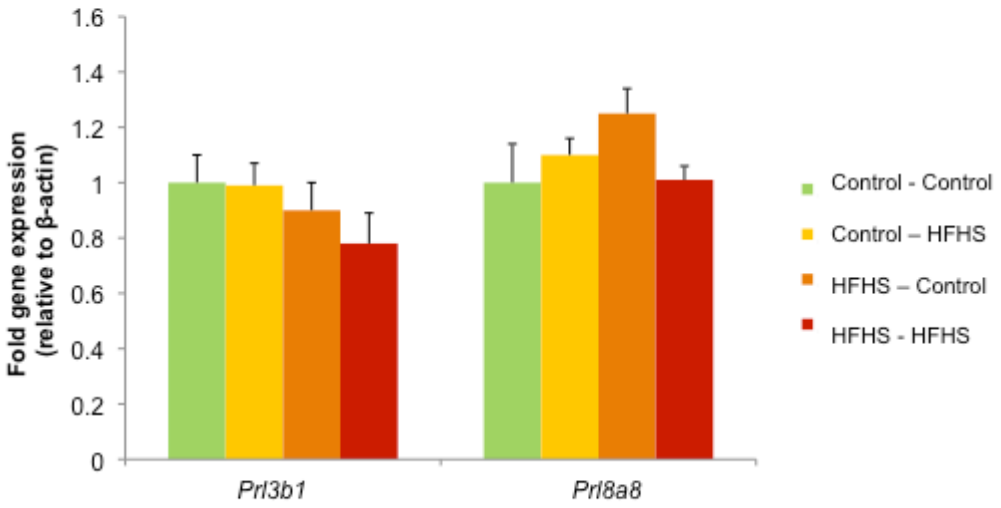


Figure 6.18: Effects of maternal high fat – high sugar (HFHS) diet on spongiotrophoblast marker gene expression. There was no significant effect of HFHS diet on placental *Prl3b1* or *Prl8a8* gene expression. Error bars represent standard error.

6.3.5. RFLP analysis

Gene expression analysis demonstrated significantly increased placental *Phlda2* expression in response to a maternal low protein diet (at both E13.5 and E18.5) and high fat diet (at E18.5) during pregnancy. Increased placental *Phlda2* expression may result from either increased expression from the maternal *Phlda2* allele or loss of imprinting at the paternal *Phlda2* allele. In order to determine the allelic origin of *Phlda2* expression in placentas from the mouse model of low protein and high fat diet, RFLP analysis was carried out. As *Phlda2* is a maternally expressed imprinted gene it was expected that basal samples would only have the maternal 100bp band. Loss of imprinting would therefore be predicted to result in the presence of both the paternal 150bp band and the maternal 100bp band.

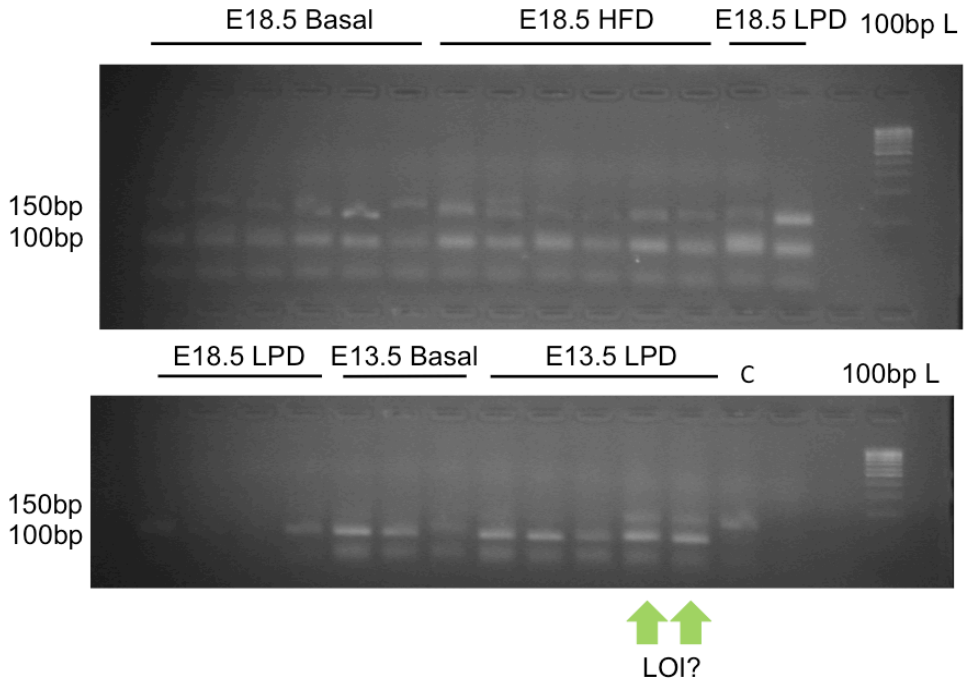


Figure 6.19: RFLP analysis. Results are shown for basal, high fat diet and low protein diet at E18.5 and for basal and low protein diet at E13.5. Expression from the maternal *Phlda2* allele is indicated by the presence of a 100bp band, and expression from the paternal allele by presence of a 150bp band. Thus loss of imprinting is suggested in samples where both bands are present. HFD = high fat diet, LPD = low protein diet, LOI = loss of imprinting, C = control sample, L = ladder.

As can be seen in Figure 6.19, at E18.5 all samples (basal, high fat diet and low protein diet) had the paternal band present, suggesting a relaxation of imprinting at E18.5 under normal conditions which may obscure any effect of maternal diet on placental *Phlda2* expression from the paternal allele. In contrast at E13.5, basal samples did not have the paternal band, suggesting that imprinting is maintained at this time point. Interestingly, two out of the five low protein diet samples at E13.5 had the paternal *Phlda2* allele indicating loss of imprinting in these samples. These results suggest that loss of imprinting of the paternal *Phlda2* allele occurs in response to low protein diet exposure during pregnancy (at E13.5), an effect which is obscured at a later time point (E18.5) as imprinting relaxes towards the end of gestation. However, this previously unreported result requires further investigation.

6.4. Discussion

This chapter further explored the effect of maternal diet before and during pregnancy on placental imprinted gene expression in a mouse model. A summary of imprinted gene expression changes resulting from maternal diet alteration is shown in Table 6.4. These results suggest that placental *Phlda2*, *Peg3* and *Peg10* expression, but not *Cdkn1c* expression, is responsive to the maternal environment.

6.4.1. Sex differences in imprinted gene expression

Previous studies have suggested differential imprinted gene expression between male and female placentas both under normal conditions (Faisal *et al*. 2014) and in response to maternal diet alteration (Radford *et al*. 2012). This study demonstrated no significant difference in placental target gene expression between male and female placentas under basal conditions. However, an even number of male and female embryos were subsequently analysed for each condition (where possible), to determine sexual dimorphism in the placental response to maternal diet alterations.

Table 6.4. Summary of placental imprinted gene expression changes following maternal diet alteration. Percentage change in expression relative to controls is shown. HFHS = high fat – high sugar.

Gene	Active Allele	Low Protein Diet	High Fat Diet	HFHS – Control diet	Control - HFHS diet	HFHS - HFHS diet
Phlda2	M	↑ 109%	↑ 53%	-	-	-
Cdkn1c	M	-	-	-	-	-
Peg3	P	↑ 24%	-	-	↑ 50%	-
Peg10	P	↓ 33%	-	-	↑ 54%	-

6.4.2. Maternal low protein diet and *Phlda2*

Maternal low protein diet had no significant effect on embryo or placental weights at E18.5, as previously reported by Vucetic *et al*. (2010). Two fold increased *Phlda2* expression was demonstrated in placentas of low protein diet pregnancies. Furthermore, although placental *Phlda2* expression was increased in both male and female low protein diet placentas, this was only statistically significant in females. No previous study has analysed placental *Phlda2* expression in response to maternal low protein diet, however these results are consistent with those of Shukla *et al*. (2011) demonstrating increased placental *Phlda2* expression in a rat model of maternal under nutrition (in the form of calorie-restriction) during pregnancy.

Moreover, *Phlda2* expression in the kidney was not significantly altered as a result of low protein diet, suggesting a placental specific response to maternal diet. Placental *Phlda2* expression was however significantly increased at E13.5 suggesting that the effect of maternal diet on gene expression was not time specific. It should be noted that the increase in placental *Phlda2* expression at E13.5 (174%) was more modest than that seen at E18.5 (209%), possibly as a result of the shorter exposure time to the low protein diet.

