Investigating genetic factors associated with complications of pregnancy

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DECLARATION

Declaration of Originality

I, Charalambos Demetriou, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

This PhD project sets out to investigate the role of genetic factors associated with fetal growth restriction and recurrent miscarriage (RM), two of the most common complications of pregnancy. This work studied large cohorts of patients collected from specialist clinics in West London with the aim of better understanding their underlying molecular aetiology.

The first part of this project focused on the paternally expressed, maternally imprinted gene, *IGF2,* which is a key growth hormone critical for *in utero* growth in mice. Its role in human fetal growth has remained ambiguous, as it has only been studied in term tissues. mRNA expression levels of *IGF2* and other genes were investigated in 260 chorionic villus samples collected at 11-13 weeks' gestation. Transcript levels of *IGF2* revealed a significant positive correlation with birth weight (P=0.009). Critically, small for gestational age neonates had significantly lower *IGF2* levels than appropriate for gestational age neonates ($P=3.6x10^{-7}$).

Next a study was undertaken to investigate a potential role for disturbed imprinting in products of conception (POC). This work first involved a detailed analysis of the POC DNA to establish levels of maternal cell contamination. POCs could then be more accurately evaluated to investigate the status of known imprinted genes. Interestingly, in a number of POCs, known maternally expressed genes were found to be paternally expressed and vice versa. This suggested that some miscarriages might be associated with or even caused by abnormal imprinting.

Two approaches were then used to study genetic factors associated with RM. The first involved a genetic association study with a placental anti-coagulant protein Annexin A5 that contains four nucleotide substitutions (M2 haplotype) in its promoter. Patient and control haplotypes were determined and compared in 500 White European pairs that had RMs and 250 control trios. Carriers of the M2 haplotype were found to exhibit higher RM risk than

non-carriers, which was in agreement with previous studies. However, this is only true for the patients who suffered with early miscarriages.

The second study involved analysis of a single family where the patient had experienced a total of 29 miscarriages but had no successful pregnancies. Next-generation exome sequencing was carried on family members to search for a potential rare genetic variant gene causative of the RM phenotype. Two candidate genes with potentially damaging mutations were investigated in more depth by sequencing them in cohorts of Asian RM patients (n=100) and White European RM patients (n=120). In one of the genes, three novel variants and one very rare SNP, which were all predicted to be damaging by different prediction programs, were identified in a total of four Asian patients. Future studies to further investigate these potential mutations, involves functional analysis of each variant such as site-directed mutagenesis and protein-protein interactions.

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ABBREVIATIONS

A	aa	amino acids
	ABI	Applied Biosystems Inc
	aCL	anticardiolipin
	ACTB	beta-actin
	AF	allele frequency
	AGA	appropriate for gestational age
	AMEL	*Amelogenin*
	ANOVA	analysis of variance
	ANXA5	*Annexin A5*
	APC	activated protein C
	aPL	antiphospholipid antibodies
	APLS	antiphospholipid syndrome
B	BMI	body-mass index
	bp	base pair
	BWA	Burrows-Wheeler Aligner
	BWS	Beckwith-Wiedemann Syndrome
C	*CD46*	*Cluster of differentiation 46*
	cDNA	complementary DNA
	CI	confidence intervals
	CpG	cytosine phosphate diester guanine
	CRL	crown-rump length
	CT	cycle threshold
	CTCF	CCCTC-binding factor
	CVS	chorionic villus sample
D	DECP	Diethylpyrocarbonate
	DLK1	*Delta-like 1 homolog*
	DMR	differentially methylated region
	DNMT	DNA-methyltransferase
	dsDNA	double stranded DNA
E	EtOH	Ethanol
	EVS	exome variant server
	EVT	extravillous trophoblast
F	FGR	fetal growth restriction
	FKBP4	*FK506 binding protein 4*
	FVL	Factor V Leiden

G	g	gram
	GA	gestational age
	GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*
	GATK	Genome Analysis Toolkit
	GR	Glucocorticoid receptor
H	HATS	histone acetyltransferases
	HEK	Human embryonic kidney
	HLA	Human leukocyte antigen
	Hsp90	heat shock protein
	HWE	Hardy-Weinberg equilibrium
I	iCGi	intronic CpG island
	ICR	imprinting control region
	IGF1 / IGF2	*Insulin growth factor 1 / 2*
	IGF1R / IGF2R	*Insulin growth factor 1 / 2 receptor*
	IGFBP	insulin-like growth factors binding protein
	IUGR	intrauterine growth restriction
K	*KCNQ1*	*potassium voltage-gated channel, KQT-like subfamily, member 1*
	KIR	killer-cell immunoglobulin-like receptors
L	LA	lupus anticoagulant
	LBW	low birth weight
	LD	linkage disequilibrium
	LGA	large for gestational age
	LOI	loss of imprinting
M	MCC	maternal cell contamination
	MCMC	Monte-Carlo Markov Chain
	MCP	membrane cofactor protein
	MEG3	*Maternally expressed gene 3*
	µg	Microgram
	MHC	major histocompatibility complex
	MIRLET7D	*Micro RNA lethal 7d*
	miRNAs	MicroRNas
	µl	Microlitre
	mM	Millimolar
	ml	Millilitre
	M-MLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
	MTHFR	*Methylene tetrahydrofolate reductase*
N	NaAc	sodium acetate

	NGS	next-generation sequencing
	NK	natural killer
O	OR	odds ratio
P	PAI-1 / PAI-2	plasminogen activator inhibitor 1 / 2
	PBS	phosphate buffered saline
	PCOS	polycystic ovaries syndrome
	PCR	Polymerase chain reaction
	PE	phosphatidylethanolamine
	PEG3	*Paternally expressed gene 3*
	PHLDA2	*Pleckstrin homology-like domain, family A, member 2*
	POC	product of conception
	PPIase	peptidyl-prolyl *cis-trans* isomerase
	PR	P_4 receptor
	PRDX6	*Peroxiredoxin-6*
	PS	phosphatidylserine
R	RM	recurrent miscarriage
	RPL19	*Ribosomal protein L19*
	RT-PCR	reverse transcription PCR
	RTqPCR	real-time quantitative PCR
S	*SERPINB2*	*Serpin peptidase inhibitor clade B member 2*
	SGA	small for gestational age
	SLC22A18	*solute carrier family 22, member 18*
	SNP	single nucleotide polymorphisms
	SNV	single nucleotide variant
	SRS	Silver-Russell syndrome
	ssDNA	single stranded DNA
T	T2D	type 2 diabetes
	TPR	tetratricopeptide repeat
U	uNK	uterine NK
	UPD	uniparental disomy
	UTR	untranslated region
W	WES	whole exome sequencing
	WGS	whole genome sequencing
	WT	wild-type
Z	*ZFP57*	*Zinc finger protein 57*

CHAPTER 1 – INTRODUCTION

The research in this PhD investigates genetic factors associated with complications of pregnancy (Fig. 1-1), in particular fetal growth restriction (FGR) and recurrent miscarriage (RM), with the latter including both early and late miscarriage. The first part of the introduction focuses on normal fetal growth, FGR and a class of genes (imprinted genes) that are involved in fetal growth. The second part of the introduction describes miscarriage and the currently known causes of RM.

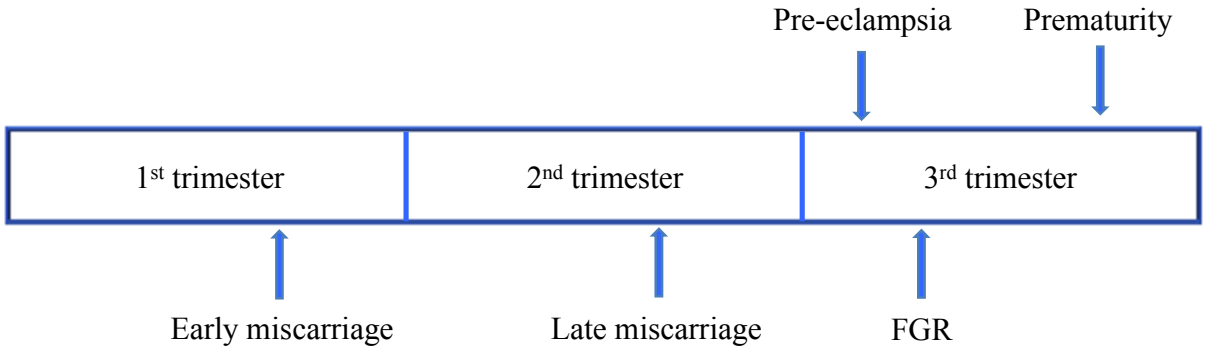


Figure 1-1: Stages of onset of common pregnancy complications.

1.1 Fetal growth and intrauterine restriction

1.1.1 Normal fetal growth

Growth is defined as the progressive increase in size. It is a complex process of multifactorial origin, as both genes and environment are involved. The normal growth of a fetus is dependent upon the balanced interaction between the mother, the fetus and the placenta, the latter being responsible for the blood flow and nutrient transportation from the mother to the fetus. Any deviation from this normal interaction can have a detrimental effect on fetal growth (Abu-Amero *et al.*, 2006).

The pattern of normal human fetal growth can be divided into three consecutive stages, often referred to as trimesters. The first trimester (weeks 0-12) is associated with rapid cell mitosis and thus an increase in cell numbers. At this stage, the fetus grows at approximately 5 g/day. The second trimester (weeks 13-27) includes hypertrophy and hyperplasia and during this stage the fetus has a growth rate of 15-20 g/day. The third trimester and final stage, which occurs from 28 weeks of gestation until term, has a fetal growth rate of 30-35 g/day and involves an increase in both cell number and size with accumulation of muscle, fat and connective tissues (Williams *et al.,* 1982; Pollack and Divon, 1992; Monk and Moore, 2004).

Each baby has a unique growth potential *in utero* due to both length of the pregnancy and physiological variations, which include the baby's gender, parity, ethnicity and maternal characteristics such as weight, height and smoking status as well as gestational age (GA). Therefore, all of these factors need to be taken into account when assessing fetal growth.

GA is considered to be the most influential marker of birth weight, and its accurate measurement is critical for the correct estimation of fetal birth weight centiles (Wilcox *et al.,* 1993). Gender also contributes to fetal growth variation, as on average, males are bigger in size and have a higher average birth weight of about 145 g than females (Hindmarsh *et al.,*

2002). It is also reported that the first pregnancy for a mother is on average 184 g smaller in birth weight than subsequent pregnancies (Hindmarsh *et al.,* 2002) therefore the influence of parity has also to be considered. This may be attributed to an improved uterine environment in subsequent pregnancies, such as a stretched uterine wall with a better circulatory system.

There is also variability in the mean birth weight among different ethnic groups. For example, the average birth weight of babies of mothers of Indian origin was found to be approximately 260 g smaller than those of European origin (Wilcox *et al.,* 1993). Fetal growth is further affected by maternal physical characteristics such as weight and height, with positive correlations being observed for heavier and taller mothers (Mongelli and Gardosi, 1995). Cigarette smoking during pregnancy has been associated with a reduction in birth weight in a dose dependent manner (Bernstein *et al.,* 2005; Cliver *et al.,* 1995). A reduction has been observed of approximately 12 to 27 g in birth weight for every cigarette per day consumed during the third trimester (Bernstein *et al.,* 2005; England *et al.,* 2001). The underlying mechanism is likely to include decreased oxygen delivery to the fetus due to increased carbon monoxide concentration and narrowing of the uterine blood vessels caused by nicotine (Pollack and Divon, 1992).

1.1.2 Fetal growth restriction

FGR (also known as intrauterine growth restriction, IUGR) is defined as the failure of a fetus to attain its expected growth potential at any gestational stage, and it is one of the most common phenotypes resulting from a disruption to the balanced interaction between the mother, the fetus and the placenta (reviewed in Abu-Amero *et al.*, 2006). Despite the established clinical significance of growth restriction, standard parameters used to describe its condition may still vary. FGR is commonly defined as an estimated birth weight being below the 10th percentile at a given GA (Resnik, 2002).

Although not synonymous, there is considerable overlap between the terms low birth weight (LBW), small for gestational age (SGA) and FGR. According to the World Health Organization, LBW is defined as a birth weight below 2500 g irrespective of the GA. SGA refers to the condition where the growth parameter of a baby falls below the 10^{th} percentile for their GA. However, these babies have a normal growth velocity *in utero*, and therefore are simply constitutionally small rather than pathologically growth restricted (Wilcox, 1983). Therefore, FGR babies can be classified as SGA, but not all SGA babies are growth restricted. It has been estimated that about 50 to 70% of SGA babies are constitutionally small (Ott, 1988). Developments in Doppler ultrasound of the maternal and fetal circulation, fetal heart rate analysis, and biophysical profile have improved the diagnosis of both FGR and SGA. SGA pregnancies often exhibit a normal fetal Doppler test, whilst FGR, due to placental disease, exhibit characteristic maternal and fetal Doppler abnormalities (Ott, 2000).

The incidence of FGR is estimated to be approximately 5-7% (Brodsky and Christou, 2004). The most common form of FGR results in asymmetric growth restriction (70-80%) and refers to a disproportionate decrease in the fetal abdominal size relative to the head; this is likely to result if growth is impaired during the last trimester (Lin *et al.,* 1991). The etiology for asymmetric FGR is mostly extrinsic and includes maternal and placental vascular factors e.g. placental insufficiency. Symmetric growth restriction (20-30%) is characterised by the proportionate decrease in both fetal head and abdominal circumference. This is likely to occur if fetal growth is impaired during the first or second trimester, thereby disturbing the cellular hyperplasia phase, leading to a proportionate reduction in all fetal organ sizes. The etiology for symmetric growth restriction is mostly intrinsic including chromosomal abnormalities and congenital malformations. Infants with symmetric FGR have a higher morbidity and mortality rate than asymmetric FGR infants (Pollack and Divon, 1992; Brodsky and Christou, 2004).

FGR is of clinical interest because it is associated not only with immediate medical problems such as hypoglycaemia, hypothermia (Doctor *et al.,* 2001) and neurological delay, but because it influences later life and is associated with common adult-onset diseases such as hypertension, type 2 diabetes (T2D) and cardiovascular disease (Barker, 1992; Ong and Dunger, 2002; Simmons, 2009). The majority of FGR infants usually demonstrate catch-up growth during the first two years of life, with most of them achieving this growth in the first six months (Hediger *et al.,* 1998). Infants with LBW have a 10 times higher risk of mortality than infants with normal birth weight (10 to 90th percentile) (McCormick, 1985; McIntire *et al.*, 1999).

1.1.3 Anthropometric measurements to predict birth weight

Accurate determination of *in utero* fetal weight is crucial. Measuring fetal body parameters allows assessment of the growth pathway of the fetus and helps in predicting and managing FGR and other pathological conditions (Brodsky and Christou, 2004). Ultrasound has been used since the 1960s as a tool for determining fetal size. Measurements are normally taken in the first trimester and these include crown-rump length (CRL), measured usually between 7 to 13 weeks. CRL is also used to provide an accurate estimation of GA. The fetal biparietal diameter that refers to the diameter between the two sides of the head, the femur length that reflects the longitudinal growth of the fetus as this is the measurement of the longest bone in the body and the fetal abdominal circumference and head circumference are also taken (Stetzer *et al.,* 2002; Loughna *et al.,* 2009). The weight of the fetus can then be estimated at any gestation with great precision through the use of equations containing different combinations of these anthropometric measurements (Stetzer *et al.,* 2002; Mu et *al.,* 2008; Loughna *et al.,* 2009).

1.1.4 Causes of FGR

FGR is a multifactorial disease with both genetic and environmental factors playing variable roles. The major cause of FGR in the Western world is placental insufficiency, whereas in developing countries, malaria infections and inadequate maternal nutrition play a larger role (Brodsky and Christou, 2004). In addition, there is a strong association between FGR and chromosomal abnormalities, as fetuses with chromosomal disorders such as trisomy 13, 18 and 21 are often growth impaired. Oxygen deprivation, as well as other maternal environmental factors such as smoking, drug abuse and alcohol consumption, are also known causes of FGR (Brodsky and Christou, 2004; Aagaard-Tillery *et al.*, 2008; Miller *et al.*, 2008). Known factors associated with FGR are summarized in Table 1-1.

Table 1-1: Maternal, Placental and Fetal Etiologies of FGR.
(table adapted from Brodsky and Christou, 2004; Monk and Moore, 2004).

Maternal	Placental	Fetal
Vascular disorders -Hypertension -Diabetes mellitus	Partial placental separation	Chromosomal abnormality -Aneuploidy
High-altitude pregnancy -Hypoxia	Abnormal trophoblast invasion	Multifactorial congenital malformations
Hypercoagulable states -Thrombophilia -Antiphospholipid antibody syndrome	Placental infarction	Intrauterine infection -Malaria -Cytomegalovirus -Rubella
Substance abuse -Smoking -Alcohol -Drugs	Placenta previa	Aberrant genomic imprinting -Uniparental Disomy -Epimutations
Malnutrition	Small placenta	Multiple gestation
Constitutionally smaller mother	Uteroplacental insufficiency	
Lack of second-trimester weight gain	Umbilical-placental vascular anomalies	
	Pre-eclampsia	

The aforementioned potential factors that may lead to FGR can influence the function of the placenta, the maternal supply of nutrients and oxygen to the fetus as well as the ability of the fetus to use this supply (Brodsky and Christou, 2004). Although many factors have been implicated in the process of fetal growth and development, the underlying molecular and cellular mechanisms regulating normal fetal growth are still poorly understood. There are many genes involved in fetal growth and development, but of particular interest in understanding fetal growth is the group of genes found in Eutherian (placental) mammals, known as imprinted genes.

1.2 Genomic Imprinting

In diploid mammals, most autosomal genes are expressed equally from the paternal and maternal alleles, resulting in biallelic expression. However, there is a small subset of genes, termed imprinted genes that show parent-specific expression. The differential expression of a gene, according to its parental origin of inheritance, is defined as genomic imprinting. That is, although both parental alleles are present, only one of them is active while the other is switched off, i.e. if the paternal allele is expressed, the maternal allele is imprinted (silenced) and vice versa (Fig. 1-2) (Hitchins and Moore, 2004; Abu-Amero *et al.*, 2006).

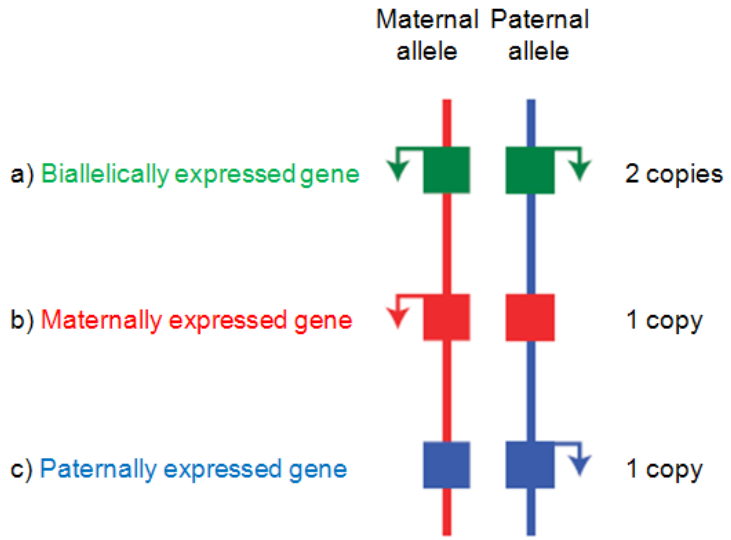


Figure 1-2: Genomic Imprinting (adapted from Hitchins and Moore, 2004).

a) As with most genes, a biallelic gene is expressed from both parental copies.
b) A maternally expressed gene is expressed specifically from the maternal allele and silenced on the paternal allele.
c) A paternally expressed gene is expressed specifically from the paternal allele and silenced on the maternal allele.

1.2.1 The discovery of imprinting

Evidence of genomic imprinting was first provided by mouse pronuclear transplantation experiments, where diploid mouse embryos were created but with the nuclear material derived solely from one parental genome (McGrath and Solter, 1984; Surani *et al.*,

1984; Barton *et al.,* 1984). These embryos contained either the paternal (androgenetic) genome or the maternal (gynogenetic) genome only. All of these embryos failed to develop post-implantation, demonstrating that diploidy is not sufficient for normal development. Moreover, both parental genomes are required, since the maternal and the paternal contributions to the embryo are not equivalent. In fact, the paternal genome appears essential for normal development of the trophoblast and the embryonic membrane, whereas the maternal genome is critical for embryogenesis (Barton *et al.*, 1984; McGrath and Solter, 1984). Subsequent mouse studies revealed that the parental origin effect was localised to specific chromosomal regions (Cattanach and Kirk, 1985; Searle and Beechey, 1990). The regions containing parent-of-origin effects were further narrowed down to clusters of genes, and in some cases to single genes, which are now known as imprinted genes.

1.2.2 The "parental conflict hypothesis"

The most widely accepted explanation for genomic imprinting is the "parental conflict hypothesis" put forward by Moore and Haig. This hypothesis is based on the conflicting interests of the two parental genomes during the offspring's growth and development. The father's contribution is concerned primarily with the development of his offspring, ensuring that it gets maximal growth and that it is the fittest, allowing the paternal genome to pass on to successive generations. The mother meanwhile, is concerned with distributing equal resources to each individual within her litter and at the same time, restricting fetal growth to ensuring her own survival and ability to reproduce in the future. This theory is strongly supported by the fact that genomic imprinting is common in placental animals, while imprinted genes are frequently monoallelically expressed specifically in the placenta, a tissue which plays a major role in the allocation of resources from the mother to the fetus (Moore and Haig, 1991).

The behaviour of several imprinting genes support this model, with the most striking example being the complementarity of the mouse genes insulin-like growth factor II (*Igf2)* and insulin-like growth factor II receptor (*Igf2r)* which show reciprocal imprinting effects in embryos. *Igf2 is* paternally expressed and encodes an embryonic growth factor and *Igf2r* in mice is maternally expressed and its product inhibits Igf2 function (Willison, 1991). Loss of function of *Igf2* results in a 40% reduction in birth weight (DeChiara *et al.*, 1991), whereas mutation of *Igf2r* on the maternal allele produces non-viable, over-sized mice (Lau *et al.*, 1994) (more details about human *IGF2* and *IGF2R* are given in Chapter 2). Another example supporting this model comes from mice with uniparental disomy (UPD). Mice with maternal UPD11 have LBW (70%) compared to their wild littermates, whereas mice with paternal UPD11 are larger (130%) (Cattanach and Kirk, 1985).

1.2.3 Uniparental disomy

UPD occurs when both homologues of a particular chromosome are inherited from a single parent. Therefore, paternal UPD can occur when there are two copies of the paternal allele and no copy of the maternal allele or vice versa. In cases of UPD for the whole chromosome, individuals appear to be cytogenetically normal, as they have a balanced number of chromosomes (Hitchins and Moore, 2004). Such UPD cases are associated with clinical disorders in humans and provide further evidence for genomic imprinting. For example, Prader-Willi syndrome can be caused by paternal UPD for chromosome 15 (pUPD15) (Robinson *et al.*, 1991), Silver-Russell syndrome (SRS) can be caused by maternal UPD for chromosome 7 (mUPD7) (Kotzot *et al.,* 1995) and Beckwith-Wiedemann Syndrome (BWS) can be caused by segmental pUPD11 (Slatter *et al.*, 1994). Both, SRS and BWS provide examples of where disrupted imprinting is associated with growth phenotypes, either growth restriction or overgrowth respectively.

SRS is a growth disorder characterised by pre- and postnatal growth retardation, limb and truncal asymmetry, lack of subcutaneous fat and macrocephaly. mUPD7 occurs in about 10% of SRS cases (Preece *et al.,* 1997). Overexpression of a growth inhibitor that is active on the maternal chromosome 7, or lack of a functional growth-promoting gene that is active on the paternal allele, could be causing SRS. Cases of SRS have been reported that have a maternal duplication of region 7p11.2-p13, a region that is homologous to the mouse-imprinted region on chromosome 11 (Monk *et al.,* 2000). Mice with maternal disomy for that region have FGR and mice with paternal disomy are overgrown. The most prevalent molecular explanation for SRS, however, is loss of methylation at the *H19* differentially methylated region (DMR), which is found in some 30-50% of SRS cases (Gicquel *et al*., 2005). In these patients, it seems most likely that the growth restriction could be caused by a decrease in *IGF2* expression, which is linked to *H19* methylation although the exact mechanism is yet to be fully understood (Frost and Moore, 2010).

BWS is an overgrowth disorder characterised by pre- or postnatal overgrowth, macroglossia, facial dysmorphisms and in some cases organomegaly and Wilms' tumour. Like SRS, the genetics of BWS is complex, and imprinting defects were implied as pUPD11p15.5 has been observed in 20% of sporadic cases (Maher and Reik, 2000). This is the same region implicated in SRS and it is known to be rich in imprinted genes (Verona *et al.,* 2003). Reciprocal to SRS, *IGF2* overexpression is strongly implicated in BWS, as in some patients it is expressed from both parental alleles. The increase in *IGF2* expression could be due to hypermethylation of *H19* DMR, which is found in about 30% of BWS cases (Cooper *et al.,* 2005). In fact, loss of imprinting (LOI) of *IGF2* is the most common molecular defect seen in sporadic BWS patients (Maher and Reik, 2000).

1.2.4 Imprinted Genes and their Characteristics

It is estimated that there are about 200 imprinted genes in the mammalian genome and so far about 100 have been identified in mouse and 50 in human (www.otago.ac.nz/IGC). A great number of imprinted genes tested so far, exhibit imprinted expression in the placenta and yolk sac (Coan *et al.*, 2005; Frost and Moore, 2010). Imprinted genes have three primary characteristics: they show monoallelic expression; they are clustered in imprinted domains that are evolutionary conserved and show an association with parental-allele-specific methylation (Hitchins and Moore, 2004) (for a list of imprinted genes highly expressed in human placenta, see Appendix A).

a) Monoallelic expression:

The major characteristic of imprinted genes is monoallelic expression and refers to the transcription of a gene from one parental allele. However, some imprinted genes have complex expression patterns, as there are some that are monoallelically expressed only in a particular organ or tissue, whereas they are biallelically expressed in other parts of the body. Moreover, imprinted genes can be monoallelically expressed during a particular developmental stage. For example, human *IGF2* is expressed specifically from the paternal allele in most fetal tissues such as the liver, but its expression during infancy becomes biallelic, so in the adult liver *IGF2* is expressed from both parental alleles (Davies, 1994).

b) Clustering into evolutionary conserved domains:

Imprinted genes tend to occur in clusters in the genome, because their silencing within a cluster is regulated by a *cis*-acting element referred to as the "imprinting centre". These regions as well as the imprinted genes are conserved between humans and mice, indicating the importance of imprinting in mammals (Hitchins and Moore, 2004). One of the most well

studied clusters in humans is at the proximal end of chromosome 11p15.5. This region shares homology to the mouse distal chromosome 7 region (Reik and Walter, 2001; Verona *et al.,* 2003).

c) Parental-allele-specific methylation:

Genomic imprinting does not depend on the DNA sequence itself, but on factors that regulate the activity of DNA such as the attachments of methyl groups. Thus genomic imprinting is an epigenetic phenomenon (Hitchins and Moore, 2004). CpG (cytosine phosphate diester guanine) islands are dinucleotide pairs found within DNA. Methyl groups can attach to cytosine residues within specific CpG islands and this can modify DNA. CpG islands at the 5'end of genes are usually unmethylated so that constitutive expression of the genes is allowed. Methylation of an allele is correlated with its transcriptional inactivation and thus methylation is a hallmark of imprinted genes (Neuamann and Barlow, 1996). For example, the *Igf2r* and *H19* promoters are methylated on the paternal allele and are thus expressed from the maternal allele (Ferguson *et al.,* 1993; Stoger *et al.,* 1993). However, exceptions do exist, as *IGF2* that is paternally expressed, is methylated on the paternal allele (Reik *et al.,* 1994). Modification is most likely taking place during gametogenesis as this is the only period when the parental genomes are separated (Barlow, 1993). For more information on *IGF2* methylation see Chapter 2 and more information on the imprinting cycle see Chapter 3.

1.3 Miscarriage

Miscarriage is the commonest complication of pregnancy and it is the spontaneous loss of a fetus before it has reached viability (Stirrat, 1990). Hence, the term miscarriage includes all the losses between the time of conception to the 24th week of gestation. There are two types of miscarriages: sporadic and recurrent. Sporadic miscarriages are experienced by at least 25% of all women and this risk increases with increasing maternal age. In fact, the risk of fetal loss increases after the age of 35 and the rate of miscarriage can reach up to 75% in women who are 45 years and older at the time of conception (Nybo Andersen *et al.,* 2000). RM is defined as three or more consecutive pregnancy losses and it affects about 1% of couples trying to conceive (Stirrat, 1990). Unlike sporadic miscarriages, RM can occur even if the fetus has no chromosomal abnormalities (Sullivan *et al.,* 2004).

Environmental factors that have been shown to affect pregnancy outcomes include cigarette smoking (Lindbohm *et al.,* 2002) and caffeine consumption (Rasch, 2003) which are both associated with a dose-dependent risk of miscarriage, the use of cocaine (Ness *et al.,* 1999) and alcohol consumption (Kesmodel *et al.,* 2002). In addition, evidence suggests that obesity is a risk factor for infertility, miscarriage (Clifford *et al.,* 1994; Lashen *et al.,* 2004) and late pregnancy complications (Sebire *et al.,* 2001).

Studies have shown that the risk of miscarriage increases after each successive pregnancy loss and it can reach 45% after three consecutive losses (Regan *et al.,* 1989). RM has been attributed to a) genetic causes, b) anatomical disorders, c) infective, d) endocrine, e) immune, f) thrombophilic disorders or unexplained causes.

1.3.1 Genetic causes

The most important cause of miscarriage before the 10th week of gestation is fetal aneuploidy, a deviation from the normal number of 46 chromosomes in humans (Jacobs and Hassold, 1987). At least half of all miscarriages are associated with cytogenetic abnormalities, where chromosome trisomies are the most frequent ones (Kalousek *et al.,* 1993). Most aneuploidies in humans arise from errors in the first meiotic division of the oocyte and an increased rate of sperm chromosomal abnormalities have been reported in couples with RM (Giorlandino *et al.,* 1998).

In addition, in about 4% of couples with RM, one partner carries either a balanced reciprocal translocation or a Robertsonian translocation (Clifford *et al.,* 1994). In the former, there is an exchange of two terminal segments from different chromosomes and in the latter there is a centric fusion of two acrocentric chromosomes. Although carriers of a balanced reciprocal translocation are phenotypically normal, 50-70% of their gametes and as a consequence, embryos are unbalanced.

Genetic defects in RM relate to skewed X chromosome inactivation in couples with a history of RM. Inactivation of the X chromosome occurs randomly early in embryogenesis where one of the parental X chromosomes becomes inactivated. Skewed X chromosome inactivation is defined as the preferential expression of the maternal or paternal X chromosome in more than 90% of peripheral leukocytes. It has been reported to occur significantly more often amongst women with RM compared with controls (Lanasa *et al.,* 1999).

1.3.2 Anatomical disorders

The frequency of congenital uterine abnormalities in the general population is unknown, but in women with RM it is estimated to be between 6% and 38%. This wide range

reflects differences in imaging techniques used and differences in diagnostic criteria (Salim *et al.,* 2003). Most uterine anomalies relate to defects in the development or fusion of the paired Müllerian ducts during embryogenesis. The septate uterus is the most common structural uterine anomaly, as approximately 20%-60% of women have a septate uterus that is associated with reproductive failure in the first and second trimester of pregnancy. The bicornuate uterus is another important uterine anomaly associated with reproductive failure (Grimbizis *et al.,* 2001).

1.3.3 Infection

Infective causes of RM remain speculative, as for any infective agent to be implicated, it has to be capable of being persistent and undetected in the genital tract and must cause few maternal symptoms. Rubella, toxoplasmosis, cytomegalovirus and herpes infection do not meet these criteria and routine screening for these diseases has now been abandoned (Regan *et al.,* 2001). However, the presence of bacterial vaginosis during the first trimester of pregnancy has been reported as a risk factor for late miscarriage in the second trimester and for early preterm birth (Hay *et al.,* 1994).

1.3.4 Endocrine abnormalities

Abnormalities in the endocrine system have been hypothesized to cause RM, but few reports withstand scrutiny. Studies have shown that well-controlled diabetes is not a risk factor for RM (Mills *et al.,* 1988). Hyperprolactinaemia on the other hand, which is the increase of prolactin that plays a role in ovulation and endometrial maturation has been reported to cause RM. Treatment with bromocriptene that suppresses the secretion of prolactin, reduces the rate of miscarriage (Hirahara *et al.,* 1998).

The incidence of polycystic ovaries is significantly higher in women with RM (40%) than women who had no history of pregnancy complications (22%) (Rai *et al.,* 2000). Miscarriage in women with polycystic ovaries syndrome (PCOS) has been associated with the plasminogen activator inhibitor 1 (PAI-1) 4G/4G genotype (Glueck *et al.,* 2000a) and with high levels of the PAI-1 gene product and activity (Glueck *et al.,* 2000b; Glueck *et al.,* 2004).

PAI-1 inhibits plasminogen activators in the fibrin degradation pathway, hence is an inhibitor of fibrinolysis, the physiological process that degrades blood clots. Elevated levels of PAI-1, due to 4G/5G polymorphisms in the PAI-1 gene promoter, result in deficient plasminogen activation and are associated with predisposition to thrombosis. Hence, plasma concentrations of fibrin, which is important in remodelling of the tissue that accompanies embryonic implantation, are also affected by the polymorphism. Women with PCOS are more likely to have PAI-1 4G/4G or 4G/5G genotypes than normal control women and women with PCOS have higher PAI-1 levels compared to controls (Diamanti-Kandarakis *et al.,* 2004; Glueck *et al.,* 2006). Moreover, the occurrence of the 4G/4G or 4G/5G genotypes in pregnant women has been shown to be clinically significant for the pathogenesis of venous thromboembolism in pregnancy, but not for early miscarriage (Schenk *et al.,* 2008).

1.3.5 Immune abnormalities

In pregnancy, hemiallogenic fetal cells remain spared from attack by the maternal immune system, posing a great unsolved enigma of immunology. From an immunological perspective, survival of the hemiallogenic fetus depends on suppression of the maternal immune response. However, no such suppression has been recorded. The current understanding of reproductive immunology, suggests a cooperative interaction between the maternal immune system and fetal antigens (Moffett-King, 2002).

Natural killer (NK) cells are the main type of maternal immune cell that populate the uterine mucosa during formation of the placenta. NK cells are lymphocytes, and although their function is not fully clear, their abundance at the time of implantation suggests that they are important for pregnancy establishment (Moffett-King, 2002).

There is a link between NK cells and pregnancy loss. Women with RM have more CD56+ NK cells in their uterine mucosa than controls and those with the highest levels, if remain untreated, have a higher rate of miscarriage in subsequent pregnancies (Clifford *et al.,* 1999; Koopman *et al.,* 2003). In addition, it has been demonstrated that women with RM have more CD16+ uterine NK cells than fertile controls. These NK cells are associated with an increased cytotoxic response and their increased number in women with RM may play a role in the rejection of the conceptus (Maybin *et al.,* 2010).

However, uterine NK cells are phenotypically different from the peripheral blood NK cells (Koopman *et al.,* 2003) and therefore testing women with RM for levels of NK cells is questionable, as no association was observed between levels of NK cells in uterine mucosa and peripheral blood and levels of NK in peripheral blood is not predictive of pregnancy outcome in women with RM (Moffett *et al.,* 2004).

1.3.5.1 Antiphospholipid syndrome

Antiphospholipid syndrome (APLS) is an established cause of RM and it is an autoimmune disease caused by the presence of circulating maternal antiphospholipid antibodies (aPL) such as anticardiolipin antibodies (IgG, IgM) and lupus anticoagulant (LA) that are directed against proteins that bind to phospholipids on plasma membranes (Rai and Regan, 2006). The prevalence of APLS in women with RM is 15% (Rai *et al.,* 1995) and women with APLS have a miscarriage rate of 90% in subsequent pregnancies if untreated (Creagh *et al.,* 1991; Rai *et al.,* 1995).

The clinical criteria used for diagnosis of APLS are a) three or more consecutive unexplained miscarriages before the 10th week of gestation, or b) one or more unexplained deaths of a morphologically normal fetus at 10 week's gestation or older or c) one or more premature births of a morphologically normal fetus at 34 week's gestation or younger associated with severe pre-eclampsia (Wilson *et al.,* 2001). Laboratory criteria include a) the presence of LA detected according to the guidelines of the International Society of Thrombosis and Haemostasis, or b) the presence of aPL of IgG and/or IgM isotype in medium or high titer, or c) presence of anti-β2-glycoprotein-1 antibody of IgG and/or IgM isotype, present in titer greater than the 99th percentile (Lim, 2009).

Antiphospholipid antibodies cause thrombosis of the placental vessels, which in turn lead to fetal loss (Salafia and Cowchock, 1997). It is suggested that the primary underlying event is platelet aggregation and maternal fibrin deposition around chorionic villi within the intervillous space (Sebire *et al.,* 2002). In addition, antiphospholipid antibodies can reduce the levels of the anticoagulant Annexin V on trophoblast cells, thus leading to hypercoagulable state in the placenta (Rand *et al.,* 1997).

Studies *in vitro* have shown that antiphospholipid antibodies interfere with the signal transduction mechanisms controlling endometrial cell decidualisation (Mak *et al.,* 2002), decrease trophoblast fusion (Di Simone *et al.,* 2002; Bose *et al.,* 2005), promote trophoblast apoptosis and impair trophoblast invasion (Bose *et al.,* 2004; Bose *et al.,* 2005). These effects of antiphospholipid antibodies on trophoblast function are reversed *in vitro* by low-molecular-weight heparin to reduce the risk of further episodes of thrombosis (Di Simone *et al.,* 1999; Bose *et al.,* 2004; Quenby *et al.,* 2004). However, a meta-analysis has shown that the best treatment for women with RM and APLS includes a combination of heparin and aspirin. This combination can significantly reduce pregnancy loss by 54% (Empson *et al.,*

2002), but such pregnancies may have an increased risk of developing later complications such as pre-eclampsia and IUGR (Backos *et al.,* 1999).

1.3.6 Thrombophilic disorders

Thrombophilia, which is defined as a tendency to thrombosis, can be inherited or acquired. The most common thrombophilia is acquired and about 40% of thrombophilic cases are inherited. Whilst the causative role of acquired thrombophilia manifest by elevated circulating antiphospholipid antibodies is widely accepted, the contribution of specific inherited thrombophilic genes is controversial (Coulam *et al.,* 2006). The first studies of the prevalence of coagulation abnormalities in women with pregnancy complications appeared in the mid-1990s, where women with familial thrombophilia, especially those with combined defects, had an increased risk of fetal loss, particularly still birth (Preston *et al.,* 1996). Although 10 thrombophilic genes have been investigated, three of these genes significantly correlated to RM <20 weeks gestation when compared with controls (Coulam *et al.,* 2006; Goodman *et al.,* 2006). These include mutations in Factor V Leiden (FVL), Factor II prothrombin and methylene tetrahydrofolate reductase (MTHFR), which are described in more detail below.

1.3.6.1 Factor V Leiden

Activated protein C (APC) is a serine protease with anticoagulant properties, which during normal haemostasis limits clot formation by inactivating clotting factors V and VIII. However, a mutation in factor V gene leads to the synthesis of a factor V molecule that is not properly inactivated by APC. APC resistance is a result of a single point mutation in factor V gene at nucleotide 1691 that codes for the APC cleavage site. This is known as the FVL mutation (G1619A) and it is the most common genetic predisposition to thrombosis (Bertina

et al., 1994). The FVL mutation causes slower cleavage (10-fold) of the factor V (Arg506Gln) by APC and therefore an increased level of thrombin gene and hence predisposition to clot formation (Kalafatis *et al.,* 1994).

Research suggests that the thrombotic state results in an exaggerated haemostatic response during pregnancy that is associated to an increased risk of systemic venous thrombosis (Dahlbäck, 1995) and can also lead to placental infarction and as a consequence fetal loss (Dizon-Townson *et al.,* 1997). The FVL mutation, which is transmitted in an autosomal dominant fashion, is common, as it is reported in about 6% of healthy Caucasian population. Heterozygosity and homozygosity of the FVL mutation is associated with a 5-10 and a 50-100 fold increased risk of thrombosis, respectively (Bloomenthal *et al.,* 2002). Case-control studies found a significant association between APC resistance/FVL carriers and late second-trimester pregnancy loss (Rai *et al.,* 1996; Grandone *et al.,* 1997).

1.3.6.2 Factor II prothrombin

The second most common inherited thrombophilia is the prothrombin (Factor II) mutation. Prothrombin is the precursor of thrombin. A nucleotide change at position 20210 (G20210A) in the 3'untranslated region (UTR) of prothrombin gene was identified in 18% of patients with familial thrombophilia. This mutation in the prothrombin gene was associated with increased prothrombin levels and a 2.8-fold increased risk of venous thrombosis (Poort *et al.,* 1996). The relative risk for early miscarriage before the 12^{th} week of gestation has been reported between two to eight times in carriers compared with controls (Coulam *et al.,* 2006).

1.3.6.2 Methylene tetrahydrofolate reductase

MTHFR catalyzes remethylation of homocysteine to methionine. Increased levels of homocysteine can result from several mutations in *MTHFR* that have been identified as risk factors for thrombosis. Homozygosity of the C677T variant of MTHFR, which converts an alanine to a valine residue, is the commonest mutation, and individuals homozygous for the mutation have significantly increased levels of plasma homocysteine (Frosst *et al.,* 1995; Kluijtmans *et al.,* 1996).