Phlda2 has a known role in the negative regulation of the endocrine lineage of the placenta (reviewed in John 2013) with the mouse model of *Phlda2* over expression demonstrating a specific loss of spongiotrophoblast cells (Tunster *et al*. 2010). Thus, given the two fold increase in placental *Phlda2* expression observed in response to maternal low protein diet, expression of the spongiotrophoblast markers *Prl8a8* and *Prl3b1* was subsequently examined. Placental *Prl8a8* and *Prl3b1* expression was not significantly altered as a result of maternal low protein diet, suggesting no concurrent effect of maternal low protein diet on spongiotrophoblast cells of the placenta.

6.4.3. Maternal low protein diet and other imprinted genes

Placental *Peg3* expression was also increased in response to maternal low protein diet, although the effect observed was more modest (24% increase). In addition, this effect was sex specific with increased expression statistically significant in male but not female placentas. Radford *et al*. (2012) similarly reported an approximate 30% increase in placental *Peg3* expression in response to maternal undernutrition during pregnancy. As in the current study, this effect was sex-specific with *Peg3* expression increased significantly in male placentas only (Radford *et al*. 2012). Increased placental *Peg3* expression in response to maternal undernutrition was proposed to reflect a fetal adaptation, stimulating maternal food intake, in response to an adverse environment (Radford *et al*. 2012).

Maternal low protein di*et al*so resulted in a significant 33% decrease in placental *Peg10* expression. Furthermore, although placental *Peg10* expression was decreased in both male and female low protein diet placentas, this was only statistically significant in females. No previous study has analysed placental *Phlda2* expression in response to maternal low protein diet, therefore these novel results provide the first evidence of an effect of maternal low protein diet on placental *Peg10* expression.

6.5.4. Maternal high fat diet

Maternal high fat diet had no significant effect on embryo or placental weights at E18.5. Gallou-Kabani *et al*. (2010) similarly demonstrated no significant effect of maternal high fat diet on embryo weights at E15.5 although placental weight was significantly reduced in response to high fat diet. However given that the number of mice studied by Gallou-Kabani *et al*. (2010) in each experimental group (209 v 184) was much higher than in the current study, it is possible that an effect of a high fat diet on placental weight could not be determined in a study of this size.

Placental *Phlda2* expression was significantly increased by 53% in placentas of high fat diet pregnancies. As with low protein diet, this difference

was statistically significant in female placentas only. No previous study has analysed placental *Phlda2* expression in response to maternal high fat diet, although Sferruzzi-Perri *et al*. (2013) did demonstrate a non-significant increase in placental *Phlda2* expression in response to maternal high fat – high sugar diet. Thus, these novel results provide the first evidence of an effect of maternal high fat diet on placental *Phlda2* expression. Furthermore, *Phlda2* expression in the kidney was not significantly altered as a result of maternal high fat diet, suggesting a placental specific response to maternal high fat diet. Finally, no concurrent change in expression of the spongiotrophoblast markers *Prl3b1* or *Prl8a8* was observed.

No significant difference was observed in expression of any other imprinted gene examined in response to maternal high fat diet.

6.4.5. Maternal high fat – high sugar diet

There was no significant effect of a maternal HFHS diet during pregnancy on embryo weight, as reported by Sferruzzi-Perri *et al*. (2013). However, embryo weight was increased in response to a HFHS before pregnancy, highlighting the importance of pre-conception diet on fetal growth. No associated effects on placental weight were observed.

There was no significant effect of maternal HFHS diet on placental *Phlda2* expression when exposure occurred pre-pregnancy, during pregnancy or both. In addition, no associated effect on spongiotrophoblast marker expression was observed. Sferruzzi-Perri *et al*. (2013) similarly demonstrated no significant difference in placental *Phlda2* expression in response to maternal high fat – high sugar diet. It should be noted for the current study that pregnancy was difficult to achieve and maintain in the HFHS-control and HFHS-HFHS mice and therefore results are based on placental gene expression in one litter. Analysis in an increased number of placentas from different litters will confirm the relationship, if any, between maternal HFHS diet and placental *Phlda2* expression.

Placental *Peg3* expression was increased in all maternal HFHS diet groups compared with controls, however this difference was only statistically significant for HFHS-Control pregnancies. Sferruzi-Perri *et al*. (2013) previously demonstrated no significant effect of maternal HFHS diet on placental *Peg3* expression, however in this study diet exposure was limited to pregnancy. Given the role of *Peg3* in positively regulating fetal growth in the mouse, it is possible that the increased placental *Peg3* expression observed underlies the increased embryo weights of the HFHS-control group.

Finally, placental *Peg10* expression was increased in all maternal HFHS diet groups compared with controls, although this difference was only statistically significant for control-HFHS mice. No previous study has examined placental *Peg10* expression in relation to a HFHS diet. However, given that expression data form the control-HFHS group is derived from a single litter, the effect of a HFHS diet during pregnancy remain to be confirmed in an increased number of placentas from different litters.

6.4.6. RFLP analysis

Bands indicating expression of *Phlda2* from the paternal allele were present in all E18.5 samples, including those where mothers were fed a basal diet during pregnancy. This suggests that a relaxation of imprinting of *Phlda2* has occurred by E18.5. Previous studies of *Phlda2* imprinting in the mouse have examined placentas only up to E14.5 (Qian *et al*. 1997; Frank *et al*. 1999; Frank *et al*. 2002). Thus, this novel result is the first evidence to support a relaxation of placental *Phlda2* imprinting towards the end of gestation in the mouse.

Imprinting was maintained in basal samples at E13.5 with only maternal *Phlda2* allelic expression observed. A proportion of the low protein diet samples (40%) demonstrated the presence of a paternal *Phlda2* allele suggesting that loss of imprinting occurs in response to a maternal low protein diet, an effect which may be obscured at a later time point (E18.5) as imprinting relaxes towards the end of gestation. Further work will involve

pyrosequencing of DNA methylation at the imprinted loci to fully determine whether loss of imprinting is responsible for the aberrant placental imprinted gene expression observed as a result maternal diet alteration.

6.4.7. Summary

In summary, the results presented in this chapter provide evidence to suggest that placental *Phlda2*, *Peg3* and *Peg10,* but not *Cdkn1c*, expression is responsive to the maternal environment. Placental *Phlda2* expression was increased at E18.5 as a result of maternal low protein diet and high fat diet during pregnancy. This study also demonstrated that placental *Phlda2* expression was increased due to a low protein diet at an earlier time point (E13.5) suggesting that the effect of low protein diet on placental *Phlda2* expression is not time specific. Furthermore, the absence of an effect of maternal low protein or high fat diet on placental *Phlda2* expression in the kidney, suggests a placental specific response to maternal diet alteration. RFLP analysis suggested that the increased placental *Phlda2* expression observed at E13.5 in response to maternal low protein diet was due to loss of imprinting but that this effect was obscured at E18.5 due to a relaxation of imprinting. Placental *Peg3* expression was also significantly increased as a result of maternal low protein diet and high fat high sugar diet during pregnancy. In contrast, placental *Peg10* expression was significantly decreased in the mouse model of low protein diet exposure but increased in response to a high fat – high sugar diet during pregnancy. Finally, placental *Cdkn1c* expression was unchanged following maternal low protein, high fat or high fat – high sugar diet, suggesting that placental expression of this gene is not responsive to the maternal environment.

CHAPTER 7: DISCUSSION

7.1. Main findings

Fetal growth restriction (FGR) is known to negatively impact on offspring health in the perinatal period and in adulthood, as well as long-term maternal health. A further understanding of the causes of growth restriction is therefore of paramount importance. This thesis examined expression of the imprinted genes *PHLDA2*, *CDKN1C*, *PEG3* and *PEG10* in relation to FGR and in relation to maternal lifestyles during pregnancy. Imprinted gene expression was also examined in relation to other complications of pregnancy that may result from inadequate maternal adaptation to pregnancy such as preeclampsia, gestational diabetes and maternal prenatal depression. This last premise was based on our understanding, from experimental animal models, that imprinted genes regulate the placental signals to the mother that induce adaptations required for a successful pregnancy.

7.1.1. *PHLDA2*

Placental *PHLDA2* expression was significantly inversely associated with birth weight and custom birth weight centiles in two independent cohorts. Apostolidou *et al*. (2007) have previously demonstrated a negative correlation between birth weight and placental *PHLDA2* expression. The results of the current study expand on these findings, demonstrating that the association between placental *PHLDA2* and birth weight remains significant in a larger cohort including both AGA and SGA pregnancies. Furthermore, this study quantified the effect of placental *PHLDA2* expression on fetal growth, demonstrating that expression accounted for 6% of variance in birth weight.

A significant two fold increase in placental *PHLDA2* expression was also demonstrated in SGA pregnancies. These results are consistent with the increase in *PHLDA2* expression previously observed in IUGR placentas (McMinn *et al*. 2006; Diplas *et al*. 2009; Kumar *et al*. 2012). Two fold *Phlda2* expression in a mouse model has been demonstrated to result in reduced fetal weights due to a gradual decrease in growth velocity during late gestation (Tunster *et al*. 2010). These data suggest that the aberrant

PHLDA2 expression observed in SGA placentas of the current study is the cause of fetal growth restriction rather than a consequence of an abnormal intrauterine environment. Furthermore, aberrant placental *PHLDA2* expression was observed in 27% of SGA placentas in this current study. McMinn *et al*. (2007) similarly reported a 25% prevalence of increased *PHLDA2* expression. This suggests that as many as a quarter of all SGA births may be due to elevated *PHLDA2* expression. Relating this finding to the number of term SGA pregnancies in the UK (approximately 53,000 in 2012), aberrant placental *PHLDA2* expression may underlie up to 14,000 SGA pregnancies in the UK per annum (Office for National Statistics 2014).

Aberrant placental *PHLDA2* expression was also observed in a small proportion of AGA pregnancies in the current study. This might suggest that these infants, which would not normally be considered growth restricted, may have failed to reach their genetic growth potential. This novel finding suggests that placental *PHLDA2* expression could be used to identify cases of *in utero* growth restriction not resulting in SGA or LBW. Full clinical characterisation of these infants, their growth patterns during pregnancy and their placentas will aid in determining the significance of these findings.

Phlda2 over expression in a mouse model is specifically associated with asymmetric fetal growth restriction (Tunster *et al*. 2014). This study provides the first evidence that placental *PHLDA2* expression may also play a role in symmetric growth restriction. Placental *PHLDA2* expression was significantly increased in symmetric SGA pregnancies and was negatively associated with head circumference at delivery. These novel results suggest that in cases of FGR characterised by perturbed *PHLDA2* expression, head sparing (the typical fetal adaptation to an adverse intrauterine environment) may be impaired. Importantly, this study does not discount a role for *PHLDA2* in human asymmetric growth restriction with a non-significant two fold increase in expression also observed in these pregnancies.

In addition, placental *PHLDA2* was significantly inversely associated with placental weight in two independent cohorts. This is consistent with the stunted placental growth observed in the mouse model of *Phlda2* overexpression (Tunster *et al*. 2010). However, previous studies have failed to replicate these findings in the human placenta (Apostolidou *et al*. 2007;

Lewis *et al*. 2012). Thus, this is the first evidence of a role for *PHLDA2* in regulating human placental growth. In addition, these results provide support for the hypothesis that aberrant *PHLDA2* expression results in FGR via a negative impact on placental growth (Jensen *et al*. 2014).

Given the role of *PHLDA2* in the control of fetal growth, it has been suggested that aberrant placental *PHLDA2* expression could serve as a biomarker to identify infants at risk of poor perinatal outcomes (Jensen *et al*. 2014). This is the first study to report on the relationship between placental *PHLDA2* expression and measures of infant wellbeing at delivery. No significant association was demonstrated between placental *PHLDA2* expression and fetal distress during delivery, umbilical cord blood pH or apgar scores. Similarly, although placental *PHLDA2* expression was almost three fold higher in infants admitted to NICU this difference was not statistically significant. Thus, the findings of the current study do not support the use of placental *PHLDA2* as a biomarker to identify at risk infants.

Based on the inverse association observed between placental *PHLDA2* expression and measures of fetal growth, it was further hypothesised that decreased expression was associated with cases of fetal overgrowth resulting in the birth of an LGA or macrosomic infant. Indeed, placental *PHLDA2* expression was significantly reduced in LGA pregnancies of the Manchester Cohort. This novel result suggests that alterations in *PHLDA2* expression may underlie complications of pregnancy associated with both extremes of birth weight.

Placental *PHLDA2* expression was also hypothesised to be altered in preeclamptic pregnancies based on the increased prevalence of FGR in these pregnancies and the development of preeclampsia-like symptoms as a result of loss of function of *Cdkn1c* (in the same imprinting cluster) in a mouse model (Takahashi *et al*. 2000; Kanayama *et al*. 2002). The current study demonstrated significantly increased *PHLDA2* in preeclamptic placentas, supporting previous reports by McMinn *et al*. (2006). In addition, these previous findings were expanded upon, with increased placental *PHLDA2* expression demonstrated in preeclamptic pregnancies with and without the presence of SGA. These novel results are the first to suggest that

placental *PHLDA2* may be involved in the pathogenesis of preeclampsia, independent of any effects on fetal growth.

It has been proposed that imprinted genes, such as *PHLDA2*, regulate fetal growth through control of mechanisms driving maternal adaptations to pregnancy (Haig 1993), such as placental hormone production (John 2013). Indeed, *Phlda2* is known to regulate the endocrine lineage of the mouse placenta (Tunster *et al*. 2010), specifically the placental lactogens. This study provides the first evidence of an inverse relationship between placental expression of *PHLDA2* and the placental lactogen *hPL*, a hormone known to control fetal growth through alterations to maternal metabolism during pregnancy. Consistent with this finding, placental *hPL* expression was significantly positively correlated with birth weight.

Inadequate adaptation of maternal metabolism to pregnancy may result in gestational diabetes, defined as glucose intolerance arising during pregnancy (Newbern and Freemark 2011). As placental *PHLDA2* expression was demonstrated to be inversely associated with *hPL* expression, a hormone known to prevent glucose intolerance during pregnancy, gene expression was analysed in a small set of GDM placentas. A non-significant two fold increase in placental *PHLDA2* expression in GDM pregnancies was demonstrated, specific to women with a high BMI. No concurrent change in placental *hPL* expression was observed. As GDM participants were compared with BMI matched controls, this suggests that the increased placental *PHLDA2* expression observed in GDM placentas was not due to any association with maternal BMI. Further research is required to confirm these interesting but preliminary findings of aberrant placental *PHLDA2* expression in GDM placentas.

Thus, the current study provided new evidence of aberrant placental *PHLDA2* expression in pregnancies complicated by FGR and PE as well as preliminary support for a possible alteration in GDM pregnancies. Therefore, identifying environmental factors responsible for altered placental *PHLDA2* expression is of chief importance as these may be amenable to intervention. This study uniquely investigated the effect of maternal lifestyle factors such as smoking, alcohol consumption, exercise and diet on human placental *PHLDA2* expression. Two maternal lifestyle factors were demonstrated to

increase *PHLDA2* expression, caffeine and a Western diet. Although, no effect of maternal caffeine consumption was observed in AGA pregnancies, placental *PHLDA2* expression was significantly increased in response to caffeine consumption in SGA pregnancies. Interestingly, these infants were also 206g smaller than SGA infants born to mother with lower caffeine consumption, although this difference was not statistically significant.

Principle component analysis revealed a Western diet pattern characterised by consumption of high fat snacks, high sugar snacks, processed meat and soft drinks and low intake of fruit, vegetables and supplements. As with caffeine, there was no effect of a maternal western diet on placental *PHLDA2* expression in AGA pregnancies. However, placental *PHLDA2* expression was significantly increased in response to a maternal Western diet in SGA pregnancies. In these pregnancies, birth weight was also significantly inversely associated with maternal western diet scores. Similarly, Knudsen *et al.* (2008) have previously demonstrated an increased risk of SGA in mothers consuming a high fat Western diet during pregnancy. Two potential explanations were proposed to interpret the novel findings of the current study. Firstly, it is possible that placental *PHLDA2* expression drives fetal growth restriction caused by environmental factors (such as increased caffeine consumption or a western diet). It is also possible that an adverse maternal environment exacerbates fetal growth restriction, via an increase in placental *PHLDA2* expression, in already compromised pregnancies. Importantly with respect to possible interventions, a maternal diet characterised by high intake of fruit and vegetables, unprocessed meat, fish and meat alternatives and a low intake of caffeine, dairy products and carbohydrates (the healthy diet pattern) was not significantly associated with birth weight or placental *PHLDA2* expression. This suggests that the healthy diet pattern was not protective of aberrant placental *PHLDA2* expression and fetal growth restriction. In summary, these novel results are the first to suggest that human placental *PHLDA2* expression may be altered by maternal caffeine consumption or a western diet pattern. Animal models will be essential in determining the mechanisms underlying these associations.