However effects of the C677T mutation on early RM are conflicting. Researchers have shown that homozygosity for the C677T mutation was associated with a 2 to 3-fold of RM (Nelen *et al.,* 1997), whereas others have shown no significant difference in prevalence between early RM patients and controls (Kutteh *et al.,* 1999). In others, the MTHFR mutation seemed to significantly increase the risk of miscarriage, when considered in combination with other thrombophilic factors (Tranquilli *et al.,* 2004).

A large meta-analysis has confirmed an association between FVL and both early and late RMs, with an odds ratio (OR) of 2.01 and 1.73, respectively, as well as an association with late non-recurrent pregnancy losses (OR=3.26). The same meta-analysis confirmed an association between the prothrombin mutation with early RM (OR=2.56) and late non-recurrent fetal loss (OR=2.30). However, MTHFR mutation was not significantly associated with fetal loss (Rey *et al.,* 2003).

Nevertheless, studies have shown that although some of the specific thrombophilic gene mutations appear not to be a risk factor for RM, when taken together, the total number of mutations is a significant risk. It looks like the additive effect of different thrombophilic mutations superimpose on the hypercoagulable state of pregnancy and this increases the risk of clotting.

1.4 Aims and Hypotheses

Chapter 2 focuses on imprinting and the role of imprinted genes on FGR. Five candidate genes, three of which are imprinted, were selected to investigate their roles on fetal growth. These consisted of the paternally expressed gene *IGF2*, two maternally expressed genes *IGF2R* and pleckstrin homology-like domain, family A, member 2 (*PHLDA2*) and genes *IGF1* and *IGF1R*. Previous studies performed by our research group have shown that *PHLDA2* expression in normal human term placentas negatively correlates with fetal growth, but no correlation was found with *IGF2* and *IGF2R* (Apostolidou *et al.,* 2007). Thus, the aim of this study was to address the role of these genes in early pregnancy on term birth weight. This was done by using real-time quantitative PCR to investigate the expression levels of *IGF1, IGF2, IGF1R, IGF2R* and *PHLDA2* in 260 chorionic villus samples, collected at 11-13 week's gestation. Since a maternally expressed gene (*PHLDA2*) controls fetal growth late in pregnancy as shown by the expression levels in term placenta, our hypothesis was that a paternally expressed gene such as *IGF2* might play an important role early in pregnancy.

Chapter 3 focuses again on imprinted genes, but this time on their potential role on miscarriage. This study involved 104 couples that had a miscarriage and whose product of conception (POC) was available. POC is tissue that is present in a fertilized gestation in a pregnancy that ended in a miscarriage. The aim of our study was to firstly detect maternal contamination levels in the 104 POCs available and then investigate the imprinting status of known imprinted genes. Genes investigated included the maternally expressed *HLA-C, H19*, *PHLDA2* and the paternally expressed *IGF2, PEG3* and *DLK1*. These genes were sequenced in the non-contaminated POCs from which DNA from both parents was available so we could detect which parental allele was expressed in these genes. Our hypothesis is that a proportion of miscarried POCs that are cytogenetically normal will have abnormal expression profiles at imprinted loci, which may be the cause of the embryonic lethality.

Chapter 4 focuses on the placental anti-coagulant protein Annexin A5 (*ANXA5*) and RMs. *ANXA5* contains four consecutive nucleotides substitutions (M2 haplotype) in its promoter. The frequency of M2 was found to be significantly higher in female RM patients than among controls (Bogdanova *et al.,* 2007). The aim of this study was to further investigate this gene in both males and females by genotyping 500 White European pairs that had more than three consecutive miscarriages. These include 300 patient couples that had early miscarriages (before 12 week of gestation) and 200 couples that had late miscarriages (after 12 week of gestation). A cohort of 250 White European control trios (mum, dad and placenta) was also genotyped. Based on previous studies, the M2 haplotype should be associated with a higher risk of RM, but our aim here was to use a larger cohort to investigate the hypothesis that the *ANXA5* M2 haplotype influences the timing of miscarriages, interacts with known risk factors, such as APLS and FVL and is also associated with the father's haplotype.

Chapter 5 focuses on identifying new candidate genes for RMs. This study involves a Bangladeshi family where the patient suffered a total of 29 early RMs. The aim of this study was to carry out next generation exome sequencing on available samples from this family pedigree to find a potential rare genetic variant gene causative of the RM phenotype. Our hypothesis is that the cause of miscarriages in this family is genetic, as the patient had three different partners but no successful pregnancies so far. Two genes, FK506 binding protein 4 (*FKBP4*) and Serpin peptidase inhibitor clade B member 2 (*SERPINB2*), with potential damaging mutations were investigated in more depth by sequencing them in 100 RM Asian patients, 120 RM White European patients and 100 Bangladeshi controls. Three novel variants and one very rare SNP in *FKBP4* were identified in a total of 4 Asian RM patients, which were all predicted to be damaging by different prediction programs. Future work involves functional studies to test pathogenicity of these variants.

CHAPTER 2 – *IGF2* EXPRESSION IN CVS

2 INTRODUCTION

As briefly described in the overview presented in Chapter 1, in Chapter 2, I will describe studies investigating the correlation between 11-13 weeks GA chorionic villus sample (CVS) mRNA levels of paternally expressed *IGF2*, maternally expressed *PHLDA2*, the polymorphic maternally expressed *IGF2R* and genes *IGF1* and *IGF1R* with birth weight. I performed all of the experiments involving the role of *IGF2*, *IGF2R* and *IGF1R*, whereas MSc students Lara Al-Olabi and Lydia Leon contributed experiments involving the role of *PHLDA2* while MSc student Jaime Stafford performed experiments involving *IGF1* under my supervision.

2.1 The IGF growth axis

The IGF growth axis includes many endocrine factors that are important for pre- and postnatal growth. These factors include insulin, insulin-like growth factors I and II (IGFI and IGFII), their corresponding receptors (IGFIR and IGFIIR) and six binding proteins (IGFBPI-VI) (Fant and Weisoly, 2001). Insulin is a hormone important for metabolism as it regulates the uptake of sugars, such as glucose by body cells. The insulin receptor is a transmembrane tyrosine kinase receptor that is activated by insulin, as well as both growth factors IGFI and IGFII (Rechler *et al.,* 1980). The proteins encoded by *IGF1* and *IGF2* are ligands that closely resemble the molecular structure and function of insulin. They constitute a functionally interrelated family of proteins referred to as the "IGF axis" which are critically linked to growth and metabolism (Fant and Weisoly, 2001).

Both IGFI and IGFII are expressed by the blastocyst at early stages of development, suggesting a role during fetal development. They circulate in fetal serum and rise throughout gestation (Bennett *et al.,* 1983; Rappolee *et al.,* 1992). Increasingly, evidence suggests that

IGF axis genes are likely to be involved in the regulation of placental nutrient transfer and fetal growth including key genes within this group that are imprinted, most notably *IGF2* (Fant and Weisoly 2001). Although both IGFI and IGFII have been detected in fetal plasma early in gestation in many species, plasma concentrations of IGFII are found to be several fold higher than those of IGFI (Gluckman and Butler, 1983). The high concentrations of IGFII in fetal serum decline within days after birth, whereas serum concentrations of IGFI rise in the postnatal period (Gluckman and Butler, 1983; Daughaday *et al.,* 1982).

The first three endogenous imprinted genes were identified in the mouse in 1991. These were the reciprocally imprinted genes *Igf2r* (Barlow *et al.,* 1991) and *Igf2* (DeChiara *et al.,* 1991), as well as *H19* (Bartolomei *et al.,* 1991). *Igf2* is found on the distal domain of mouse chromosome 7 and *Igf2r* on the proximal domain of mouse chromosome 17. These two genes confer reciprocal function in fetal growth as IgfII is a growth promoter and IgfIIr is responsible for IgfII's sequestration from the circulation, thus a negative regulator of IgfII (reviewed by Coan *et al.*, 2005). A number of animal studies support the role of insulin growth factors in intrauterine growth and some of these studies are summarised below.

Baker *et al.* showed that $Igf1^{-/-}$ mutant mice are infertile and have a retarded growth rate. Their relative size was 60% of normal at the end of gestation and their retarded growth continued after birth as they were still only 30% of normal adult weight. Three other classes of mutant embryos also had distinct differences in size. The relative weight of $Igf2^{p-}/Igf1r^{-/-}$ double mutants was approximately 30% of normal and the relative weight of $Igf1r^{-/-}$ mutant and $Igf2^{p-}$ mutant were 45% and 60% of normal, respectively. It is worth mentioning that these mutations affected the embryos' growth after embryonic day 10.5, since younger mutant embryos did not differ in size from their wild-type (WT) littermates (Baker *et al.*, 1993).

Wang *et al.* showed that mice carrying maternal inheritance of an *Igf2r* null allele as well as mice homozygous for the inactive allele are generally lethal at birth and these mutants are about 30% larger compared to their WT littermates. Observations from mice lacking both *Igf2r* and *Igf2* suggest that the overgrowth phenotype was due to an excess of IgfII, as the introduction of an *Igf2* null allele rescued the *Igf2r* mutant mice (Wang *et al.*, 1994).

Expression of *Igf2* in mice is driven from four distinct promoters. The fetal promoters P1-P3, that control expression of *Igf2* in fetal tissues and the placental-specific promoter (P0) that is specifically expressed in the labyrinthine trophoblast of the placenta (Moore *et al.,* 1997). Humans also have an *IGF2* P0 promoter but this is not placental-specific, indicating only a partial conservation of imprinting regulation of *Igf2/IGF2* between humans and mice (Monk *et al.,* 2006a).

Constancia *et al.* reduced IgfII action specifically in the placenta by deleting *Igf2* (P0). Mice that inherited the deletion paternally had placental growth restriction and the degree of deficiency remained constant throughout the gestational stages; at birth mutant P0 mutant mice were 69% of normal birth weight. They also showed that the deletion reduced the growth of the placenta and that the supply of nutrients across the placenta was influenced, as the mean weight of P0 mutant placenta was 68% of WT placentae at late gestation and the permeability for nutrients was decreased (Constancia *et al.*, 2002). Therefore, disruption of the *Igf2* (P0) in mice is associated with both late gestation IUGR and placental growth restriction.

2.1.1 *IGF2*

The first evidence for the imprinted role of *Igf2* in embryonic growth came from the work of DeChiara and his colleagues in 1990, when mutations at the *Igf2* gene locus were introduced in the mouse germ line. Heterozygous mice that inherited the inactive copy of the *Igf2* gene from the father were about 40% smaller than their WT littermates and remained smaller throughout their lives. Moreover, homozygous mutants, in whom both alleles were disrupted, appeared to have no phenotypic differences in size when compared to heterozygous growth-deficient mice. When the inactive *Igf2* was transmitted maternally, heterozygous mice were phenotypically normal (DeChiara *et al.*, 1990).

The difference in size between those mice that inherited the mutated allele maternally (M-heterozygotes) and those that inherited the mutated allele paternally (P-heterozygotes) can be attributed to genomic imprinting. *Igf2* is paternally expressed as M-heterozygotes that carry the intact paternal allele are of normal size, whereas P-heterozygotes that carry the intact maternal allele are growth-deficient (DeChiara *et al.*, 1991). Two years after DeChiara provided evidence that mouse *Igf2* is paternally expressed, it was shown that the human *IGF2* is also expressed paternally (Giannoukakis *et al.*, 1993). Imprinting mechanism regulating *IGF2* are shown in Figure 2-2.

Observations of pUPD11 in some patients with BWS provided indirect evidence that human *IGF2* is subject to parental imprinting, as a double dose of *IGF2* could explain some of the syndromes' characteristics. In addition, overgrowth in BWS occurs only in the tissues where *IGF2* is expressed. Sparago *et al.* (2004) reported that microdeletions in the human *H19* DMR on chromosome 11p15.5 inherited maternally, resulted in hypermethylation and hence silencing of the *H19* DMR. This in turn resulted in upregulation of *IGF2* due to the loss of *IGF2* imprinting, showing that *IGF2* is important in the overgrowth and tumour predisposition of BWS. On the other hand, in SRS that is characterised by growth retardation,

loss of methylation at the *H19* DMR is observed in 30-50% of cases (Gicquel *et al.,* 2005). Growth restriction in these patients could be caused by a decrease in *IGF2* expression, which is linked to *H19* methylation, again suggesting a role of *IGF2* in human growth.

2.1.2 ***IGF2R***

IGF2R/M6P (insulin-like growth factor 2 receptor/mannose 6-phosphate) maps to human chromosome 6q24.1- 24.3. IGFIIR is a multifactorial receptor involved in intracellular lysosomal enzyme trafficking and IGFII degradation, cell growth and signal transduction (Jirtle, 1999). *Igf2r* deficiency in mice causes increased levels of *Igf2* and results in abnormalities including cleft palate, cardiac abnormalities and fetal overgrowth. Epigenetic changes in gene regulation that result in decreased levels of *Igf2r*, lead to large offspring syndrome, thus suggesting an important role in growth (Young *et al.,* 2001).

Mice embryos that inherit a mutated *Igf2r* gene from their fathers are viable and develop into normal adults. However, mice that have inherited the non-functional *Igf2r* from their mothers, do not express IgfIIr in their tissues, have increased levels of IgfII, are larger than their littermates and die around birth. Therefore *Igf2r* is maternally expressed and paternally imprinted (Lau *et al.*, 1994).

However, *IGF2R* is not imprinted in humans, as both paternal and maternal alleles are expressed early in fetal development (Killian *et al.*, 2001). This is was the first demonstration for the presence of differential imprinting between mouse and human genes. One explanation for this might be due to the structural differences between the mouse and human *IGF2R* locus or more likely imprinting was lost during evolution (Kalscheuer *et al.*, 1993). The current consensus is that imprinting of *IGF2R* is demonstrated in less than 10% of individuals and even this may not be functionally relevant for those individuals (Monk *et al.,* 2006b).

2.1.3 ***IGF1***

Insulin-like growth factor 1 (IGFI) located on chromosome 12, stimulates cell growth and proliferation, and inhibits apoptosis via activation of the AKT signaling pathway. Downstream elements of this pathway regulate various crucial cell processes such as proliferation, differentiation, apoptosis, and metabolic homeostasis. IGFI exerts its growth properties on almost every cell in the body (Klammt *et al.,* 2008). As previously mentioned, IGFI is structurally related to insulin, and is able to bind to the insulin receptor although with a lower affinity than insulin itself. *IGF1* and its primary binding receptor *IGF1R* are not imprinted. A homozygous partial deletion of *Igf1r* in mice results in stunted height and weight, as well as disruption to the pubertal growth spurt. A complete inactivation of *Igf1r* is lethal in the neonatal period (Klammt *et al.,* 2008). In contrast to *Igf2* deficient mice, growth restriction continued into the postnatal period in the *Igf1* mutants (Le Roith *et al.,* 2001).

2.1.4 ***IGF1R***

IGF ligand signalling is mediated by IGFIR. *IGF1R* gene is located on chromosome 15 and encodes a cell surface tyrosine kinase that shares amino acid (aa) sequence homology with the insulin receptor (Le Roith *et al.,* 2001). IGFIR is recognised by both IGFI and IGFII, although IGFI is binding with a 15-20 fold higher affinity. IGFIIR does not appear to share any homology to IGFIR as they differ in structure and peptide binding specificity. IGFIIR strongly binds IGFII but rarely recognizes IGFI (Kornfeld, 1992). In fact, IGFII can bind to all three cell surface receptors (insulin receptor, IGFIR and IGFIIR), but it binds to IGFIIR with the highest affinity (Rechler and Nissley, 1985; Filson *et al.*, 1993).

2.2 ***PHLDA2***

PHLDA2 is a maternally expressed, paternally imprinted gene in both humans and mice (Qian *et al.,* 1997). It is located on the imprinting cluster of human chromosome 11p15.5, and its orthologous region on distal chromosome 7 in mice (Paulsen *et al.,* 1998). *PHLDA2* is highly conserved among vertebrates and consists of two exons and a single 223 bp intron and encodes a small protein consisting of 152 aa with a Pleckstrin-homology domain (Qian *et al.,* 1997). *PHLDA2* expression is highly tissue specific. Its expression is the highest in the placenta, and is expressed throughout gestation in human placenta correlating with its continuous growth (Qian *et al.,* 1997; Frank *et al.,* 1999).

Involvement of *Phlda2* in growth and development of the placenta was demonstrated by knockout mouse models with a maternal germline deletion that was associated with a significant increase in placental size during mid to late gestation (Frank *et al.,* 2002). Studies that have investigated the human placental expression of *PHLDA2* report increased expression in SGA pregnancies and a negative association with birth weight (McMinn *et al.,* 2006; Apostolidou *et al.,* 2007; Ishida *et al.,* 2012). Collectively, this indicated that *Phlda2* is a putative growth suppressor, consistent with the role of maternally expressed genes in the parental conflict hypothesis (Moore and Haig, 1991).

2.3 Imprinted gene clusters

2.3.1 Human chromosome 11p15.5: *KCNQ1* domain

An imprinting control region (ICR) is a specialized region of DNA that is differentially methylated in parental alleles. The imprinted expression of the paternally expressed *IGF2* gene alongside the co-localised maternally expressed *H19* has been extensively investigated. Expression of both genes is controlled by the ICR1 located upstream of the *H19* gene on chromosome 11p15.5. This region contains one of the most intensely studied imprinted gene clusters, which is divided into two regulatory independent domains. The centromeric *KCNQ1* (potassium voltage-gated channel, KQT-like subfamily, member 1) domain that contains several imprinted genes including *PHLDA2* (Fig. 2-1) and the telomeric *IGF2/H19* domain (Fig. 2-2) (reviewed by Ishida and Moore, 2013).

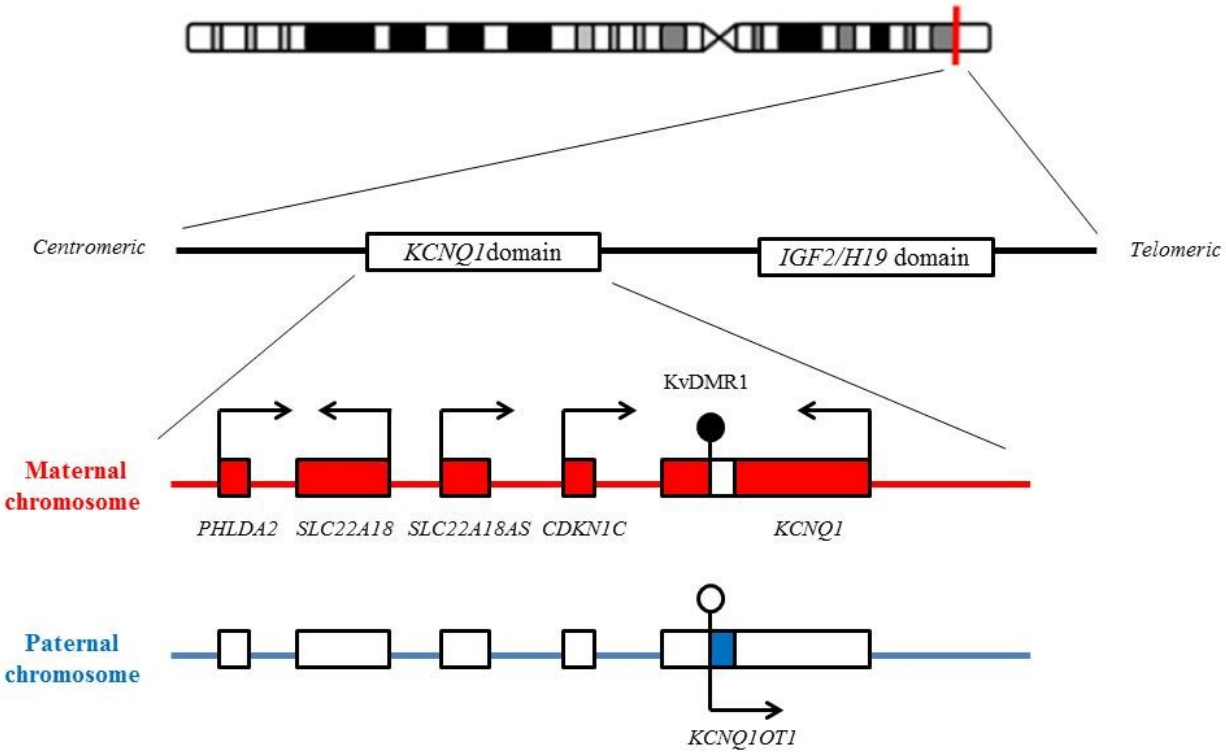


Figure 2-1: Schematic representation of imprinted gene clusters on human chromosome 11p15.5. Maternally expressed genes are indicated as red boxes with arrows showing transcription and direction. Paternally expressed genes are indicated as blue boxes. *PHLDA2* lies on chromosome 11p15.5 *KCNQ1* domain, which is under control of the kvDMR1. Black circle represents methylated KvDMR1 and white circle represent unmethylated KvDMR1.

2.3.2 Human chromosome 11p15.5: *IGF2/H19* domain

ICR1 which is methylated exclusively on the paternal allele, works as an insulator and binds to a CCCTC-binding factor (CTCF) in a parent-specific manner. CTCF binds the unmethylated ICR1 on the maternal allele and this prevents the *IGF2* gene promoter from interacting with enhancers found upstream from the *H19* gene. This result in silencing of the maternal *IGF2* gene while *H19* gene is expressed (Fig. 2-2A). Therefore the insulator is unmethylated and active on the maternal chromosome. On the paternal allele, DNA methylation prevents CTCF from binding, the enhancer acts beyond its previous boundary and this allows continued expression of *IGF2*, while at the same time it prevents expression of *H19* (Fig. 2-2B). Thus, the insulator is methylated and inactive in the paternal chromosome (reviewed by Miozzo and Simoni, 2002; Demars *et al.*, 2010; Shi *et al.*, 2011).

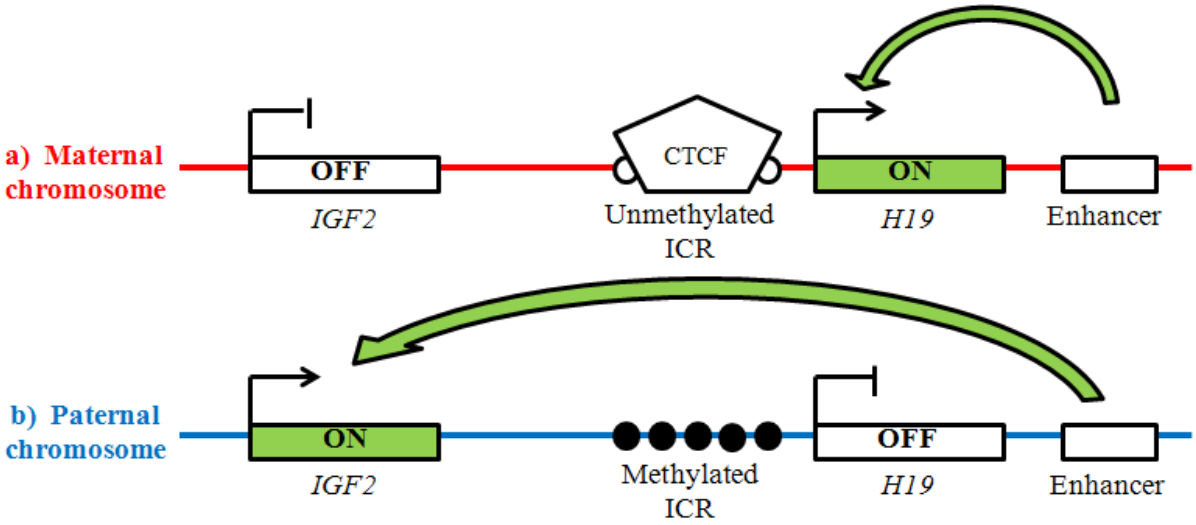


Figure 2-2: The imprinting mechanism of maternally expressed *H19* and paternally expressed *IGF2* in humans (figure taken from Charalambos Demetriou MSc thesis).
The two genes *H19* and *IGF2* share an enhancer region, downstream of *H19*.

A) The CTCF protein binds to the unmethylated maternal ICR (white circles). This prevents the promoters located in the *IGF2* gene from interacting with the enhancers so *IGF2* transcription is silenced. *H19* is transcriptionally active in this case.

B) The paternal ICR is methylated (black circles) and this prevents binding of CTCF. Enhancers can now contact the promoters of *IGF2*, allowing this gene to be transcribed. *H19* is silenced in this case.

2.3.3 Mouse chromosome 17: *Igf2r* domain

Another related and notable mechanism of imprinting involves the mouse *Igf2r* gene. *Igf2r* is maternally expressed and encodes insulin-like growth factor II receptor. An intronic CpG island (iCGi) located within intron 2 of the *Igf2r* gene helps to maintain the imprinting signal. On the maternal chromosome, the promoter of *Igf2r* is unmethylated and active when the iCGi is methylated (Fig. 2-3A). On the paternal chromosome, the promoter is methylated and thus inactive, and the iCGi is unmethylated leading to the production of an antisense transcript. Thus *Igf2r* is paternally imprinted (Fig. 2-3B). The fact that deletion of the iCGi leads to absence of the antisense transcript and biallelic expression of *Igf2r*, demonstrates that iCGi is not required for production of *Igf2r* but for the synthesis of the antisense strand (Wutz *et al.*, 1997; Biliya and Bulla, 2010).

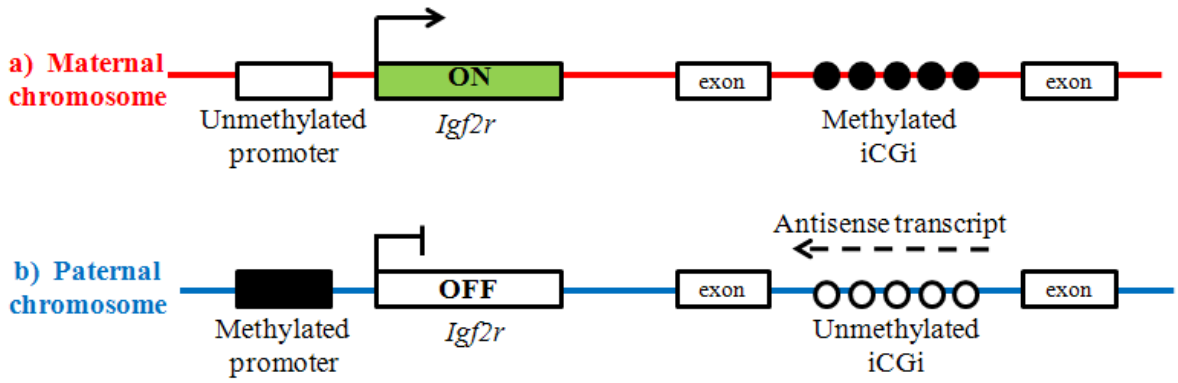


Figure 2-3: The imprinting mechanism of maternally expressed *Igf2r* in mice (figure taken from Charalambos Demetriou MSc thesis).

A) The promoter of the *Igf2r* gene on the maternal chromosome is active when iCGi is methylated and hence *Igf2r* is expressed maternally.

B) iCGi is unmethylated on the paternal chromosome, leading to the production of an antisense transcript. The promoter of the *Igf2r* gene is methylated and inactive and thus *Igf2r* is paternally imprinted.

Note: A description of the mechanism of imprinting of mouse *Igf2r* is described here instead of human *IGF2R*, because as described in section 2.1.2 above, *IGF2R* is not imprinted in 90% of humans.

2.4 Previous work

The analyses of human placental expression of *IGF1*, *IGF1R, IGF2* and *IGF2R* have previously been confined to term placental samples obtained at the time of birth. *IGF1* under-expression was observed in term placental samples from FGR pregnancies (Koutsaki *et al.,* 2011), while mutations in the *IGF1R* gene led to abnormalities in the function of IGF1 receptors that may also slow down intrauterine growth in humans (Abuzzahab *et al.,* 2003).

Studies on *IGF2* in term placenta have reported conflicting results. Some demonstrated no correlation between *IGF2* expression and birth weight (Apostolidou *et al.,* 2007) whereas others have variably shown that in FGR pregnancies compared to controls, *IGF2* expression is either increased (Abu-Amero *et al.,* 1998), decreased (McMinn *et al.,* 2006; Koukoura *et al.,* 2011), or similar (Street *et al.,* 2006; Antonazzo *et al.,* 2008), including at the protein level (Laviola *et al.,* 2005). Moreover, studies have either shown no significant relationship between IGFII cord serum levels and size at birth (Klauwer *et al.,* 1997), or a positive effect on birth weight (Smerieri *et al.,* 2011). In others, IGFII cord blood levels were significantly correlated with birth weight only when its interaction with IGFIIR was taken into account (Ong *et al.,* 2000).

Although the precise roles by which fetal growth is promoted by insulin are not completely defined, it is clear that insulin can stimulate growth via multiple mechanisms. These include promoting transport of nutrients, storage of fat and an influence on cellular proliferation by its interaction with other growth factors and hormones (Fant & Weisoly, 2001).

2.5 Aims

The profound role of the IGFI and IGFII pathways in the regulation of fetal growth have been established largely based on experiments using the mouse as a model (DeChiara *et al.,* 1990; Wang *et al.,* 1994; Klammt *et al.,* 2008). Analysis in humans has been hampered by the lack of available tissue to study during the course of pregnancy and has therefore relied on the use of term placenta, at a time when this tissue has become redundant and therefore the levels of gene expression may no longer reliably reflect the needs of the in utero growth of the baby. To fully assess the role of these growth factors and their receptors, we have focused on an earlier developmental time point when these genes are much more likely to measure functionally relevant expression levels.

The aim of this study was to measure the mRNA levels of *IGF1*, *IGF2*, *IGF1R*, *IGF2R* and *PHLDA2* in a large collection of CVS (n=260) using real-time quantitative PCR technology. Chorionic villi are tiny finger-shaped growths found in the placenta. The genetic material in chorionic villus cells is fetal in origin so by analysing CVS, which were collected at 11-13 weeks' of gestation, direct information about the fetus can be obtained. A critical time period for fetal growth occurs at the end of first trimester and progresses through the second trimester of pregnancy. CVS was a convenient sampling method as it is the only route that directly allows early access to first trimester placental genome and it is in regular use for the purposes of prenatal testing for genetic abnormalities (Evans and Andriole, 2008).

The expression levels of the genes of interest were calculated relative to *L19*, a housekeeping gene, expression of which does not change throughout gestation in term placentae and were then correlated with birth weight. Confounding factors such as fetal sex, GA, parity, maternal body-mass index (BMI) and smoking status were taken into consideration when the statistical analysis was carried out.

2.6 MATERIALS AND METHODS

2.6.1 Subjects

CVS was performed at 11-13 weeks of gestation in 260 singleton pregnancies that subsequently resulted in normal live births at term. The samples were collected from women undergoing CVS for prenatal diagnosis of chromosomal defects at King's College Hospital London. The excess tissue samples used for this study were obtained from women agreeing to participate in research, which was approved by the Hospital's Ethics Committee.

Demographic characteristics were recorded including maternal age, racial origin, smoking status, parity and BMI as well as pregnancy outcomes such as gestational age at delivery, sex and birth weight (full data in Appendix B). Other birth parameters such as placental weight were not available, nor were blood or tissue samples from the newborn baby. The pregnancies were subdivided according to the birth weight of neonates into SGA with birth weight <10^{th} percentile, large for GA (LGA) with birth weight >90^{th} percentile and appropriate for GA (AGA). Information to define these pregnancies as pathological FGR was not available, so for this study they were all considered as SGA.

2.6.2 Preparation of DNA and RNA from chorionic villus samples

DNA and RNA were extracted using the iPrep Purification Instrument (Invitrogen), either by use of the iPrep™ ChargeSwitch® gDNA Tissue Kit, or iPrep™ PureLink™ Total RNA and Trizol® Plus RNA kit including DNAse treatment, according to the manufacturer's instructions. These kits are for use specifically with the iPrep™ Purification Instrument. They are based on charge dictated by the pH of the surrounding medium, which allows selective binding of nucleic acids to magnetic beads. Purification is achieved through a three-step bind-wash-elute procedure. The kits provide prefilled cartridges containing all of the necessary reagents for isolating DNA or RNA from tissue samples.

2.6.3 DNA and RNA concentration

Concentrations of DNA and RNA of samples were measured using the NanoDrop™ 1000 Spectrophotometer (Thermo Scientific). The Nanodrop verifies the concentration and the purity of the samples by adding 1 µl of the DNA. Nucleic acid absorbs at a wavelength of 260 nm, and the ratio of absorbance at 260 nm and 280 nm was used to examine the purity of DNA and RNA samples. A ratio of ~1.8 and ~2.0 for DNA and RNA, respectively, indicated a high level of purity. RNA integrity can have an important influence on experiments designed to measure mRNA expression levels, and it has been suggested that samples with a 260/280 ratio higher than 1.8 are most suitable for gene expression measurement (Becker *et al.,* 2010). Therefore, only RNA samples with the 260/280 ratio in the range of 2 ± 0.2 were used for this analysis.

2.6.4 Primer design

Primer sequences for the genes of interest were obtained using National Centre for Biotechnology Information (NCBI) reference sequences (http://www.ncbi.nlm.nih.gov/gene). The primers were designed using the Primer3 software (://frodo.wi.mit.edu/primer3/). The size of each primer was between 18 to 22 nucleotides long, GC content was between 40 to 60% and annealing temperatures did not differ by more than 10 ^{0}C. Primer annealing temperatures were optimised using gradient polymerase chain reaction (PCR).

2.6.5 Polymerase Chain Reaction

PCR is a method used for the selective amplification of a specific target DNA sequence (Mullis *et al.,* 1986). PCR requires a pair of oligonucleotide primers approximately 18-22 bp in length that are complementary to the specific sequences on each DNA strand flanking the target region. The double stranded template DNA is initially separated by high

temperatures, and subsequent cooling allows the primers to anneal to complementary sequences. These primers are extended by a thermostable DNA polymerase to form the opposite strand. The new double stranded DNA (dsDNA) is then denatured and the process repeated, resulting in the exponential amplification of the specific DNA fragment. A standard Bioline PCR reaction is shown in Table 2-1.

Table 2-1: Standard Bioline PCR reaction mix.

Reagent	Volume (µl) per reaction
NH_4 Reaction Buffer (10x)	2
$MgCl_2$ (50 mM)	0.75
dNTPs (2.5 mM)	1.8
Forward primer (50 ng/µl)	1
Reverse primer (50 ng/µl)	1
Taq DNA polymerase	0.2
Betaine (5 M)	6
DEPC-treated water	6.25
DNA or cDNA template (50-100 ng)	1

All reactions were carried out in a Veriti® 96-Well Thermal Cycler (Applied Biosystems - ABI) with a heated lid set at 100 °C to prevent evaporation. A standard PCR cycle consisted of an initial denaturation at 94 °C for 5 minutes, and 35-40 cycles of: denaturation at 94 °C for 30 seconds, annealing at 58-62 °C for 30 seconds and extension at 72 °C for 30 seconds, and a final extension at 72 °C for 2 minutes. 5 µl of PCR products were used to confirm product size and quality on an agarose gel.

2.6.6 Agarose gel electrophoresis

Agarose gel electrophoresis is used to estimate the size and integrity of PCR products. It involves the migration of DNA fragments depending on their molecular weight/size. As DNA molecules have an overall negative charge, in the presence of an electric field they move towards the positively charged anode. 1 g of UltraPure^{TM} agarose powder was mixed with 100 ml of TAE buffer (1% agarose gel). The solution was microwaved until the agarose powder had completely dissolved and 1 µl of ethidium bromide (10 mg/ml) was added to the solution once it had cooled down and no vapour was visible. The solution was then poured into a gel tray and left to solidify. 5 µl of commercially available Blue/Orange Loading Dye (Promega) was mixed with 5 µl of PCR product and loaded on the gel. 5 µl of a 100 bp DNA ladder (Promega) was used as a size marker. The gel tank was run at 90 volts for 30 minutes and the gels visualised on a Genosmart ultraviolet machine.

2.6.7 Reverse transcription PCR

In order to synthesise complementary DNA (cDNA) for real-time expression analysis and imprinting analysis, reverse transcription PCR (RT-PCR) was performed. RT-PCR uses the enzyme reverse transcriptase to catalyse the formation of cDNA from an RNA template. A first strand of cDNA was synthesised by reverse transcription of the total RNA extracted from CVS with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT). 100 ng of each RNA sample was diluted with DEPC-treated water to a total volume of 10.25 µl. The mix was initially heated at 72 °C for 5 minutes to melt any secondary structures of the single stranded RNA (ssRNA) and then cooled to 37 °C in a thermal cycler. The mixture that was then added for reverse transcription is shown in Table 2-2.

Table 2-2: Standard reverse transcription reaction mix.

Reagent	Volume (µl) per reaction
M-MLV RT Buffer (5x)	4
dNTPs (10 mM)	2
Random Primers (500 µg/ml)	1
M-MLV Reverse transcriptase (200 units/µl)	0.5
RNasin RNase inhibitor (40 units/µl)	0.25
DEPC-treated water	2

9.75 µl of the RT reaction mix was added to each sample followed by incubation at 37 °C for one hour before heating at 80 °C for 10 minutes to stop the reaction by denaturing the enzyme. A negative control was used in each run, where water was used in the place of RNA. 1 µl of cDNA samples was used to confirm the conversion of RNA to cDNA by performing PCR with beta-actin (*ACTB*) housekeeping gene primers. *ACTB* primers (shown in Table 2-3 below) were designed to amplify both genomic DNA (product size: 407 bp) and cDNA (273 bp) by flanking a relatively short intron (134 bp) in order to detect any genomic contamination in the RNA samples, as well as to confirm the conversion of RNA to cDNA.

Table 2-3: Control primers designed for PCR to check the integrity of cDNA.

Gene	Primer sequence 5' to 3'	Tm (°C)	Product size (bp)	Accession number	Notes
ACTB	F: gtcttcccctccatcgtg R: ggtcatcttctcgcggttg	60	273	NM_001101	for cDNA

PCR products were then electrophoresed to check for the presence of good quality products and the absence of genomic contamination. cDNA samples were then stored at -20 °C for future use.

2.6.8 Real-time quantitative PCR

Real-time quantitative PCR (RTqPCR) is a technique that detects and quantifies an amplified PCR product in 'real time'. This can be achieved by the incorporation of a fluorescent reporter dye; Power SYBR Green (ABI) was used in this experiment. SYBR Green binds to dsDNA but not to ssDNA. When it is bound to dsDNA it fluoresces brightly, so as more DNA is synthesised, more dye will bind and the fluorescence will increase (Ma *et al.*, 2006). This increase in the fluorescence is then measured by a sensitive detector that monitors the fluorescence in each well of the plate and therefore this enables the expression of a gene to be quantified at any time during amplification.

Relative quantification allows relative target quantity comparisons between the samples, with expression of each sample normalised to that of an endogenous control gene. The "comparative Ct method" was used in this study, which relies on the assumption that the amplification of the target and endogenous control genes are equally efficient. The PCR efficiency of the primers was tested by producing a standard curve for each pair by performing an RTqPCR using five serial 1:10 dilutions of CVS cDNA pool as a template. The RTqPCR reactions were run in triplicate on 96-well plates (microAmp Fast Optical 96-well reaction plates) (ABI). Each reaction contained 10 µl of template and 15 µl of SYBR Green master mix (Table 2-4).

Table 2-4: Standard SYBR Green reaction mix.

Reagent	Volume (µl) per reaction
Power SYBR® Green PCR Master Mix	12.5
Forward primer (50 ng/µl)	1
Reverse primer (50 ng/µl)	1
DEPC-treated water	0.5

For this experiment a cDNA pool was also prepared by mixing 1 µl from each of the cDNA samples. This was used for inter-calibration control between plates. The template contained either a 10-fold dilution of the cDNA sample of interest, a 10-fold dilution of the cDNA pool or DECP-treated water (acting as a blank). The quantitative expression analysis of the genes of interest, *IGF1*, *IGF1R*, *IGF2, IGF2R* and *PHLDA2* as well as the endogenous control gene *ribosomal protein L19 (RPL19),* was determined by RTqPCR in the 260 CVSs. Primers used for quantitative analysis are shown in Table 2-5.

Table 2-5: Primers designed for RTqPCR in chorionic villus samples.

Gene	Primer sequence 5' to 3'	Tm (°C)	Product size (bp)	Accession number	Notes
IGF1	F: ggaggctggagatgtattgc R: acttgcttctgtcccctcct	60	197	NM_000618	for cDNA
IGF1R	F: ccaagggtgtggtgaaagat R: tccatgatgaccagtgttgg	60	187	NM_000875	for cDNA
IGF2	F: cgagagggacgtgtcgacc R: ggactgcttccaggtgtcata	60	97	NM_000612	for cDNA
IGF2R	F: ccggcgtgctctgga R: ccagagggtcacagtggaaga	60	189	NM_000876	for cDNA
PHLDA2	F: ccatccccgcagcccaaacc R: ccacgtcctagcctcggtcc	60	83	NM_003311	for cDNA
RPL19	F: gcggaagggtacagccaat R: caggctgtgatacatgtggcg	60	140	NM_000981	for cDNA

All primer sets were free of primer-dimer products. The RTqPCR assays were run in triplicate for each sample, with the gene of interest and housekeeping gene run on the same 96-well plates. A control pool of CVS cDNA was also included on each plate. The plates were sealed with Optical Adhesive Covers (ABI) and spun briefly to remove air bubbles, then placed in the StepOne Plus™ Real-Time PCR Systems (ABI) to be analysed. The amplification reaction was programmed for an initial incubation at 50 °C for two minutes, polymerase activation at 95 °C for 10 minutes, and 40 cycles of denaturation at 95 °C for 15 seconds, and annealing and extension step at 60 °C for 1 minute. Each real time PCR reaction

was subjected to melt curve analysis in order to confirm the fidelity of the target sequence amplification.

After amplification, quantitative expression levels were obtained using the StepOne software (version 2.1). The software measures the cycle number at which the fluorescence crosses an arbitrary line, the threshold. This crossing point gives the C_T value (cycle threshold). The increment of fluorescent signal at each cycle point is measured as ΔRn, which is calculated as: ΔRn (cycle) = Rn (cycle) - Rn (baseline), where normalised reporter (Rn) is the fluorescence of the reporter dye divided by that of passive reference which produces consistent fluorescent signal.