Finally, a mouse model was used to examine the effects of maternal diet on fetal growth and imprinted gene expression, independent of other

lifestyle factors. Maternal low protein diet resulted in significantly increased placental *Phlda2* expression at two time points (E13.5 and E18.5) although no effect on fetal or placental weights was observed. Interestingly, this effect was significant only in female placentas. To the author's knowledge, no previous study has analysed placental *Phlda2* expression in response to a low protein diet. These results are therefore the first to show that low protein diet may alter placental *Phlda2* expression. As *Phlda2* expression in the kidney was not significantly altered this suggests a placental specific response to a maternal diet. Human placental *PHLDA2* expression was increased in mothers reporting consuming protein rich foods less than once a day, however this increase was modest and non-significant. Further research using a FFQ specifically designed to assess protein intake, will aid in determining the relevance to humans, of the observed increase in mouse placental *Phlda2* expression in response to a low protein diet.

Maternal high fat diet in a mouse model also resulted in significantly increased placental *Phlda2* expression, although no associated effects on fetal or placental weight was observed. In contrast, there was no significant effect of maternal high fat – high sugar diet on placental *Phlda2* expression, suggesting a specific effect of increased fat consumption. This study is the first to provide evidence of increased placental *Phlda2* expression in response to a high fat diet in an animal model. Importantly, these results aid interpretation of observations on maternal lifestyle effects on human placental gene expression. While a maternal western diet (characterised by increased consumption of high fat foods) was associated with increased human placental *PHLDA2* expression, it is not possible from human studies alone to establish causation. The increased placental *Phlda2* expression observed as a result of a high fat diet in the mouse model, suggests that the association between a maternal western diet in humans and aberrant placental *PHLDA2* expression may be causative.

In summary, the findings of the current study support previous reports of a role for placental *PHLDA2* in negatively regulating fetal growth. In addition, this study uniquely contributes to the current knowledge of *PHLDA2* in a number of ways. Novel findings include evidence supporting a role for *PHLDA2* in symmetric FGR, placental growth and placental hormone

production. In addition, aberrant placental *PHLDA2* expression was demonstrated for the first time in LGA pregnancies and in PE pregnancies with or without FGR. Finally, this study demonstrated a previously unreported association between human placental *PHLDA2* expression and maternal caffeine consumption and a maternal western diet, with work in a mouse model further highlighting an effect of a maternal low protein and high fat diet. Given the potentially large number of SGA pregnancies caused by aberrant placental *PHLDA2* expression, interventions aimed at preventing adverse lifestyles associated with altered expression will be of paramount importance.

7.1.2. *CDKN1C*

There was no significant association between placental *CDKN1C* expression and birth weight or custom birth weight centiles in the two independent cohorts examined, after controlling for mode of delivery. Diplas *et al*. (2009) similarly reported no significant difference in placental *CDKN1C* expression in IUGR placentas. However, in general, previous studies of human placental *CDKN1C* expression in relation to fetal growth have yielded conflicting results (McMinn *et al*. 2006; Diplas *et al*. 2009; Rajaraman *et al*. 2010). The current study demonstrated a significant difference in placental *CDKN1C* expression between labouring and non-labouring placentas and therefore proposes that conflicting results in previous studies may have arisen due to failure to control for mode of delivery. In conclusion, this study does not support a role for placental *CDKN1C* in the control of human fetal growth. Although *Cdkn1c* over expression is associated with fetal and postnatal growth retardation in a mouse model (Andrews *et al.* 2007), it is possible that this represents a mouse-specific effect or that the association in humans is relatively modest such that it cannot be detected in a cohort of this size.

Imprinted genes have been proposed to regulate the spongiotrophoblast cells of the mouse placenta (John 2013). The spongiotrophoblast cells serve as the endocrine lineage of the placenta,

producing hormones such as placental lactogens. *Cdkn1c* over expression in a mouse model negatively affects the spongiotrophoblast cells and decreases expression of placental lactogens. This study provides the first evidence that this may also be true in humans, with placental *CDKN1C* expression inversely correlated with *hPL* expression.

Little is known about whether placental *CDKN1C* is responsive to environmental stimuli. In this study, placental *CDKN1C* expression was demonstrated to be significantly increased in overweight and obese participants. Moore *et al*. (2015) have previously reported no significant correlation between maternal weight and placental *CDKN1C* expression, although maternal BMI was not analysed. Thus, these novel findings provide the first evidence of an association between raised maternal BMI and aberrant *CDKN1C* expression.

In summary, the findings of the current study do not support a role for placental *CDKN1C* in the control of fetal growth. This study does however uniquely contribute to the current knowledge of *CDKN1C*, demonstrating an association between maternal BMI and placental *CDKN1C* expression.

7.1.3. *PEG3*

Placental *PEG3* expression was not significantly associated with birth weight or custom birth weight centiles in two independent cohorts. This is in line with four previous studies demonstrating no significant association between placental *PEG3* expression and measures of fetal growth (McMinn *et al*. 2006; Diplas *et al*. 2009; Kumar *et al*. 2011; Moore *et al*. 2015). Thus, the findings of the current study do not support a role for *PEG3* in the control of human fetal growth. Although embryo weight was decreased (by 10%) in a mouse model of *Peg3* loss of function (Li *et al*. 1999; Kim *et al*. 2013), it is possible that the relatively small effect observed on fetal growth cannot be detected in a cohort of this size.

Placental *PEG3* expression was however demonstrated to be significantly decreased in mothers with increased symptoms of prenatal depression or diagnosed depression during pregnancy in two independent cohorts. *Peg3* has been demonstrated in a mouse model to be required for

the induction of nurturing and nest building behaviour in rodents (Li *et al*. 1999; Curley *et al*. 2004; Champagne *et al*. 2009; Chiavegatto *et al*. 2012). The novel results reported in the current study are the first evidence that placental *PEG3* expression is associated with maternal mood in humans. This is consistent with the hypothesis that imprinted gene expression, via regulation of placental hormone production, may contribute to maternal psychological adaptation to pregnancy with inadequate adaptation manifesting as maternal mood disorders.

Placental *hPL* expression was also demonstrated for the first time to be significantly decreased in mothers with prenatal depression in the two cohorts. The lactogenic hormone hPL is key to the induction of maternal behaviour in animals (Bridges *et al*. 1985; Bridges *et al*. 1990; Bridges and Freemark 1995; Bridges *et al*. 1997) and maternal serum levels been demonstrated to be reduced in mothers with symptoms of postnatal depression (Abou-Saleh *et al*. 1998; Ingram *et al*. 2003; Groer and Morgan 2007. In the current study, a significant positive correlation was observed between placental *PEG3* and *hPL* expression in the larger of these two cohorts, consistent with the reduction in placental lactogen expression observed in the mouse model of *Peg3* loss of function (Broad and Keverne 2011; Kim *et al*. 2013).

These novel results, combined with data from previous studies in animal models, suggest that placental *PEG3* expression contributes to the induction of maternal psychological adaptation to pregnancy, via control of placental hPL production. However, *PEG3* is also known to be responsive to environmental stimuli. For example, placental *Peg3* has been demonstrated to be increased in response to maternal undernutrition (Radford *et al*. 2012) but decreased in response to transient maternal starvation (Broad and Keverne 2011). Similarly, this study demonstrated increased placental *Peg3* expression in response to maternal low protein diet during pregnancy and a high fat – high sugar diet before pregnancy in a mouse model. It is therefore also possible that placental *PEG3* and subsequent placental hormone production is responsive to maternal depression (or alterations in maternal lifestyle associated with depression) thereby establishing a cycle of aberrant placental gene expression, placental hormone production and increased

symptoms of maternal depression. Further studies on larger cohorts, including for example measures of maternal care such as mother-infant bonding, are required to corroborate the findings of the current study while additional animal studies will be important in further establishing cause and effect relationships.

Although anxiety and depression are co-morbid, placental *PEG3* and *hPL* expression was not significantly altered in women with high trait anxiety symptoms. O'Donnell *et al*. (2012) and Blakeley *et al*. (2013) similarly reported distinct effects of maternal anxiety and depression on placental gene expression. It is therefore possible that placental *PEG3* and *hPL* expression is differentially associated with maternal depression and anxiety, although this remains to be confirmed in a larger cohort.