The threshold is automatically set by the machine and it is set high enough so that the reaction crosses the line due to amplification rather than noise. When seen in the logarithmic view the threshold is on the linear part of the reaction (Fig. 2-4A). In the regular view the threshold is close to the bottom of the curve (Fig. 2-4B). The same threshold is used for all the samples in each plate.

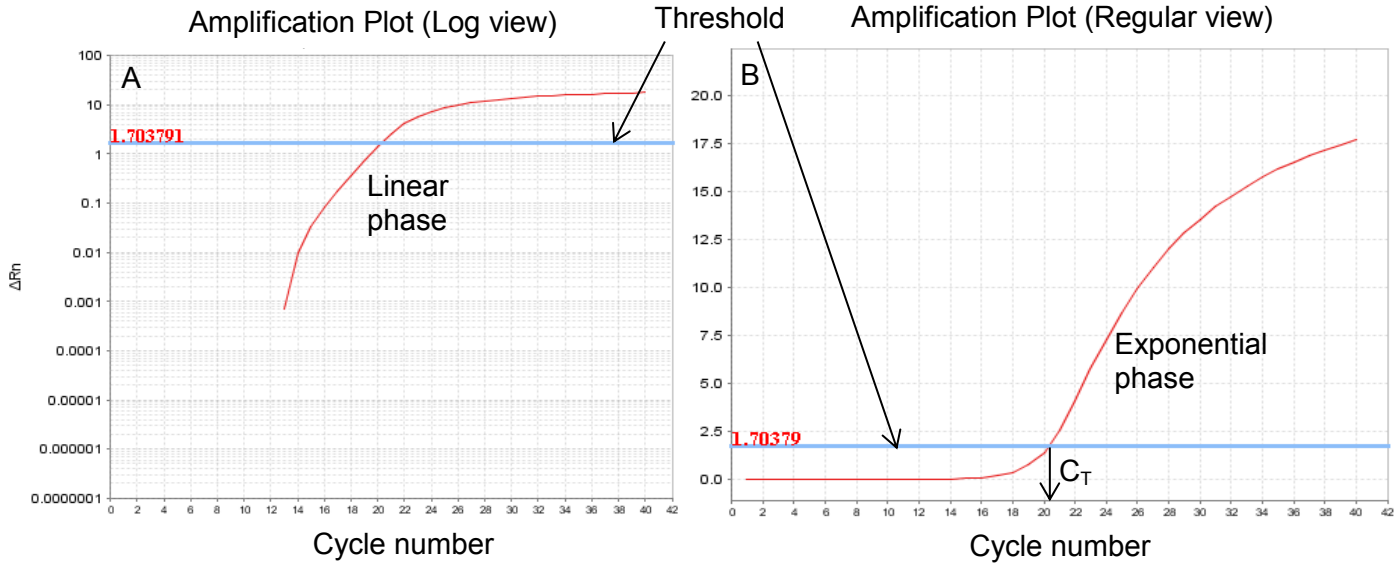


Figure 2-4: Graphical representation of RTqPCR threshold data. The exponential phase in (B) corresponds to the linear phase of the amplification plot in (A). In the log view (A) the threshold is on the linear part of the reaction, whereas in the regular view (B) the threshold is close to the bottom of the curve. Cycle number is shown on the x-axis and ΔRn is shown on the y-axis.

When determining the difference in the C_T values (ΔC_T), the control C_T value is subtracted from the target C_T value.

ΔC_T = average target C_T (gene of interest) – average control C_T (endogenous gene)

Although the C_T value for each sample triplicate should be the same, variations do occur mainly to pipetting errors. C_T values for each sample were individually checked and any C_T value that was not within one cycle of the other two repeats, was excluded from the analysis. The relative quantity of the target gene was calculated using the formula $2^{\wedge -\Delta Ct}$.

2.6.9 *RPL19* endogenous control gene

To accurately compare mRNA expression levels of genes of interest between each sample, these expression levels need to be normalised to that of a stable, endogenous control gene. This procedure provides a relative expression level. Housekeeping genes are often used as endogenous controls since their expression is assumed to be constant in most tissues. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is one of the most frequently used endogenous control gene for RTqPCR. However, in placental tissues, *GAPDH* levels have been found to vary considerably during the pregnancy (Patel *et al.,* 2002). In this study we used the *RPL19* housekeeping gene as an RTqPCR endogenous control as data from our group has previous demonstrated consistent results with placental tissues. We chose not use a second endogenous control gene since the amount of CVS tissue was extremely limiting.

2.6.10 Sequencing

PCR products were sequenced by the dideoxy chain termination method (Sanger *et al.,* 1977). In a Thermo-Fast® 96-well PCR plate, 3-4 µl of PCR products were purified and precipitated by adding an equal volume of microCLEAN. The plate was covered with MicroAmp® Clear Adhesive Film, vortexed and left for 5 minutes at room temperature,

followed by centrifugation step at 4000 rpm for 40 minutes at room temperature. The microCLEAN was removed by centrifugation of the plate set upside down on a tissue at 600 rpm for 1 minute. Sequencing mixture was then added to the precipitate (Table 2-6).

Table 2-6: Standard Big Dye Terminator sequencing reaction mix.

Reagent	Volume (µl) per reaction
BigDye® Terminator v1.1	1
BigDye® Terminator v1.1 Buffer (5x)	2
Betaine (5 M)	2
Forward or Reverse primer (50 ng/µl)	0.5
DEPC-treated water	4.5

The sequencing program consisted of an initial incubation step at 96 °C for 1 minute, and 34 cycles of: incubation at 96 °C for 30 seconds, 53 °C for 15 seconds, 60 °C for 4 minutes and at 20 °C for 2 minutes. The sequencing products were then precipitated by adding 50 µl of precipitation mix (5 ml of 3M sodium acetate (NaAc) at pH 5.2, 125 ml of 100 % Ethanol (EtOH) and 40 ml of Milli-Q water) in each sample. The plate was left at room temperature for 20 minutes and it was then centrifuged at 3000 rpm for 40 minutes at 10 °C. The solution was removed and the pellets were washed with 50 µl of 70% EtOH and spun at 3000 rpm for 10 minutes at 10 °C. The EtOH was removed by spinning the plate upside-down at 300 rpm for 1 minute and the resulting pellets were resuspended in 10 µl of 1:10 1x TE Buffer. The plate was then run on the ABI 3730xl DNA Analyzer by staff in the Regional Diagnostic Laboratories (Barclay House). The read-out was then analysed by me using Sequencher™ v4.8 (Gene Codes Corporation).

2.6.11 Imprinting analysis

Imprinting analysis of *IGF2*, *IGF2R* and *PHLDA2* in CVS was carried out by DNA sequence analysis of expressed single nucleotide polymorphisms (SNP) in CVS genomic DNA after amplification using specific primers shown in Table 2-7.

Table 2-7: Primers designed for sequencing genomic DNA and cDNA in CVS for imprinting analysis.

Gene	Primer sequence 5' to 3'	Tm (°C)	Product size (bp)	Accession number	Notes
IGF2	F: aacaccccacaaaagctcag R: tgcatggattttggttttca	58	425	NM_000612	for both gDNA and cDNA
IGF2R	F: gaaacacaaaacctacgacc R: agaacccaaaagagccaacc	60	163	NM_000876	for gDNA
IGF2R	F: gaaacacaaaacctacgacc R: cctttggagtacgtgacaac	60	242	NM_000876	for cDNA
PHLDA2	F: caaaccccgcacgccatgag R: gatttatttgcaatgggcacag	60	483	NM_003311	for both gDNA and cDNA

Informative DNA samples were heterozygous for *IGF2* A/G (rs680), *IGF2R* A/G (rs1805075) or *PHLDA2* A/G (rs1056819) SNPs (http://www.ncbi.nlm.nih.gov/projects/SNP/), where one allele is inherited from the mother and the other inherited from the father. Corresponding cDNA from the informative samples was then screened. If a single peak was detected in the cDNA sequence, the gene must be monoallelically expressed, with one of the parental alleles silenced (imprinted). If both alleles were detected in the resulting sequence, it was biallelically expressed and therefore not imprinted.

2.6.12 Statistical analysis

Standard multiple linear regression models were used to examine the correlation of *IGF1*, *IGF1R*, *IGF2, IGF2R* and *PHLDA2* relative expression values to birth weight after adjustment for gestational age at delivery, sex, parity, maternal age, smoking status and maternal BMI. Any outliers more than 2 standard deviations from the mean were removed. The analysis of variance (one-way ANOVA) was used to compare the gene expression means between the three different categorical birth weight groups (SGA, AGA, LGA) taking into consideration any confounding factors. Statistical package "R" (version 2.13.0) was used for the analyses and for calculating adjusted R-squared (R^2) values. Significance was defined as $p<0.05$.

2.7 RESULTS

2.7.1 Subjects

A total of 260 CVSs from King's College Hospital were used in this study. The clinical details about the pregnancy from both the mother and the baby at term are shown in Table 2-8.

Table 2-8: Medical details of participants in the CVS cohort.

Maternal information:	
Pre-pregnancy weight (Kg)	63 ± 7
Height (cm)	153.4 ± 8.6
Maternal BMI	27.3 ± 6
Maternal age (years)	34.3 ± 10.3
Smoking	Yes: 24 (9 %)
	No: 236 (91%)
Parity	0: 90 (35 %)
	>1: 170 (65 %)
Baby's information:	
Birth weight (g)	3,515 ± 690
Gestational age at birth (days)	279.4 ± 9
Sex	Males: 142 (55%)
	Females: 118 (45 %)
Ethnicity:	
White European	226 (87 %)
Black	13 (5 %)
Asian	10 (4 %)
Oriental	8 (3 %)
Unknown	3 (1%)

Mean ± standard deviations.

2.7.2 Analysis of mRNA levels in CVS cohort

In order to perform quantitative expression analysis using RTqPCR, RNA was first isolated from each CVS and reversed transcribed to cDNA. The housekeeping gene *ACTB* was used to confirm the presence of cDNA and the absence of genomic contamination, as the band size (incorporating an intron) would be much larger if genomic DNA was present (Fig. 2-5).

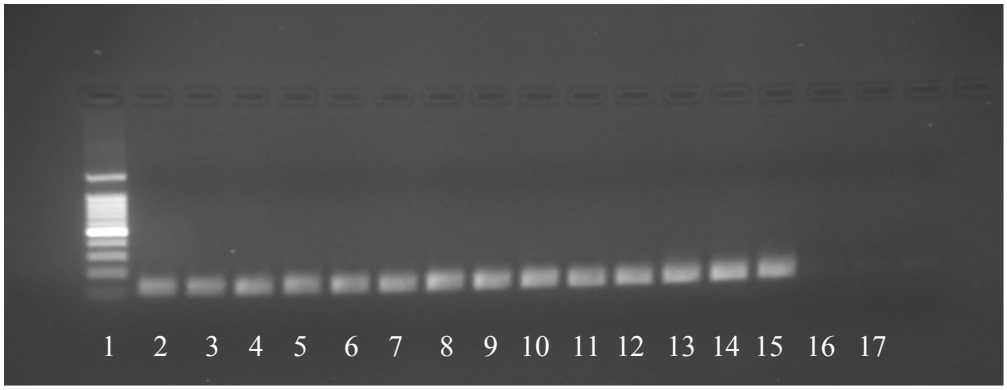


Figure 2-5: ***ACTB*** **cDNA amplification.**
Lane 1: 100 bp DNA marker; lane 2: sample 2593; lane 3: sample 2454; lane 4: sample 2457; lane 5: sample 2512; lane 6: sample 2522; lane 7: sample 2567; lane 8: sample 2570; lane 9: sample 2588; lane 10: sample 2611; lane 11: sample 2670; lane 12: sample 2697; lane 13: sample 2702; lane 14: sample 2713; lane 15: sample 2715; lane 16: RT negative control; lane 17: PCR blank.

Next, the relative mRNA levels of *IGF1, IGF2*, *IGF1R, IGF2R, PHLDA2* and *L19* were quantified in the 260 CVSs using the StepOnePlus Real-Time PCR system and analysed by the StepOne software (version 2.1). In order to determine the relative expression of the genes of interest in each sample, levels of each gene were compared to the ubiquitously expressed housekeeping gene *L19 (*Fig. 2-6). *L19* was chosen as an endogenous control because it encodes a ribosomal protein that has a constant expression in all cell types (Thellin *et al.*, 1999).

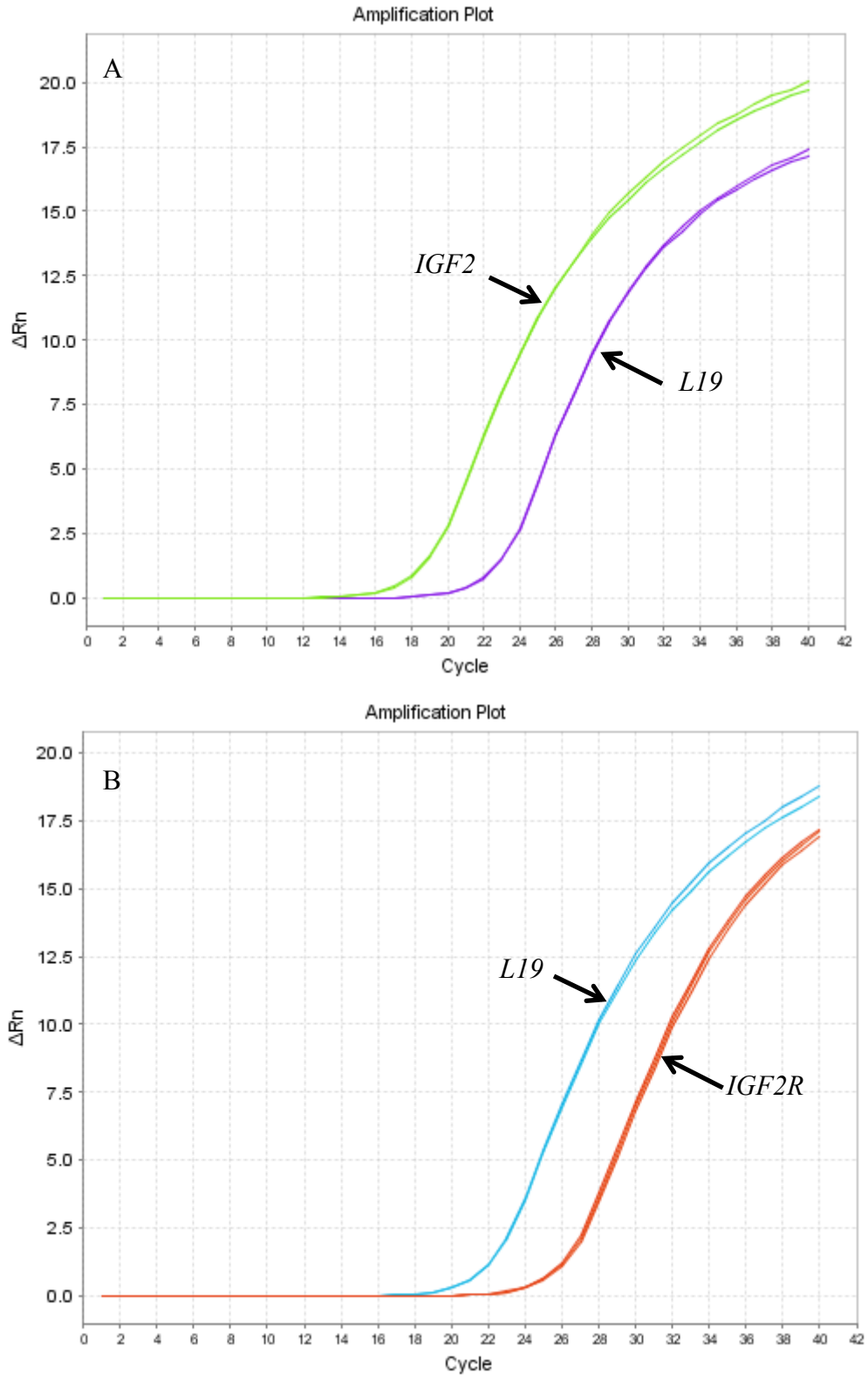


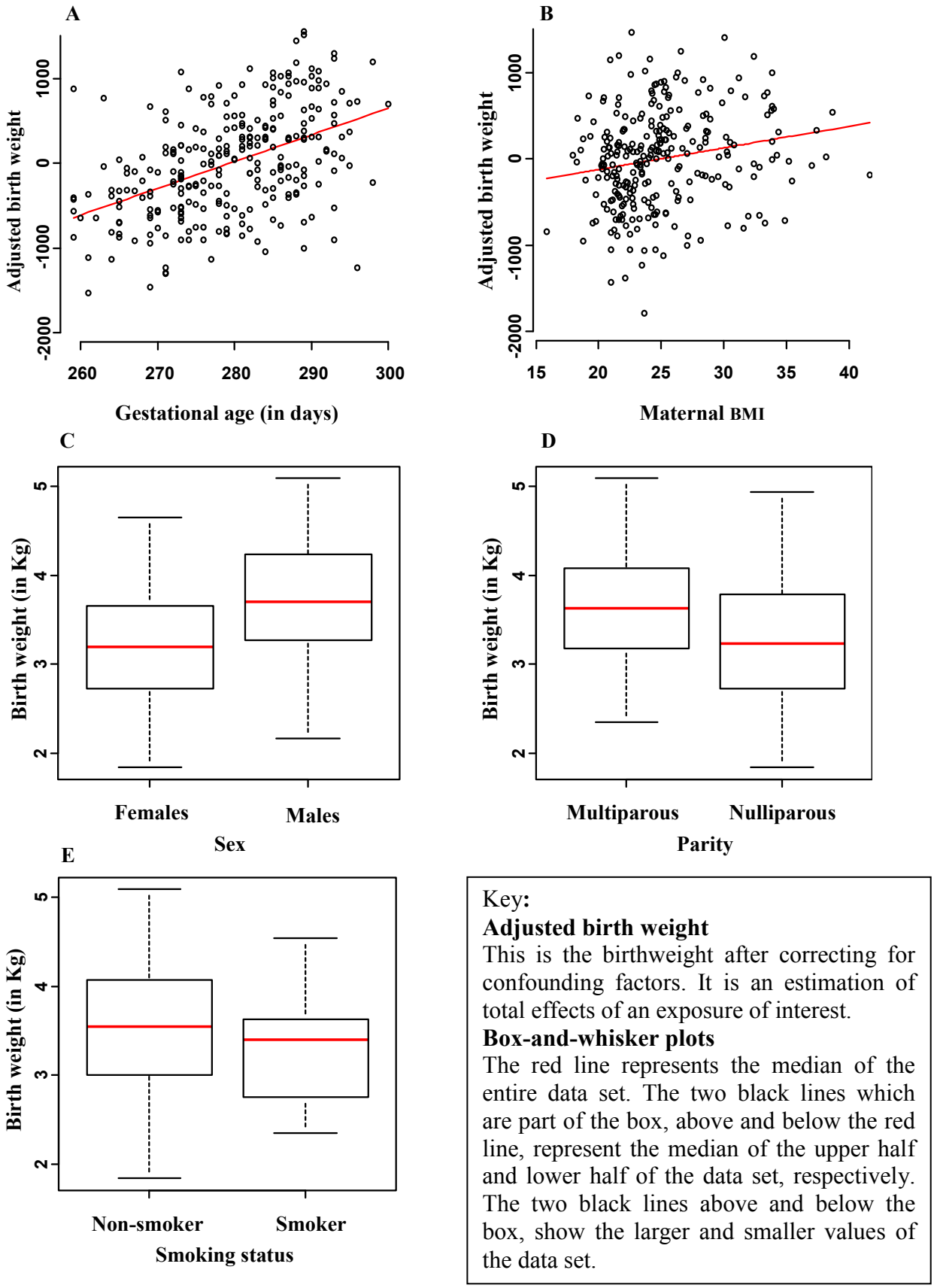
Figure 2-6: Amplification plots.
Plots are shown for two out of the five genes tested. (A) *IGF2* and *L19*, sample 3400; (B) *IGF2R* and *L19*, sample 1885. The amplification plots shown are representative of the sample selection as all CVSs consistently showed relatively higher expression of *IGF2* compared to *L19*; CVSs also showed relatively lower expression of *IGF2R* compared to *L19*. The Rn (normalised reported) value is the fluorescent signal from SYBR Green normalized to the signal of the passive reference dye. The y-axis represents the ΔRn (delta Rn) value, which is the Rn value of an experimental reaction minus the Rn value of the baseline signal generated by the instrument. The x-axis represents the cycle number.

2.7.3 Confounding factors – The influence of GA, maternal BMI, sex, parity and smoking status on fetal birth weight

In this study, we have investigated the relationship between *IGF1*, *IGF2*, *IGF2R*, *IGF1R* and *PHLDA2* mRNA levels in first-trimester placental tissue with the birth weight of the resultant term new born babies. We correlate this data taking into account the carefully recorded birth parameters that may have a confounding effect (GA, sex, parity, maternal BMI and smoking status; Fig. 2-7).

As expected by Wilcox *et al.,* 1993, there was a strong positive association between GA at term and birth weight, as babies that are born at a later GA are heavier ($P=4.8\times10^{-15}$; Fig. 2-7A). In addition, maternal BMI was positively correlated to fetal birth weight, which is in agreement with Mongelli and Gardosi (1995) who showed that heavier and taller mothers give birth to heavier offspring (P=0.0012; Fig. 2-7B). There was also an association between fetal sex and birth weight, as males had a higher birth weight than females ($P=1.4\times10^{-7}$; Fig. 2-7C). In addition, parity showed a significant association with birth weight, as women that had previous pregnancies (multiparous) had heavier babies than those women that had their first pregnancy (nulliparous) ($P=7.5\times10^{-5}$; Fig. 2-7D). These associations between birth weight and sex or parity are all in agreement with Hindmarsh *et al.,* 2002.

Although studies have shown that cigarette smoking during pregnancy is associated with a reduction in birth weight (Bernstein *et al.,* 2005), in our cohort we did not find a significant association between birth weight and maternal smoking status (P=0.33; Fig. 2-7E). This could be due to the low percentage (9%) of smokers in this cohort. Moreover, no association was observed between birth weight and different ethnic groups, probably because 87% of the samples used were of the same ethnic group (White-Europeans).



Key:

Adjusted birth weight

This is the birthweight after correcting for confounding factors. It is an estimation of total effects of an exposure of interest.

Box-and-whisker plots

The red line represents the median of the entire data set. The two black lines which are part of the box, above and below the red line, represent the median of the upper half and lower half of the data set, respectively. The two black lines above and below the box, show the larger and smaller values of the data set.

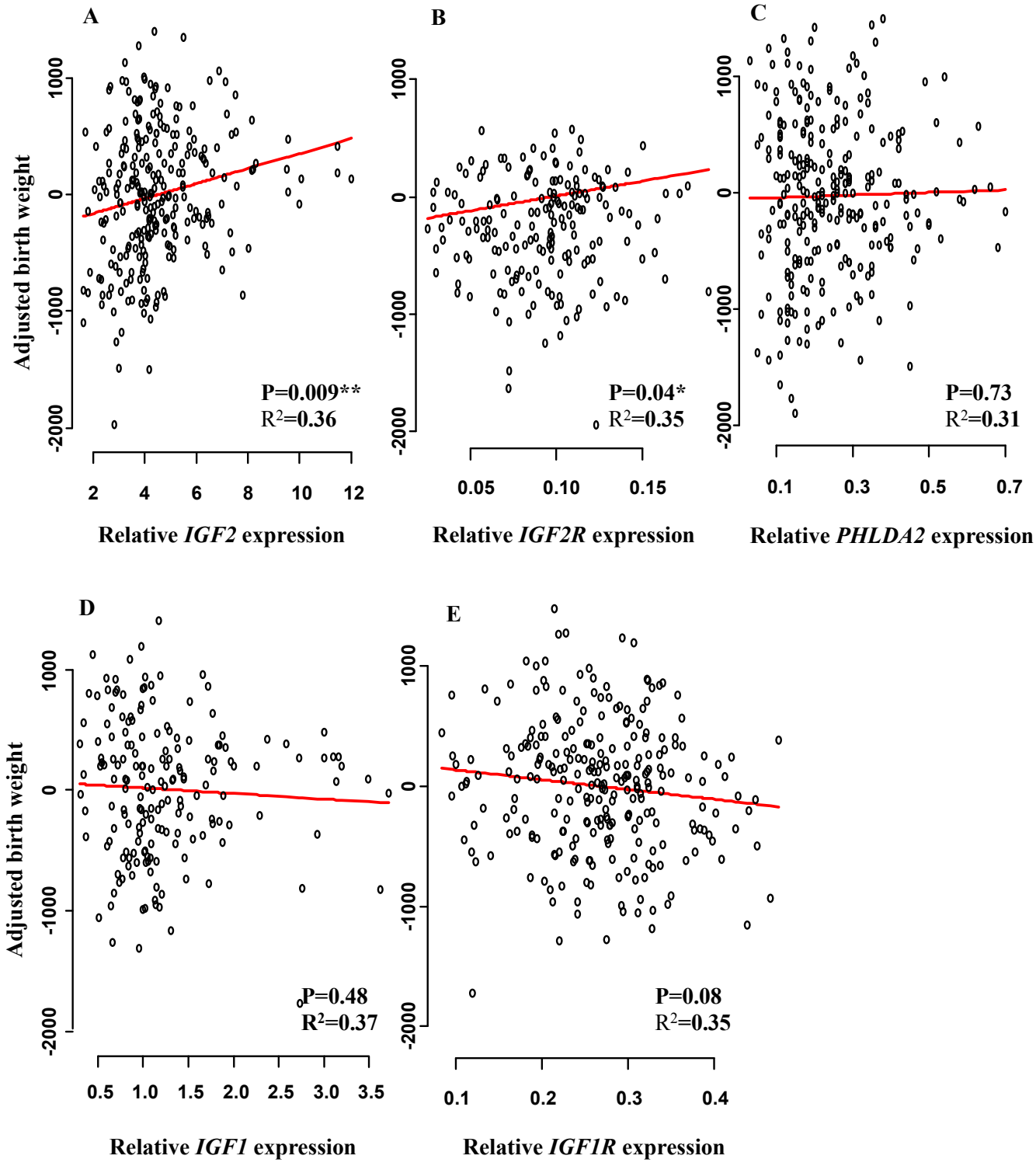
Figure 2-7: Confounding factors.

Correlation of birth weight with GA ($P=4.8\times10^{-15}$; Fig. 2-7A), with maternal BMI ($P=0.0012$; Fig. 2-7B), with sex ($P=1.4\times10^{-7}$; Fig. 2-7C), with parity ($P=7.5\times10^{-5}$; Fig. 2-7D) and with maternal smoking status ($P=0.33$; Fig. 2-7E) (Figure from Demetriou *et al.,* 2014).

2.7.4 Correlation between expression of genes of interest in CVS and birth weight

Regression analysis was performed after standardization to the endogenous control gene *L19,* in relation to birth weight corrected for confounding factors. Significant associations were observed for expression of *IGF2* (P=0.009) (Fig. 2-8A) and *IGF2R* (P=0.04) (Fig. 2-8B) between CVS tissue and birth weight. Importantly the range of *IGF2* in relative expression units was from 1.5-12 with one relative expression unit being equivalent to a change in birth weight of 63 g. No association was found for *PHLDA2* (P=0.73) (Fig. 2-8C), *IGF1* (P=0.48) (Fig. 2-8D) or *IGF1R* (P=0.08) (Fig. 2-8E) between CVS tissue and birth weight.

As both IGF1 and IGF2 bind to the receptor IGF1R but only IGF2 binds to IGF2R we decided to look at this relationship between the ligands and their receptors and any correlation they might have with birth weight. No association was found for the ratio of *IGF1 to IGF1R* (P=0.76) (Fig. 2-9A) or the ratio of *IGF2 to IGF2R* (P=0.93) (Fig. 2-9B) between CVS tissue and birth weight. However, the ratio of *IGF2 to IGF1R* (P=0.005) (Fig. 2-9C) was significantly associated with birth weight.



<u>Figure 2-8:</u> mRNA expression levels of *IGF2*, *IGF2R*, *PHLDA2*, *IGF1* and *IGF1R* in chorionic villi. The expression levels of *IGF2*, *IGF2R, PHLDA2, IGF1* and *IGF1R* after standardization to the control gene *L19,* in relation to birth weight corrected for parity, sex, GA at term, maternal BMI and smoking status. Significant associations were observed for CVS expression of A) *IGF2* (P=0.009) and B) *IGF2R* (P=0.04). No significant association was found for C) *PHLDA2* (P=0.73), for D) *IGF1* (P=0.48) or for E) *IGF1R* (P=0.08) (Figure from Demetriou *et al.,* 2014).

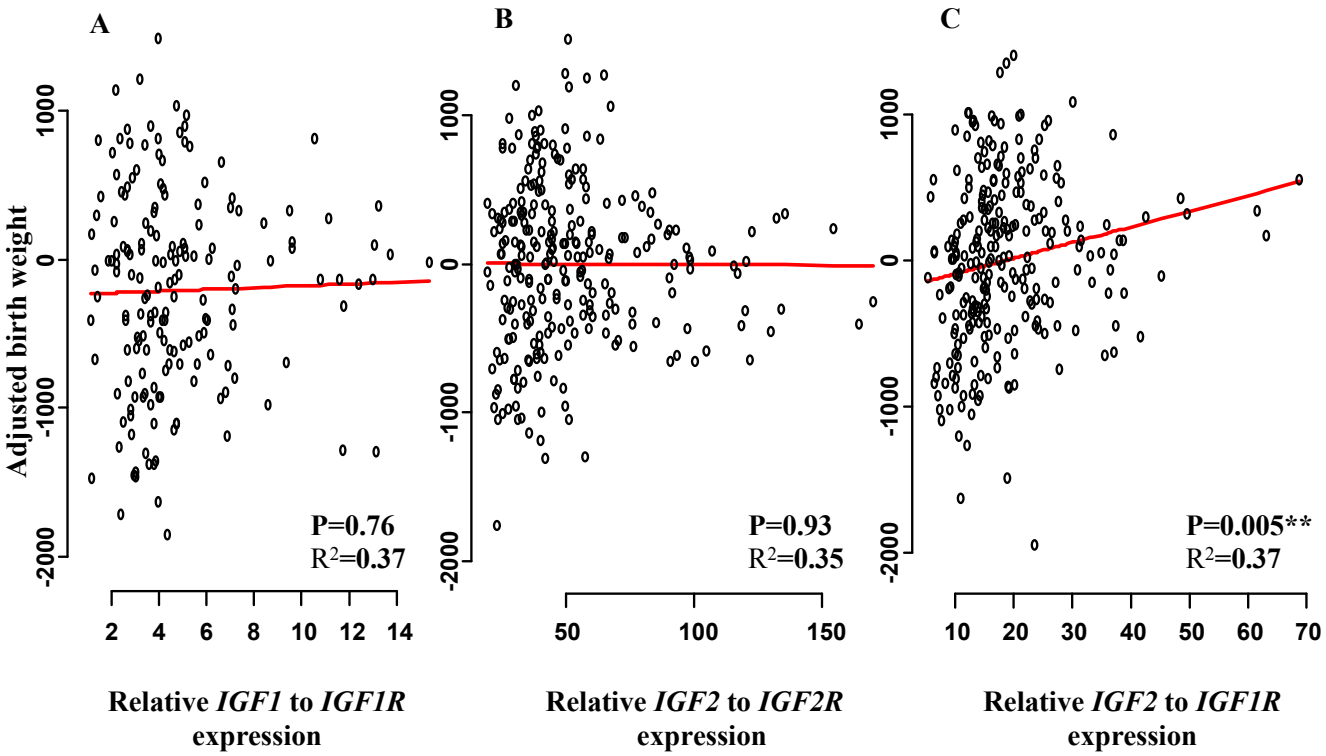


Figure 2-9: mRNA expression levels of *IGF1/IGF1R*, *IGF2/IGF2R* and *IGF2/IGF1R* in chorionic villi. The expression levels of *IGF1/IGF1R, IGF2/IGF2R* and *IGF2/IGF1R* after standardization to the endogenous control gene *L19,* in relation to birth weight corrected for parity, sex, GA at term, maternal BMI and smoking status. No significant association was found for A) the ratio of *IGF1* to *IGF1R* (P=0.76), or for B) the ratio of *IGF2* to *IGF2R* (P=0.93). Significant association was observed for CVS expression of C) the ratio of *IGF2* to *IGF1R* (P=0.005) and birth weight (Figure from Demetriou *et al.,* 2014).

To investigate the whole growth spectrum pregnancies were subdivided into small for gestational age (SGA; n=50) with birth weight <10th percentile, large (LGA; n=65) with birth weight >90th percentile and appropriate (AGA; n=145) (Table 2-9).

Table 2-9: Samples subdivided into groups according to the birth weight centile.

BW Centile	Class	Sample Number	Mean BW (g)	P-value
<10th	SGA	50	2564	P <2x10^{-16***}
10th – 90th	AGA	145	3455	P <2x10^{-16***}
>90th	LGA	65	4382	

SGA: small for gestational age (GA); AGA: appropriate for GA; LGA: large for GA

In the SGA group compared to the AGA group, *IGF2* expression was reduced by statistically significant amounts ($P=3.6x10^{-7}$), despite no significant differences in the expression levels of individual receptors (*IGF2R,* P=0.76 or *IGF1R,* P=0.15). In the LGA group *IGF2R* expression was found to be higher than in the AGA group (P=0.02) and *IGF1R* expression was lower than in the SGA group (P=0.05) (Fig. 2-10).

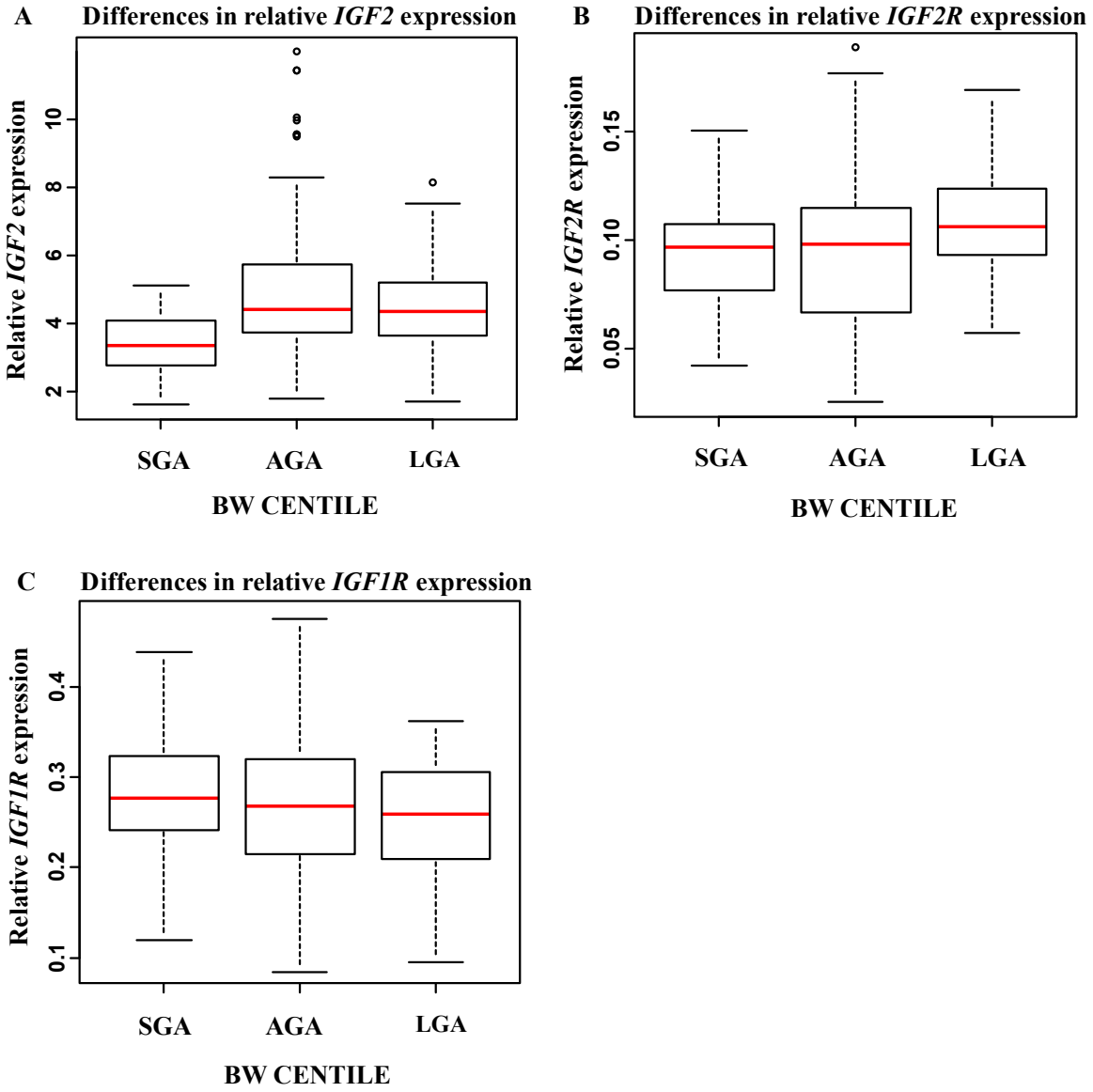
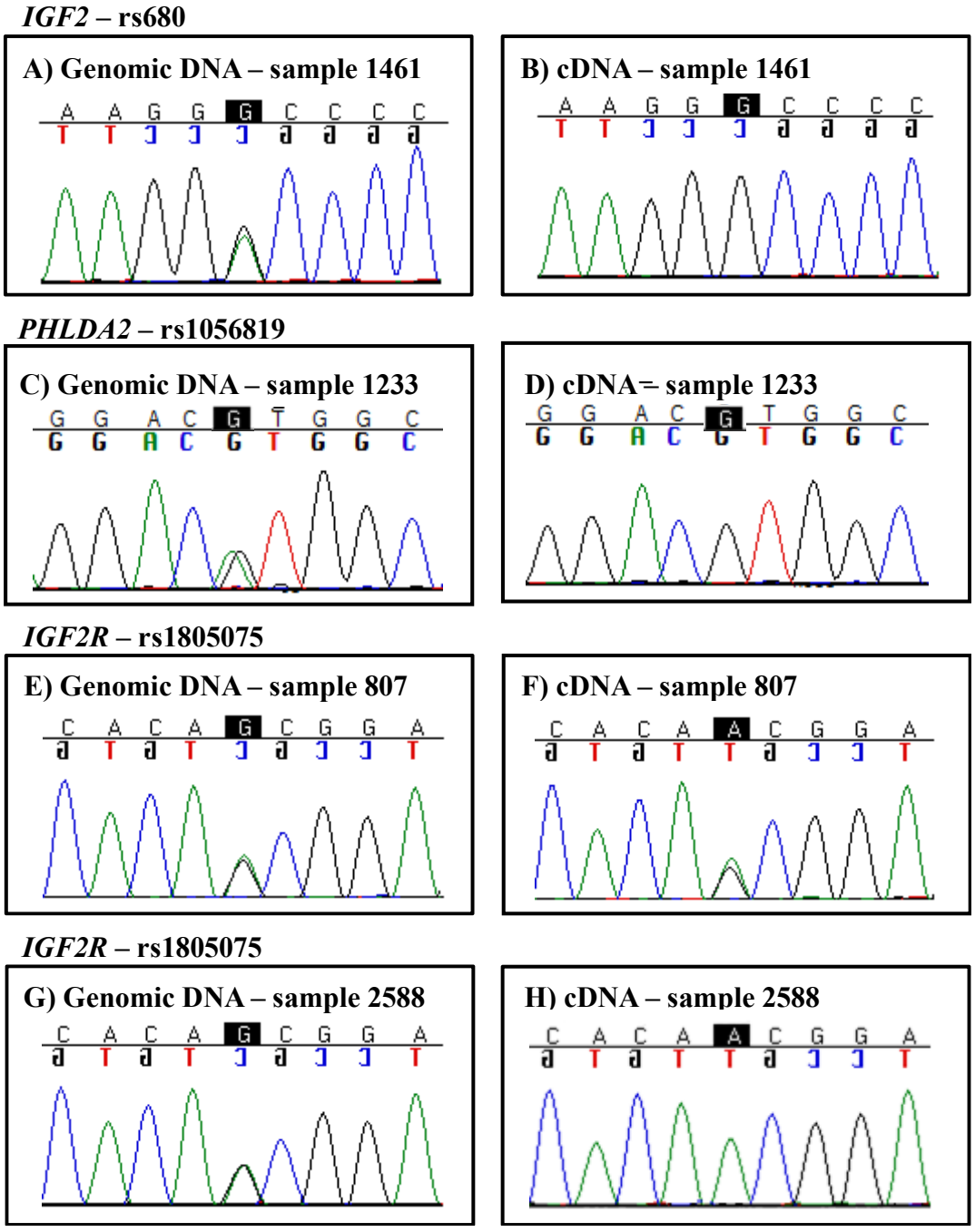


Figure 2-10: mRNA expression levels of *IGF2*, *IGF2R*, and *IGF1R* in chorionic villi according to BW centile. Relative *IGF2*, *IGF2R*, and *IGF1R* expression levels in the pregnancies with SGA, AGA and LGA neonates. In the SGA group, compared to the AGA group, A) *IGF2* expression was lower ($P=3.6x10^{-7}$), but there was no significant difference in the expression levels of B) *IGF2R* (P=0.76) or C) *IGF1R* (P=0.15). In the LGA group B) *IGF2R* expression was higher than in the AGA group (P=0.02) and C) *IGF1R* expression was lower than in the SGA group (P=0.05) (Figure from Demetriou *et al.,* 2014).

2.7.5 Imprinting analysis in CVS

Given that *IGF2* and *PHLDA2* are both imprinted in mouse and human (DeChiara *et al.,* 1990; Qian *et al.,* 1997; Giannoukakis *et al.,* 1993), we wanted to confirm that monoallelic expression was maintained in CVS material, since reversion to biallelic expression may have an impact on mRNA levels. Furthermore, in contrast to the mouse, where it is fully imprinted (Monk *et al.,* 2006), *IGF2R* is reported to be monoallelically expressed in only 10% of individuals in the human population. We therefore wished to investigate for any potential skewing. The transcribed SNPs rs680 (*IGF2*), rs1805075 *(IGF2R*) and rs1056819 (*PHLDA2)* were used to report on allele-specific expression and thus determine imprinting status of *IGF2*, *IGF2R* and *PHLDA2*.

Genomic DNA extracted from the CVS from 200 patients were tested for heterozygosity at these SNPs, with 40 samples found to be informative at rs680 for *IGF2,* 24 samples at rs1805075 for *IGF2R* and 21 samples at rs1056819 for *PHLDA2.* The 40 informative *IGF2* individuals and the 21 informative *PHLDA2* individuals were all found to be monoallelically expressed. For the 24 samples informative for *IGF2R*, 21 (88%) were biallellically expressed for *IGF2R* and 3 (12%) were monoallelic, which is similar to the expected population frequency (Fig. 2-11). Thus, skewed monoallelic/biallelic expression was unlikely to be a factor reflected in the relative expression levels associated with birth weight.



<u>Figure 2-11:</u> Imprinting analysis of *IGF2*, *PHLDA2* and *IGF2R*.
Representative Sanger sequencing chromatograms are shown from one of the 40 informative CVS samples for the *IGF2* A/G polymorphism (rs680) (A, B), one (from 21) for the *PHLDA2* A/G polymorphism (rs1056819) (C, D) and two (from 24) for the *IGF2R* A/G polymorphism (rs1805075) (E, F, G, H). Heterozygous genomic CVS DNA sequence is shown in A), C), E) and G). While monoallelic expression from the corresponding cDNA samples are shown in B), D) and H). Monoallelic expression in cDNA was found in all informative *IGF2* and *PHLDA2* samples as well as 12% of *IGF2R* samples. Biallelic expression, as shown in F), was found in the majority (88%) of informative cDNA samples for *IGF2R* (Figure from Demetriou *et al.,* 2014).

2.8 DISCUSSION

Previous studies that have linked *IGF2* expression to birth weight have shown that *IGF2* in term placenta is decreased in FGR pregnancies compared to controls (Koukoura *et al.,* 2011), as well as in SGA pregnancies (Guo *et al.,* 2008). However, the first trimester *IGF2* expression data reported in this study, provides the first evidence for the role of this paternally expressed imprinted gene as an *in utero* fetal growth enhancer this early in human pregnancy (Demetriou *et al.,* 2014). This is in agreement with results previously described in animal studies (DeChiara *et al.,* 1990; Constancia *et al.,* 2002). In mouse experiments, for example, paternally expressed *Igf2* was reported to control 40% of fetal growth, while the maternally expressed gene *Igf2r* limited growth (Wang *et al.,* 1994; Lau *et al.,* 1994). In humans, *IGF2* is maternally imprinted and thus a paternal-expression driven growth promoter. However, in humans, *IGF2R* is polymorphic for its imprinting status as 90% of individuals show biallelic expression (Monk *et al.,* 2006). This suggests a possible shift in the mechanism of its regulation of expression away from that used in the mouse. Interestingly, here, the samples showing monoallelic expression of *IGF2R* had similar levels of expression to those showing biallelic expression.

As fetal size is subject to both positive and negative regulation, *IGF2* and *IGF2R* can apply opposite forces on the fetus, thus achieving a fine degree of control over the growth process. As levels of *IGF2* increase in the fetus, the levels of *IGF2R* can also increase, resulting in clearance of IGFII from tissue fluids and plasma, correcting and maintaining levels of IGFII in the circulation. This acts as a fine-tuning rheostat designed to prevent babies from overgrowth in order to maintain a normal growth trajectory. This is also supported by the fact that no association was observed between the *IGF2/IGF2R* expression levels and birth weight, as the ligand and the receptor work tightly together to regulate growth. The same applies for IGF1 and its regulating receptor IGF1R (Fig. 2-9).

In this study, expression of *IGF1* in chorionic villi was not associated with birth weight outcome (Fig. 2-8D) These results directly contradict findings from previous studies involving mouse models which highlighted IGF1 in the regulation of both pre- and postnatal growth (Le Bouc *et al.,* 2003). A strong possibility that IGF1 is indeed relevant to intrauterine growth in the later stages of pregnancy remains, as it is preparing the baby for a postnatal life. While fetal chorionic samples in our cohort proved an opportunity to assess correlation between gene expression and fetal growth in early gestation (11-13 weeks), this narrow window might not be the best to investigate *IGF1* expression, as IGFI does not appear in the fetal circulation until later (Demendi *et al.,* 2011).

Although previous studies have shown a negative association of term placental *PHLDA2* with birth weight (Apostolidou *et al.,* 2007; Ishida *et al.,* 2012), in our study no statistically significant association was observed between *PHLDA2* expression levels in CVS and birth weight (Fig. 2-8C). This suggests that maternally expressed *PHLDA2* is suppressing the baby's growth later in pregnancy rather than early on. Previous studies investigating term placental *IGF2* expression have shown inconclusive results. However, in our study a statistically significant association was observed between *IGF2* expression levels in CVS and birth weight, suggesting that the paternal genome is promoting the baby's growth earlier in pregnancy (Fig. 2-8A). Therefore, the two parental genomes appear to be acting at different times during pregnancy to control fetal weight. This supports the idea that whilst increased fetal growth is important early on, it must still require careful regulation by the mother to ensure a successful birth.

In our study, SGA neonates had significantly lower *IGF2* expression levels compared to AGA neonates, but no differences were observed between the levels of the receptors *IGF2R* and *IGF1R* (Fig. 2-10). This highlights the importance of the *IGF2* ligand rather than the receptor level in determining size of the neonate in SGA pregnancies. This is in

agreement with previous studies investigating *IGF2* mRNA levels in the placentas from SGA pregnancies (Guo *et al.,* 2008). However, other studies have reported no differences in *IGF2* expression levels in term placenta between FGR pregnancies and controls (Street *et al.,* 2006; Antonazzo *et al.,* 2008) or increased *IGF2* levels in FGR pregnancies (Abu-Amero *et al.,* 1998). Possible explanations for these conflicting results could be the fact that term tissue was used or the fact that pathological FGR pregnancies were investigated, whereas in our study SGA pregnancies were used. In addition, as previous studies have relied on a smaller sample size, for example 16 FGR samples compared to 20 controls (Street *et al.,* 2006), these studies may not have had sufficient power to observe significant differences between the two different groups.

To better understand the causes and consequences of FGR as a human pregnancy complication, we focused on available tissue from the first trimester, as this time window is more likely to accurately reflect the *in utero* growth potential. We used a larger sample size and we investigated the whole growth spectrum including not only SGA but also LGA neonates. Extending the investigation of these genes to samples displaying macrosomic birth weight revealed further correlations between growth parameters and gene expression. In this study, LGA neonates had higher *IGF2R* expression levels compared to AGA neonates and lower *IGF1R* levels compared to SGA babies. Similar results have been reported in placenta tissue for *IGF1R* mRNA levels alone (Iniguez *et al.,* 2011). This suggests that fetuses with high birth weight are producing more *IGF2R* and correspondingly less *IGF1R*, which could remove the growth promoting effect *IGF2*. This balance may represent another important compensatory mechanism in response to fetal overgrowth (summarised in Table 2-10). This is also supported by the fact that a significant association was observed between the ratio of *IGF2* to *IGF1R* and birth weight, suggesting that the levels of IGF1R in LGA neonates decrease as their IGF2 levels increase, to avoid any further increase in size.

<u>Table 2-10:</u> Differences in the expression levels of *IGF2, IGF2R* and *IGF1R* in SGA and LGA neonates compared to AGA neonates.

Early in pregnancy at 11-13 weeks' gestation			
SGA neonates		LGA neonates	
Reduced	*IGF2*	Similar	*IGF2*
Similar	*IGF2R*	Elevated	*IGF2R*
Similar	*IGF1R*	Reduced	*IGF1R*

The "small baby syndrome hypothesis" proposed by Barker *et al.,* (1993) suggests that there is an inverse linear relation between birth weight and T2D. This is also supported by a large meta-analysis (Whincup *et al.,* 2008). The mechanisms by which birth weight is related to T2D is still under debate; some researchers claim that this relationship reflects long-term consequences of under-nutrition *in utero* (Barker *et al.,* 1993), whereas others do not see under-nutrition as playing a significant role (Hofman *et al.,* 2004). Interestingly, specific fetal *IGF2* paternal haplotypes are linked to higher maternal glucose levels that increase the risk of gestational diabetes (Petry *et al.,* 2011). Nevertheless the amount of IGF2 available from the placenta early in the first trimester is likely to be a major factor in promoting growth.

An important aim for antenatal care is the prediction, detection and treatment of abnormal fetal growth. It is known that variation in size at birth results from interaction between fetal genetic factors and the maternal genetic and uterine environment. In this study we also implicate the important role of the father's genes in fetal growth *in utero*.

2.9 FUTURE WORK

IGF2 mRNA levels in adult human peripheral blood leukocytes – a pilot study

Maternal circulating IGF-proteins increase during pregnancy. IGFII levels are highest from the end of the first trimester and persist through the remainder of pregnancy, remaining much higher than IGFI (Gargosky *et al.* 1990). Previous data has shown that imprinted genes are not highly expressed in adult blood compared to more relevant fetal tissues. However, *IGF2* was among those few that could be readily detected (Frost *et al.,* 2010).

In this pilot study, *IGF2* mRNA levels were measured using RTqPCR in peripheral blood leukocytes of 23 males, 13 non-pregnant females and 41 pregnant females (samples were previously collected by Dr Andy Duncan, UCL). An unpaired two-tailed t-test was used to compare the difference in the *IGF2* expression levels between the three groups. Pregnant females had significantly higher *IGF2* expression levels compared to both males and non-pregnant females (P=0.008 and P=0.02, respectively), suggesting that *IGF2* mRNA levels are higher during pregnancy (Fig. 2-12).

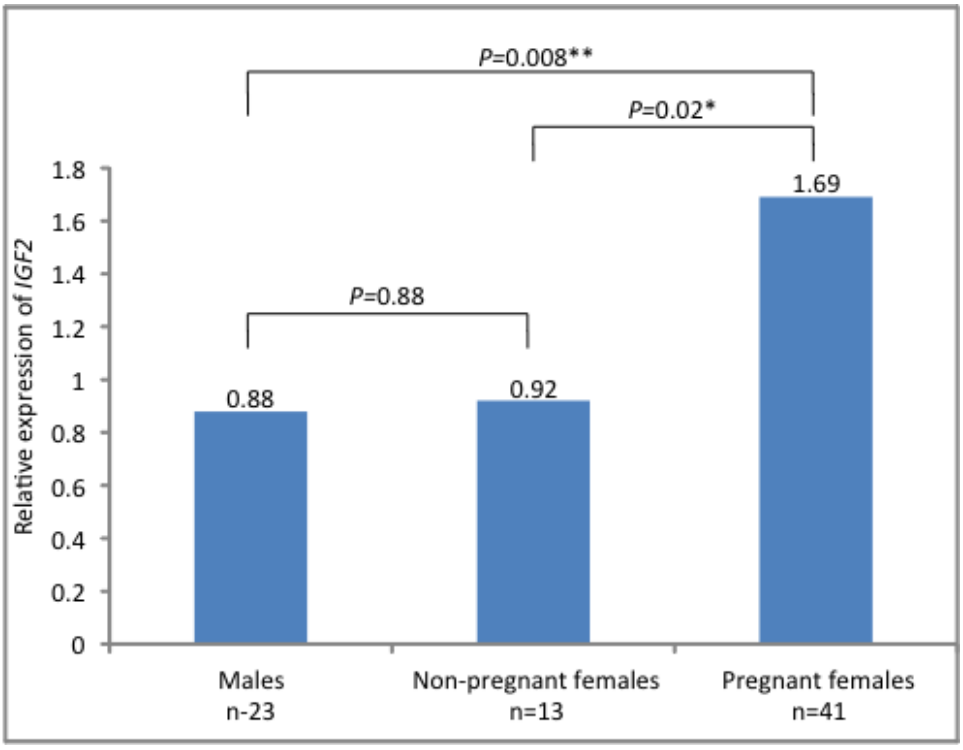


Figure 2-12: Relative expression levels of *IGF2* in adult blood. Pregnant females had significantly higher *IGF2* expression levels compared to both males and non-pregnant females (P=0.008 and P=0.02, respectively).

Interestingly, a study in pregnant guinea pigs demonstrated that administration of IGFII during early pregnancy increased the volume of the placenta devoted to exchange by 30%, as well as the transfer of nutrients from the mother to the fetus. Infusion of IGFII increased the number of viable fetuses by 26% and fetal weight by 11% but did not affect maternal body composition. Moreover, they showed that maternal IGFII in early gestation acts in part via the IGFIIR to enhance the functional development of the placenta and nutrient delivery and hence promote fetal growth (Sferruzzi-Perri *et al.,* 2006, 2007, 2008). As the IGF axis in guinea pigs is thought to function in a very similar way to that of humans, one might predict that increasing maternal IGFII abundance in an early pregnancy in humans may also enhance fetal growth and viability at term. Therefore, more samples from pregnant females are now being collected to investigate whether *IGF2* mRNA levels detected in maternal blood are correlated with birth weight at term and whether maternal *IGF2* can be used as a biomarker in predicting SGA neonates.

Understanding the developmental role, function and parent-of-origin effect of critical imprinted genes during human fetal growth will make an important contribution to effective clinical evaluation of growth disorders and their association with longer term, metabolically related, health risks such as T2D. Determination of paternal *IGF2* expression in the first trimester, potentially in maternal blood, may act as a predictor of fetal growth trajectory for later in the pregnancy. An early growth biomarker could be invaluable to alert Obstetricians and Neonatologists towards closer ‘at risk’ pregnancy surveillance.

CHAPTER 3 – IMPRINTING ANALYSIS OF MISCARRIED PRODUCTS OF CONCEPTION

3 INTRODUCTION

Fetal growth involves a complex interaction between genes and the environment. Of particular interest is one group of genes that are found almost exclusively in placental mammals, known as imprinted genes many of which have growth regulatory functions. As described in Chapter 1, genomic imprinting is an epigenetic form of gene regulation that gives rise to differential expression or silencing of a given allele, depending upon its maternal or paternal inherited origin. The imprinting mechanism is complicated and still not completely understood. Although the regulation of imprinted expression is cluster specific, several common features shared by imprinting clusters have been described, including enrichment of CpG sites which are normally subject to epigenetic modifications. These modifications include histone modifications associated with repressive or active chromatin conformation, DNA acetylation and DNA methylation (Lewis and Reik, 2006).

Histone acetylation is the process brought about by histone acetyltransferases (HATs) that acetylate the histone tails of the nucleosome, as part of gene regulation. Acetylation neutralizes the positively charged lysine residues of the histone N termini, thus decreasing their affinity for DNA. As a result, the condensed chromatin is transformed into a more relaxed structure that is associated with greater levels of gene transcription (Lee *et al.,* 1993).

However, of these mechanisms of epigenetic variation the most widely studied and best understood is methylation. The difference between an active and an inactive gene is the presence of 5'-methylcytosine bases within CpG islands that lie upstream of the gene. Allele specific methylation causes certain genes to be only active on one of the two parental chromosomes (Lee *et al.,* 1993).

The sequences contributing to differences in DNA methylation between the parental alleles are known as DMRs. DMRs can have different properties, as some DMRs are methylated resulting in an active gene copy, whereas others are methylated causing inactivation. In addition, methylation of DNA in some DMRs is introduced in the parental germ cells and maintained in all tissues and during all developmental stages, whereas others show tissue-specific patterns as well as changes during development. At imprinting loci, two types of DMRs have been described. One of them is known as a germline DMR that acquires methylation during gametogenesis and the other is known as a somatic DMR that becomes methylated after fertilization and depends on the presence of the germline DMR (Lewis and Reik, 2006; Edwards and Ferguson-Smith, 2007).

3.1 The imprinting cycle

Most imprinted genes studied to date are involved in embryonic development and fetal growth (Reik and Walter, 2001). Genomic imprints change in characteristic ways during the life cycle of an organism and have three major stages in their life cycle: establishment, maintenance, and erasure (Fig. 3-1). The paternal and the maternal epigenetic marks are heritable to the daughter cells, but must reset in each generation to establish parental specific imprints. In mammals, the parental genomes undergo two rounds of genome-wide DNA methylation reprogramming during the life cycle of imprints.

Erasure:

The first round of reprogramming that occurs in the primordial germ line involves sequential demethylation and remethylation steps where the old imprints are erased. These steps occur to remove the existing parental methylation patterns and establish new sex-specific imprints in the gametes (Constancia *et al.,* 1998; Lee *at al.,* 2002). The epigenetic

imprints of the new organism are erased, followed by the acquisition of new imprints according to the sex of the germ line, making sure that the sex dependent imprints can be expressed in later embryonic development (Reik and Walter 2001).

Establishment:

Establishing epigenetic imprints involves a complex procedure involving reprogramming of the entire genome. This reprogramming is very important, as accurate imprints must be passed from one generation to the next. All cells contain two sets of chromosomes: one from the father, hence male imprints and one from the mother, hence female imprints. When these chromosomes are passed on to the next generation, the two sets in the specific germ cells are reprogrammed to contain male or female imprints exclusively (Biliya and Bulla, 2010).

Sex-specific imprints are established during the development of germ cells into sperm or eggs (gametogenesis). The genomic imprints get implanted into the male and female germ line and then go through further development ready to be passed on to the offspring. In males, the genomic imprints become fully established and finish developing in prospermatagonia by the neonatal stage. In females, the imprints are implanted to the germ line layers of the oocyte and then completely established by the time the oocyte is fully developed (Davis *et al.,* 2000; Li *et al.,* 2004; Li and Sasaki, 2011).

The second round of reprogramming occurs after fertilization, and involves genome-wide demethylation in the early embryo prior to implantation. Post-implantation, global remethylation takes place. The somatic methylation patterns for normal development are thus created, a process that is essential for the activation and silencing of specific genes (Reik and Walter, 2001). Although is not yet clear which enzymes are responsible for *de novo* methylation, in mammals three functional DNA-methyltransferase (DNMT) enzymes have

been described; DNMT1, DNMT3a and DNMT3b (Hata *et al.,* 2002; Kaneda *et al.,* 2004).

Maintenance:

Once the imprints are completely ready to be expressed they are passed on to the zygote through fertilization and maintained throughout the development and adult life. The methylation imprints at germline DMRs escape from the global epigenetic reprogramming that occurs in the pre-implantation embryos. This reprogramming includes the active de-methylation of the paternal genome and the subsequent passive de-methylation of both parental genomes. Only imprinted genes are protected against this demethylation, thus maintaining their germline methylation marks and parent-specific activities throughout development of the new organism. The mechanism that protects ICRs from genome-wide demethylation is not well characterized, but zinc finger protein 57 (Zfp57), has been shown to be involved in the post-fertilisation maintenance of DNA-methylation imprints at multiple ICRs (Li *et al.,* 2008). In somatic cells, imprints are maintained and are modified during development. The imprints are eventually read thus resulting in parent-specific gene expression (Reik and Walter 2001, Morgan *et al.,* 2005).

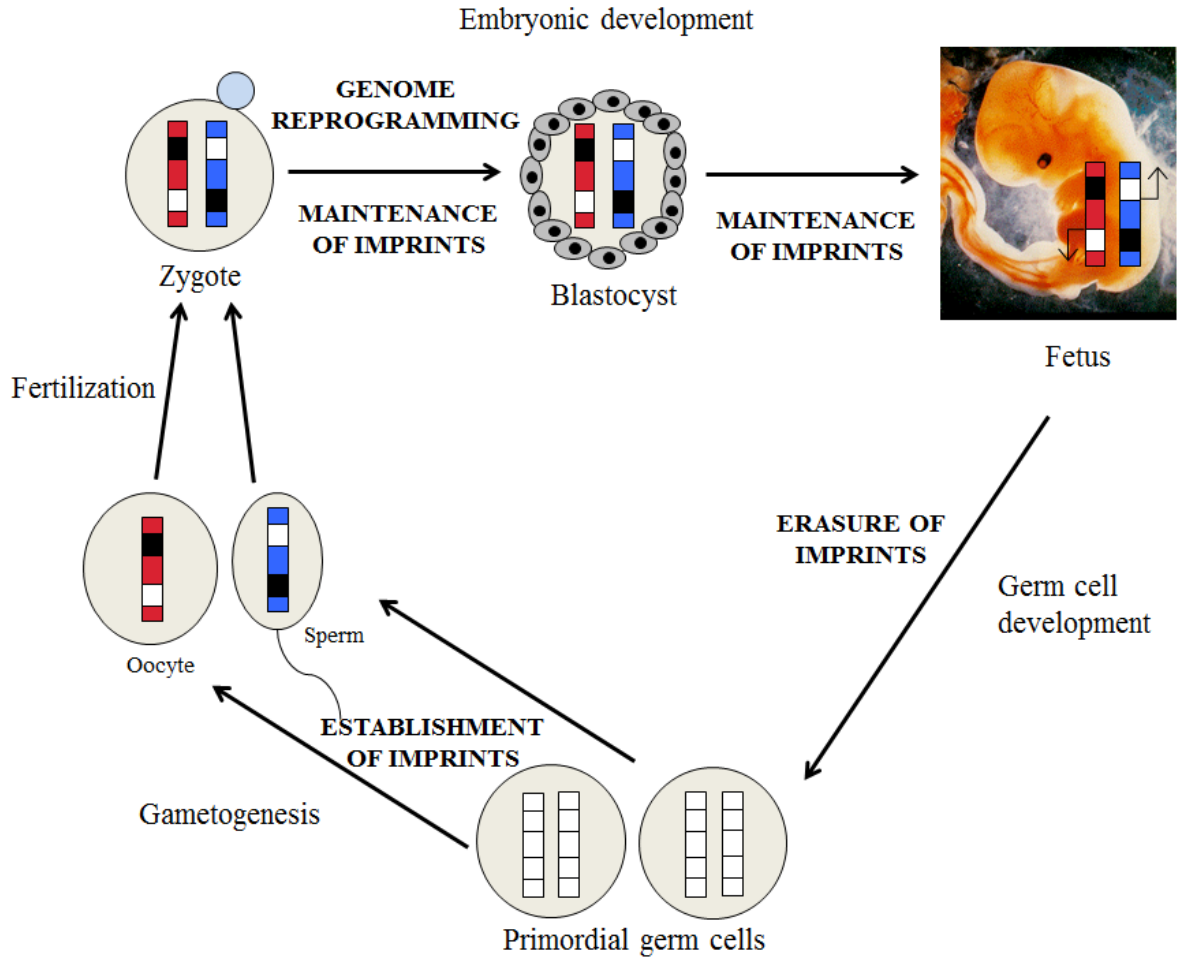


Figure 3-1: Life cycle of genomic imprints.
The maternal and paternal methylation imprints are established during the development of germ cells into sperm or eggs (gametogenesis). The two germline genomes are combined at fertilization and in the early embryo, parent-specific genome reprogramming takes place. Imprints are maintained as chromosomes duplicate and segregate in the developing organism. In the germ cells of the new organism, DNA methylation patterns are erased at an early stage. This is followed again by establishment at a later stage of germ cell development, thus completing the imprinting cycle. In somatic cells, imprints are maintained and modified during development.

3.2 Genes investigated

Known imprinted genes include *IGF2, PHLDA2,* paternally expressed gene 3 *(PEG3),* delta-like 1 homolog *(DLK1)* and *H19*. *IGF2* is paternally expressed, maternally imprinted in both mouse (Willison, 1991) and human (Giannoukakis *et al.,* 1993). Studies that have investigated the human expression of *IGF2* report decreased levels in SGA pregnancies and a positive association with birth weight early in pregnancy (Demetriou *et al.,* 2014). *PHLDA2* is maternally expressed, paternally imprinted, in both humans and mice (Qian *et al.,* 1997). Studies that have investigated the human placental expression of *PHLDA2* report increased levels in SGA pregnancies and a negative association with birth weight (Apostolidou *et al.,* 2007; Ishida *et al.,* 2012). For more information on *IGF2* and *PHLDA2* see Chapter 2 sections 2.1.1 and 2.2, respectively.

3.2.1 Paternally expressed gene 3

PEG3 was the first imprinted gene to be discovered in human chromosome 19q13.4 locus, a region of conserved synteny with mouse proximal chromosome 7 (Murphy *et al.,* 2001). *PEG3* encodes a large Kruppel-type (C2H2) zinc-finger protein, most of which are believed to function as transcription factors (el-Baradi and Pieler, 1991; Pieler and Bellefroid, 1994). Although the overall genomic structure of *PEG3* is well conserved between mice and humans, some species-specific changes exist. For instance, mouse Peg3 contains two proline-rich domains whereas human PEG3 lack one of these domains (Kim *et al.,* 2004). *PEG3* is ubiquitously expressed and imprinted in a wide variety of human fetal tissues including the fetal adrenal gland, heart, liver, lung, kidney, brain and fetal and term placenta (Murphy *et al.,* 2001). Both male and female mice carrying the disrupted paternal copy of *Peg3* are viable and fertile but suffer from placental and fetal growth restriction (Li *et al.,* 1999).

3.2.2 Delta-like 1 homolog

DLK1 is a paternally expressed gene located on the human chromosome 14q32 imprinting cluster, approximately 90 kb away from the reciprocally imprinted, maternally expressed gene 3 (*MEG3*). *DLK1* is highly conserved throughout vertebrates with the predicted aa sequence of DLK1 sharing 86% identity with mouse DLK1 (Laborda *et al.,* 1993). *DLK1* encodes a transmembrane glycoprotein containing six epidermal growth factor-like repeat motifs in the extracellular domain (Laborda *et al.,* 1993) involved in adipogenesis (Sul*,* 2009). *DLK1* is ubiquitously expressed in fetal tissues, including the fetal kidney, lung, liver, adrenal gland, brain and fetal and term placenta, where it is also shown to exhibit monoallelic expression (Wylie *et al.,* 2000). *Dlk1*-null mice show high perinatal lethality, pre- and postnatal growth restriction followed by obese phenotype (Moon *et al.,* 2002), suggesting its role as a growth promoter.

3.2.3 *H19*

H19 is maternally expressed, paternally imprinted and encodes a noncoding RNA in humans and mice (Bartolomei *et al.,* 1991). The imprinting manner of *H19* was determined by inducing *H19* mutation in mice. Offspring who inherited the *H19* mutation from the mother were 27% heavier than those who inherited it from the father. Additionally, there was no effect of paternal inheritance, which reflects the paternal silencing of this gene (Leighton *et al.,* 1995). Biallelic expression of *H19* has been reported in some cases at the early stage of normal pregnancy before the 10^{th} week of gestation (Yu *et al.,* 2009).

3.3 Human leucocyte antigens in human pregnancy

Placenta trophoblast cells form the interface between the mother and her conceptus and are the primary fetal cells that are exposed to the maternal immune system. The ability of the genetically distinct allogeneic fetus to escape immune rejection by avoiding alloreactivity has been described as an immunological paradox (Moffett-King, 2002). The main molecules involved in allorecognition by T-cells and NK cells belong to the highly polymorphic major histocompatibility complex (MHC) (reviewed by Moffett and Loke, 2006).

The MHC is a 5 Mb region on the short arm of chromosome 6 that contains the most highly polymorphic genes in the human genome. The human MHC is named the human leukocyte antigen (HLA) complex and genes of this complex are the principal cause of acute graft rejection following human transplantation. Rejection is stimulated by HLA differences between the transplant recipient and donor.

There are two HLA classes, class-I and class-II. There are six HLA class I loci that express protein products; three classical molecules (HLA-A, HLA-B and HLA-C) and three non-classical (HLA-E, HLA-F and HLA-G). The three classical molecules present antigens to CD8 T-cells and the three HLA class II (HLA-DP, HLA-DQ and HLA-DR) molecules present antigens to CD4 T-cells (Moffett-King, 2002). Class I molecules are expressed on a wide range of cells where they serve as ligands for receptors of NK cells (Parham *et al.,* 2012).

The two main trophoblast subpopulations are the villous trophoblast that covers the villous tree and contacts maternal blood in the intervillous space and the extravillous trophoblast (EVT) that invades the decidua and myometrium (Moffett-King, 2002; Moffett and Loke, 2006). The villous trophoblast that is exposed to maternal blood lacks both class I and class II molecules. The EVT cells of the placenta are the only fetal cells that contact the maternal circulation and have the capacity to stimulate maternal lymphocyte responses

against paternal HLA antigenic differences. These cells, express the non-polymorphic HLA-E and HLA-G molecules as well as the polymorphic HLA-C molecules (Apps *et al.,* 2009). HLA-C molecules are highly polymorphic with 106 different alleles known, encoding 750 proteins (IPD-HLA database).

To date, the only potential trophoblast NK-cell ligands that have been found are the MHC class-I molecules HLA-C, HLA-E and HLA-G (Ellis *et al.,* 1990; Kovats *et al.,* 1990; King *et al.,* 2000). The NK-cell receptors for HLA-C are members of the killer-cell immunoglobulin-like receptors (KIR) family. These receptors are encoded by a polymorphic family of 15 genes in the leukocyte-receptor complex, and contribute to the immunogenetics of human placentation (Moffet and Loke, 2006; Barrow and Trowsdale, 2008). Although all three (HLA-C, HLA-E and HLA-G) are ligands for NK cell receptors, it is the interaction between HLA-C and KIR that have the greatest immunogenetic potential to vary the course of pregnancy (Male *et al.,* 2011).

In pregnant women, uterine NK (uNK) cells, which represent up to 70% of the leukocyte population in first trimester decidua (King *et al.,* 1991), express a higher proportion of KIRs that are specific for HLA-C than peripheral blood NK cells do. This suggests that the range of the NK-cell receptors is skewed towards recognizing HLA-C in the uterus. This KIR expression is highest earlier in gestation and slowly declines over the first trimester (Hiby *et al.,* 1997; Verma *et al.,* 1997). Both *KIR* and *HLA-C* are highly polymorphic thus paternally derived fetal HLA-C and maternal uNK-cell KIRS should interact during pregnancy. The fact that each pregnancy involves different combinations of HLA-C molecules and maternal KIRs suggests the possibility that some combinations would not be optimal for implantation and that these pregnancies might fail as a consequence.

3.3.1 Imprinting of *HLA-C*

A recent study by Fogarty *et al.,* (unpublished, manuscript in preparation) showed that the *HLA-C* is temporally imprinted and maternally expressed in CVS at 12 weeks of gestation, becoming biallelically expressed in term placenta and adult blood. *HLA-C* expression was also absent in complete hydatidiform mole tissue where only the paternal genome is present. Given the genetic diversity of HLA alleles and the advantage associated with heterozygosity in HLA loci (Hughes and Yeager 1998), it is highly unlikely that couples will have an identical genotype for *HLA-C.* Therefore, in most pregnancies paternally inherited *HLA-C* alleles will be different from the maternal alleles.

Classical immunological paradigms describe the rejection of allogeneic tissues due to the detection of non-self MHC antigens by circulating immune cells. It is possible that in some cases, the conceptus expresses the *HLA-C* alleles inherited from the father that are different from the maternal alleles, and the maternal immune system recognizes the foreign paternal HLA-C. Based on this, we decided to investigate the imprinting of *HLA-C* in our POC cohort.

3.4 Aim and Hypothesis

Miscarriage is the most common pregnancy complication with up to 50% of women experiencing one or more early miscarriages (Rai and Regan, 2006). The selective imprinting of *HLA-C* during early human development may have a major functional significance in maintaining the human pregnancy and any failures or disruptions of this imprinting could be involved in the pathology of miscarriages.

The aim of our study was to investigate products of conception material, i.e. tissue that is present in a fertilized gestation in a pregnancy that ended in a miscarriage, for imprinting anomalies. This study focused on investigating the imprinting status of the

maternally expressed *HLA-C, H19*, *PHLDA2* genes and the paternally expressed *IGF2, PEG3* and *DLK1* genes from which DNA from both parents was available so we could detect which parental allele is expressed in these genes. Our hypothesis is that a proportion of miscarried POC that are cytogenetically normal will have abnormal expression profiles at imprinted loci, possibly causing embryonic lethality. Before this study commenced, it was first necessary to investigate the fetal origin of the POC to determine whether maternal tissue contamination might interfere with this analysis, and if so to estimate the levels of contamination present in each POC sample.

3.5 MATERIALS AND METHODS

3.5.1 Subjects

Samples were collected from the RM Clinic at St Mary's Hospital, Imperial College London. Ninety-nine trios, consisting of the mother, father and a sample from POCs were used in this study. Blood was collected from individuals agreeing to participate in the research project approved by the Hospital's Ethics Committee. Tissues from the POC were collected from the uterus of each of these women after miscarriage. Five further trios were available from the Baby Bio Bank (Abu-Amero *et al.,* 2014).

3.5.2 Preparation of DNA and RNA from samples

Parental DNAs were extracted from blood using the iPrep™ PureLink™ gDNA Blood Kit using the iPrep Purification Instrument based on the manufacturer's instructions (Invitrogen). DNA from POC tissue was extracted using the iPrep™ ChargeSwitch® gDNA Tissue Kit and RNA from the POC was extracted using the iPrep™ Total RNA kit without DNAse treatment.

3.5.3 Sex determination of POCs

A previous study has shown that the majority of POCs are maternal decidua, as maternal cell contamination (MCC) was present in 90% of the 46, XX POCs (Jarrett *et al.,* 2001). The contamination appeared to completely obscure the fetal material in 92% of these specimens. Therefore, to exclude possible cases of MCC in POCs, some researchers have used only samples with a normal male karyotype for their experiments (Doria *et al.,* 2010).

We therefore also decided to determine the gender of our 104 POCs and use only males for this study. Sex determination was done by PCR analysis of the X-Y homologous *Amelogenin* gene (*AMEL*) (Akane *et al.,* 1991). The gene has homologous sequences on both

the X and the Y chromosome; however, a small deletion in the *AMELY* homologue makes it possible to distinguish between them. Amplification of a segment of this gene using the pair of primers shown in Table 3-1 reveals both *AMELX*- and *AMELY*-specific bands at the same time, with the smaller band being indicative of the Y chromosome (Fig. 3-2).

Table 3-1: Primers designed for sequencing *AMEL*.

Gene	Primer sequence 5' to 3'	Tm (°C)	Product size (bp)	Accession number	Notes
AMELX *AMELY*	F: ctgatggttggcctcaagcctgtg R: taaagagattcattaacttgactg	58	977 788	NM_182680 NM_001143	for gDNA

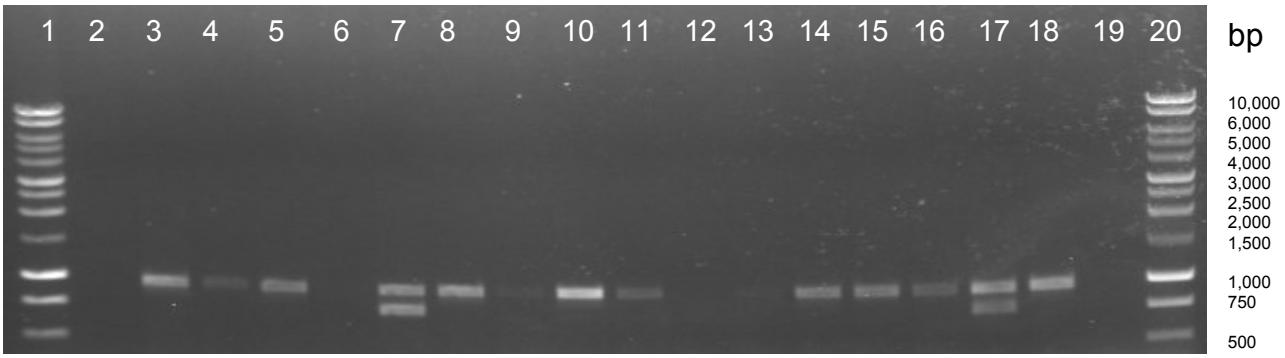


Figure 3-2: Detection of an X-Y homologous locus by PCR.
Two bands are observed in males (lanes 7 and 17) and a single band is observed in females (lanes 3, 4, 5, 8, 9 10, 11, 14, 15, 16, 18). Lanes 1 and 20 contain DNA marker (1kb) and lanes 2 and 19 are water blanks.

Out of the 104 POCs tested, nine failed to amplify, twelve POCs were male and the remaining 83 were female. However, we cannot exclude the possibility that those POCs that were determined as males were 100% free of MCC or that those POCs that were determined as females were 100% maternal decidua. Therefore we decided to use a second test to detect which of these POCs were maternally contaminated.

3.5.4 Checking for maternal cell contamination in POCs and paternity

The AmpFISTR Profiler Plus™ ID PCR Amplification kit (ABI) was used to check for the presence of MCC in the 104 POCs. This is a fluorescent kit that co-amplifies the repeat regions of 9 highly polymorphic short tandem repeat loci: D7S820 (Green *et al.,* 1991), D21S11 (Sharma and Litt, 1992), FGA (Mills *et al.,* 1992), vWA (Kimpton *et al.,* 1992), D3S1358 (Li *et al.,* 1993), D8S1179 (Oldroyd *et al.,* 1995), D18S51 (Urquhart *et al.,* 1995), D5S818 and D13S317 (Hudson *et al.,* 1995). A segment of the *AMEL* gene is also amplified. One primer of each locus-specific primer pair is labelled with the 5-FAM, JOE or NED dyes, which are detected as blue, green and yellow, respectively, on the ABI prism instruments. The standard AmpFISTR PCR reaction mix is shown in Table 3-2 and information on the loci tested using this kit is shown in Table 3-3.

Table 3-2: A standard AmpFISTR PCR reaction mix.

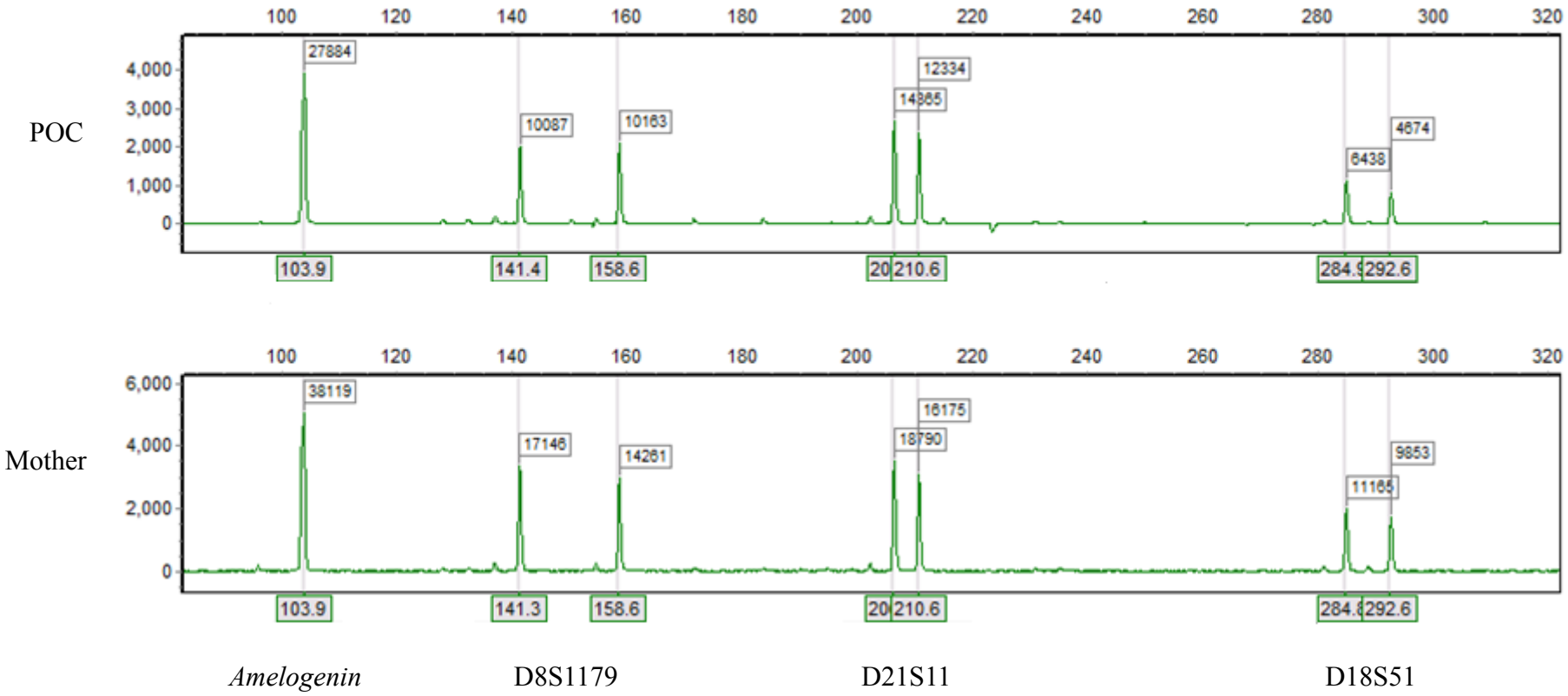
Reagent	Volume (µl) per reaction
AmpFISTR PCR Reagent Mix	4.2
AmpFISTR Profiler Plus ID Primer set	2.2
AmpliTaq Gold DNA polymerase	0.2
DEPC-treated water	3.6
DNA (1-3 ng/µl)	0.4

Table 3-3: Loci tested using the AmpFISTR Profiler Plus™ ID PCR Amplification kit.