7.1.4. *PEG10*

Results from two independent cohorts support a role for placental *PEG10* in the positive regulation of fetal growth, albeit with different lines of evidence. In the Manchester Cohort placental *PEG10* expression was significantly positively associated with birth weight and expression significantly decreased in SGA pregnancies. This is consistent with the positive correlation reported between umbilical cord *PEG10* expression and birth weight (Lim *et al*. 2012). Moreover, this study provides the first evidence to suggest that placental *PEG10* expression may play a role in asymmetric growth restriction. Although not significant, *PEG10* expression was reduced only in asymmetric SGA placentas. Placental *PEG10* expression was also significantly associated with ultrasound measures of fetal growth including abdominal circumference and femur length. In contrast placental *PEG10* expression was not significantly associated with ultrasound measurement of head circumference or with head circumference at delivery. This is consistent with a role for placental *PEG10* expression in asymmetric growth restriction, which is characterised by reduced length and abdominal circumference but relative sparing of the head. These novel results are intriguing but require further replication in a larger cohort.

While placental *PEG10* expression was not significantly reduced in SGA pregnancies of the Wales Cohort, additional lines of evidence did suggest a role for placental *PEG10* in the positive regulation of fetal growth. In this cohort, placental *PEG10* expression was significantly increased in LGA and macrosomic placentas; these novel results provide the first evidence to suggest a role for aberrant placental *PEG10* expression in pregnancies complicated by fetal overgrowth. Discrepancies between the two cohorts may arise for a number of reasons such as different proportions of AGA, SGA and LGA pregnancies in the two cohorts, differences in the control population to which gene expression is compared and/or that the Manchester Cohort is comprised of a population of pregnancies also complicated by reduced fetal movements. Although demonstrated in different ways in the two cohorts, this study provides the first evidence of a positive association between placental *PEG10* expression and fetal growth, specifically growth of the femur and abdomen during pregnancy. Furthermore, this association is suggested to be causal given the growth retardation observed in the mouse model of *Peg10* loss of function (Ono *et al.* 2006).

Placental *PEG10* expression was also demonstrated to significantly positively correlated with apgar scores at 1 and 5 minutes after delivery. These novel results may be explained by the positive association demonstrated between placental *PEG10* expression and fetal growth, with infants reaching their full growth potential expected to exhibit better outcomes at delivery. Further research is required to determine whether placental *PEG10* expression can be used as a biomarker to predict adverse outcomes at delivery.

Given the role of *PEG10* expression in the control of fetal growth, it is important to identify maternal lifestyle factors associated with aberrant placental *PEG10* expression, as these may be amenable to intervention. However, few studies have examined whether placental *PEG10* is responsive to environmental stimuli. In this study, placental *PEG10* expression was demonstrated for the first time to be significantly increased in response to a carbohydrate rich diet in the mother. The increased placental *PEG10* expression observed may underlie the positive association between

frequency of consumption of carbohydrate-rich foods and custom birth weight centiles demonstrated in this study. In addition, maternal low protein diet resulted in decreased placental *Peg10* expression and a maternal high fat – high sugar diet associated with increased placental *Peg10* expression in a mouse model. These novel results suggest that *PEG10* expression is responsive to maternal diet in both the human and mouse placenta.

In summary, the findings of the current study support a role for human placental *PEG10* in the positive regulation of fetal growth. In addition, this study contributes to the current knowledge of *PEG10*, demonstrating a specific association with fetal abdominal and femur growth and highlighting a role for aberrant *PEG10* expression in asymmetric growth restriction. Finally, placental *PEG10* expression was uniquely demonstrated to be responsive to environmental stimuli, with expression significantly associated with maternal diet in humans (specifically carbohydrate consumption) and in a mouse model (both low protein and a high fat – high sugar diet).

7.2. Strengths and limitations

One of the strengths of this study was the thorough optimisation of the placental dissection protocol. No guidelines currently exist on placenta sampling, which would ensure consistency between studies (Burton *et al*. 2014). In addition, a number of factors including time to sampling, sampling site, mode of delivery and fetal sex are known to affect placental gene expression (Burton *et al*. 2014). This study demonstrated that placental samples taken within two hours of delivery were of sufficient quality for qPCR analysis in line with previous reports (Fajardy *et al*. 2009; Avila *et al*. 2010). For the first time, placental *PHLDA2* expression was demonstrated to vary significantly according to placental sampling site, with increased expression observed at the distal edges of the placenta compared with sites closer to the umbilical cord. This finding highlights the importance of consistency in placental sampling and most likely has implications for the design of future studies. Another novel finding was that of increased placental *CDKN1C* expression in labouring compared with non-labouring placentas. This result

may explain conflicting results previously reported with respect to placental *CKDN1C* expression and fetal growth (McMinn *et al*. 2006; Diplas *et al*. 2009; Rajaraman *et al*. 2010) and has implications for the interpretation of results in future studies. Thus, the dissection protocol optimisation carried out as part of this study ensured that good quality, consistent and comparable gene expression data was obtained from the placentas sampled.

Another advantage of the current study was the wealth of information available on the study participants. Clinical information necessary for interpretation of the study results was provided, including parity, gestational age, ethnicity, prescribed medication and mode of delivery, as specifically recommended for studies of the human placenta (Nelson and Burton 2011). This comprehensive characterisation of the study cohort enables identification of potential confounding factors and therefore strengthens study results (Nelson and Burton 2011).

The use of custom birth weight centiles in the identification of growth restricted pregnancies was another strength of this study. Custom birth weight centiles are adjusted for maternal height, weight, ethnicity, parity, fetal sex and gestational age and therefore provide an indication of the optimal growth potential of each individual fetus (Gardosi 1992). Use of custom birth weight centiles has been demonstrated to better identify infants at risk of adverse outcomes (De Jong *et al*. 1998; Clausson *et al*. 2001; Gardosi 2009). Previous studies of imprinted genes and fetal growth have used population growth centiles or birth weight as the outcome measure. Using custom birth weight centiles in the current study uniquely identified a role for aberrant imprinted gene expression in pregnancies complicated by pathological growth restriction rather than constitutional smallness.

Finally, another advantage in the analysis of maternal lifestyle effects on placental gene expression was that only Caucasian women were included in this part of the study. This was done so as to limit possible genetic variation (in comparison to ethnically diverse populations) and therefore focus on environmental factors and their effects on placental imprinted gene expression and fetal growth. This is particularly important given that differences in culture may impact on maternal lifestyle during pregnancy and the known ethnic differences in birth weight and pregnancy complications.

Furthermore, a recent study analysed SNPs in Caucasian participants in different regions of the UK and demonstrated a distinct genetic cluster in Wales, differentiating Wales from the rest of the UK (Leslie *et al.* 2015). In light of these findings and given that 78% of participants in the current study were Caucasian-Welsh, it is likely that genetic diversity in this population was further reduced.

There are however a number of limitations to this study which should be considered. Firstly, although described as strength, including only Caucasian participants in the study of maternal lifestyle effects on placental gene expression could limit the relevance of these findings to other ethnic groups. Studies of large populations with greater ethnic diversity will aid in determining whether the findings reported underlie complications of pregnancy in other ethnic groups.

Another limitation of the current study is that only one housekeeping gene was used for normalisation of placental target gene expression data. It has been argued that due to intrinsic variation in housekeeping gene expression, use of a single housekeeping gene can result in inaccuracies in expression data normalisation (Vandesompele *et al.* 2002). Therefore, it has been suggested that target gene expression should be normalised to the average expression of three housekeeping genes (Vandesompele *et al*. 2002). However, it should be noted that the housekeeping gene used in the current study, *YWHAZ*, has been demonstrated in a number of studies to be stably expressed in the human placenta of normal pregnancies (Meller *et al.* 2005; Cleal *et al.* 2009; Cleal *et al.* 2010) and in pregnancies complicated by IUGR (Murthi *et al.* 2008).

With respect to the participant questionnaire, another important study limitation is the reliance on maternal self-report data, particularly information on diet during pregnancy and symptoms of depression and anxiety. Therefore, where possible data from the participant questionnaire was compared with that in the participant medical notes (e.g. smoking, alcohol consumption and illegal drug use) and found to be highly correlated, as previously reported in other studies (Rice *et al.* 2007). Another limitation was that participant questionnaires were completed and returned by only 80% of the cohort such that lifestyle data is missing for a number of participants.