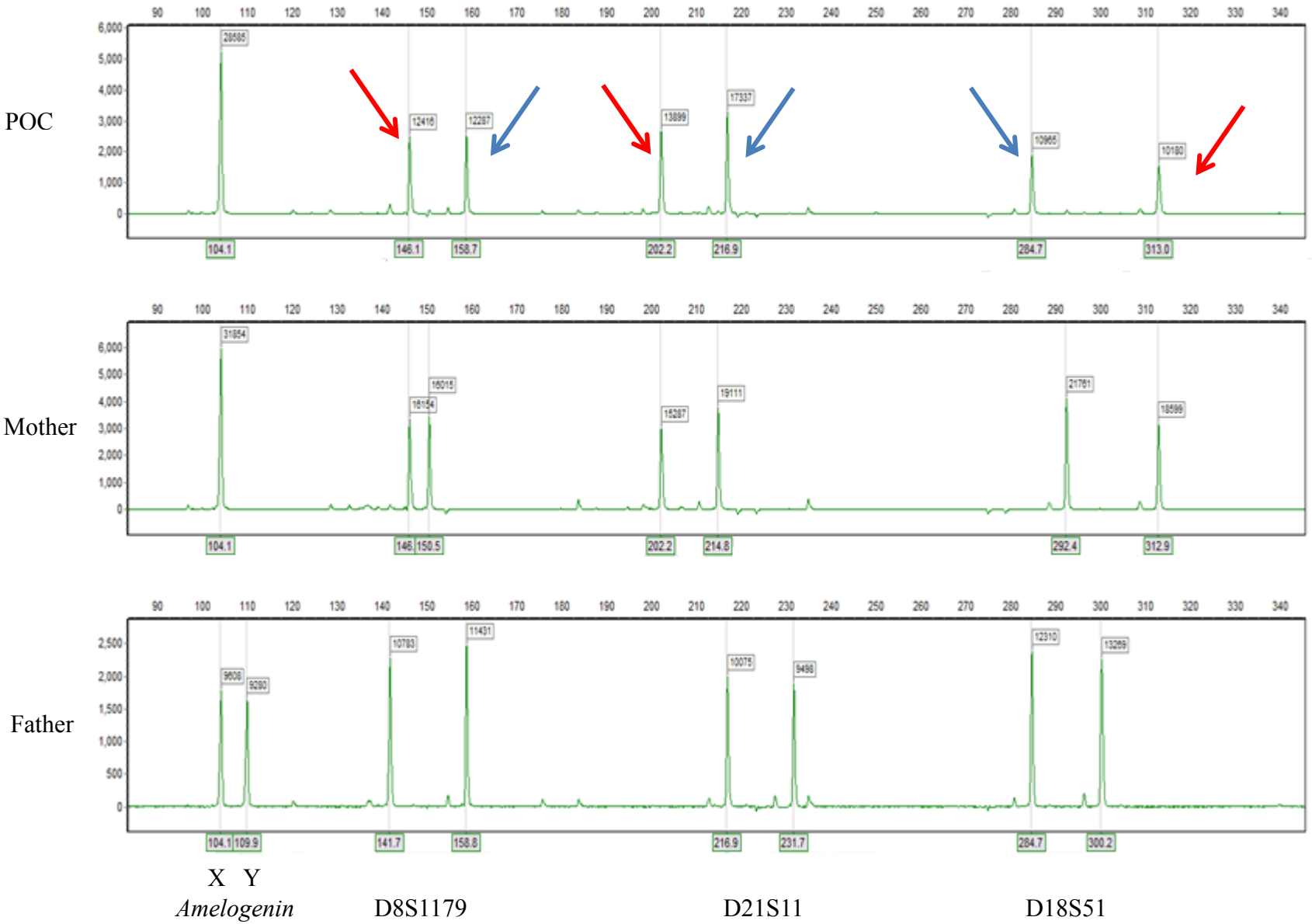
Locus Designation	Chromosome Location	Number of alleles	Size range (bp)	Dye Label
D3S1358	3p	8	114-142	5-FAM
VWA	12p12-pter	11	157-197	5-FAM
FGA	4q28	14	219-267	5-FAM
AMEL	X:p22.1-p22.3	1	107	JOE
	Y:p11.2	1	113	JOE
D8S1179	8	12	128-168	JOE
D21S11	21	22	189-243	JOE
D18S51	18q21.3	21	273-341	JOE
D5S818	5q21-q23	10	135-171	NED
D13S317	13q22-q31	8	206-234	NED
D7S820	7q	10	258-294	NED

All reactions were carried out in a Veriti® 96-Well Thermal Cycler (ABI). A standard PCR cycle consisted of an initial denaturation at 95 °C for 11 minutes, and 28 cycles of: denaturation at 94 °C for 1 minute, annealing at 59 °C for 1 minute and extension at 72 °C for 1 minute, and a final extension at 60 °C for 45 minutes. A running mix was then prepared that included 13.5 µl of Hi-Di formamide (ABI) and 0.5 µl of GeneScan™ 500 ROX™ size standard (ABI), per reaction. Using a 96-well plate, 14 µl of running mix was aliquot into each well and 1 µl of relevant PCR product was added to each well. Samples were then run on an ABI 3130.

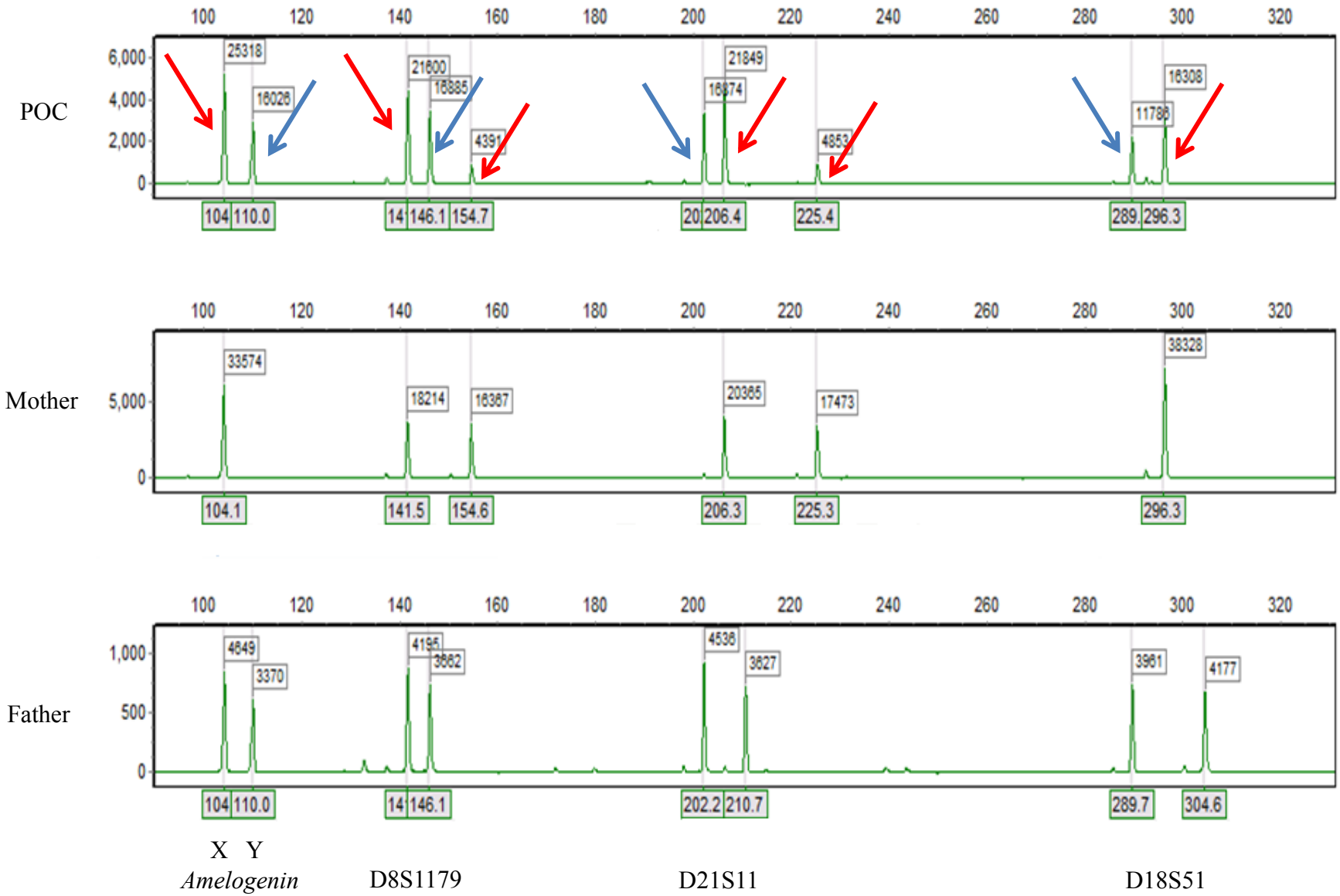
All POCs were tested for maternal contamination. This was performed by simultaneously analysing DNA from both the mother and the POC (Fig. 3-3A). POCs that were free of contamination or partially contaminated were then tested alongside paternal DNA to confirm paternity (Fig. 3-3B and 3-3C).



<u>Figure 3-3A:</u> Electropherogram showing the AmpFISTR Profiler Plus ID PCR Amplification kit results, used to detect levels of contamination in products of conception. The x-axis is showing the loci tested. This graph is showing three (D8S1179, D21S11 and D18S51) out of the nine loci tested using this kit as well as the *AMEL* gene. The number in the grey box under each peak refers to the allele size and the number in the white box above each peak refers to the area under the peak. The y-axis is showing the level of intensity. You can change the intensity level and this does not change the allele size or the area under the peak. In this example, this POC is actually maternal decidua (100% contamination) as it contains exactly the same alleles as the mother (identical allele sizes).



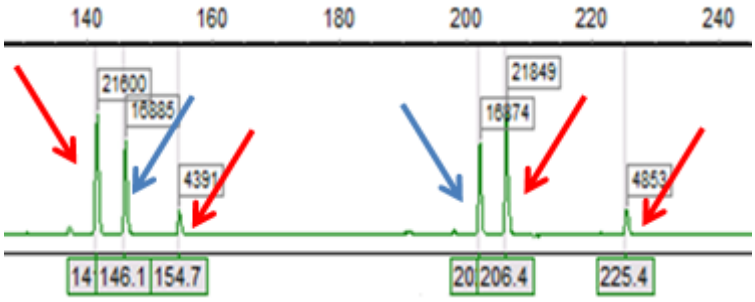
<u>Figure 3-3B:</u> Using the AmpFISTR Profiler Plus ID PCR Amplification kit to detect levels of MCC in the POC as well as paternity. This POC is female as it contains only the X allele. This POC is free of maternal contamination, as in the loci shown here (D8S1179, D21S11 and D18S51), two peaks are detected, one from the mother (indicated by the red arrows) and one from the father (indicated by the blue arrows).



<u>Figure 3-3C:</u> Using the AmpFISTR Profiler Plus ID PCR Amplification kit to detect levels of MCC in the POC as well as paternity. This POC is male as it contains both X and Y alleles. However it is partially maternally contaminated, as in the loci D8S1179 and D21S11, two peaks are detected from the mother (indicated by the red arrows) and one peak is detected from the father (indicated by the blue arrows). The level of contamination in this POC was calculated to be around 21%.

3.5.5 Results of maternal cell contamination test

Out of the 104 mothers and POCs tested, nine POCs failed to amplify and six POCs did not match the mother. Out of the remaining 89 POCs, 66 were 100% maternally contaminated (maternal decidua). Only seven POCs were free of MCC while 16 POCs had an average of 47% MCC. The area under the peak was used to calculate the level of MCC in these 16 POCs (Thiede *et al.,* 1999). An example is shown in Figure 3-4 below (part of Fig. 3-3C above).



MCC= 100 x [4391/(4391+16885)]=20.6% MCC= 100 x [4853/(4853+16874)]=22.3%

Figure 3-4: Using the area under the peak to calculate MCC levels in each POC sample. The area under the peak of the extra maternal allele (in this case the smaller peak indicated by the red arrow) is divided by the sum of the area under the peak of the paternal allele (indicated by the blue arrow) and the area under the peak of the extra maternal allele. The average contamination level in this specimen is 21.5 % [(20.6+22.3)/2].

Of the 23 POCs that were either free of maternal contamination or were partially contaminated, the paternal DNA was used to check for paternity. All 23 fathers matched the POCs (Fig. 3-5). The RNA samples of these 23 POCs were also checked for the presence of maternal cells as we suspected that the levels of contamination could have been different due to the fact that a different part of the POC tissue was used for the RNA extractions. The fact that no DNase was used in the RNA extraction, allowed us to test for this as we expected some DNA to be present in the RNA. Out of the seven non-contaminated DNA POC samples, two had partial maternal contamination (average 17%) in the RNA and five were

free of contamination. Out of the 16 partially contaminated DNA POC samples, one RNA sample was fully contaminated and the remaining 15 had an average of 49% contamination.

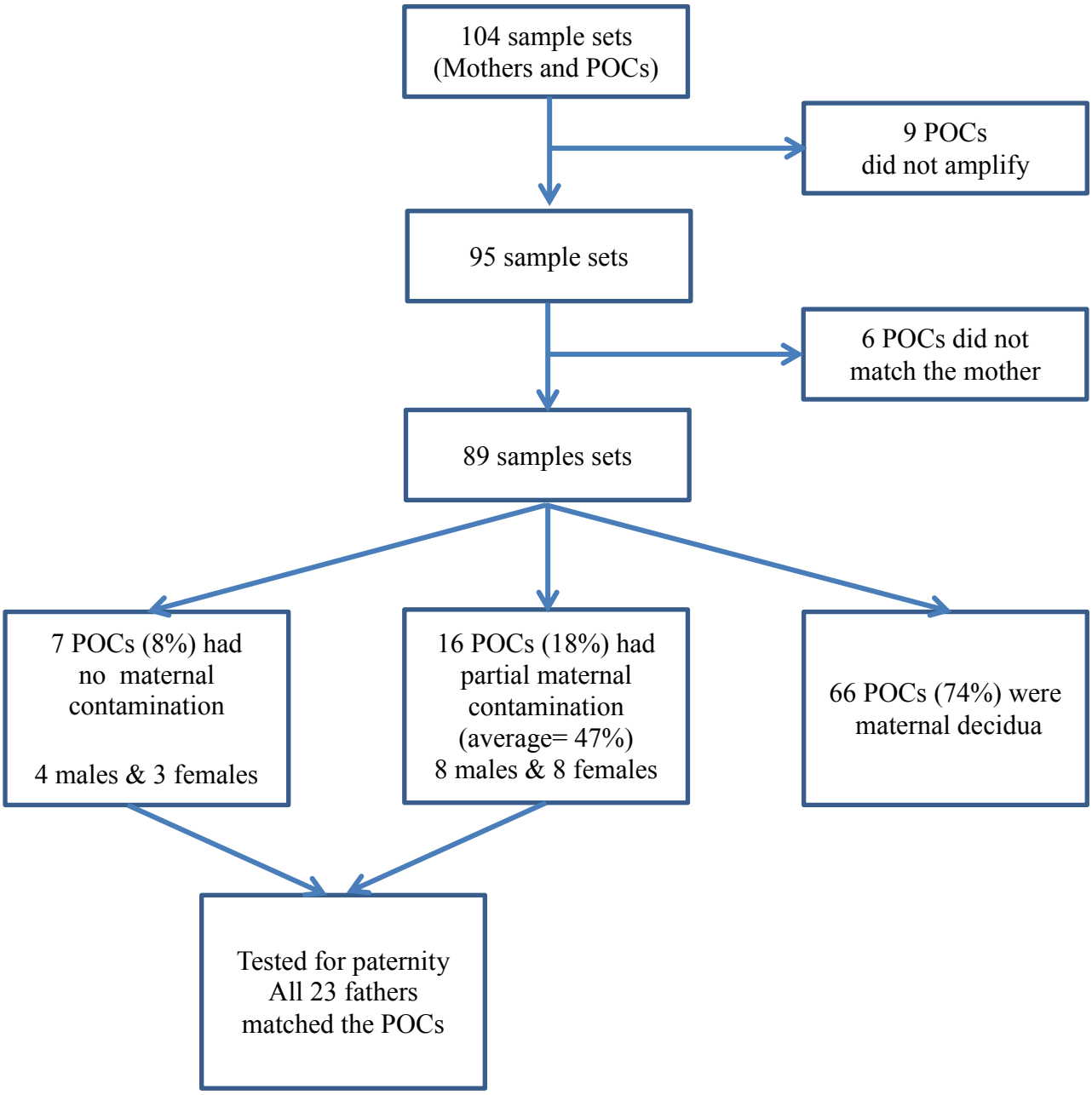


Figure 3-5: POCs were tested for MCC and paternity.
Out of the 104 POCs tested, nine did not amplify and six did not match the mother. Out of the remaining 89 POCs, 66 were fully contaminated. Only seven POCs (four males and three females) were free of MCC and 16 POCs (eight males and eight females) had an average of 47% contamination. All 23 POCs tested for paternity, matched the father.

3.5.6 Reverse transcription

All of the 22 RNA samples from POCs that were free of MCC or were partially contaminated, as well as 20 RNA from maternal decidua were reverse transcribed to cDNA. For protocol see section 2.6.7 in Chapter 2.

3.5.7 Polymerase Chain Reaction

Genomic DNA from the 22 trios and the 20 mothers that had their decidua reverse transcribed as well as cDNA from the 22 POCs and the 20 maternal decidua were amplified by PCR before sequencing. Primers used are shown in Table 3-4 below:

Table 3-4: Primers designed for sequencing gDNA and cDNA for imprinting analysis.

Gene	Primer sequence 5' to 3'	Tm (°C)	Product size (bp)	Accession number	Notes
HLA-C	F: cgcttaccatgactgacctg R: gaaggttctcaggtctttatttgc	58	469	NM_002117	for gDNA
HLA-C	F: gctctgatgagtctctcatcact R: gaaggttctcaggtctttatttgc	60	450	NM_002117	for cDNA
IGF2	F: aacaccccacaaaagctcag R: tgcatggattttggttttca	58	425	NM_000612	for both gDNA and cDNA
H19	F: ctcacccaccgcaattcatt R: ccctggatgctgtactgtct	60	340	NR_002196	for both gDNA and cDNA
PHLDA2	F: caaaccccgcacgccatgag R: gatttatttgcaatgggcacag	60	483	NM_003311	for both gDNA and cDNA
PEG3	F: gaaccctgtcaatatctttttgag R: catgagaaccacttcaacaaac	57	263	NM_006210	for both gDNA and cDNA
DLK1	F: cacctgcgtggatgatgag R: gatggtgaagcagatggcctg	58	283	NM_003836	for both gDNA and cDNA

For protocol see section 2.6.5 in Chapter 2.

3.5.8 Sequencing

For protocol see section 2.6.10 in Chapter 2.

3.5.9 Imprinting analysis

Imprinting analysis of *HLA-C* in maternal decidua and analysis of *HLA-C*, *IGF2, H19*, *PHLDA2, PEG3* and *DLK1* in POC trios was carried out by DNA sequence analysis of expressed SNPs. Corresponding cDNA samples were screened in individuals heterozygous for SNPs in *HLA-C* (rs1049853 C/T, rs1049724 G/A, rs1049709 A/G, rs1065711 T/C, rs1049650 G/C and rs1094 A/G), *IGF2* (rs680 A/G, rs74050124 C/T), *H19* (rs2067051 A/G), *PHLDA2* (rs1056819 A/G), *PEG3* (rs1055359 A/G) and *DLK1* (rs1802710 C/T) (http://www.ncbi.nlm.nih.gov/projects /SNP/).

3.6 RESULTS

3.6.1 Imprinting status of *HLA-C* in maternal decidua

Genomic DNA samples extracted from blood from 20 females whose POC sample was found to be maternal decidua, were tested for heterozygosity at the *HLA-C* SNPs. Eight of these samples were informative for at least one SNP. Upon sequencing the respective cDNA, all samples were biallelically expressed for *HLA-C* (Fig. 3-6). Therefore, it could be confirmed that *HLA-C* is biallelically expressed in maternal decidua tissue.

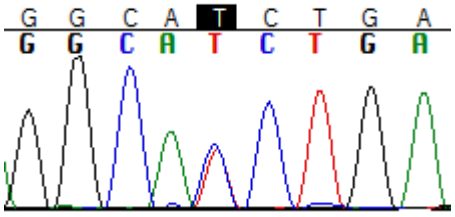
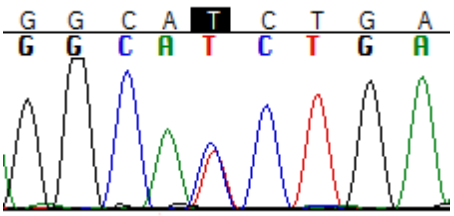
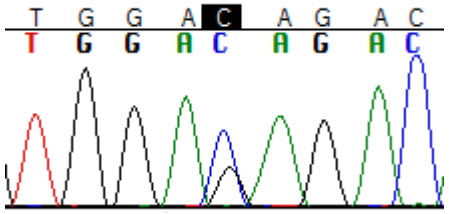
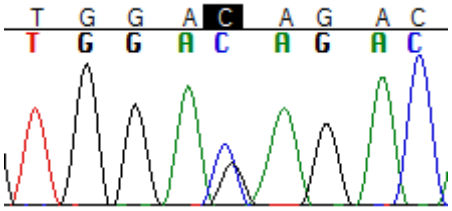
	HLA-C	
Lab ID	**A)** Genomic DNA - rs1065711	**C)** cDNA from decidua - rs1065711
69 Mother		
	B) Genomic DNA - rs1049650	**D)** cDNA from decidua - rs1049650
83 Mother		

Figure 3-6: Imprinting analysis of *HLA-C* in maternal decidua tissue. Representative Sanger sequencing chromatograms are shown from two of the eight informative samples for the *HLA-C* T/C polymorphism (rs1065711) (A) and the *HLA-C* G/C polymorphism (rs1049650) (B). Biallelic expression was observed in all the samples tested (C, D).

3.6.2 Imprinting status of *HLA-C, IGF2, H19, PHLDA2, PEG3* and *DLK1* in POCs.

Genomic DNA extracted from the 22 POCs that had either no maternal contamination or had partial contamination was used to test for heterozygosity at the *HLA-C*, *IGF2*, *H19*, *PHLDA2, PEG3* and *DLK1* SNPs. All samples were informative for at least one of these genes. Twenty-one samples were informative at different SNPs for *HLA-C*, fourteen were informative for *IGF2*, twelve were informative for *H19*, five were informative for *PHLDA2,* seven were informative for *PEG3* and nine were informative for *DLK1* (Table 3-5).

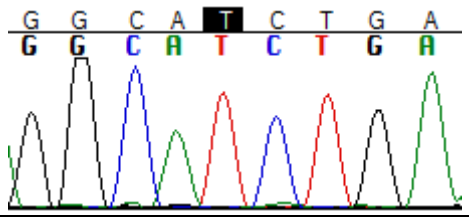
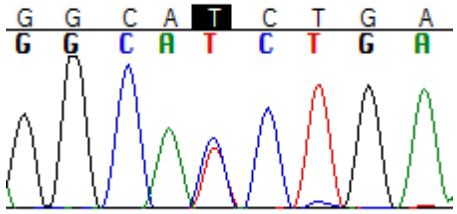
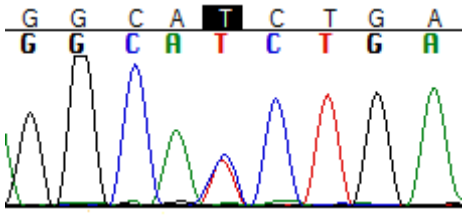
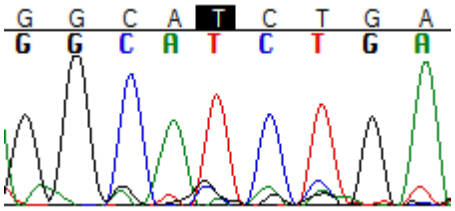
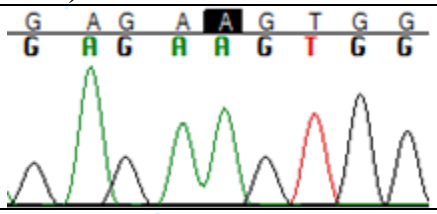
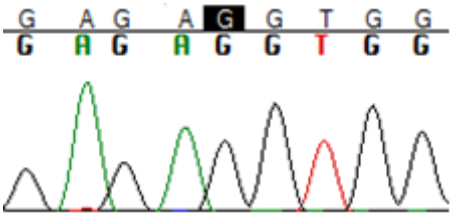
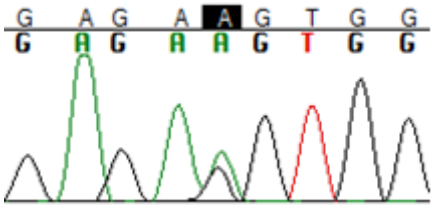
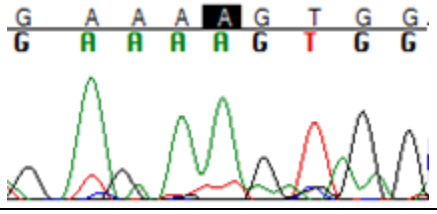
Table 3-5: Table showing the 22 informative POCs for the *HLA-C*, *IGF2*, *H19*, *PHLDA2, PEG3* and *DLK1* SNPs.

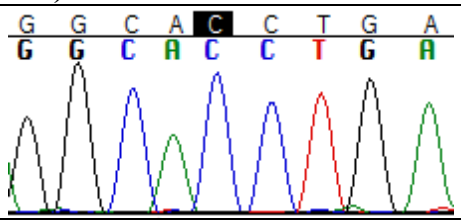
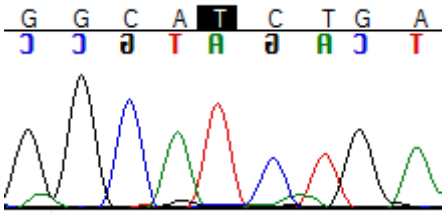
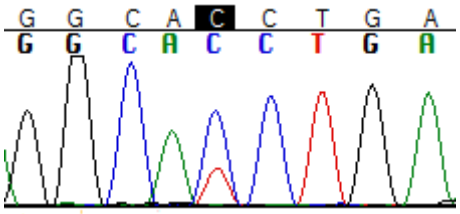
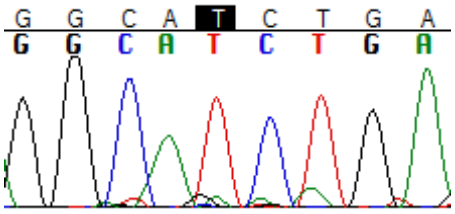
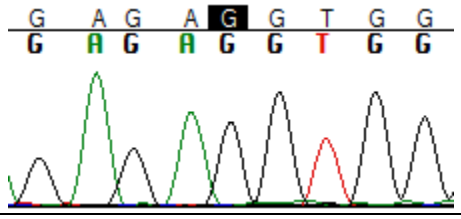
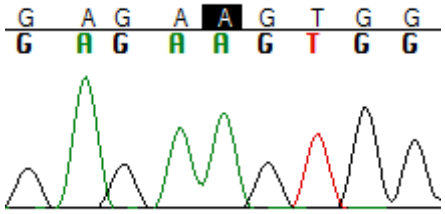
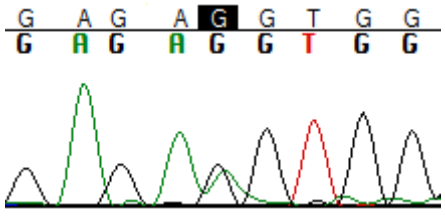
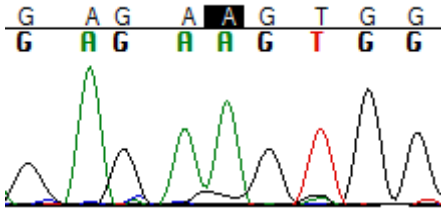
	HLA-C						***IGF2***		***H19***	***PHLDA2***	***PEG3***	***DLK1***
POC No	rs1049853	rs1049724	rs1049709	rs1065711	rs1049650	rs1094	rs680	rs74050124	rs2067051	rs1056819	rs1055359	rs1802710
5	C/T						A/G		A/G			
36 BBB*						A/G	A/G		A/G			
43				T/C	G/C	A/G	A/G			A/G		
45				T/C	G/C		A/G			A/G		
67				T/C		A/G						T/C
72					G/C							
84			A/G				A/G				A/G	T/C
102		G/A						C/T	A/G			
119 BBB*	C/T						A/G		A/G		A/G	
120				T/C			A/G				A/G	T/C
129			A/G			A/G			A/G			
131					G/C		A/G			A/G	A/G	T/C
138									A/G		A/G	T/C
145				T/C		A/G						
151				T/C		A/G						
158	C/T				G/C				A/G	A/G		
163		G/A							A/G			T/C
201				T/C		A/G	A/G		A/G		A/G	T/C
215			A/G				A/G		A/G			
217			A/G				A/G		A/G			
219		G/A					A/G		A/G	A/G		T/C
220				T/C	G/C	A/G	A/G				A/G	T/C
Total	3	3	4	8	6	8	13	1	12	5	7	9

*BBB: samples from Baby Bio Bank

3.6.2.1 Imprinting status of *HLA-C* in POCs

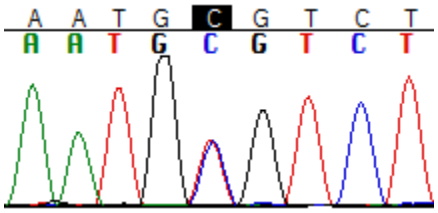
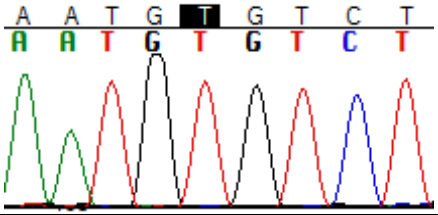
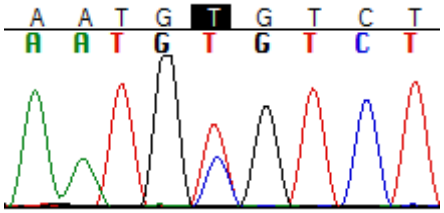
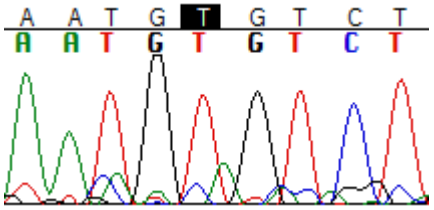
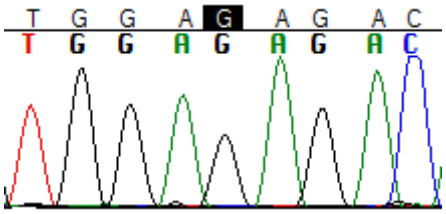
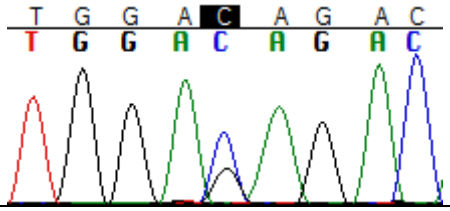
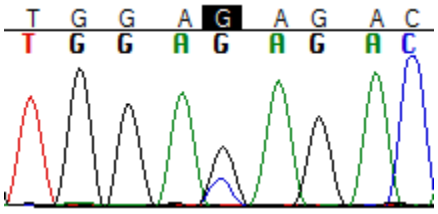
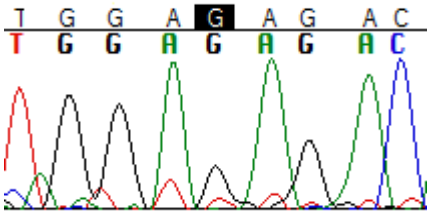
All 21 samples that were informative for *HLA-C* were monoallelically expressed; ten were maternally expressed, paternally imprinted and eight were paternally expressed, maternally imprinted (Fig. 3-7). The parental origin for three POC samples could not be determined as both parents were heterozygotes at the same SNP.

	HLA-C	
Lab ID	**A)** Genomic DNA - rs1065711	**C)** cDNA from POC - rs1065711
43 Mother		
43 Father		
43 POC		
	B) Genomic DNA - rs1094	**D)** cDNA from POC - rs1094
43 Mother		
43 Father		
43 POC		

	HLA-C	
Lab ID	**E)** Genomic DNA - rs1065711	**G)** cDNA from POC - rs1065711
145 Mother		
145 Father		
145 POC		
	F) Genomic DNA - rs1094	**H)** cDNA from POC - rs1094
145 Mother		
145 Father		
145 POC		

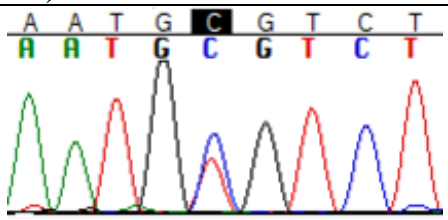
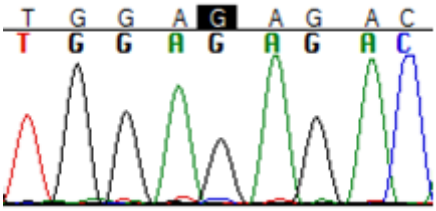
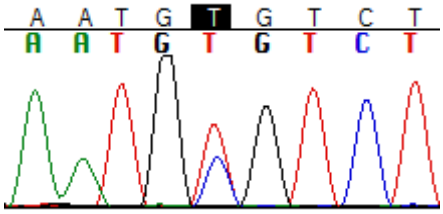
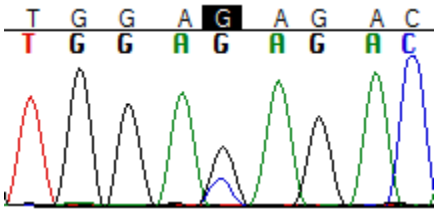
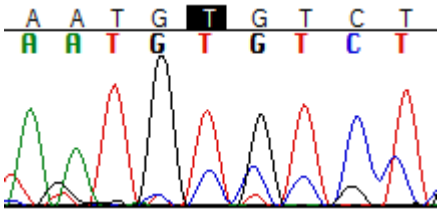
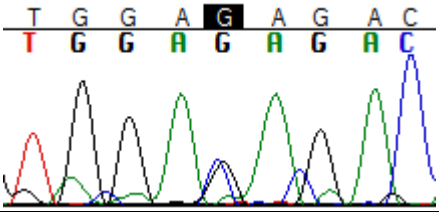
<u>Figure 3-7:</u> Imprinting analysis of *HLA-C* in POCs. Representative Sanger sequencing chromatograms are shown from two of the 21 informative POC samples for the *HLA-C* T/C polymorphism (rs1065711) (A, E) and the *HLA-C* A/G polymorphism (rs1094) (B, F). Monoallelic expression was observed in all of the samples tested. However ten POC samples expressed the maternal allele (C, D) while eight samples expressed the paternal allele (G, H). The parental origin for three POC samples could not be determined as both parents were heterozygotes at the same SNP.

However, it was interesting to note that some POC samples gave conflicting imprinting results. For example, POC 45 that was informative heterozygous for the two *HLA-C* SNPs appeared to be paternally expressed, maternally imprinted at the first SNP and maternally expressed, paternally imprinted at the second SNP (Fig. 3-8).

	HLA-C	
Lab ID	**A)** Genomic DNA – rs3176007	**C)** cDNA from POC – rs3176007
45 Mother		
45 Father		
45 POC		
	B) Genomic DNA – rs1049650	**D)** cDNA from POC – rs1049650
45 Mother		
45 Father		
45 POC		

<u>Figure 3-8:</u> Imprinting analysis of *HLA-C* in POCs that gave conflicting results. Representative Sanger sequencing chromatograms are shown for a sample that gave conflicting results for the *HLA-C* T/C polymorphism (rs3176007) (A) and the *HLA-C* G/C polymorphism (rs1049650) (B). Monoallelic expression was observed in both SNPs. However, *HLA-C* in this sample appears to be paternally expressed for the first SNP, as the T allele inherited from the father was expressed (C). The same POC appears to be maternally expressed at the second SNP, as the G allele inherited from the mother was expressed (D).

To investigate this further, we decided to investigate the POC genomic DNA at different levels of MCC (Fig. 3-9).

Lab ID	**A)** Genomic DNA – rs3176007	**D)** Genomic DNA - rs1049650
45 POC 75% MCC		
	B) Genomic DNA – rs3176007	**E)** Genomic DNA - rs1049650
45 POC 65% MCC		
	C) Genomic DNA – rs3176007	**F)** Genomic DNA - rs1049650
45 POC 32% MCC		

<u>Figure 3-9:</u> POC genomic DNA at different levels of MCC. Sanger sequencing chromatograms are shown for POC sample 45 for the *HLA-C* T/C polymorphism (rs3176007) and the *HLA-C* G/C polymorphism (rs1049650), at different levels of MCC, that is at 75% (A,D), 65% (B,E) and 32% (G,F).

At 65% MCC, POC 45 appeared to be heterozygous for rs3176007 (Fig. 3-9B). However at higher levels of MCC (75%), the peak of the C allele inherited from the mother was higher (Fig. 3-9A), whereas at lower levels of MCC (32%) the peak of the C allele drops

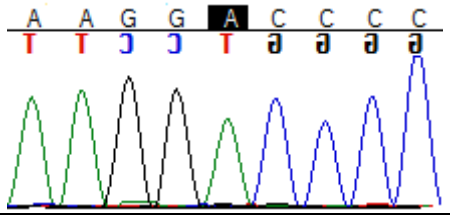
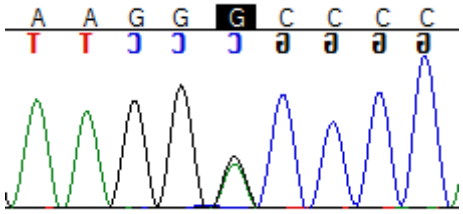
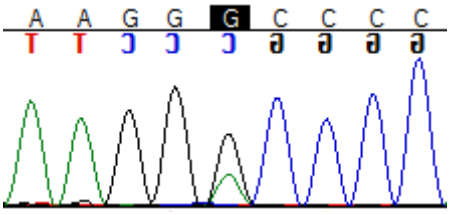
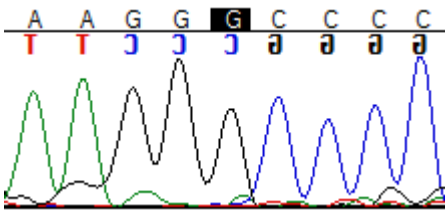
(Fig. 3-9C), suggesting that the sample is in fact homozygous T/T for rs3176007 and that the C allele from the mother appeared in the chromatogram because of the contamination.

For rs1049650, at 75% MCC it appeared that this POC inherited a G allele from both parents (Fig. 3-9D). However when the levels of contamination drop to 65% a small peak of the C allele that was inherited from the father (Fig. 3-9E) was observed and this peak becomes even higher when the levels of contamination drop to 32% (Fig. 3-9F), suggesting that POC 45 was in fact heterozygous G/C for rs1049650. Therefore in POC 45, *HLA-C* was maternally expressed, as rs1049650 is the informative SNP.

Taking this analysis in to consideration, the imprinting status of *HLA-C* in POCs was found to be abnormal, with 38% of the samples demonstrating expression from the paternal allele. Given this unexpected result, it was decided to investigate additional imprinted genes to establish if this was unique to *HLA-C*, or a more global imprinting anomaly.

3.6.2.2 Imprinting status of *IGF2* in POCs

All fourteen samples that were informative for *IGF2* were monoallelically expressed; seven were paternally expressed, maternally imprinted and four were maternally expressed, paternally imprinted (Fig. 3-10). The parental origin for three POC samples could not be determined as both parents were heterozygotes at the same SNP. Therefore, the imprinting status of *IGF2* in many of the POCs was considered abnormal, with 29% of the samples showing expression from the maternal allele.

	IGF2	
Lab ID	**A)** Genomic DNA – rs680	**C)** cDNA from POC – rs680
5 Mother		
5 Father		
5 POC		

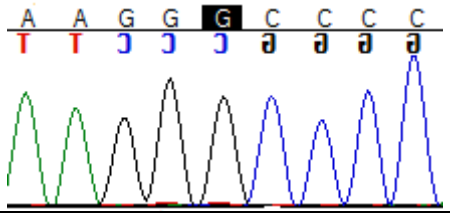
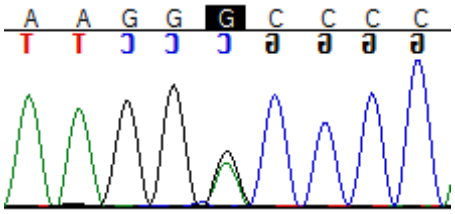
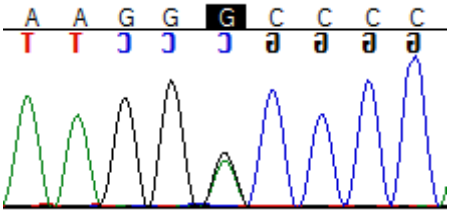
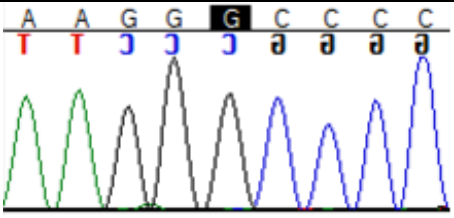
	IGF2	
Lab ID	**B)** Genomic DNA – rs680	**D)** cDNA from POC – rs680
43 Mother		
43 Father		
43 POC		

Figure 3-10: Imprinting analysis of *IGF2* in POCs. Representative Sanger sequencing chromatograms are shown from two of the fourteen informative POC samples for the *IGF2* A/G polymorphism (rs680) (A, B). Monoallelic expression was observed in all of the samples tested. However seven POC samples expressed the paternal allele (C) while four samples expressed the maternal allele (D). The parental origin for three POC samples could not be determined as both parents were heterozygotes at the same SNP.