Importantly, it is not possible to know whether participants not completing the participant questionnaire were those with a particularly adverse lifestyle during pregnancy. Finally, the FFQ used in the current study was non-quantitative and did not include as comprehensive a list of food items as FFQs used in previous studies of maternal diet during pregnancy, which typically include around 100 food items (for example Robinson *et al*. 1996; Moore *et al*. 2004; Rao *et al*. 2001). Use of dietary records would have provided more detailed, quantitative information on maternal diet during pregnancy, however this method is more time consuming and is associated with reduced return rates (Thompson and Subar 2008).

Another limitation, particularly with respect to analysis of placental imprinted gene expression in pregnancy complications (Chapter 4) is the relatively small sample size. For example, only 16 GDM placentas and 22 PIH/PE placentas were available for analysis. In particular, placental dissection rates were lower for these pregnancies complications (67% for GDM and 71% for PIH/PE pregnancies) compared with dissection rates in the overall cohort (84%). This may reflect the increased risk of maternal and fetal complications around the time of delivery in these pregnancies, such that placental sampling was not a key priority. As a result, caution must be taken when interpreting results from these samples and replication is required in a larger cohort.

Finally, use of placental *hPL* and *PGH* expression as a proxy for placental hormone production could be argued as another potential weakness. Koutsaki *et al*. (2011) have previously demonstrated differences between placental gene expression and maternal serum levels of these placental hormones, which was proposed to be due to posttranscriptional and/or posttranslational modifications. In contrast, it could be argued that placental gene expression is more constant than maternal serum levels, which may vary according to factors such as time of day, stress and maternal lifestyle. The associations observed in this study between placental imprinted gene and hormone gene expression must be further examined in a cohort where maternal serum levels of these hormones can be measured. This is of clinical relevance since levels of these hormones in the maternal blood may

serve as a biomarker for inadequate maternal adaptation to pregnancy, such as in cases of maternal depression.

7.3. Future work

There are a number of potential future studies arising from the findings of this thesis, which have been discussed in each individual chapter. In addition, there are three main areas of interest that warrant further investigation.

Firstly, fetal growth restriction is known to negatively impact on long term offspring health, increasing the risk of certain diseases in adulthood such as hypertension, cardiovascular disease and type II diabetes (Godfrey and Barker 2001). Interestingly, in the mouse model of *Phlda2* over expression, offspring are growth restricted at birth but show increased adiposity and impaired glucose tolerance in adulthood (from the thesis of S Tunster). This suggests that aberrant placental *Phlda2* expression causes fetal growth restriction and programmes an increased risk of disease in adult life. No study to date has analysed long term child outcomes resulting from human fetal growth restriction characterised by aberrant placental *PHLDA2* expression. Long term follow up of child health including measures of postnatal catch up growth, glucose tolerance and adiposity will determine whether aberrant placental *PHLDA2* expression at birth can predict future risk of disease in later life. This would be beneficial in informing postnatal care of the child given that the increased risk of adult disease (such as type II diabetes) is greatest in growth restricted infants that subsequently grow up in a nutrient rich environment (Gluckman *et al.* 2005). Long term effects of the growth restriction resulting from *Peg10* loss of function have not been examined due to the embryonic lethality observed in this mouse model. However, given the role of placental *PEG10* in the positive regulation of fetal growth demonstrated in this study, analysis of long term child outcomes associated with aberrant placental *PEG10* expression may also be of interest.

Secondly, this thesis suggests a possible role for imprinted genes in maternal adaptation to pregnancy. Inadequate adaptation to pregnancy may

result in lasting effects on the mother. For example, a number of studies have demonstrated that mothers carrying SGA infants or suffering from preeclampsia during pregnancy are at an increased risk of cardiovascular disease in later life (Bukowski *et al*. 2012; Pariente *et al*. 2013; Catov *et al*. 2011; Ngo *et al*. 2015; Hutcheon *et al*. 2011). Similarly, women suffering from gestational diabetes during pregnancy are at an increased risk of developing type II diabetes after pregnancy (Hartling *et al*. 2013). It is therefore of interest to follow up not just the infants of pregnancies characterised by aberrant imprinted gene expression but also their mothers. This will establish whether placental gene expression at delivery can predict future maternal disease risk and thereby inform postnatal care. This may be particularly relevant in the case of maternal mood disorders. In the current study placental *PEG3* and *hPL* expression were demonstrated to be significantly reduced in pregnancies complicated by maternal prenatal depression. Follow up of mothers in the postnatal period will determine whether placental *PEG3* and *hPL* expression can predict the subsequent development of postnatal depression.

Finally, a particular area of interest is the development of biomarkers for fetal growth restriction in order to enable early detection, decrease rates of false FGR diagnosis and to provide optimal prenatal care of the pregnant mother. This is similarly true for other complications of pregnancy including preeclampsia, gestational diabetes and maternal mood disorders. The finding of altered imprinted gene and placental hormone gene expression in these pregnancy complications, suggests a possible foundation on which to further explore development of a biomarker. Although, Demetriou *et al*. (2014) reported no significant correlation between *PHLDA2* expression in first trimester chorionic villus samples and birth weight at delivery, this cohort did not examine prediction of other pregnancy complications and therefore does not discount the possibility of using placental imprinted gene expression in chorionic villus samples in the early detection of pregnancy complications such as PE/PIH and GDM. Moreover, results from this thesis support the proposed regulation of placental hormone production by imprinted genes (John 2013). Future studies examining placental imprinted gene expression in relation to maternal serum hormone levels will aid in determining whether

serum levels of placental hormones can be used as a biomarker for aberrant placental imprinted gene expression and therefore predict complications of pregnancy.

7.4. Concluding remarks

In conclusion, this thesis provides new evidence for important but differing roles of the imprinted genes *PHLDA2*, *PEG3* and *PEG10* in the control of fetal growth and maternal adaptation to pregnancy. The data presented supports negative regulation of human fetal growth by placental *PHLDA2* expression and positive regulation by placental *PEG10* expression, uniquely expanding on the current understanding of these associations. In contrast, the current study did not support a role for *PEG3* in the control of human fetal growth but instead suggested an involvement of this gene in maternal adaptation to pregnancy with aberrant gene expression observed in pregnancies complicated by maternal depression. Furthermore, this study provided evidence that the imprinted genes *PHLDA2*, *CDKN1C*, *PEG3* and *PEG10* are responsive to environmental stimuli, in particular maternal diet, in both human pregnancies and in a mouse model. Thus, this thesis highlights the importance of imprinted genes in achieving a successful pregnancy for both mother and fetus and the possible role of maternal lifestyle in influencing this.

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Appendix 1: Participant Information Sheet

Participant Information Sheet

Version 4.1
Date: 14.05.13
REC ref no. 10/WSE02/10

Study title: Examining imprinted gene expression in human placenta.

Part 1 of information sheet

Invitation paragraph:

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will give you the information sheet and answer any questions you have. We'd suggest this should take about 10 minutes. Talk to others about the study if you wish.

Ask us if there is anything that is not clear.

What is the purpose of the study?

We want to look at the factors in the placenta that effect growth of the baby. We know of a group of genes (instructions) that are important for building the placenta. If these "instructions" are incorrect or misread, then normal growth and development does not happen. We will look at these instructions in a large number of placentae, including yours, so that we can understand them better. This will help us predict which pregnancies might need closer monitoring and more support from our Obstetrics team to ensure a safe delivery and a long and healthy life for babies born in the future.

What is the placenta?

The placenta is the afterbirth. After your baby is born, more contractions push the placenta out through the vagina. The placenta at this stage is also called the afterbirth. Normally it is destroyed.

What does the placenta do?

The placenta is the organ that links you to your baby via the umbilical cord. The job of the placenta during pregnancy is to pass oxygen and food from your blood supply to the baby. Waste products from the baby, such as carbon dioxide, are returned along the umbilical cord back to the placenta and then into your bloodstream.

Why do we need to collect the placenta?

Following the birth of your baby your placenta is normally destroyed. By collecting placentae and studying them, we can hope to learn what is needed for a normal, healthy pregnancy and what may cause a difficult pregnancy. For example, we would like to learn more about why some babies are born smaller or larger than average or why some women develop pre-eclampsia or gestational diabetes.

Why have I been invited?

Any pregnant women can participate. As of April 2007 a new legislation came into effect (The EU Directive for Tissue and cells), which states that consent for the use and testing of your placenta must be obtained prior to the birth of your baby or before labour is so advanced that you do not have time to make an informed decision(which includes active labour).

This ensures that you have had plenty of time to ask us any questions you have relating to the collection, testing and storage of your placenta. We encourage you to make an informed decision about donating your placenta.

Do I have to take part?

It is up to you to decide to join the study. We describe the study in this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

What will happen to me if I take part?