3.6.2.3 Imprinting status of *PHLDA2* in POCs

All five samples that were informative for *PHLDA2* were monoallelically maternally expressed (Fig. 3-11). Therefore, the imprinting status of *PHLDA2* in POCs was considered normal.

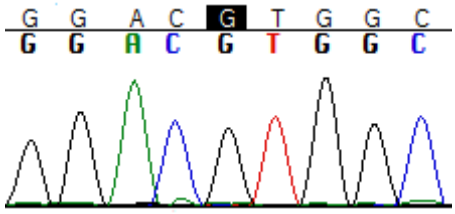
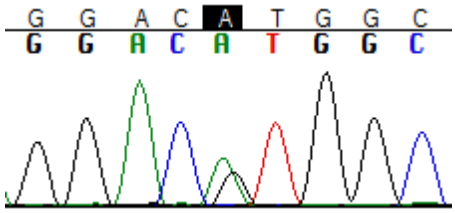
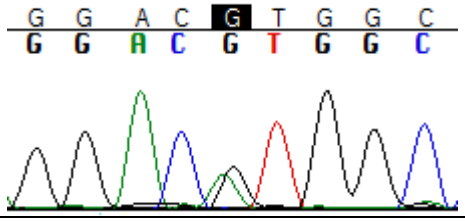
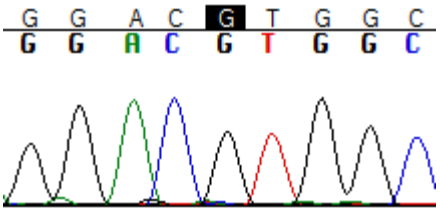
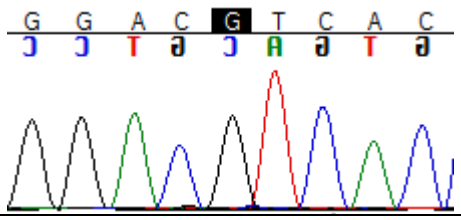
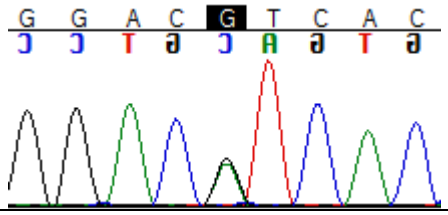
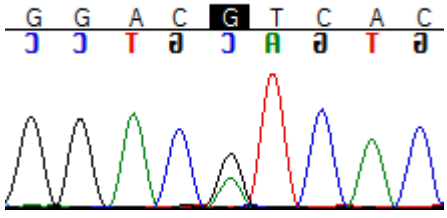
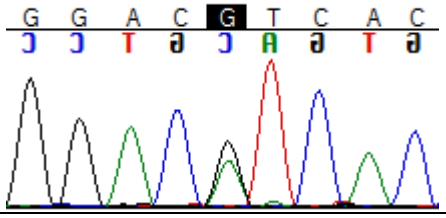
	PHLDA2	
Lab ID	**A)** Genomic DNA – rs1056819	**B)** cDNA from POC – rs1056819
43 Mother		
43 Father		
43 POC		

Figure 3-11: Imprinting analysis of *PHLDA2* in POCs. Representative Sanger sequencing chromatograms are shown from one of the five informative POC samples for the *PHLDA2* A/G polymorphism (rs1056819) (A). Monoallelic expression was observed in all of the five samples tested. Three samples expressed the maternal allele (B). The parental origin for two POC samples could not be determined as both parents were heterozygotes at the same SNP.

3.6.2.4 Imprinting status of *H19* in POCs

Twelve samples were informative for *H19*. Ten samples showed monoallelic expression and two samples showed biallelic expression. Seven samples were maternally expressed, paternally imprinted (Fig. 3-12). The parental origin for three samples could not be determined as both parents were heterozygotes at the same SNP. This mixed imprinting status of *H19* with 17% of samples showing biallelic expression and the remained showing monoallelic expression from the maternal allele, was considered to be normal (Yu *et al.,* 2009).

	H19	
Lab ID	**A)** Genomic DNA - rs2067051	**C)** cDNA - rs2067051
102 Mother		
102 Father		
102 POC		

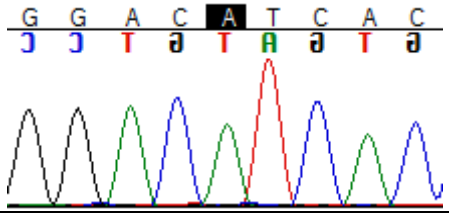
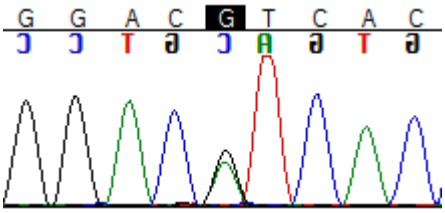
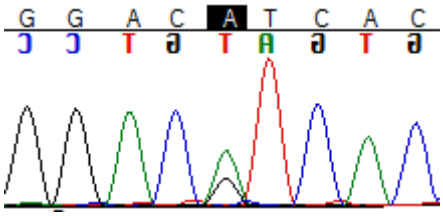
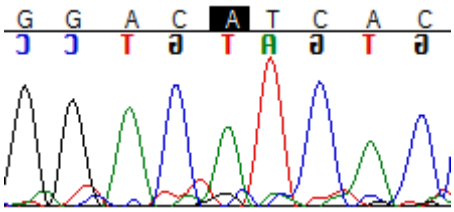
	H19	
Lab ID	**B)** Genomic DNA - rs2067051	**D)** cDNA - rs2067051
215 Mother		
215 Father		
215 POC		

Figure 3-12: Imprinting analysis of *H19* in POCs. Representative Sanger sequencing chromatograms are shown from two of the twelve informative POC samples for the *H19* A/G polymorphism (rs2067051) (A, B). Biallelic expression was observed in two out of the twelve samples tested (C). Monoallelic expression was observed in all the remaining ten samples. Seven samples expressed the maternal allele (D). The parental origin for three samples could not be determined as both parents were heterozygotes at the same SNP.

3.6.2.5 Imprinting status of *PEG3* in POCs

Seven samples were informative for *PEG3*. However, for two of these samples a clear cDNA chromatogram could not be obtained. The remaining five informative samples were monoallelically expressed (Fig. 3-13). Expression from the paternal allele indicated that the imprinting status of *PEG3* in POCs was as expected.

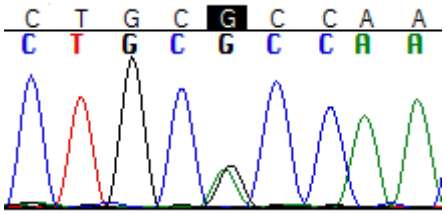
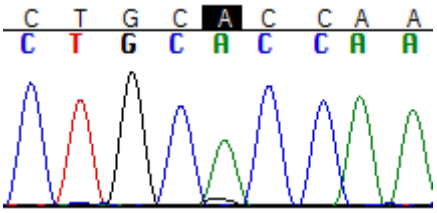
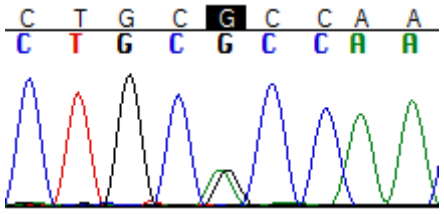
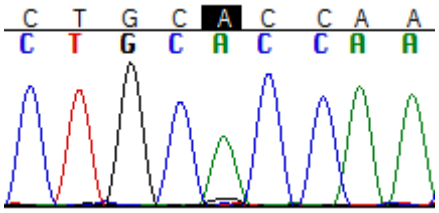
	PEG3	
Lab ID	**A)** Genomic DNA – rs1055359	**B)** cDNA from POC – rs1055359
220 Mother		
220 Father		
220 POC		

Figure 3-13: Imprinting analysis of *PEG3* in POCs. Representative Sanger sequencing chromatograms are shown from one of the five informative POC samples for the *PEG3* A/G polymorphism (rs1055359) (A). Monoallelic expression was observed in all of the five samples. Two samples expressed the paternal allele (B). The parental origin for three POC samples could not be determined as both parents were heterozygotes at the same SNP.

3.6.2.6 Imprinting status of *DLK1* in POCs

Nine samples were informative for *DLK1*. However, only one generated a clear cDNA chromatogram (Fig. 3-14). This is most likely because *DLK1* is not highly expressed early in pregnancy. This sample was monoallelically paternally expressed as expected.

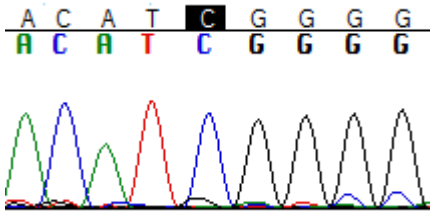
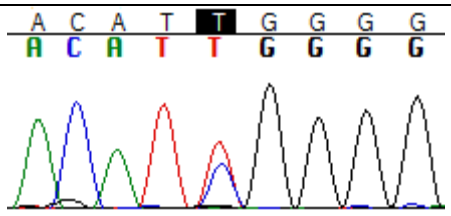
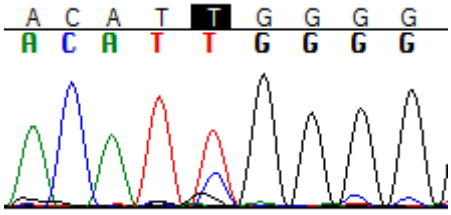
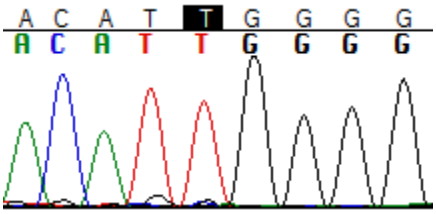
	DLK1	
Lab ID	**A)** Genomic DNA – rs1802710	**B)** cDNA from POC – rs1802710
219 Mother		
219 Father		
219 POC		

Figure 3-14: Imprinting analysis of *DLK1* in POCs. Sanger sequencing chromatograms are shown from the one POC sample for the *DLK1* C/T polymorphism (rs1802710) (A). Monoallelic expression from the paternal allele was observed in this sample (B).

Table 2-6 summarizes the imprinting status of the maternally expressed *HLA-C, PHLDA2* and *H19* and the paternally expressed *IGF2, PEG3* and *DLK1* in the 22 POCs tested. In summary, two of the genes tested showed abnormal expression in POCs, as 38% of samples showed paternal expression of *HLA-C* and 29% showed maternal expression of *IGF2*, whereas the rest of the genes were expressed as normal. Combining all the data together, 11 of the 22 POCs (50%) showed abnormal expression for at least one imprinted gene tested.

Table 3-6: A summary of the imprinting status of the genes *HLA-C, IGF2, PHLDA2, H19, PEG3* and *DLK1* in the 22 POC samples tested.

POC no	Sex	Cont. in DNA	Cont. in RNA	*HLA-C* (mat)	*IGF2* (pat)	*PHLDA2* (mat)	*H19* (mat)	*PEG3* (pat)	*DLK1* (pat)
5	Female	NO cont	NO cont	PAT	PAT		BIALLELIC		
36 BBB	Male	NO cont	NO cont	PAT	MAT		MONO		
43	Female	NO cont	NO cont	MAT	MAT	MAT			
45	Male	65%	32%	MAT	MONO	MAT			
67	Male	41%	70%	MAT					?
72	Female	67%	71%	MAT					
84	Male	38%	40%	MONO	MAT			PAT	?
102	Female	NO cont	NO cont	MAT	MAT		BIALLELIC		
119 BBB	Male	29%	36%	MAT	MONO		MONO	MONO	
120	Male	70%	43%	MAT	PAT			MONO	?
129	Female	28%	11%	MAT			MAT		
131	Female	53%	63%	PAT	PAT	MONO		?	?
138	Male	NO cont	NO cont				MAT	?	?
145	Female	31%	70%	PAT					
151	Female	32%	52%	MAT					
158	Male	23%	66%	PAT		MAT	MONO		
163	Male	45%	15%	PAT			MAT		?
201	Male	NO cont	13%	PAT	PAT		MAT	MONO	?
215	Male	NO cont	20%	MONO	MONO		MAT		
217	Male	40%	40%	MAT	PAT		MAT		
219	Female	67%	56%	MONO	PAT	MONO	MAT		PAT
220	Female	69%	65%	PAT	PAT			PAT	?

KEY	
MAT	Maternally expressed
PAT	Paternally expressed
MONO	Monoallelic expression
BIALLELIC	Biallelic expression
?	No amplification of cDNA
POC no	POC has at least one abnormal expression of one imprinted gene

3.7 DISCUSSION

For this study, POCs were collected from pregnancies that had miscarried. The samples used here had no chromosomal abnormalities detected. However, one can argue that as a high percentage of POCs had MCC, this could be in fact reflecting the maternal karyotype and not the fetal in some of the cases. Assuming that in those POCs that had no contamination or partial contamination, the normal karyotype detected was that of the fetus, we hypothesize that something else must have contributed to the failure of these pregnancies. We focused our investigation on imprinted genes, as these genes are important for gene expression, gene regulation and development. As there is only one functional allele, imprinted genes can be susceptible to lethal mutations. Lethality could be either due to changes in the DNA sequence of the active allele, or because of epimutations. Epimutations, such as altered methylation patterns in imprinted genes, lead to heritable changes in the gene without changing its DNA sequence, and can cause an inactive gene to become active or vice versa (Ubeda and Wilkins, 2008).

In this study, we investigated the imprinting status of three maternally (*HLA-C, PHLDA2, H19*) and three paternally (*IGF2, PEG3, DLK1*) imprinted genes. We suggest that in some of these failed pregnancies, abnormal imprinting could be the cause of the miscarriage, as aberrant imprinting disturbs development and possibly *HLA-C* allorecognition of the new organism.

The fact that only 8% of the POCs tested were free of MCC highlights the importance of checking for maternal contamination in POCs before conducting any experiments. It is not adequate to simply choose male POCs as we can see that even in males, different levels of MCC were detected (Fig. 3-5).

In summary, 38% of POCs tested showed an abnormal imprinting for *HLA-C*, as eight samples showed monoallelic expression from the paternal allele and ten showed the normal

maternal monoallelic expression. As 16 samples used in this study had some levels of MCC, one could argue that the imprinting status of *HLA-C* observed in some of these POCs is actually the imprinting status of the mother. However, by showing that *HLA-C* in maternal decidua is not imprinted and that it is in fact biallelically expressed (Fig. 3-6), rules out that assumption.

In addition, 29% of samples showed abnormal imprinting of *IGF2*, as four samples showed monoallelic expression from the maternal allele and seven showed the normal monoallelic paternal expression. This data is unequivocal since three of these samples with maternal expression of *IGF2* were found to be completely free of contamination and *IGF2* is a known paternally expressed gene (Giannoukakis *et al.,* 1993). Although, biallelic expression of *IGF2* is seen in adult liver (Davies, 1994), to our knowledge, this is the first time maternal expression of *IGF2* has been observed in humans. Whether maternal expression levels of *IGF2* would be any different from the paternal expression is not known, as in both cases only one allele is expressed. Further work, such as real-time PCR, needs to be performed to investigate whether there are any differences in *IGF2* expression levels between maternal and the normal paternally expressed samples.

Imprinting status of the remaining genes tested (*H19*, *PHLDA2* and *PEG3*) was normal. Definitive conclusions about *DLK1* could not be made as only one sample was successfully amplified, which showed normal paternal expression. Overall, out of the 22 POCs used in this study, eleven (50%) had an abnormal imprinting expression of either *HLA-C* or *IGF2* (Table 2-6). In conclusion, the disruption of imprinting in particular the expression of *HLA-C* from the paternal allele and the expression of *IGF2* from the maternal allele may have been involved in the pathology of these miscarriages. Expression of *IGF2* has been shown to be involved in fetal growth early in pregnancy (Demetriou *et al.,* 2014), hence any deviations from the normal expression and expression of the maternal allele could impair

normal fetal development. HLA-C is implicated in allorecognition during human pregnancy and recently has been shown to be maternally expressed in the fetus (Fogarty *et al.,* unpublished data, manuscript in preparation), which can explain why the mother does not reject the allogeneic fetus. However, as we have shown in our study, if the paternal allogeneic allele is no longer repressed, it means that the conceptus could be detected by the maternal immune system as a foreign entity, and hence rejected. However, since a woman is considered to be pregnant only after implantation has occurred, this suggests that allosuppression must have taken place after implantation.

Regulation of MHC expression in the conceptus is observed in other species too, as in the mouse, only one polymorphic MHC class I gene is expressed in the trophoblast, although both parental alleles are expressed (Madeja *et al.,* 2011). In the rat however, experiments have shown that MHC class I is imprinted and paternally expressed in the placenta (Kanbour-Shakir *et al.,* 1993). These differences between species reflect the differences in placentation and reproductive strategies. Humans reproduce less frequently than rodents and have longer gestational periods hence prolonged exposure of alloantigens to maternal cells in human pregnancy. The fact that *HLA-C* is biallelically expressed in the latter stages of pregnancy, when the pregnancy is well established, suggests that the process of implantation and early placental development are at the highest risk of allorecognition and this risk is decreased by silencing the foreign paternal allele.

It is possible that a high proportion of miscarriages can be explained by gene deregulation at imprinted loci leading to early embryonic lethality. The early stage of development is a time of genome-wide epigenetic remodelling and if incomplete, can lead to aberrant expression of genes and possibly failure to maintain normal expression. In conclusion, embryonic lethality in these cases could be due to the failure to maintain normal expression of imprinted genes, especially that of *HLA-C*.

3.8 FUTURE WORK

As previously described, erasure, establishment and maintenance of methylation imprints at imprinting centres take place during germ cell and embryonic development. The pre-implantation embryonic genome losses its DNA-methylation through demethylation beginning in the zygote, followed by *de novo* DNA-methylation around implantation time (Reik and Walter, 2001). It is likely that embryonic fatality is due to faulty demethylation and *de novo* acquisition of DNA-methylation after fertilisation, hence due to reprogramming errors in the life cycle of imprints.

It would therefore be of considerable interest to investigate methylation patterns in these POCs by bisulphite sequencing, a method used to detect and quantify DNA methylation. We can investigate for example the *IGF2/H19* DMR and see whether POCs that show maternal expression of *IGF2*, have abnormal methylation on the maternal inherited allele. In addition, we can use bisulphite sequencing to identify the DMR of *HLA-C* and investigate methylation patterns in the available POCs. In addition, real-time quantitative PCR could be carried out in the POCs whose expression of either *HLA-C* or *IGF2* is expressed from the opposite parental allele. These can be compared to the POCs whose expression of these two genes was normal, to see if any differences in expression levels are observed. Finally, a larger collection of POCs should also be established and analysed to confirm the reproducibility of these findings.

CHAPTER 4 – ROLE OF ANNEXIN A5 (*ANXA5*) IN RECURRENT MISCARRIAGE

4 INTRODUCTION

As described in more detail in the Introduction in Chapter 1, RM is defined as having three or more consecutive pregnancy losses and it affects about 1% of couples trying to conceive (Stirrat, 1990). RM is not only associated with higher rates of morbidity but is also associated with complications later in pregnancy including fetal growth restriction, prematurity and pre-eclampsia (Rai and Regan, 2006). A number of these miscarriages occur due to chromosomal abnormalities in the fetus and the chance of this taking place increases as the maternal age rises (Nybo *et al.,* 2000).

In addition to the risk factors detailed in Chapter 1, such as APLS and FVL, ANXA5 has been implicated in this disorder. This chapter deals with investigating this gene and its relevance to RM.

4.1 Annexins and ANXA5

Annexins were discovered approximately 35 years ago and have a unique core domain that is made up of four similar repeats, each about 70 aa long. These repeats usually contain a characteristic 'type 2' motif for binding calcium ions, hence annexins are traditionally thought of as calcium-dependent phospholipid-binding proteins. However more recent work suggests more complex functions, as annexins interact with various cell-membrane components that are involved in the structural organisation of the cell, adhesion mechanisms, intracellular signalling and ion fluxes (Moss and Morgan, 2004).

The twelve human annexin genes range in size from 15 to 96 kb and are dispersed throughout the genome on chromosomes 1, 2, 4, 5, 8, 9, 10 and 15 (Fernandez and Morgan, 2003). The *ANXA5* gene is found on human chromosome 4q27 and consists of 13 exons and 12 introns. The gene spans 29 kb and encodes a single transcript of approximately 1.6 kb and a protein product of 320 aa with a molecular weight of about 35 kDa. The region genomic locus encompassing the promoter is very GC rich (73% GC) (Cookson *et al.,* 1994).

ANXA5 is an anti-coagulant protein that occurs on normal placental villi. Due to its ability to bind to anionic phospholipids that are found on platelets, it impedes aggregation and hence it is thought to function as an inhibitor of coagulation (Thiagarajan and Tait, 1990). Moreover, due to its affinity to phosphatidylserine (PS) and phosphatidylethanolamine (PE), ANXA5 is used as a probe to detect cells that are undergoing apoptosis, as these cells express PS and PE on their cell surface (Koopman *et al.,* 1994). ANXA5 is a ubiquitous, but not abundantly expressed protein, manifesting highest levels in liver, kidney and placenta (Morgan *et al.,* 1998). ANXA5 abundance appears to be reduced in the placental trophoblast in the presence of aPL (Rand *et al.,* 1994). However, the ANXA5 knockout mouse lacks any clear phenotype, perplexing theories about its function in anticoagulation (Brachvogel *et al.,* 2003).

Bogdanova *et al.*, 2007 reported the presence of two variant *ANXA5* promoter haplotypes in addition to the WT; the M1 and M2 haplotypes, which are common in the normal population. The M1 haplotype comprises of two nucleotide substitutions (1A→C and 27T→C) and the M2 haplotype comprises of four substitutions (-19G→A, 1A→C, 27T→C and 76G→A) that are in linkage disequilibrium (LD) with each other.

The frequency of the M2 haplotype was found to be significantly higher in RM patients than among controls (Bogdanova *et al.,* 2007). In fact, subsequent studies have shown that the M2 haplotype is present in 11% of the Japanese population (Miyamura *et al.,*

2011), 15% of the Caucasian population and 30% in RM patients (Tüttelmann *et al.,* 2013; Tiscia *et al.,* 2009). No significant association of the M1 haplotype was found with RM. Other studies have also shown an association between the M2 haplotype and pre-eclampsia or gestational hypertension (Tiscia *et al.,* 2009), as well as being a risk factor for fetal growth restriction (Chinni et al., 2009) and small for gestational age babies (Tiscia *et al.,* 2012).

Reporter gene assays demonstrated a 60% reduction in the *ANXA5* promoter activity when the M2 haplotype was present in comparison to the WT promoter (Bogdanova *et al.* 2007). Therefore, in patients carrying the M2 haplotype, the anti-coagulant properties of ANXA5 are reduced and may lead to a hypercoagulable state in the intervillous space, potentially explaining an increased risk of RM. In addition, a study on *ANXA5* expression in RM placentas demonstrated that mRNA levels are reduced regardless of the parental origin of the M2 haplotype (Markoff *et al.,* 2010).

4.2 Aim and Hypothesis

The aim of this study was to replicate and further elucidate the role of the M2 *ANXA5* haplotype and its association with RM. In order to do that we genotyped a large cohort of white European couples with RM (n=500) and a control cohort where the couple had a normal pregnancy and no history of miscarriage (n=241).

Our primary hypothesis was that the M2 haplotype would be associated with a higher risk of RM as shown in previous studies. However, by using a larger cohort we could also investigate the influence of the *ANXA5* M2 haplotype on the timing of miscarriages, to assess the male risk and to investigate interactions with known risk factors, such as APLS and FVL.

4.3 MATERIALS AND METHODS

4.3.1 Subjects

DNA samples and medical records for this study were obtained from two independent cohorts described below. The Recurrent Miscarriage Clinic at St Mary's Hospital, part of Imperial College Healthcare NHS Trust, contains blood samples from over seven thousand women and their partners who agreed to participate in research with signed, informed consent. A total of 996 white European samples (501 female patients and 495 male partners) were used in this study. Patients with uterine anomalies, polycystic ovaries (Rotterdam, 2004) and fetal and parental chromosomal abnormalities were excluded. APLS was confirmed by the presence of LA antibodies and/or aCL antibodies and b2-glycoprotein IgG and IgM autoantibodies (Wilson *et al.,* 2001). Patients were broadly divided into different classes according to the gestational period in which the miscarriage occurred. A total of 310 females and 309 male partners that had 3 or more early miscarriages (before 12^{th} week of gestation) and no late miscarriages were classified as "early miscarriage patients". A total of 191 females and 186 males that had at least 1 late miscarriage (after 12^{th} week of gestation) were classified as "late miscarriage patients". The study was approved by the Imperial College Hospital Ethics Committee (REC ref EC0081).

A total of 241 control trio (mother, father and placenta) samples were used in this study who were recruited from the Moore cohort. The Moore cohort consists of 306 white European trios recruited at Queen Charlotte and Chelsea Hospital, London between 2003 and 2004 (Apostolodou *et al.,* 2007). Inclusion criteria for control couples required at least one successful pregnancy and no previous history of miscarriage. All placental samples were collected from singleton pregnancies that resulted in live-birth term deliveries. From each placenta, four pieces of tissue were dissected near the umbilical cord insertion point, washed three times in phosphate buffered saline (PBS) to eliminate traces of maternal blood, snap

frozen in liquid nitrogen and stored at -80 °C until use. The study was approved by the Hammersmith and Queen Charlotte's and Chelsea Hospital Trust ethics committee (Registration number: 2001/6029).

The PopGen control group of 533 randomly selected German individuals (UKSH, Kiel) was also used as a control population. These samples were previously genotyped by Professor Arseni Markoff's research group in Germany (Bogdanova *et al.,* 2007) and were used as an additional control group for our study.

4.3.2 DNA extraction from blood and placenta

DNA samples from the Moore cohort (parental blood and placenta) were available as previously described (Apostolidou *et al.,* 2007). DNA from patients from the RMC was extracted from blood using the iPrep™ PureLink™ gDNA Blood Kit using the iPrep Purification Instrument based on the manufacturer's instructions (Invitrogen, UK).

4.3.3 Polymerase Chain Reaction

Genomic DNA from patients was amplified by PCR before sequencing. Primers used to amplify the promoter region of *ANXA5* are shown in table 4-1 below.

Table 4-1: Primers designed for sequencing the promoter region of *ANXA5*.

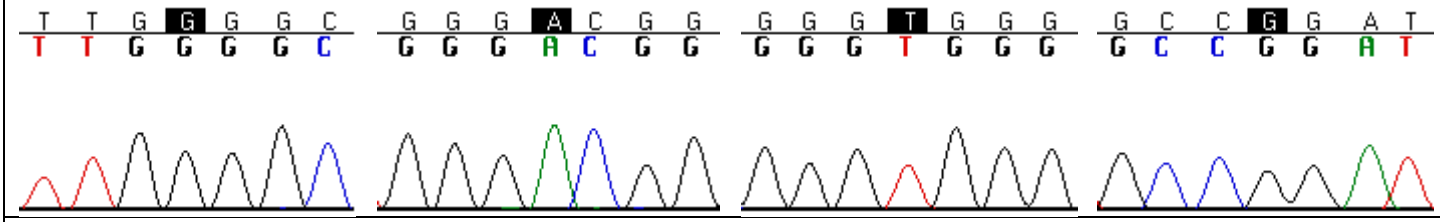
Gene	Primer sequence 5' to 3'	Tm (°C)	Product size (bp)	Accession number	Notes
ANXA5	F: ccgagccctggacagctccc R: gccccgcgaccacgctctcctc	60	496	NM_001154	for DNA

The first 50 female patients were sequenced after PCR was carried out using a standard Taq polymerase (BIOLINE). It was a concern that all 50 patients appeared to be

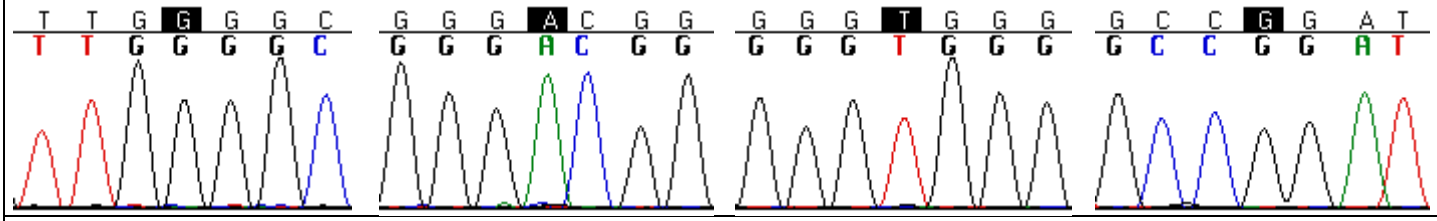
wildtype since it had previously been reported that about 30% of RM patients carry the M2 haplotype (Bogdanova *et al.,* 2007). The region encompassing the promoter of *ANXA5* is very GC rich (Cookson *et al.,* 1994), so it was decided to replicate the experiment using HotStarTaq DNA polymerase (Qiagen) (Table 4-2). The same patients were amplified and sequenced and this time, different alleles were readily detected, with 12 patients found to carry the M2 haplotype. This result was found to be reproducible and we concluded that HotStar Taq was capable of more robust amplification and reduced allelic dropout in this difficult, GC rich region (Fig. 4-1).

A) Three patients samples sequenced following PCR carried out with Bioline Taq DNA polymerase

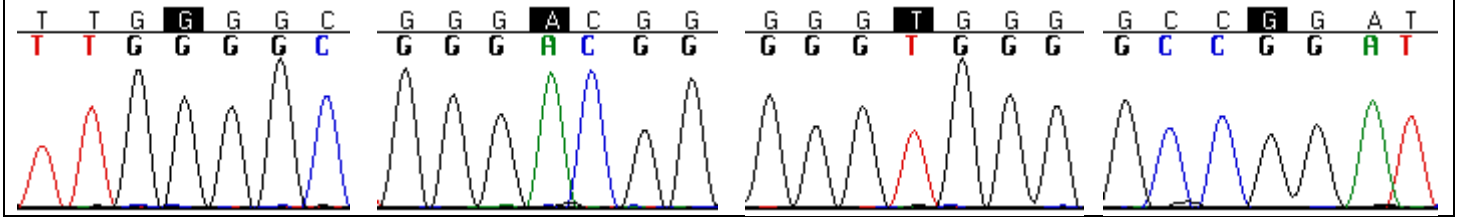
Sample 5948 female – Wildtype (GATG)



Sample 5949 female – Wildtype (GATG)

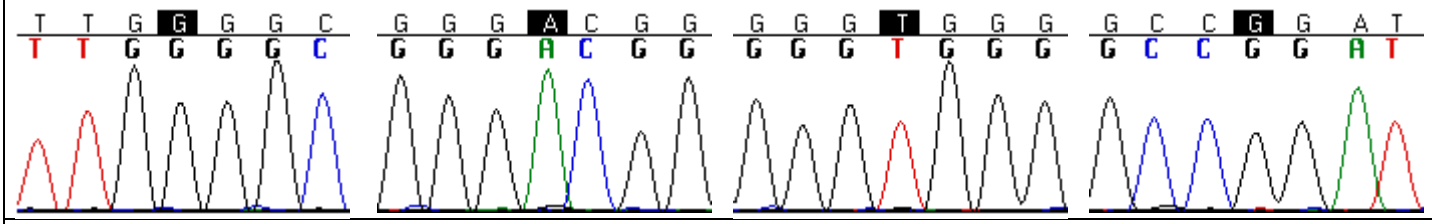


Sample 5950 female – Wildtype (GATG)

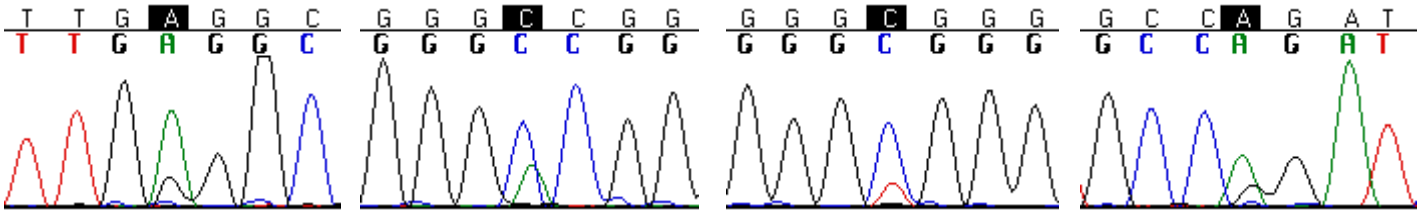


B) The same three patients samples sequenced following PCR carried out with HotStar Taq DNA polymerase

Sample 5948 female – Wildtype (GATG)



Sample 5949 female – M2 heterozygote (ACCA)



Sample 5950 female – M1 heterozygote (GCCG)

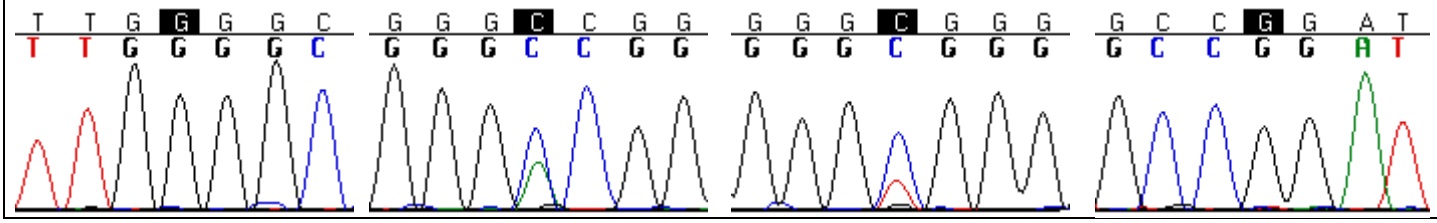


Figure 4-1: Sanger sequencing chromatographs of the *ANXA5* promoter region. Chromatographs are shown for 3 patients, all of who were sequenced after PCR was carried out using the regular Taq DNA polymerase (BIOLINE) (A) and the high fidelity HotStar DNA polymerase (Qiagen) (B).

Table 4-2: A standard HotStar PCR reaction mix.

Reagent	Volume (µl) per reaction
10 x PCR buffer	2
dNTPs (2.5 mM)	1.8
Forward primer (50 ng/µl)	1
Reverse primer (50 ng/µl)	1
HotStar Taq DNA polymerase	0.5
Q-solution (5 M)	6
DEPC-treated water	6.7
DNA or cDNA template (50-100 ng)	1

All reactions were carried out in a Veriti® 96-Well Thermal Cycler (ABI) with a heated lid set at 100 °C to prevent evaporation. A standard PCR cycle consisted of an initial denaturation at 96 °C for 15 minutes, and 35 cycles of: denaturation at 96 °C for 30 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 30 seconds, and a final extension at 72 °C for 2 minutes. 5 µl of PCR products were used to confirm product size and quality on an agarose gel.

4.3.4 Agarose gel electrophoresis

For protocol see section 2.6.6 in Chapter 2.

4.3.5 Sequencing

For protocol see section 2.6.10 in Chapter 2.

Sequencing/genotyping results of patients and controls are shown in Appendix C.

4.3.6 Statistical analysis

When two groups are under observation, two measures can be used to describe the comparative likelihood of an event happening. These are the odds ratio (OR) and the relative risk (RR). Both are two different statistical concepts, although they are closely related to each other. OR is defined as the ratio of the odds of an event occurring in one group to the odds of it occurring in another group. RR is the risk of an event (or of developing a disease) relative to exposure.

OR and RR with 95% confidence intervals (CI) were calculated using an online software package (http://www.hutchon.net/ConfidOR.htm). Departure from Hardy-Weinberg equilibrium (HWE) was assessed using a Monte-Carlo Markov Chain (MCMC) implementation of an exact test, part of the Genepop package (http://genepop.curtin.edu.au/). Deviations from HWE were investigated by Professor Arseni Markoff.

4.4 RESULTS

A possible association between RM and the M2 haplotype was investigated by genotyping RM patient and control cohorts (Table 4-3). Similar to the Muenster control group (Bodganova *et al.,* 2007), female controls in the Moore cohort were shown to deviate from HWE (MCMC P=0.03). This was due to an excess of M2 homozygotes (lack of heterozygotes), possibly because of positive ascertainment bias as miscarriage occurs in about 10% of women worldwide. In contrast, male controls were in HWE (MCMC P=0.79). Since the prevalence of the M2 haplotype was similar between female and male controls, frequencies were combined (Table 4-4). The association between the M2 haplotype and RM was also tested by comparison to an independent White-European patient cohort comprising of PopGen controls (Bodganova *et al.,* 2007) (Table 4-3).

The analysis showed a similar prevalence of the M2 haplotype between female and male partners of the RM group. The allele frequency (AF) of female and male partners of the early RM group was 0.134 and 0.141, respectively and that of the late RM group was 0.094 and 0.108 for females and males, respectively (Table 4-4).

Table 4-3: Genotype frequencies of *ANXA5* gene promoter haplotypes in White European RM patients and two control groups.

	All miscarriage patients		Early miscarriage patients		Late miscarriage patients		Moore Controls		PopGen Controls	
Genotype	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
WT/WT	622 (62.4%)	617.8	385 (62.2%)	377.6	237 (62.9%)	240.2	320 (66.4%)	320.4	415 (77.9%)	411.8
WT/M1	136 (13.7%)	139.5	71 (11.5%)	78.9	65 (17.2%)	60.7	71 (14.7%)	71.8	35 (6.6%)	37
M1/M1	10 (1.0%)	7.8	6 (0.9%)	4.1	4 (1.1%)	3.8	7 (1.5%)	4	1 (0.2%)	0.8
WT/M2	189 (19.0%)	193.9	126 (20.4%)	132.9	63 (16.7%)	60.8	75 (15.6%)	73.5	72 (13.5%)	76.5
M2/M2	18 (1.8%)	15.1	13 (2.1%)	11.6	5 (1.3%)	3.8	6 (1.2%)	4.1	5 (0.9%)	3.5
M1/M2	21 (2.1%)	21.9	18 (2.9%)	13.9	3 (0.8%)	7.7	3 (0.6%)	8.2	5 (0.9%)	3.4
Total	996	996	619	619	377	377	482	482	533	533

Expected: genotype frequency expected at Hardy-Weinberg equilibrium

Table 4-4: Genotype distributions of female/male partners in the RM cohort and Moore controls.

	All RM patient couples		Early RM patient couples		Late RM patient couples		Moore control couples	
Genotype	Females	Males	Females	Males	Females	Males	Females	Males
WT/WT	305 (60.9%)	317 (64.1%)	186 (60.0%)	199 (64.4%)	119 (62.3%)	118 (63.4%)	159 (66.0%)	161 (66.8%)
WT/M1	79 (15.8%)	57 (11.5%)	44 (14.2%)	27 (8.7%)	35 (18.3%)	30 (16.1%)	34 (14.1%)	37 (15.4%)
M1/M1	6 (1.2%)	4 (0.8%)	3 (1.0%)	3 (1.0%)	3 (1.6%)	1 (0.6%)	5 (2.1%)	2 (0.8%)
WT/M2	95 (18.9%)	94 (19.0%)	65 (21.0%)	61 (19.7%)	30 (15.7%)	33 (17.7%)	38 (15.8%)	37 (15.4%)
M2/M2	8 (1.6%)	10 (2.0%)	6 (1.9%)	7 (2.3%)	2 (1.05%)	3 (1.6%)	4 (1.6%)	2 (0.8%)
M1/M2	8 (1.6%)	13 (2.6%)	6 (1.9%)	12 (3.9%)	2 (1.05%)	1 (0.6%)	1 (0.4%)	2 (0.8%)
Total	501	495	310	309	191	186	241	241
M2 AF	0.119	0.128	0.134	0.141	0.094	0.108	0.098	0.089
Combined *M2* AF	0.123		0.137		0.101		0.093	

The AF of the M2 haplotype was found to be higher among all patients (0.123) than among Moore controls (0.093) or PopGen controls (0.082). Consequently, carriers of M2 face a 1.3 times higher risk of RM than non-carriers (OR 1.41, 95% CI 1.07 - 1.86) compared to the Moore controls and a 1.5 higher risk (OR 1.63, 95% CI 1.24 - 2.16) in comparison to the PopGen controls (Table 4-5A). As shown in previous studies, the M1 haplotype was not associated with a higher RM risk.

Because of the noted similar M2 frequency in RM couples, female and male subjects were pooled together and divided into two different groups according to the timing of miscarriage; early miscarriage patients (n=619) and late miscarriage patients (n=377). In early miscarriage patients, the AF of M2 was 0.137 that contributes a RR of 1.4 (OR 1.61, 95% CI 1.21 - 2.17) as compared to Moore controls and a RR of 1.6 (OR 1.87, 95% CI 1.39 - 2.51) when compared to PopGen controls (Table 4-5B). In late miscarriage patients, the AF of M2 was 0.101 and no significant difference was observed between late miscarriage M2 carriers in either Moore (P=0.60) or PopGen (P=0.17) controls (Table 4-5C).

Patients were stratified according to those that had no live births (n=495) and those that had at least 1 live birth (n=352). Relative risks for M2 carriers that either had or did not have a live birth compared to the Moore controls were 1.5 (OR 1.60, 95% CI 1.15 - 2.24) and 1.4 (OR 1.53, 95% CI 1.12 - 2.09), respectively and 1.6 (OR 1.86, 95% CI 1.33 - 2.61) and 1.6 (OR 1.8, 95% CI 1.25 - 2.59) when compared to the PopGen controls (Table 4-5A). The same relative risks were observed for early RM M2 carriers when compared to Moore controls (Table 4-5B), but no significant difference was observed between late RM M2 carriers and Moore controls (Table 4-5C). More importantly, no significant differences were observed between early RM patients who are M2 carriers whether they had a live birth or not.