You will be asked to complete a short questionnaire about your pregnancy. Any questions that you feel unhappy answering can be left blank. This information will be given a unique identification number so that your details are kept confidential.

Once your baby has been born, your midwife will confirm that consent has been given and will pass your placenta to our staff, along with your signed consent form. Small pieces of the placenta are then placed in collection tube. We need to collect only a few small pieces the size of a thumb tip. The rest will be destroyed by the normal Hospital procedures.

Your donation is given a unique identification number. All the information provided to us uses this number only. Your details are therefore kept confidential. We will not know your name or address, just the babies weight and a few details about your pregnancy i.e head and abdominal measurements of baby and any medical problems you have experienced. We will collect the details about the pregnancy and newborn at the time of birth and then the details will be anonymised therefore we will not be able to collect any further information and can not identify you after wards.

We expect study to last approximately 1 year.

Expenses and payments?

Your placental sample will be collected at the time of birth. Therefore it will not cost you any money. We do not pay for your donation.

What are the side effects of any treatment received when taking part?

The procedures we use to collect a placental sample are safe and risk-free for both you and your baby. This is because the collection of the placenta happens AFTER the baby is born, AFTER the cord is cut and AFTER the placenta has been delivered. Donating samples of your placenta does not interfere with the management of labour and delivery, or with the aftercare of you or your baby.

What are the possible disadvantages and risks of taking part?

There are no disadvantages of taking part in the research as we will only take the sample from the placenta after the delivery of placenta before it is sent for destroying destruction as per hospital policy.

What are the possible benefits of taking part?

We cannot promise the study will help you in current pregnancy, but the information we get from this study we can learn what genetic activity in the placenta is needed for a normal, healthy pregnancy and what may cause a difficult pregnancy. For example, we would hope to learn more about why some babies are born smaller or larger than average or why some women develop pre-eclampsia or gestational diabetes.

What if there is a problem?

Any complaint about the way you have been dealt with during the study you will be addressed. The detailed information on this is given in Part 2.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

<u>Part 2 of the information sheet</u>

What if relevant new information becomes available?

As this study is anonymous we will not be able to contact you.

What will happen if I don't want to carry on with the study?

If you withdraw from the study by just informing your midwife and we will not use your placental sample for the study. You can withdraw from the study before the placenta is passed on to the research team, after which the samples will not be identifiable.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (contact number at the end). If you remain unhappy and wish to complain formally, you can do this [e.g. NHS Complaints Procedure].

What will happen to any samples I give?

The placenta will be stored for a short time (weeks) and then we will prepare RNA and DNA (in order to look at the genes) from it. This will be stored for the duration of the study. These samples will be anonymous and we cannot identify you or your baby from them.

What If a participant, who has given informed consent, loses capacity to consent during the study?

No further data or tissue will be collected once the placental samples have been taken. It will not be possible to identify the tissue related to the participant if she loses the capacity to consent during the study after the samples have been taken. If between giving consent and the donation of the tissue sample, the woman looses capacity to consent she will be withdrawn from the study and placental tissue samples will not be taken. as the samples will be anonymised after they have been taken.

What will happen to the results of the research study?

This is an anonymous study. We will not know who you are. We will not have your name or address. This means we cannot contact you to tell you anything about your placenta. We aim to publish the results of our research in scientific journals, and will also provide feedback via the hospital newsletter, and local media.

Who is organising and funding the research?

University Hospital Wales & University of Cardiff (Biosciences)

Who has reviewed the study?

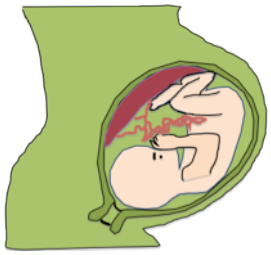
All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by South East Wales Research Ethics Committee.

6.2.11 Further information and contact details

Mr R Penketh (Consultant Obstetrician & Gynaecologist)

Email: Richard.Penketh@wales.nhs.uk

Appendix 2: Participant Consent Form



Centre Number:

REC reference number: 10/WSE02/10

CONSENT FORM VERSION 4

Date: 15.12.11

Title of Project: Examining imprinted gene expression in human placenta.

Name of Researcher

Please initial box

1. I confirm that I have read and understand the information sheet dated **15.12.11 (version 4)** for the above study, have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that only relevant sections of my medical notes and data collected during the study may be looked at by research team members, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in research. I give permission for these individuals to have access to my records, but the data retrieved will be anonymised. ☐

4. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person taking consent	Date	Signature

When completed: 1 for participant; 1 for researcher site file: 1(original) to be kept in medical notes.

Appendix 3: Participant Questionnaire

Study ID:

Date:

Participant Questionnaire

Version 1

15.12.11

REC Ref no: 10/WSE02/10

Study of Imprinted Gene Expression in the Placenta

Thank you for answering these questions. It would help us if you could answer all the questions, however if you feel unhappy about answering any of them, please feel free to leave them blank. All information is kept in the strictest confidence and will not affect the standard of care you receive.

Where known, please provide us with the answers to the following questions :

About You

- **To which of these ethnic groups do you consider you belong?** *(Please tick)*

  Caucasian (White) ☐ Indian/Pakistani/Bangladeshi ☐ Middle Eastern ☐

  Far Eastern ☐ African/Afro-Caribbean ☐

  South American/Hispanic ☐ Mixed (*please specify*) ☐ ……

  Do not wish to say ☐ Other *(Please specify)* ☐ ……

- **What is your country of birth?** *(Please tick)*

  Wales ☐ England ☐ Ireland ☐ Scotland ☐ Other ☐

- **What is your age?** …….....

- **What was your weight before pregnancy?** ……..... **kg or** ……..... **lb**

- **What is your height?** ……..... **m or** ……..... **ft**

- **Towards term (37 weeks), we will ask for your final weight** which will be recorded here by yourself or your midwife as ……..... **kg or** ……..... **lb** **at** …….. **weeks**

- **Do you know your birthweight?** *(Please tick)*

  Yes ☐ it was ……..

  No ☐

- **What is your highest level of education?** *(Please tick)*

  Left before GCSE ☐ GCSE/O levels ☐ A levels ☐ University ☐

About your family

- **To which of these ethnic groups does your baby's father belong?** *(Please tick)*

  Caucasian (White) ☐ Indian/Pakistani/Bangladeshi ☐ Middle Eastern ☐

  Far Eastern ☐ African/Afro-Caribbean ☐

  South American/Hispanic ☐ Mixed (*please specify)* ☐……

  Do not wish to say ☐ Other *(Please specify)* ☐……

- **What is your baby's father's country of birth?** *(Please tick)*

  Wales ☐ England ☐ Ireland ☐ Scotland ☐ Other ☐

- **What is your family income per year before deductions?** *(Please tick)*

  <£18,000 ☐ £18,000 - £25,000 ☐ £25,000 - £43,000 ☐

  >£43,000 ☐ Do not wish to say ☐

About your pregnancy

- **How many weeks pregnant are you today?** …….....

- **Is this pregnancy a natural or assisted conception?** *(Please tick)*

  Natural ☐ Assisted *(IVF)* ☐ Do not wish to say ☐

- **Did you smoke in the three months <u>before</u> you found out you were pregnant?**

  (Please tick) Yes (daily) ☐ Yes (Occasionally but not every day) ☐ No ☐

- **Have you smoked <u>during</u> your pregnancy?** *(Please tick)*

  During the **first 12 weeks**: Yes (daily) ☐ Yes (Occasionally but not every day) ☐

  No ☐

  After 12 weeks: Yes (daily) ☐ Yes (Occasionally but not every day) ☐

  No ☐

- **Did you drink alcohol in the three months <u>before</u> you found out you were pregnant?**

  Yes, almost every day ☐ Yes, once or twice a week ☐

  Yes, once or twice a month ☐ Yes, once every couple of months ☐ No, never ☐

- **Have you had alcohol <u>during</u> your pregnancy?**

  During the **first 12 weeks:**

  Yes, almost every day ☐ Yes, once or twice a week ☐

  Yes, once or twice a month ☐ Yes, once every couple of months ☐ No, never ☐

  After 12 weeks:

  Yes, almost every day ☐ Yes, once or twice a week ☐

  Yes, once or twice a month ☐ Yes, once every couple of months ☐ No, never ☐

- **Have you taken any illicit drugs during your pregnancy? Yes / No**

  If yes, what kind of drugs did you take? ……

- **Have you done any strenuous exercise during your pregnancy? Yes / No**

(Strenuous exercise could be for example: running, jogging, squash, swimming, aerobics, cycling or football done for at least 30 minutes, at least once a week)

After you found out you were pregnant, how often did you eat the following foods? For each food type, please tick one box that best describes how often you ate this food. If this is different to before you found out you were pregnant, please circle in the right hand column whether you ate more or less of this food before. If this was not different, please leave the right hand column blank.