In order to investigate whether *ANXA5* promoter mutations interact with other general thrombophilic factors in terms of RM risk, patients were divided into those patients who were

positive for known risk factors (APLS, FVL or LA) (n=265) and those who were negative (n=466). The relative risks compared to the Moore cohort for M2 carriers who were positive for known risk factors was 1.4 (OR 1.57, 95% CI 1.10 - 2.26) and for those who were negative was 1.3 (OR 1.45, 95% CI 1.05 – 1.99), while compared to the PopGen controls was 1.6 (OR 1.80, 95% CI 1.25 - 2.59) and 1.5 (OR 1.68, 95% CI 1.22 - 2.30), respectively (Table 4-5A). Slightly higher relative risks were observed for early RM M2 carriers when compared to both control groups (Table 4-5B), while no significant difference was observed between late RM M2 carriers and controls (Table 4-5C). The relative risks became even higher when patients that were positive only for APLS were compared to controls, as the AF of M2 increased to 0.142 for all patients and to 0.163 for early RM, with an M2 carriage of 30.4%. However, no significant differences were observed between M2 carriers of RM patients that were positive for known risk factors and those that were negative.

The highest RR for M2 haplotype (1.7) carriage was observed in patients who had early fetal losses before the 12th week of gestation and who were also positive for APLS (OR 2.07, 95% CI 1.35 - 3.20) when compared to the Moore controls and increased to 2.0 (OR 2.41, 95% CI 1.56 - 3.71) when compared to PopGen controls. This is generally in agreement with Tüttelmann *et al.,* 2013, where the highest relative risk of the M2 haplotype was observed in women that had early fetal losses.

Interestingly, in our study, out of 29 couples where both parents were M2 carriers, 23 couples had only early miscarriages (average 4.3 miscarriages), 3 couples had both early and late miscarriages and 3 couples had only late miscarriages.

<u>Table 4-5:</u> Relative risks and odds ratios for the M2 haplotype between A) all RM patients and controls, B) early RM patients and controls and C) late miscarriage patients and controls, stratified into different sub-groups.

	Recurrent miscarriage patients				against Moore Controls			against PopGen Controls		
		N	M2 AF	M2 carriage	RR	OR; 95CI	P-value	RR	OR; 95CI	P-value
A)	All RM patients	996	0.123	22.9%	1.3	1.41; (1.07 – 1.86)	**0.02**	1.5	1.63; (1.24 – 2.16)	**0.0005**
All RM patients	No live births	495	0.130	24.4%	1.4	1.53; (1.12 – 2.09)	**0.007**	1.6	1.78; (1.30 – 2.43)	**0.0003**
	At least 1 live birth	352	0.139	25.3%	1.5	1.60; (1.15 – 2.24)	**0.006**	1.6	1.86; (1.33 – 2.61)	**0.0003**
	Positive for known risk factors	265	0.136	24.9%	1.4	1.57; (1.10 – 2.26)	**0.02**	1.6	1.80; (1.25 – 2.59)	**0.002**
	Positive for APLS	240	0.142	25.8%	1.5	1.65; (1.14 – 2.40)	**0.008**	1.7	1.92; (1.32 – 2.78)	**0.0006**
	Negative for known risk factors	466	0.124	23.4%	1.3	1.45; (1.05 – 1.99)	**0.02**	1.5	1.68; (1.22 – 2.30)	**0.001**
B)	Early RM patients (< 12 weeks of gestation)	619	0.137	25.4%	1.4	1.61; (1.20 – 2.17)	**0.002**	1.6	1.87; (1.39 – 2.51)	**0.0001**
Early RM patients	No live births	382	0.131	24.9%	1.4	1.57; (1.13 – 2.18)	**0.008**	1.6	1.82; (1.31 – 2.53)	**0.0004**
	At least 1 live birth	237	0.148	26.2%	1.5	1.68; (1.16 – 2.44)	**0.007**	1.7	1.95; (1.34 – 2.83)	**0.0005**
	Positive for known risk factors	148	0.159	29.7%	1.7	2.00; (1.31 – 3.05)	**0.001**	1.9	2.33; (1.52 – 3.56)	**0.0001**
	Positive for APLS	138	0.163	30.4%	1.7	2.07; (1.35 – 3.20)	**0.0009**	2.0	2.41; (1.56 – 3.71)	**0.0001**
	Negative for known risk factors	316	0.136	25.3%	1.4	1.61; (1.14 – 2.27)	**0.007**	1.6	1.86 (1.32 – 2.64)	**0.0004**
C)	Late RM patients (> 12 weeks of gestation)	377	0.101	18.8%	1.1	1.10; (0.78 – 1.56)	0.60	1.2	1.27; (0.90 – 1.81)	0.17
Late RM patients	No live births	113	0.128	23.0%	1.3	1.42; (0.86 – 2.33)	0.17	1.5	1.64; (1.00 – 2.70)	**0.05**
	At least 1 live birth	115	0.122	23.5%	1.3	1.45; (0.89 – 2.38)	0.14	1.5	1.69; (1.03 – 2.76)	**0.04**
	Positive for known risk factors	117	0.107	18.8%	1.1	1.10; (0.65 – 1.85)	0.73	1.2	1.27; (0.76 – 2.14)	0.36
	Positive for APLS	102	0.113	19.6%	1.1	1.16; (0.67 – 1.99)	0.60	1.3	1.34; (0.78 – 2.31)	0.29
	Negative for known risk factors	150	0.100	19.3%	1.1	1.14; (0.71 – 1.81)	0.59	1.3	1.32; (0.82 – 2.11)	0.25

Table 4-6: Genotype distributions of Moore control trios.

Genotype	All controls	Female controls	Male controls	All Placentas	Female Placentas	Male Placentas
WT/WT	320 (66.4%)	159 (66.0%)	161 (66.8%)	146 (60.6%)	72 (61.5%)	74 (59.8%)
WT/M1	71 (14.7%)	34 (14.1%)	37 (15.4%	44 (18.3%)	22 (18.8%)	22 (17.7%)
M1/M1	7 (1.5%)	5 (2.1%)	2 (0.8%)	1 (0.4%)	0 (0%)	1 (0.8%)
WT/M2	75 (15.6%)	38 (15.8%)	37 (15.4%)	43 (17.8%)	21 (18.0%)	22 (17.7%)
M2/M2	6 (1.2%)	4 (1.6%)	2 (0.8%)	5 (2.1%)	2 (1.7%)	3 (2.4%)
M1/M2	3 (0.6%)	1 (0.4%)	2 (0.8%)	2 (0.8%)	0 (0%)	2 (1.6%)
Total	482	241	241	241	117	124
AF *M2*	0.093	0.098	0.089	0.114	0.107	0.121

4.5 DISCUSSION

Previous studies have shown that polymorphisms in the promoter region of the *ANXA5* gene are significantly associated with RM. Women with the M2 haplotype have a higher risk of fetal loss than non-carriers. This was shown initially in a population of 70 German women with RM (Bogdanova *et al.,* 2007), and reproduced in cohorts of 103 Italian (Tiscia *et al.,* 2009), and 243 Japanese women (Miyamura *et al.,* 2011). Recently these findings have been confirmed in populations of 243 German and 236 Bulgarian patients with RM (Tüttelmann *et al.,* 2013).

Findings from our study on 501 White-European females and 495 male partners are in agreement with previous studies, as we have shown that carriers of M2 exhibit a higher RM risk than non-carriers. In this study we show that the haplotype confers about the same relative risk to carriers of both sexes, suggesting that paternal M2 carriage confers an equal risk for RM as M2 carriage in mothers and that both maternal and paternal promoter alleles of the *ANXA5* gene contribute equally to the lower expression levels seen in the M2 carriers. This confirms a pilot study that showed that the M2 haplotype confers the same relative risk to carriers of both sexes, based on 30 couples (Rogenhofer *et al.,* 2012), and a more recent study based on 109 couples (Tüttelmann *et al.,* 2013). Collectively, this finding provides strong evidence that both partners in RM couples should be screened for carriage of the M2 haplotype.

Most importantly, our data demonstrate that M2 is associated with ‘early’ RM and not with ‘late’ RM. In addition the M2 haplotype appears to be enriched in the subgroup of early RM patients that are positive for known risk factors (FVL and APLS) in comparison to those that are negative, although the observed difference is not statistically significant. Filtering out the FVL positive individuals from this group results in M2 carriage rate of almost 31% for

APLS patients. This is in general agreement with the proposed function of the *M2/ANXA5* as genetic predisposition for the development of obstetric APLS (Bogdanova *et al.,* 2012).

Reduced levels of ANXA5 have been observed in women with APLS, who suffered RM and these may contribute to the placental thrombosis observed in these patients (Rand *et al.,* 1994). However, even in the absence of aPL, reduced *ANXA5* expression would probably lead to a hypercoagulable state in the intervillous placental space. The fact that no association was seen between the M2 haplotype and late miscarriages implies that reduced levels of ANXA5 may be used as a marker only for early miscarriages. It also suggests that the effect of coagulation of this specific gene on late pregnancy miscarriage is not the main cause for the loss.

ANXA5 is most abundantly enriched at the syncytiotrophoblast surface covering the placenta villi (Krikun *et al.,* 1994). This is consistent with its role as an anticoagulant, playing an important role in the maintenance of a hemodynamic balance during pregnancy. Since reduced *ANXA5* expression is seen in M2 carriers, the onset of this pathology during the course of pregnancy is an important question. The classic inherited thrombophilia conditions associated with pregnancy loss generally lead to late events that are rare in comparison with early miscarriages. However, the M2 haplotype of *ANXA5* emerged as a marker for patient groups with early recurrent miscarriages. When binding to phosphatidylserine surfaces, ANXA5 forms unique two-dimensional crystals which are essential for the dynamics of membrane repair in living cells (Bouter *et al.,* 2011). Repair of syncytiotrophoblast membranes should progressively activate upon enhanced mechanical stimuli, parallel to the dramatic increase of the uteroplacental and intervillous blood flow after the 10^{th} week of gestation (Burton *et al.,* 1999). This occurs when the growing fetus and placenta are exposed to a more normal oxygen concentration environment. Therefore, the finding of a significant

association of the *ANXA5* M2 haplotype with fetal losses early in pregnancy correlates well with this biological hypothesis.

In Bogdanova *et al.,* (2007) reporter gene assays have shown that the M2 haplotype decreases the activity of the promoter of this anti-coagulant gene, potentially leading to fetal loss. However, data in our study from the control placentas is suggestive that this M2 haplotype may be advantageous at some point in pregnancy. Although not statistically significant, the AF of M2 was found to be higher in control placentas (0.114) as compared to the parents (0.093). This was true for placentas of both female (0.107) and male fetuses (0.121) (Table 4-6). In fact, out of 47 control couples where one parent was WT and the other was an M2 carrier, M2 was inherited by 30 offspring. If the M2 haplotype was completely disadvantageous, one might have expected it to disappear or at least diminish in frequency over generations due to natural selection.

Another example of a possible survival advantage of a prothrombotic phenotype is the FVL mutation. The high population frequency of FVL mutation despite the disadvantages associated with it such as APC resistance, increased risk of venous thrombosis and miscarriage (Bertina *et al.,* 1994; Dahlbäck, 1995), suggests some sort of evolutionary advantage for carriers, such as aversion of sepsis (Kerlin *et al.,* 2003). In addition there may be protection from bleeding, as APC-resistant women were characterized by less intrapartum blood loss than non APC-resistant women (Lindqvist *et al.,* 1998). Findings from a more recent study also suggest that FVL as well as prothrombin G20210A might have conveyed a survival advantage (Lussana *et al.,* 2012). It has been also shown that men carrying the FVL mutation display an increased sperm count (Cohn *et al.,* 2010) and a higher fecundity rate (van Dunne *et al.,* 2006).

The high prevalence of the *ANXA5* M2 haplotype in the population raises the question whether carriers may also have a survival advantage. It is possible that the hypercoagulable

state might have an evolutionary benefit, for example to avoid excessive bleeding in the mother and hence reduce the risk of intrapartum bleeding complications.

In conclusion, this study strongly suggests that the *M2/ANXA5* haplotype is a risk factor for early miscarriages that occur before the 12th week of gestation. The M2 haplotype confers about the same relative risk to carriers of both sexes, suggesting a role of *ANXA5* expression levels in the fetus and/or the extraembryonic membranes. It appears that the M2 haplotype is not associated with the presence of other known risk factors and that M2 carriers may have a survival advantage.

As a final comment on the methodology employed here, although Sanger sequencing using Taq DNA polymerase is known to be a very robust technique, in this study, the regular Taq polymerase failed to amplify both alleles in the *ANXA5* promoter region. This resulted in M2 carriers wrongly being classified as wildtype. The lack of heterozygotes was suspicious and it was only when Hotstar Taq DNA polymerase was tested on the same samples that it was possible to conclude that allelic dropout was taking place. Hotstar Taq is a higher fidelity enzyme and uses a modified protocol, involving heating at 96°C for a longer period to ensure complete denaturation of the dsDNA. In addition, the protocol contains Q-solution that enables more efficient amplification of difficult templates including templates that have a high degree of secondary structure or are GC rich. Therefore, when sequencing targets that are hard to amplify, testing an alternative DNA polymerase should be considered.

4.6 FUTURE WORK

As described in Chapter 1, a meta-analysis has shown that the best treatment for women with RM and APLS included a combination of heparin and aspirin, as this combination reduced pregnancy loss by 54% (Empson *et al.,* 2002), as aspirin and heparin helped to reduce the risk of thrombosis. ANXA5 is an anti-coagulant protein and the M2 haplotype has been shown to reduce the *ANXA5* promoter activity by 60% in comparison to WT (Bogdanova *et al.* 2007). This suggests that the anti-coagulant properties of ANXA5 are reduced, leading to a hypercoagulable state in the intervillous space.

Future work would therefore include a prospective survey of our female RM patients to see if any had undergone heparin or aspirin treatment throughout any of their pregnancies and whether that had resulted in a successful pregnancy leading to birth. Ideally, we could investigate whether M2 carriers were more successful if they were treated with heparin or aspirin compared to those M2 carriers that were not treated.

CHAPTER 5 - WHOLE-EXOME SEQUENCING IN A RECURRENT MISCARRIAGE FAMILY

5 INTRODUCTION

As previously described in Chapter 1, miscarriage is the commonest complication of pregnancy and it is the spontaneous loss of a fetus before it has reached viability (Stirrat, 1990). Studies have shown that the risk of miscarriage increases after each successive pregnancy loss and can reach as high as 45% after three consecutive losses (Regan *et al.,* 1989). RM has been attributed to genetic causes, anatomical disorders, infection, endocrine, immune and thrombophilic disorders. However, in the majority of patients, the cause of RM remains a clinical dilemma, since even after detailed investigation most miscarriages remain unexplained.

Chapter 5 describes a genomics approach to identify a new candidate gene for RM. This study focuses on a single family where the patient suffered a total of 29 early RMs, all of which were unexplained.

5.1 The family pedigree

The patient (II-3) and her family (Fig. 5-1) originate from Bangladesh but live in London, and she is under the care of the Recurrent Miscarriage team at St Mary's Hospital, Imperial College London. This patient, who is now 46 years old, had a total of 29 miscarriages all of which were early, between 9-11 weeks of gestation, and had no live births. She had 17 miscarriages with her first husband (II-1), five miscarriages with her second husband (II-2) and seven miscarriages with her third husband (II-4). All of these pregnancies were naturally conceived and the miscarriages were unexplained as the patient tested normal for APLS and LA and negative for the FVL mutation. The patient's mother had three

successful pregnancies and three late miscarriages all of which were in the second or third trimester. The patient's brothers (II-5 and II-6), each had two successful pregnancies.

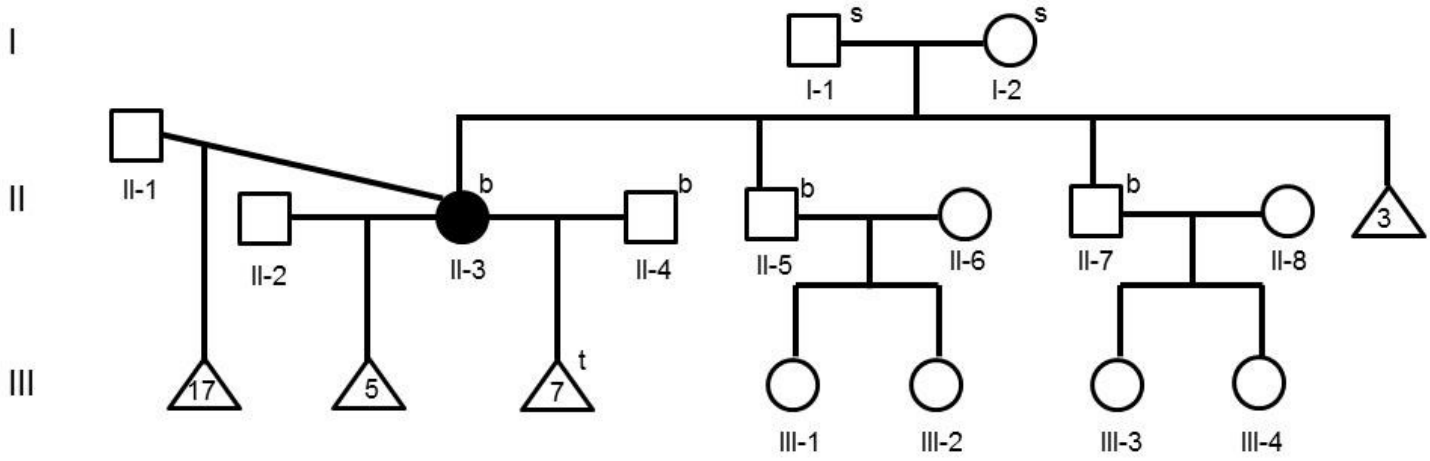


Figure 5-1: Family pedigree.
The affected patient (II-3) had a total of 29 early miscarriages: 17 with her first husband (II-1), five with her second husband (II-2) and seven with her third husband (II-4). Her two brothers (II-5 and II-7) had each two successful pregnancies. Her mother (I-2) had suffered three late miscarriages. Blood was collected from individuals marked with a "b", buccal swab was collected from individuals marked with an "s" and POC tissue was available from one of the miscarriages, marked with a "t".

5.2 Whole Exome Sequencing

Striking revolutions in gene identification strategies have been derived from the development of next-generation sequencing (NGS) technologies at both the exome and genome level. These technologies rely on a combination of template preparation and sequencing, along with genome alignment and assembly methods. The main advantage of NGS is the massive amount and coverage of sequence data generated. Although NGS is much cheaper than Sanger sequencing per base, it is still expensive on a genome scale, although the cost has dramatically reduced as the methodologies improve and become more widely available. Several reviews have focused on the technical details of these new technologies (Mardis, 2008; Metzker, 2010; Coonrod *et al.,* 2013).

As the majority of all single base mutations causing monogenic disorders are located in exons or in splice sites, sequencing of an exon enriched fraction of the genome, the exome, would detect most mutations causing monogenic diseases, whilst significantly reducing the amount of DNA to be covered (Rabbani *et al.,* 2014). Whole exome sequencing (WES), which is still much cheaper than whole genome sequencing (WGS), is currently the main approach used, as researchers tend to look at the exome fraction of the genome first. Obviously, WES will miss those mutations located in non-coding regions, but it will probably take some time before WGS is performed cheaply and routinely. With this will come larger capacity computer storage as well as the appropriate tools to better evaluate the importance and frequency of these types of mutations than are currently available.

The starting material used for NGS is usually whole genomic DNA and the initial preparatory step is the same for exome and genome libraries, which is to generate a "library" of DNA fragments. Different methodologies may be used for library preparation, as some platforms require fragment amplification and others are capable of reading single DNA-molecules. Exon enrichment is performed prior to sequencing, using a number of methods involving fragment capture, which are also frequently updated. Commercial in-solution exome capture kits are currently the most commonly used methods and kits differ in targeted capture areas (part of the exome covered), type of capture probes and their sequence composition, according to the company (Coonrod *et al.,* 2013). The sequencing reaction is then performed; different platforms such as the Illumina Genome Analyzer and Life Technologies SOLiD instruments are commercially available and use significantly different sequencing concepts and chemistries (Metzker, 2010).

Analysing reads from NGS in order to develop a list of variants in relationship to the reference sequence involves multiple steps. After sequence base call files are generated, they are converted to a common file format, usually a FASTQ file format, for storage and

subsequent analyses. Multiple alignment-to-reference software such as the Burrows-Wheeler Aligner (BWA) is then used to map reads to a best-fit location on the reference sequence (Li and Durbin, 2010). Mapped reads for the entire data set are stored in a binary alignment file format called BAM and these reads can then be visualised in the Integrative Genomics Viewer (Robinson *et al.,* 2011).

Nucleotides in aligned sequenced reads are then used to infer the presence and zygosity of variants in a process known as variant calling. The Genome Analysis Toolkit (GATK) is a program commonly used to call variants and this task produces SNPs and insertions and deletions (indels) (McKenna *et al.,* 2010). To call variants, different parameters are considered, which include the ratio of reference to alternate allele bases in the reads, read coverage, base quality and read mapping scores. After alignment is complete, variants are annotated for further analysis, usually using the variant annotation tool ANNOVAR (Wang *et al.,* 2010). ANNOVAR annotates single nucleotide variants (SNVs) and indels, such as examining their functional consequence on genes, finding variants in conserved regions, or identifying variants reported in SNP databases such as dbSNP or 1000 Genomes Project.

Once a list of variants has been generated and annotated, the process of candidate gene identification can begin. With WES, approximately 15 to 20 thousand changes from the reference will be observed in coding regions. Bioinformatics tools are then required to narrow down variant lists to a small subset of variants in candidate genes. The major challenge is the interpretation of the results where a complex network of different combinations of filtering steps is possible and requires optimisation for each project. An increasing number of user-friendly software designed to help in this filtering task and customise analyses parameters for each study have been developed and commercialised, such as the Ingenuity Variant Analysis software (www.ingenuity.com).

5.3 Literature assessment of candidate genes

5.3.1 FK506 binding protein 4 (*FKBP4*)

FKBP4 maps to chromosome 12 (12p13.33) and consists of ten exons that spans approximately 9 kb of genomic DNA (Scammell *et al.,* 2003). *FKBP4* encodes FKBP52, which is a member of the immunophilin protein family FK506-binding proteins (FKBPs) that can bind to the immunosuppressive drug FK506. Immunophilins play a role in immunoregulation and basic cellular processes involving protein folding and protein trafficking. FKBPs range in size from 12 to 135kDa (Galat, 2004) but all share a common domain for immunosuppressant ligand binding. However, they differ in their FK506 affinity, in other domain structures and their subcellular localization, suggesting that each FKBP may have a distinct function.

FKBP52 is common to several vertebrate species and the respective mRNA transcripts are expressed in a variety of human tissues such as brain, heart and placenta (Peattie *et al.,* 1992). Human FKBP52 contains 458 aa and is composed of 4 distinct domains (see Fig. 5-11) (Callebaut *et al.,* 1992; Lebeau *et al.,* 1992).

Domain I: aa 1 to 149 includes the N-terminal portion of FKBP52 and consists of the FK506-binding site, which contains peptidyl-prolyl *cis-trans* isomerase (PPIase) activity (Chambraud *et al.,* 1993). PPIase catalyses the conversion of prolyl-peptide bonds from *trans*- to *cis*-proline, often a rate-limiting step in protein folding (Schiene and Fischer, 2000). The PPIase activity of FKBP52 is inhibited by FK506 and rapamycin, whose immunosuppressant drug action is mediated partly by binding to immunophilins (Schreiber, 1991; Peattie *et al.,* 1992).

Domain II: aa 149 to 267 contains the PPIase-like domain, which shares 32% sequence homology to domain I, but exhibits no PPIase activity (Callebaut *et al.,* 1992; Chambraud *et al.,* 1993). Domain II contains an ATP/GTP binding sequence (Le Bihan *et al.,* 1993).

Domain III: aa 273 to 379 possesses three tetratricopeptide repeat (TPR) domains, that serve as binding sites for 90-kDa heat shock protein (Hsp90), which is also a component of the steroid receptor complexes (Radanyi *et al.,* 1994).

Domain IV: The C-terminal domain of FKBP52 is a short helix that contains a putative binding site for calmodulin (Massol *et al.,* 1992).

A more recent study has shown that FKBP52 interacts directly with tubulin. More specifically, the region of FKBP52 located between aa 267 and 400 is required for tubulin binding. Evidence is provided that FKBP52 prevents tubulin polymerization and that aas 375 to 458 are necessary for its microtubule depolymerization activity. The property of FKBP52 as a novel regulator of microtubule dynamics suggests that it could contribute to neuronal differentiation (Chambraud *et al.,* 2007).

The fact that FKBP52 associates with hsp90 in steroid receptor complexes, suggested a role in the nuclear translocation of steroid hormone receptors such as the glucocorticoid receptor (GR). The hormone-free GR is localized in the cytoplasm and it translocates to the nucleus after steroid binding (Picard and Yamamoto, 1987). Studies suggest that the hsp90-based chaperone system and the hsp90-binidng FKBP52 are involved in the movement of the GR to the nucleus (Davies *et al.,* 2002). In fact, *in vivo* studies have shown that FKBP52 is incorporated into steroid receptor complexes by binding to hsp90 via the TPR domain and it then facilitates translocation of the activated receptor to the nucleus via PPIase domain binding to dynein (Galigniana *et al.,* 2001).

5.3.3.1 *FKBP4* and implantation

One of the requirements for implantation and pregnancy maintenance in Eutherian animals (animals that have a placenta) is progesterone signaling, which acts through the nuclear P_4 receptor (PR) to activate transcription of genes that are involved in ovulation, implantation and pregnancy maintenance (Dey *et al.,* 2004). FKBP52 is also a cochaperone that binds to the PR to optimise progesterone P_4-PR signaling (Davies and Sanchez, 2005).

A critical role for FKBP4 in mouse implantation was shown by Tranguch *et al.,* (2005), where knockout mice (*Fkbp4*$^{-/-}$) were created. Both male and female mice lacking *Fkbp4* were infertile. Further experiments showed that infertility in the female mice was not due to a failure in ovulation or fertilization, but as a result of implantation failure or pregnancy failure following implantation. While this implantation failure phenotype was observed in both CD1 and C57BL6/129 knockout mice, daily P_4 supplementation rescued implantation with subsequent decidualization in CD1 *Fkbp4*$^{-/-}$ females but was ineffective in rescuing implantation in C57BL6/129 *Fkbp4*$^{-/-}$ females (Tranguch *et al.,* 2007). Further experiments by the same group found that uterine levels of an antioxidant, peroxiredoxin-6 (PRDX6) were significantly lower in *Fkbp4*$^{-/-}$ mice and that these mice having reduced uterine PRDX6 levels were susceptible to oxidative stress leading to implantation failure even with P_4 supplementation (Hirota *et al.,* 2010).

5.3.2 Serpin peptidase inhibitor, clade B member 2 (*SERPINB2)*

SERPINB2, also known as placental plasminogen activator inhibitor 2 (PAI-2), is located on chromosome 18q21.3 and it is a member of the serine protease inhibitor superfamily. PAI-2 is a coagulation factor that inactivates tissue plasminogen activator and urokinase plasminogen activator (Kawano *et al.,* 1970)

PAI-2 is only detectable during pregnancy (Kruithof *et al.,* 1984) and the main physiological producers of PAI-2 are activated monocytes and macrophages (Wohlwend *et al.,* 1987). Secreted or released PAI-2 has been detected in biological fluids such as umbilical cord blood, pregnancy plasma and amniotic fluid (Lecander and Astedt, 1987; Booth *et al.,* 1988). During pregnancy fibrinolytic activity declines, but following delivery, it quickly returns to normal levels (Kruithof *et al.,* 1995; Astedt *et al.,* 1998). This is due to increases in PAI-2. Although PAI-2 antigen is undetectable in human plasma, it increases during late pregnancy (Kruithof *et al.,* 1987; Hunt *et al.,* 2009) and this increase is largely due to PAI-2 secretion by the placental trophoblast cells (Kruithof *et al.,* 1995; Brenner, 2004).

Two forms of PAI-2 have been described with variations in three aa at positions Asn120, Asn404 and Ser413 in form A and Asp120, Lys404 and Cys413 in form B. Although the functional consequences of these aa residue changes are unknown, form A has been associated with APLS (Vázquez-Del *et al.,* 2007), whereas form B is linked to preterm birth (Gibson, *et al.,* 2007).

It has been suggested that during pregnancy, the physiological role of PAI-2 is to protect against premature placental separation and secure homeostasis at birth, as reduced plasma levels of PAI-2 correlate with FGR and pre-eclampsia (Reith *et al.,* 1993; Astedt *et al.,* 1998; Brenner, 2004). In general, *SERPINB2* has been linked to pregnancy as there are some studies supporting a role for PAI-2 in fibrinolysis and proteolysis, but its role was not fully characterized (Lee *et al.,* 2011). An impaired plasmin-dependent proteolysis in women

may favour recurrent abortion by promoting fibrin deposition in early placental circulation and/or by limiting trophoblast development (Gris *et al.,* 1993).

5.3.3 Cluster of differentiation 46 *(CD46)*

CD46 complement regulatory protein, also known as membrane cofactor protein (MCP), is a type I membrane protein which in humans is encoded by the *CD46* gene. This gene is found in a cluster on chromosome 1q32 with other genes encoding structural components of the complement system. Alterations in complement regulation have been associated with pregnancy complications including pregnancy loss (Girardi *et al.,* 2006). Activation of the complement cascade early in pregnancy has been associated with pre-term birth and pre-eclampsia (Lynch *et al.,* 2008; Lynch *et al.,* 2011). Mutations in the complement regulatory proteins that regulate the complement cascade have been indicated as risk factors for various pregnancy outcomes, including pre-eclampsia. CD46 has been implicated in pregnancy complications with over 20 mutations identified in the gene (Fang *et al.,* 2008). The data however, is controversial, with polymorphisms of the human CD46 gene were associated with RMs in some studies (Wang *et al.,* 2006) but not in others (Heuser *et al.,* 2011).

5.3.4 Micro RNA lethal 7d *(MIRLET7D)*

MicroRNas (miRNAs) are short (21-24 nucleotide) non-coding RNAs found in many organisms, but only a few have been functionally characterized. MiRNAs are involved in post-transcriptional regulation of gene expression in multicellular organisms by affecting both the stability and translation of mRNAs. It is estimated that miRNAs account for about 1% of genes in higher eukaryotic genomes and that miRNAs regulate about 30% of genes (Griffiths-Jones, 2004; Brown and Sanseau, 2005).

Analysis of mRNA expression by microarray has shown that many genes are significantly changed in mouse uterus between implantation sites and inter-implantation sites (Reese *et al.,* 2001). A miRNA microarray in the mouse uterus has revealed that 8 miRNAs were up-regulated at implantation sites, half of which belonged to the *let-7* family (Hu *et al.,* 2008). One of these was miRNA *let-7d*. *MIRLET-7D* is highly expressed in human endometrium and represents one of the exceptional cases of a miRNA with perfect sequence conservation over distant species (Pasquinelli *et al.,* 2000). A known role of *let-7* is the repression of genes that promote cell division and proliferation, as it has been shown to reduce tumour growth in mouse models of lung cancer (Esquela-Kerscher *et al.,* 2008).

5.4 Aim and hypothesis

Our hypothesis is that the cause of miscarriages in this family is genetic. We also hypothesise that the unsuccessful mother is the affected proband (rather than the POCs or involvement of the male partner) since she has had no successful pregnancies so far, with three different partners. The aim of this study was therefore to carry out next generation exome sequencing on available samples from this family pedigree with a view to identifying a rare genetic variant causal of the RM phenotype. Two genes, *FKBP4* and *SERPINB2*, with potential damaging mutations were identified and investigated in more depth. This included sequencing them in 100 Asian RM patients, 120 White European RM patients and 100 Bangladeshi controls. Three novel variants and one very rare SNP in *FKBP4* were identified in a total of 4 Asian RM patients, which were all predicted to be damaging by different prediction programs.

5.5 MATERIALS AND METHODS

5.5.1 Subjects

Blood samples were available from the RM patient with the 29 miscarriages, her third husband and her two brothers. POC tissue was also available from her most recent miscarriage. Buccal swabs were collected from the patient's parents.

100 Asian female patients from the same RM cohort at St Mary's Hospital and 120 White-European female patients that were used in chapter 3 were also used in this study. Inclusion criteria were that the patients had three or more RMs and no live births. 100 Bangladeshi controls with at least one live birth were also used in this study. These samples were provided by Prof Maria Bitner-Glindzicz (Institute of Child Health – UCL).

5.5.2 Preparation of DNA from samples

For extraction of DNA from blood and POC tissue see section 3.5.2 in Chapter 3. DNA from buccal swabs was extracted using the Isohelix Buccal DNA isolation kit (Cell Projects) according to the manufacturer's instructions.

The 100 Bangladeshi DNA samples were whole genome amplified before being used as controls by using the Genomiphi V2 DNA Amplification Kit (GE Healthcare) according to manufacturer's instructions.

5.5.3 Whole exome sequencing

Five samples from the family (Figure 5-1) were sent for WES (samples marked with b and t). This was done in collaboration with GOSgene (UCL genomic partner http://www.ucl.ac.uk/ich/services/lab-services/gosgene). Genomic DNA was subjected to array capture with the SureSelect Human Exon Kit v.4 (Agilent Technologies) according to the manufacturer's instructions. Adapters were ligated, and paired-end sequencing was

performed on an Illumina HiSeq 2000, which generated 2 x 50 bp reads. The mean exome coverage was 81 to 91-fold, and 98% and 86% of the target sequence was covered at least 10 and 30 times, respectively. Sequencing reads were aligned to the reference human genome (GRCh37/hg19, UCSC Genome Browser) with the BWA consensus, variant bases were called with GATK and variants were annotated with ANNOVAR.

5.5.4 Ingenuity Analysis Variant Server

Ingenuity Analysis Variant Server is a web-based functional analysis tool for comprehensive omic data. Analysis using Ingenuity was performed by me, Dr William Hywel and Dr Estelle Chanudet (Gosgene). The model we used was a dominant model that considered the RM patient as an affected individual and her two brothers and husband as unaffected. Four Asian females provided by Gosgene were used as additional unaffected controls.

Filters used using Ingenuity were:

1) **Confidence filter:** kept variants that have a call quality of at least 20 in any case or in any control and a read depth of at least 10 in any sample.
2) **Common variants filter:** excluded variants that are observed in a population with an allele frequency of at least 3% in the 1000 Genomes Project, 3% in the public Complete Genomics genomes and are present in dbSNP.
3) **Predicted deleterious filter:** kept variants that are experimentally observed to be pathogenic, likely pathogenic or uncertain significance. Kept variants that are associated with a gain of function or loss of function (frameshift, in-frame indel, missense, stop-codon) and not predicted tolerated by PolyPhen-2 or SIFT.

4) **Genetic Analysis filter:** kept variants that are homozygous, heterozygous compound heterozygous, haploinsufficient and occur in the case sample and exclude variants that occur in at least 1 of the control samples.
5) **Biological context filter:** kept only variants that are known or predicted to affect genes implicated in the following diseases, processes, pathways or phenotypes: implantation, thrombosis or miscarriage.

Therefore, based on variant changes that were present in the patient but not present in the remaining samples, and based on genes that were linked to miscarriage, thrombosis or implantation, a list of genes was prioritized using the software program Ingenuity. A complete list of the variants and genes prioritized can be found in Appendix D.

5.5.5 Sequencing candidate genes

For protocol see section 2.6.10 in Chapter 2. Primers used for sequencing variants in candidate genes *CD46* and *MIRLET7D*, as well as the whole *FKBP4* and *SERPINB2* genes are shown in Table 5-1.

Table 5-1: Primers designed for sequencing candidate genes in RM patients.

Gene	Primer sequence 5' to 3'	Tm (°C)	Product size (bp)	Accession number	Notes
CD46	F: tcgtttctttttggtttgaagtc R: gccaatatctctttgctcagg	60	250	NM_002389	for gDNA
MIRLET7D	F: gaaacaaaactcaaagaacatgacc R: caccaaagcaaagtagcaagg	56	227	NR_029481	for gDNA
FKBP4 exon 1	F: cccgccgtctctagaaagtt R: cctcggtgccttaaacgac	60	575	NM_002014	for gDNA
FKBP4 exon 2	F: gtgctggtgccccctttc R: gcttgtgacagcttgtgtcc	56	265	NM_002014	for gDNA
FKBP4 exon 3	F: ttcaggagcactgtttgagc R: gtctccaagaagcaggaagg	56	307	NM_002014	for gDNA
FKBP4 exon 4	F: tgtttcactgcccatgagttag R: gagaacggaagtgtcttgcc	60	260	NM_002014	for gDNA
FKBP4 exon 5	F: tcctcagtcacctgccctc R: acaaacagctggttctacaattc	56	296	NM_002014	for gDNA
FKBP4 exons 6-7	F: agcctggagcaggttaagtg R: tctcagtcaccaagggaagg	60	407	NM_002014	for gDNA
FKBP4 exon 8	F: tgtctgcagggtataagagcc R: agacatgctggcagctcac	62	324	NM_002014	for gDNA
FKBP4 exon 9	F: ggtacctttggaacccagtc R: tagccaggttaagtggaggg	60	377	NM_002014	for gDNA
FKBP4 exon 10	F: gacagatgtaggagaatgggtgt R: gtgacccctgattgtgcta	62	985	NM_002014	for gDNA
SERPINB2 exon 1	F: aaaatgccatgtgggagg R: aataccagcatgagggaagag	60	197	NM_002575	for gDNA
SERPINB2 exon 2	F: gacctcacccaaaatgttacc R: gcataaaacaagagcagttctcc	60	317	NM_002575	for gDNA
SERPINB2 exon 3	F: ttaaaagttcagtaaatccgtgg R: ggctttaagacaggaagattgc	62	243	NM_002575	for gDNA
SERPINB2 exon 4	F: ccatggcttaagaactatcttgtt R: gtttgggatccagccattt	60	238	NM_002575	for gDNA
SERPINB2 exon 5	F: ccatggcttgtttggtatg R: ggctaactaagttcatggatgg	60	258	NM_002575	for gDNA
SERPINB2 exon 6	F: cagaagattcagtaagtaatttcacag R: tctgtctatatcttgtcaaaagccc	60	277	NM_002575	for gDNA
SERPINB2 exon 7	F: ccaatcctcctttatgtctaattg R: catctcatctcacgaaaggtagg	60	304	NM_002575	for gDNA
SERPINB2 exon 8	F: tttccttctttctaatacttgctgt R: tgggtagcagaagttgttcag	62	600	NM_002575	for gDNA
SERPINB2 exon 9	F: tttcggcagattttcctcac R: cttggctccttcatcctgtc	60	723	NM_002575	for gDNA

5.5.6 Real-time quantitative PCR

The quantitative expression analysis of gene *FKBP4* (primers shown in Table 5-2) in different tissues was determined by RTqPCR. For protocol see section 2.6.8 in Chapter 2. Tissues tested included maternal decidua from the exome patient that carried the A16E variant, maternal decidua from miscarried pregnancies and from termination of pregnancies. We also used chorion tissue (CVS pool – Chapter 2) and tissue from products of conception (POC pool – Chapter 3).

Table 5-2: Primers designed for RTqPCR of gene *FKBP4* in different human tissues.

Gene	Primer sequence 5' to 3'	Tm (°C)	Product size (bp)	Accession number	Notes
FKBP4 Real-time	F: accagctcccggataaacg R: tgatgtccactccctccatg	60	110	NM_002014	for cDNA

5.6 RESULTS

5.6.1 Ingenuity analysis Variant Server

Although five samples were sent for exome sequencing, the POC was found not to be useful as the sequence data showed it to be identical to the mothers DNA and therefore that it was maternal decidua, not of fetal origin. DNA sequences were uploaded into the Ingenuity programme and filters were applied, revealing the best candidate gene as *FKBP4* (Fig. 5-2). This gene was the only one selected at the "Biological Context" filter. This was because it has been reported in the literature to be associated with infertility in mice (Tranguch *et al.,* 2005; Tranguch *et al.,* 2007; Hirota *et al.,* 2010).

Nevertheless, we also chose to investigate three other genes (*SERPINB2*, *MIRLET7D* and *CD46*) out of the 34 genes that passed the "Genetic analysis" filter, as we cannot truly exclude any of the genes in this list just because there are no reports directly associating these genes with implantation, thrombosis or miscarriage. These three genes were chosen because there are some studies suggesting a role in processes involved in pregnancy and the variants identified in these genes were novel as they haven't been reported before in SNP databases such as dbSNP, Exome Variant Server (EVS), UK10K or 1000 Genomes. Sanger sequencing was then applied on the original DNAs to confirm the validity of the variant changes detected in these genes.