Example: If you typically ate chocolate 4 times each week during your pregnancy (but ate less before you found you were pregnant) you would complete the row as shown:

	More than once/day	Once/day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Before you found out you were pregnant
Chocolate			✔			More / Less

Fruit and Vegetables

	More than once/day	Once/day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Before you found out you were pregnant
Fresh Fruit						More / Less
Dried fruit e.g. raisins, dried apricots						More / Less
Salad and Cooked vegetables						More / Less

Dairy and Carbohydrates

	More than once/day	Once/day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Before you found out you were pregnant
Milk						More / Less
Cheese, yoghurt						More / Less
Bread, cereals, potatoes, rice, pasta						More / Less

Meat, Meat alternatives and Fish

	More than once/day	Once/day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Before you found out you were pregnant
Meat alternatives e.g. beans, peas, tofu, soy						More / Less
Meat (unprocessed) e.g. steak, ham, chicken slices						More / Less
Meat (processed) e.g. Burgers, sausages, fried chicken						More / Less
Fish/shellfish						More / Less

Snacks

	More than once/day	Once/day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Before you found out you were pregnant
Chocolate						More / Less
Chips, Crisps						More / Less
Cakes, biscuits, ice cream						More / Less
Take away meals e.g. Chinese food, Curry						More / Less

	More than once/day	Once/day	2-3 times per/week	Once in 2 weeks	Never/ Rarely	Before you found out you were pregnant
Soft drinks						More / Less
Caffeine e.g. tea, coffee, power drinks						More / Less

Appendix 4: Data Collection Form

Study ID:

Mother Information: General		Mother Information: Current Pregnancy	
Maternal weight at booking	……. (week:) BMI ……. (week:)	**Prescribed medication (incl. week)**	
Maternal age at delivery		**Bleeding**	Yes / No (Week)
Mother Information: Obstetric History		**Concern over fetal growth**	Yes / No (Week)
Previous no. pregnancies		**IUGR/SGA Diagnosis**	Yes / No (Week)
Previous no. live births		**Ultrasound: Amniotic Fluid Index**	Normal / High / Low (Week)
Previous Stillbirth	Yes / No	**Ultrasound anomalies**	Yes / No
Previous LBW baby (<2.5kg)	Yes / No	**Doppler Anomalies**	Yes / No
Previous Macrocosmic baby (>4kg)	Yes / No	**Ultrasound: SGA centile**	Yes / No Centile (Week)
Illegal Drug use	Yes / No (Week)	**Ultrasound: LGA centile**	Yes / No Centile (Week)
Smoking	Yes / No (Week)		
Alcohol	Yes / No (Week)		

Current Pregnancy: Preeclampsia		Delivery Time	
Diagnosis PE	Yes / No (Week)	**Maternal Weight at Delivery**	(Week)
Previous PE Pregnancy	Yes / No (Week)	**Mode of Delivery (& Indication)**	
Hypertension	Yes / No (Week)	**Complications during Delivery**	
Proteinuria	Yes / No (Week)	**Baby Information**	
Treatment for PE	Yes / No (Week)	**Gestational Age**	
	Treatment:	**Sex of Baby**	
Current Pregnancy: Gestational Diabetes		**Arterial Cord Blood pH**	
Abnormal Glucose Tolerance Test	Yes / No (Week)	**Apgar Scores**	1 min……. 5 min …….
Diagnosis GDM	Yes / No (Week)	**Birthweight**	BW: Centile:
Previous GDM Pregnancy	Yes / No	**Head Circumference**	
Management	Diet Yes / No		
	Exercise Yes / No		
	Medication Yes / No		
Delivery Information			
Delivery Date			

Appendix 5: Placenta Proforma

Placenta Proforma

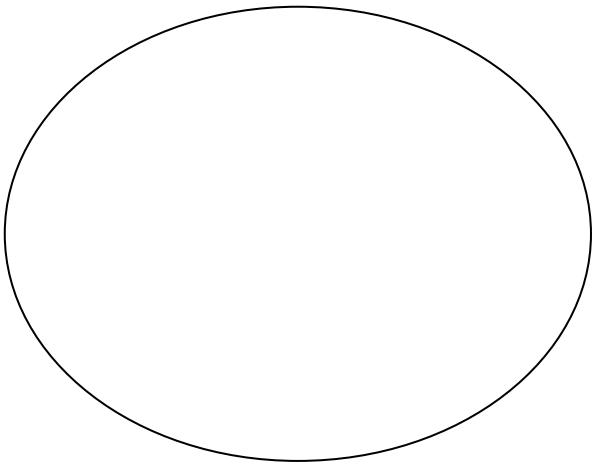
Study ID:

Date:

Time of Delivery: **Time dissection completed:**

Type of Delivery:

Please note sampling sites:



Total placental weight:

Gross placental abnormalities:

Date and time collected and stored in Biosi 3:

Appendix 6: STAI

DIRECTIONS: A number of statements which people have used to describe themselves are given below. Read each statement and then tick in the appropriate box on the right to indicate how you **generally** feel. There are no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe how you **generally** feel, even before pregnancy

		Almost never	Sometimes	Often	Almost always
1.	I feel pleasant	4	3	2	1
2.	I feel nervous and restless	1	2	3	4
3.	I feel satisfied with myself	4	3	2	1
4.	I wish I could be as happy as others seem to be	1	2	3	4
5.	I feel like a failure	1	2	3	4
6.	I feel rested	4	3	2	1
7.	I am "calm, cool and collected"	4	3	2	1
8.	I feel that difficulties are piling up so that I cannot overcome them	1	2	3	4
9.	I worry too much over something that really doesn't matter	1	2	3	4
10.	I am happy	4	3	2	1

11.	I have disturbing thoughts	1	2	3	4
12.	I lack self-confidence	1	2	3	4
13.	I feel secure	4	3	2	1
14.	I make decisions easily	4	3	2	1
15.	I feel inadequate	1	2	3	4
16.	I am content	4	3	2	1
17.	Some unimportant thought runs through my mind and bothers me	1	2	3	4
18.	I take disappointments so keenly that I can't put them out of my mind	1	2	3	4
19.	I am a steady person	4	3	2	1
20.	I get in a state of tension or turmoil as I think over my recent concerns and interests	1	2	3	4

Appendix 7: EPDS

Appendix X: Edinburgh Postnatal Depression Scale

Please UNDERLINE the answer which comes closest to how you have felt in the **past week**, not just how you feel today. Here is an example already completed:

I have felt happy:

Yes, all the time

Yes, most of the time

No, not very often

No, not at all

This would mean: I have felt happy most of the time in the past few days.

Please complete the other questions in the same way. Do not take too long over it and make sure you answer all the questions.

IN THE PAST WEEK

1. I have been able to laugh and see the funny side of things:

As much as I always could	0
Not quite so much now	1
Definitely not so much now	2
Not at all	3

2. I have looked forward with enjoyment to things

As much as I ever did	0
Rather less than I used to	1
Definitely less than I used to	2
Hardly at all	3

3. I have blamed myself unnecessarily when things went wrong

Yes, most of the time	3
Yes, some of the time	2
Not very often	1
No, never	0

4. I have been anxious or worried for no good reason

No, not at all	0
Hardly ever	1
Yes, sometimes	2
Yes, very often	3

5. I have felt scared or panicky for no very good reason

Yes, quite a lot	3
Yes, sometimes	2
No, not much	1
No, not at all	0

6. Things have been getting on top of me

Yes, most of the time I haven't been able to cope at all	3
Yes, sometimes I haven't been coping as well as usual	2
No, most of the time I have coped quite well	1
No, I have been coping as well as ever	0

7. I have been so unhappy that I have had difficulty sleeping

Yes, most of the time	3
Yes, sometimes	2
Not very often	1
No, not at all	0

8. I have felt sad or miserable

Yes, most of the time	3
Yes, quite often	2
Not very often	1
No, not at all	0

9. I have been so unhappy that I have been crying

Yes, most of the time	3
Yes, quite often	2
Only occasionally	1
No, never	0

10. The thought of harming myself had occurred to me

Yes, quite often	3
Sometimes	2
Hardly ever	1
Never	0