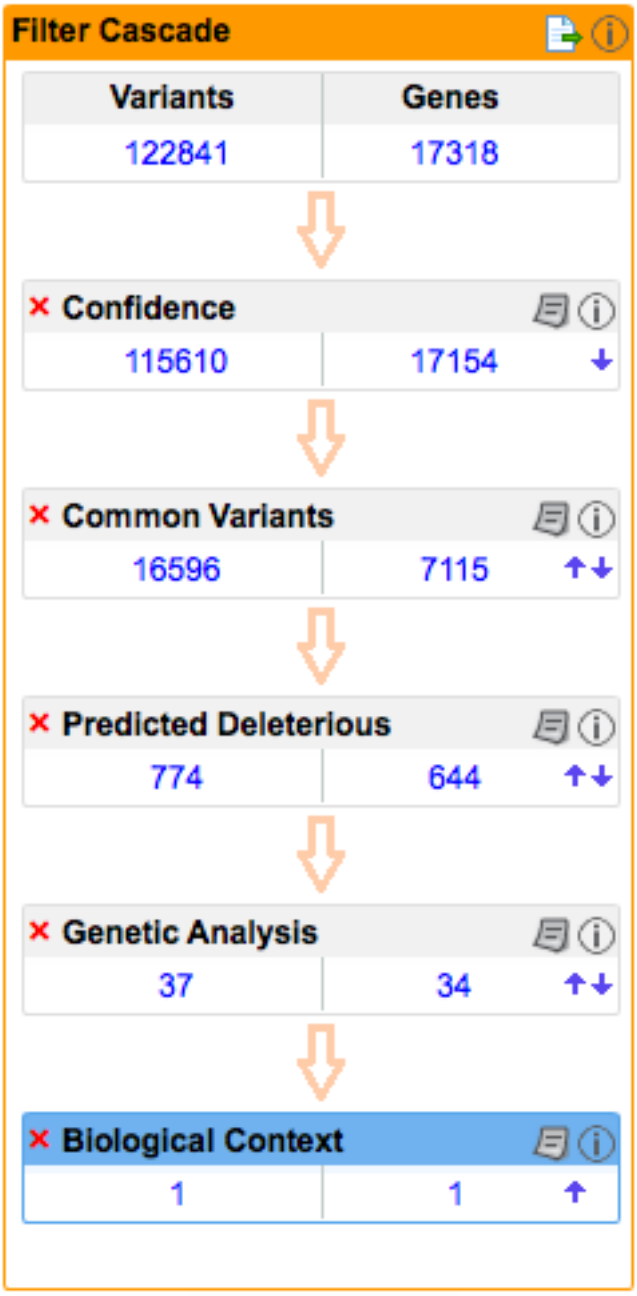


Figure 5-2: Ingenuity analysis Variant Server filters.
Filters applied to this study using software program Ingenuity revealed one variant in one candidate gene, *FKBP4* and 37 variants in 34 candidate genes.

5.6.2 Candidate genes

5.6.2.1 *FKBP4*

The female patient had a novel missense heterozygous change (c.47C>A) in *FKBP4* at position chr12:2904352 that causes an alanine at position 16 to change to glutamic acid (p.A16E). This change was confirmed by Sanger sequencing and was not present in the other family members checked (Fig. 5-3).

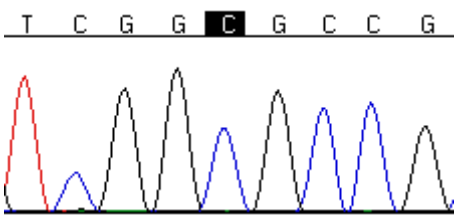
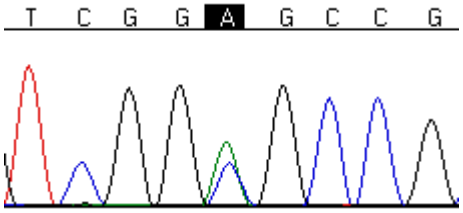
	FKBP4
Husband & 2 brothers Wildtype	
Patient c.47C>A	

Figure 5-3: Sanger sequencing chromatogram showing the C/A change at position chr12:2904352 in gene *FKBP4*, present only in the RM patient.

5.6.2.2 ***SERPINB2***

The female patient had a novel missense heterozygous change (c.328C>G) in *SERPINB2* at position chr18:61564364 that causes leucine at position 110 to change to valine (p.L110V). This change was confirmed by Sanger sequencing and was not present in the other family members checked (Fig. 5-4).

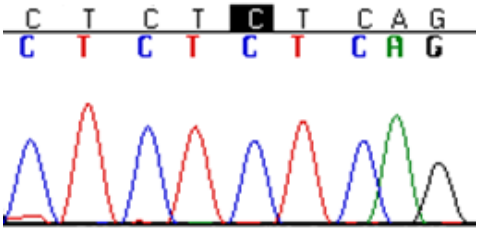
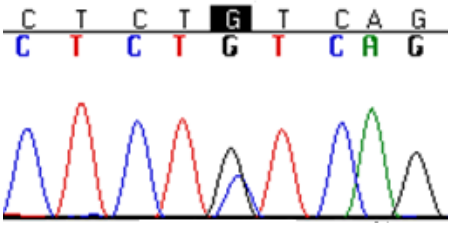
	SERPINB2
Husband & 2 brothers Wildtype	
Patient c.328C>G	

Figure 5-4: Sanger sequencing chromatogram showing the C/G change at position chr18:61564364 in gene *SERPINB2*, present only in the RM patient.

5.6.2.3 *CD46*

The female patient had a novel 16 bp deletion (ACTTCTCTCTGAGAAG) in *CD46* at position chr1:207963638. This change was confirmed by Sanger sequencing and was not present in the other family members checked (Fig. 5-5). The deletion (c.1168_1183del) spanned the stop codon TGA at the 3' end of the coding region. This would result in loss of three aas (threonine, serine, leucine), but would result in the incorporation of glutamic acid and arginine before the next stop codon TGA.

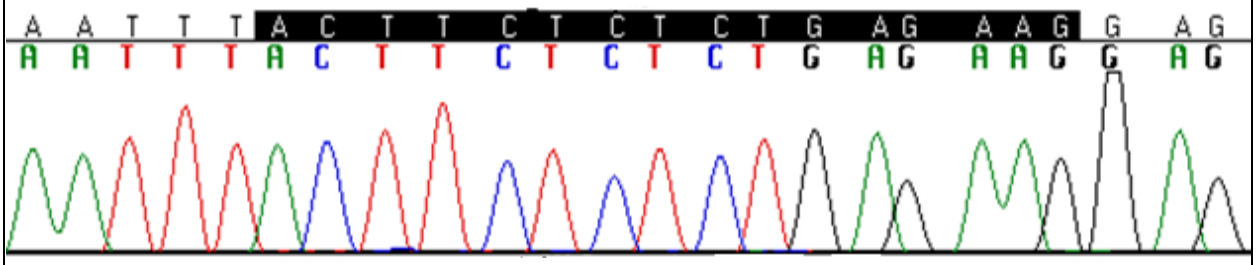
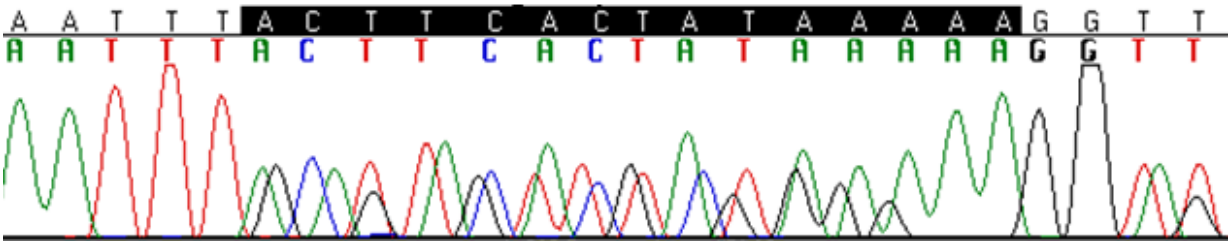
	CD46
Husband & 2 brothers Wildtype	
Patient c.1168_1183del	

Figure 5-5: Sanger sequencing chromatogram showing a 16 bp deletion at position chr1:207963638 in gene *CD46*, present only in the RM patient.

5.6.2.4 ***MIRLET7D***

The female patient had a novel AC insertion in the microRNA *LET-7D* at position chr9:96941126. This change was confirmed by Sanger sequencing and was not present in the other family members checked (Fig. 5-6).

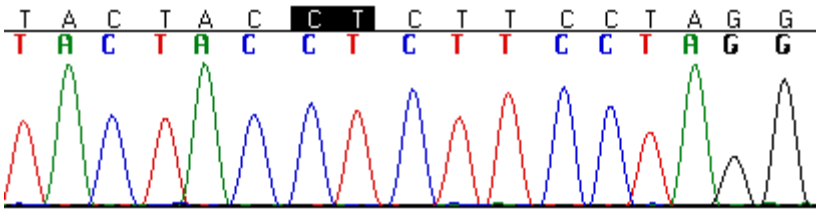
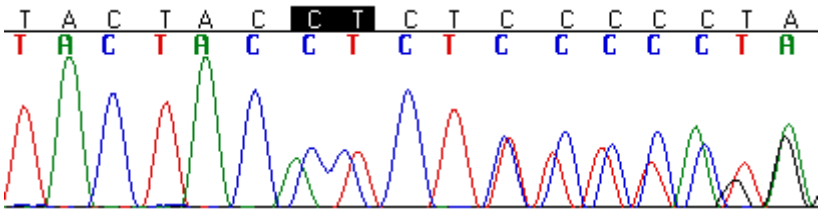
	MIRLET7D
Husband & 2 brothers Wildtype	
Patient AC insertion	

Figure 5-6: Sanger sequencing chromatogram showing the AC insertion at position chr9:96941126 in microRNA *LET-7D*, present only in the RM patient.

5.6.3 Sequencing variants in candidate genes in RM and control cohorts

After confirmation of these four novel changes in the RM patient, we sequenced the regions of these genes in 100 Bangladeshi controls (parents that had successful pregnancies) as we wanted to check whether these variants were polymorphisms found specifically in the Bangladeshi population. However, no controls had these novel changes therefore none of these variants could be excluded.

We then decided to check the presence of these variants in RM patients, including 100 patients from Asia and 120 White European patients, all of whom had no successful pregnancies. However no patients were found to carry these novel variants. The results are summarized in Table 5-3 below.

Table 5-3: Sequencing results of the four variants in the four candidate genes.
These novel variants are only present in the RM patient and not present in other family members, or in the two RM cohorts (100 Asians and 120 White-European RM patients) and Bangladeshi controls.

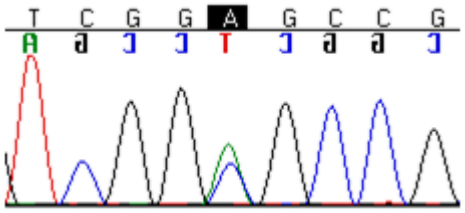
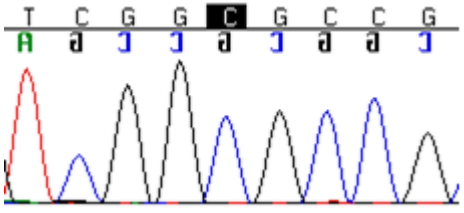
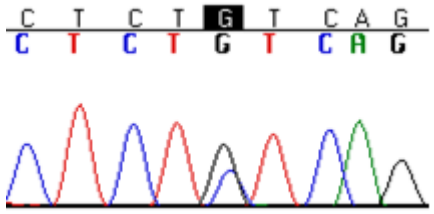
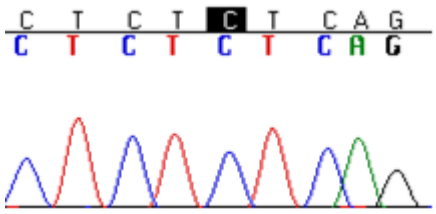
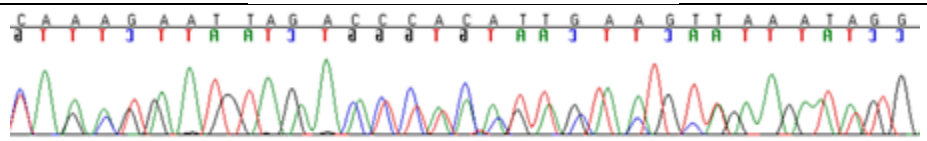
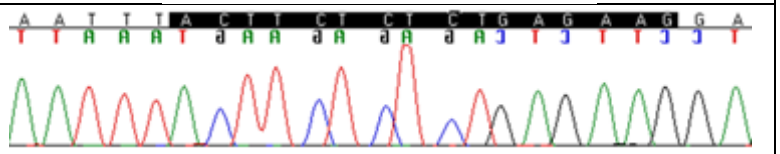
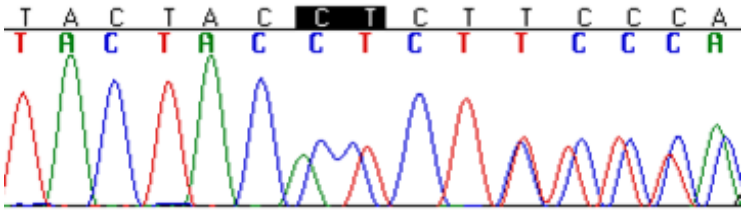
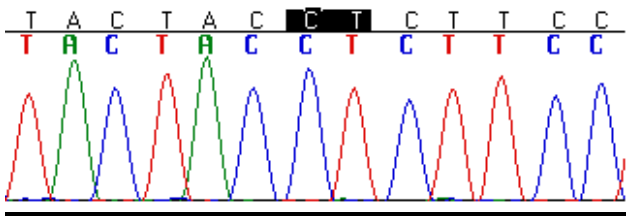
Gene/novel variant	RM patient	Family members	100 Asian patients	120 White-Europ. patients	100 Bangladeshi controls
FKBP4/ c.47C>A	✓	✗	✗	✗	✗
SERPINB2/ c.328C>G	✓	✗	✗	✗	✗
CD46/ c.1168_1183del	✓	✗	✗	✗	✗
MIRLET7D/ AC insertion	✓	✗	✗	✗	✗

5.6.4 Grandparental DNA

The four variants in the four candidate genes were then sequenced in the grandparents since we were able to obtain DNA from the patient's parents (individuals I-1 and I-2) using buccal swabs. The grandmother had the wildtype allele for all four variants, whereas the grandfather had the same alleles as his daughter for all four variants (Table 5-4).

Table 5-4: Grandparental DNA

Sanger sequencing results of the four variants in the four candidate gene indicate that these variants present in the RM patient were all inherited from her father, as her father was heterozygote for all four variants, whereas the patient's mother was wildtype.

	Grandfather	Grandmother
FKBP4		
SERPINB2		
CD46		
MIRLET7D		

Based on these results, we cannot exclude any of the four candidate genes, as all the variants present in the RM patient were inherited from her father and it is possible that the variants do not manifest when present in a male.

5.6.5 Sequencing *SERPINB2* and *FKBP4* genes in RM patients

Next, *SERPINB2* and *FKBP4* were investigated in more depth by sequencing the coding regions of each gene in the RM Asian cohort, the RM White European cohort and the Bangladeshi controls to check if any novel missense variants are identified in these patients. A number of different prediction methods were used to predict pathogenicity of any novel missense changes found in *SERPINB2* and *FKBP4* (Thusberg *et al.,* 2011).

5.6.5.1 Sequencing gene *SERPINB2*

SERPINB2 (chr18:61538926-61571124) has nine coding exons and Table 5-5 below summarizes the variants found in the patients and controls sequenced. A more detailed table showing chromatograms, protein changes and prediction scores is presented in Appendix E.

Table 5-5: Table summarizing the variants found in RM patients and controls in gene *SERPINB2.*

Exon	SNP database	SNP type	Asian RM patients	White European RM patients	Bangladeshi controls
5'UTR	rs2288288		13 het, 2 homo	2 het	11 het
Exon 4	NOVEL	missense	Exome patient	none	none
Exon 5	rs6106	synonymous	2 het	1 het	none
Exon 5	rs6105	synonymous	17 het, 3 homo	14 het	15 het, 2 homo
Exon 6	rs143202684	missense	1 het	none	2 het
Exon 7	rs138446596	missense	1 het	none	2 het
Exon 8	rs6102	synonymous	22 het, 4 homo	30 het, 6 homo	37 het, 3 homo
Exon 8	NOVEL	synonymous	1 het	none	none
Exon 8	rs6103	missense	31 het, 8 homo	30 het, 6 homo	35 het, 15 homo
Exon 8	rs6104	missense	31 het, 8 homo	32 het, 6 homo	35 het, 10 homo
3'UTR	NOVEL		3 het	none	1 het
3'UTR	rs12102		4 homo	1 het, 5 homo	1 het, 13 homo

het: heterozygotes, homo: homozygotes

No novel missense variants in *SERPINB2* were identified in patients, other than the one identified in the exome patient (highlighted in yellow), as most of them were known SNPs.

5.6.5.2 Sequencing gene *FKBP4*

FKBP4 (chr12:2904119-2914576) has ten coding exons and Table 5-6 below summarizes the variants found in the patients and controls sequenced. A more detailed table showing chromatograms, protein changes and prediction scores is presented in Appendix E.

Table 5-6: Table summarizing the variants found in RM patients and controls in gene *FKBP4*.

Exon	SNP database	SNP type	Asian RM patients	White European RM patients	Bangladeshi controls
Exon 1	NOVEL	missense	Exome patient	none	none
Exon 3	NOVEL	synonymous	1 het	none	1 het
Exon 3	rs145385559 (very rare)	missense	1 het	none	none
Exon 5	reported but rs number unknown	missense	3 het	1 het	1 het
Exon 5	rs56196860	missense	1 het	8 het	none
Exon 9	reported but rs number unknown	synonymous	1 het	none	none
Exon 9	NOVEL	missense	1 het	none	none
Exon 9	NOVEL	missense	1 het	none	none
3'UTR	rs1803818		1 het	none	none
3'UTR	NOVEL		2 het	none	none
3'UTR	rs116573718		1 het	none	none

het: heterozygotes, homo: homozygotes

In addition to the novel missense variant in *FKBP4* identified in exon 1 of the original patient, two more novel missense variants were identified in two Asian patients in exon 9, as well as one more very rare missense variant in exon 3 (reported in only two out of 13006 alleles) (Table 5-6). Each of the variants identified in these patients appear to be unique, as they are not present in the 1000 Genomes, dbSNP, EVS and UK10K datasets.

5.6.6 Variants identified in *FKBP4*

5.6.6.1 c.374A>G / p.N125S

A very rare SNP in gene *FKBP4*, reported in only two out of 13006 alleles, was detected in an Asian patient (5510) who had three early miscarriages. The female patient had a missense heterozygous change (c.374A>G) at position chr12:29047018 that causes asparagine at position 125 to change to serine (p.N125S) (Fig. 5-7).

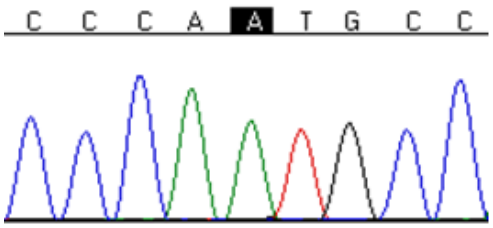
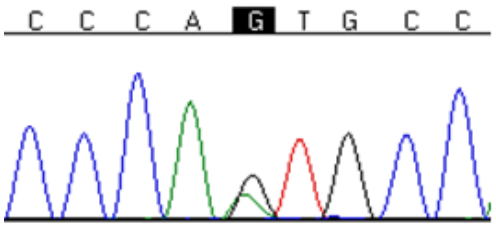
	FKBP4
Wildtype	
Patient 5510 c.374A>G	

Figure 5-7: Sanger sequencing chromatogram showing the A/G change at position chr12:2907018 in gene *FKBP4*, present in patient 5510.

5.6.6.2 c.1142A>T / p.Q381L

A novel variant in gene *FKBP4*, not reported before, was detected in an Asian patient (5644) who had six early miscarriages. The female patient had a missense heterozygous change (c.1142A>T) at position chr12:2910392 that causes glutamine at position 381 to change to leucine (p.Q381L) (Fig. 5-8).

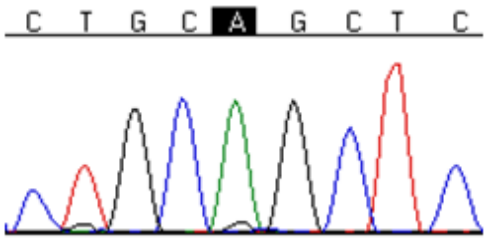
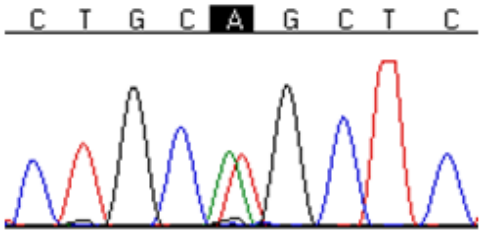
	FKBP4
Wildtype	
Patient 5644 c.1142A>T	

Figure 5-8: Sanger sequencing chromatogram showing the novel A/T change at position chr12:2910392 in gene *FKBP4*, present in patient 5644.

5.6.6.3 c.1196G>A / p.R399Q

A novel variant in gene *FKBP4*, not reported before, was detected in an Asian patient (6040) who had three early miscarriages. The female patient had a missense heterozygous change (c.1196G>A) at position chr12:2910446 that causes arginine at position 399 to change to glutamine (p.R399Q) (Fig. 5-9).

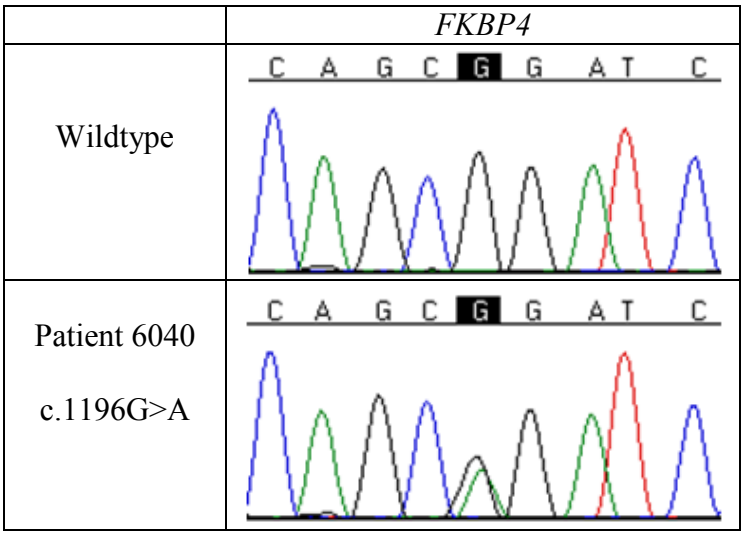


Figure 5-9: Sanger sequencing chromatogram showing the novel G/A change at position chr12:2910446 in gene *FKBP4,* present in patient 6040.

5.6.7 Conservation of *FKBP4* among species

Amino acid sequence conservation across different species was investigated using Clustal W2 (Larkin *et al.,* 2007), a multiple sequence alignment program for DNA or proteins. This protein shows a high degree of conservation between species and the four variant residues are at highly conserved positions across mammals, although were not conserved in the chicken (Fig. 5-10).

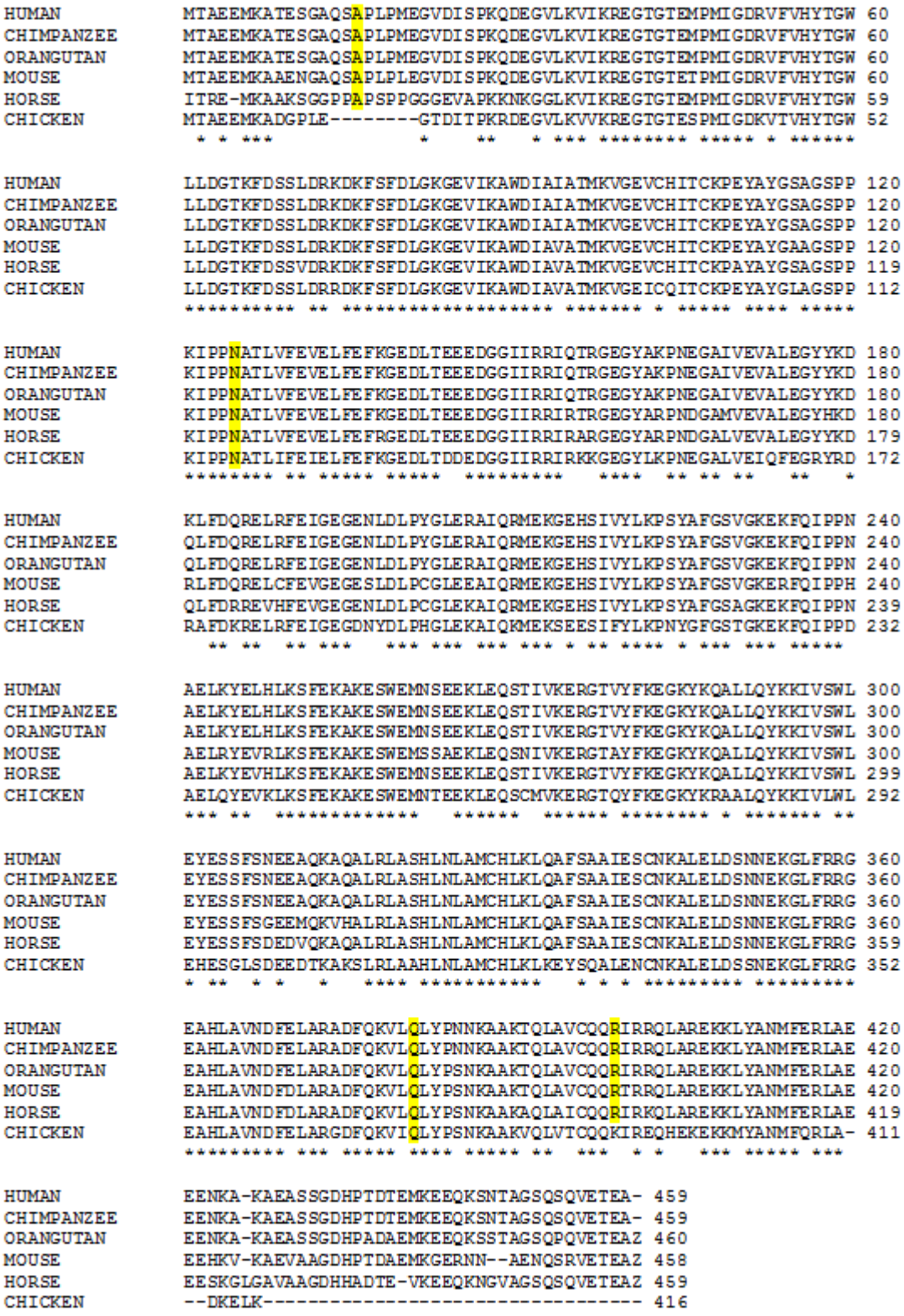


Fig. 5-10: Clustal W2 alignment of the human FKBP52 protein and its orthologues in selected species. The aa residues at each of the identified variants (p.A16E, p.N125S, p.Q381L and p.R399Q) are highlighted in yellow. Asparagine (N) at position 125 and glutamine (Q) at position 381 are conserved in the selected species. Alanine (A) at position 16 and Arginine (R) at position 399 are conserved across mammals, but not in chicken.

5.6.8 Prediction of pathogenicity

Next, we used *in silico* tools to predict the effect that these variants might have on FKBP52 (Table 5-7). These tools used sequence homology to predict whether a substitution affects protein function. Residues that are conserved in the protein family are expected to be important for function.

Table 5-7: *In silico* pathogenicity prediction of the four *FKBP4* missense variants identified in Asian RM patients.

Position Variant *FKBP4*	**Polyphen-2** (Adzhubei *et al*., 2010)	**PROVEAN** (Choi *et al.,* 2012)		**SIFT** (Ng and Henikoff, 2001)	**nsSNPAnalyzer** (Bao *et al.,* 2005)
		PROVEAN	**SIFT**		
12:2904352 c.47C>A p.A16E	Benign	Neutral	Damaging	Tolerated	Neutral
12:2907018 c.374A>G p.N125S	Benign	Deleterious	Tolerated	Damaging	Disease
12:2910392 c.1142A>T p.Q381L	Benign	Deleterious	Damaging	Damaging	Disease
12:2910446 c.1196G>A p.R399Q	Possibly damaging	Neutral	Damaging	Damaging	Neutral

Although most *in silico* tools predicted the p.N125S, the p.Q381L and the p.R399Q variants to be damaging, only one predicted that the p.A16E variant identified in the initial RM patient to be damaging. The different prediction tools use different algorithms but it is not known which, if any, is the most reliable for any given sequence. It is interesting to note that SIFT in Provean gives a different result from the original SIFT in several cases.

Polyphen-2 for example uses multiple sequence alignment of homologous proteins to compare the property of the wild-type ancestral allele and the property of the mutant allele (Adzhubei *et al.,* 2010). SIFT for example bases its prediction on sequence data alone and does not depend on knowledge of protein structure or function (Ng and Henikoff, 2001).

nsSNPAnalyzer on the other hand, combines multiple sequence alignment and protein structure analysis to identify disease-associated SNPs (Bao *et al.,* 2005). Prediction by PROVEAN is not only based on the aa residue at the position of interest, but also on the quality of sequence alignment derived from the neighbourhood flanking sequences (Choi *et al.,* 2012).

Based on the fact that mouse knockout models of *FKBP4* are infertile and the fact that 3 novel and one rare missense variants were identified in 4 RM patients that were predicted to be damaging by a number of prediction tools, we decided that this gene was worth pursuing further in laboratory based functional tests. These tests could include protein-protein interaction studies, such as pull-down assays and are described in more depth in section 5.8 below.

A schematic representation of the four FKBP52 protein domains as well as an illustration on where about in these domains the four potential damaging variants and three common polymorphisms are based is shown in Fig. 5-11 below.

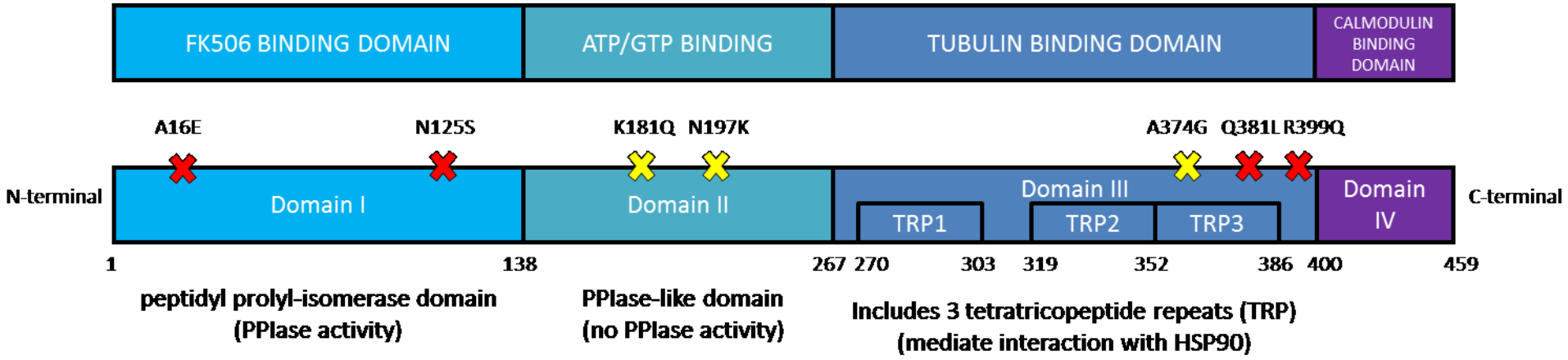


Figure 5-11: Schematic representation of FKBP52 protein.
FKBP52 protein has four domains (described in section 5.3.1). A red cross indicates the position of the four potential mutant variants identified in the four Asian patients (p.A16E, p.N125S, p.Q381L and p.R399Q). A yellow cross indicates the position of common polymorphisms that were used as controls. p.K181Q and p.N197K are two common variants that were detected in our RM cohort. SNP rs143021274 (p.A374G) was chosen from the EVS database as an additional control since this polymorphism is close to the two variants identified in RM patients.

5.6.9 *FKBP4* mRNA expression in human tissues

We analysed *FKBP4* mRNA expression using RTqPCR in several human tissues (Fig. 5-12). These included tissue from maternal decidua from the exome patient that carried the c.47C>A variant, maternal decidua from miscarried pregnancies and from termination of pregnancies. We also used chorion tissue (CVS pool) and tissue from products of conception (POC pool). No significant differences were observed in the relative expression levels of *FKBP4* between the different tissues tested.

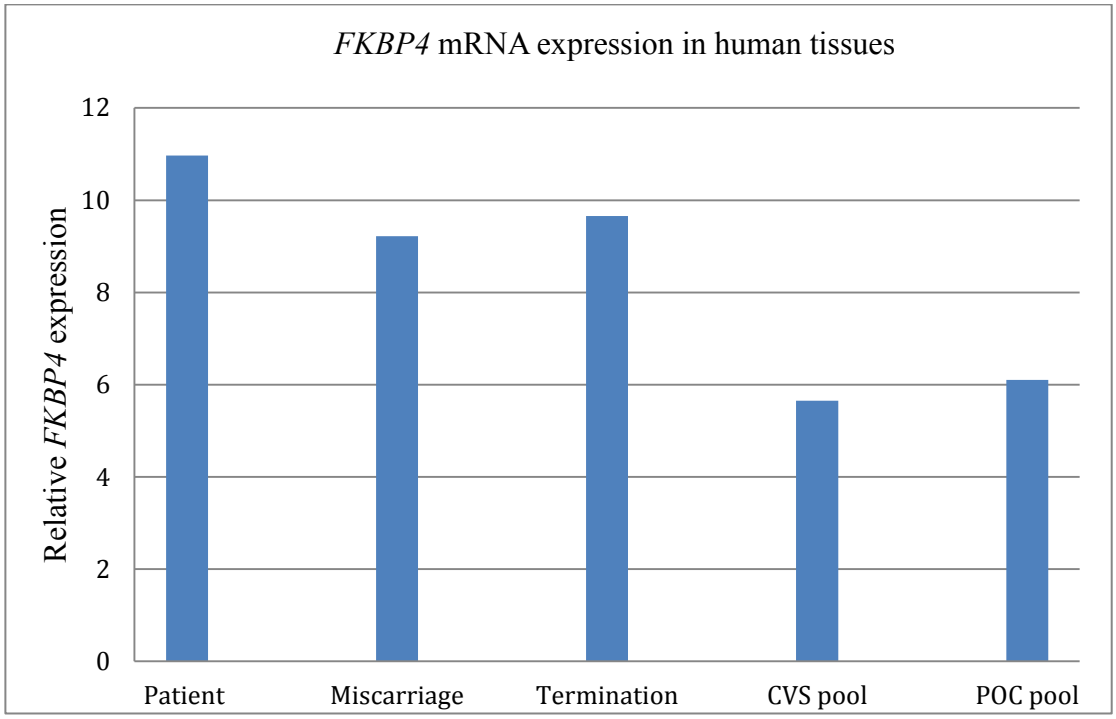


Figure 5-12: **RT-qPCR mRNA analysis of gene *FKBP4* in selected tissues.** These were maternal decidua from patient, from miscarriages, from termination of pregnancies, from chorionic villi and product of conceptions. mRNA levels for *FKBP4* were quantified relative to the levels of the endogenous gene *L19*.

5.7 DISCUSSION

Infertility and spontaneous pregnancy losses are a persistent problem to women's health. The establishment of pregnancy depends on successful implantation, where a complex series of interactions occurs between the uterus and blastocyst. Early loss of pregnancy is a significant clinical problem for women and their health care providers.

In this study, a woman with a severe history of miscarriages was investigated. Exome sequencing was carried out and Ingenuity analysis variant server was used to analyse the data obtained. *FKBP4* was predicted as the best candidate gene, containing a novel missense variant (c.47C>A; p.A16E) in the female patient but which was not present in the other family members tested and neither in the four Asian female controls provided by GOSgene. Three more candidate genes (*SERPINB2*, *CD46* and *MIRLET-7D*) were chosen for further investigation.

Segregation analysis was performed by PCR and Sanger sequencing in the parents. This revealed that all four variants were inherited from the patient's father and none were de novo. This could be consistent for an inheritance model that suggests that the RM effect is primarily expressed in the mother as an environment that is not compatible with implantation rather than in the fetus. A male (i.e. the mother's father) could carry such an allele for this without expressing the phenotype.

Genes *FKBP4* and *SERPINB2* were sequenced in our Asian and White-European RM cohorts. Although the novel missense variant identified in gene *SERPINB2* (c.328C>G; p.L110V) in the exome patient was predicted to be damaging by almost all prediction programs, no other novel missense variants were identified in this gene in our RM cohorts. Two novel missense variants (c.1142A>T; p.Q381L and c.1196G>A; p.R399Q) in *FKBP4*, which are in a domain that interacts with tubulin, were identified in two Asian patients, as well as a very rare missense variant (c.374A>G; p.N125S) in a third patient. These novel

variants were not present in the Bangladeshi controls tested, were not reported in any of the SNP databases, are conserved amongst mammals and are predicted deleterious or damaging by most prediction programs used. Functions of FKBP52 include regulation of steroid hormone receptor function (Schülke *et al.,* 2010) and protection of pregnancy from oxidative stress through regulation of peroxiredoxin-6 levels (Hirota *et al,* 2010). Therefore, gene *FKBP4* is currently the leading candidate, as there is good evidence in the literature that *FKBP4* is linked to pregnancy and potentially pregnancy failure (Tranguch *et al.,* 2005; 2007).

The fact that no significant differences in *FKBP4* expression were observed when real-time quantitative PCR analysis was carried out between the maternal decidua that carried the c.47C>A variant and wildtype decidua does not surprise us (Fig. 5-12). As patients carry a point variant that leads to a missense change we do not expect the expression levels of the mRNA to change. That would be probably the case if a patient carried a stop-codon mutation.

5.8 FUTURE WORK

Preliminary data

Site-directed mutagenesis was carried out in a FLAG_*FKBP4*_pcDNA3 plasmid, provided by Dr Felix Hausch (Max-Planck-Institute of Psychiatry, Germany) (März *et al.,* 2013) to introduce mutations in the protein. In addition to the four potential damaging mutations (p.A16E, p.N125S, p.Q381L, p.R399Q), three common polymorphisms (p.K181Q, p.N197K and p.A374G) were also introduced to be used as controls along with the wildtype protein. Cell culture experiments were performed with HEK293 cells, derived from human embryonic kidney cells, plasmid DNA was transfected in these cells and

proteins extracted. Western blot analysis was performed to detect the FKBP52 protein in the sample of cell extracts using a FLAG antibody (Fig 5-13).

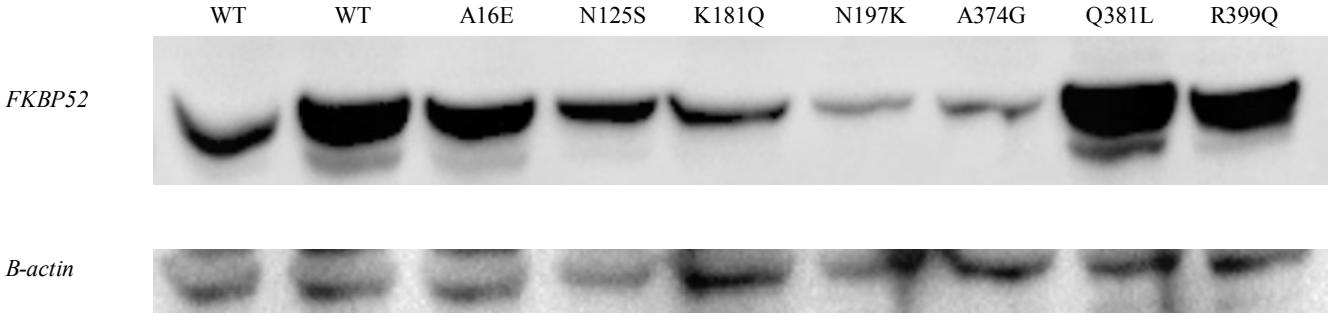


Figure 5-13: Western blot showing the presence of FKBP52 in HEK 293 cells. Top panel is FKBP52 (52 kDa) and bottom panel is the ACTB house keeping gene loading control.

Future work will involve functional studies to investigate whether the four *FKBP4* variants identified in four patients are indeed damaging mutations. A mutation may lead to alterations in the structure, folding or stability of the protein therefore preventing or altering the normal function of the protein. Therefore, experiments will include protein-protein interaction studies, such as pull-down assays or immunoprecipitation to firstly confirm the interaction between wildtype FKBP52 protein and proteins such as tubulin or FKBP506 and then test whether the potential mutations introduced affect the normal interaction of FKBP52 with proteins.

We can also use mass spectrometry to investigate protein-protein interactions, i.e. which proteins physically interact with FKBP52. This methodology will also allow us to test whether the proteins carrying the potential harmful mutations bind to different proteins than the wildtype FKBP52 or those carrying the normal polymorphisms. In order to do this, anti-FLAG magnetic beads can be used to pull the FLAG-FKBP52 proteins from the total protein mixture, so that we only isolate the FKBP52 protein and whatever proteins are bound to it.

SUMMARY

This PhD project investigated the role of genetic factors associated with FGR and RM, two of the most common complications of pregnancy by studying large cohorts of patients using a number of different techniques such as real-time PCR, Sanger sequencing and WES. Part of this PhD focused on imprinted genes such as the paternally expressed *IGF2* and the maternally expressed *PHLDA2* investigating their role in fetal growth as well as potential links between abnormal imprinting and failed pregnancies.

The role of *IGF2* in human fetal growth was investigated in a large cohort of CVS samples (early placenta tissue). Transcript levels of *IGF2* revealed a significant positive correlation with birth weight (P=0.009), providing evidence for the role of this gene as an *in utero* growth enhancer early in human pregnancy. In addition, SGA neonates had significantly lower *IGF2* levels than AGA neonates ($P=3.6x10^{-7}$). The fact that no association was observed between the *PHLDA2* levels in CVS and birth weight, although *PHLDA2* is a known negative regulator of birth weight in term tissue, suggests that this maternally expressed gene is suppressing the baby's growth later in pregnancy rather than early on. Therefore, the two parental genomes appear to be acting at different times during pregnancy to control fetal weight.

Another study explored a potential role for disturbed imprinting in POCs. Detailed analysis of the POC DNA to establish levels of MCC revealed that a large percentage of samples (74%) were in fact maternal decidual tissue rather than of fetal origin. Non-contaminated or partially contaminated POCs were then selected to investigate the status of known imprinted genes, including the newly identified maternally expressed gene *HLA-C.* Interestingly, in a number of POCs, known maternally expressed genes were found to be paternally expressed and vice versa. In summary, 38% of POCs tested showed an abnormal imprinting for *HLA-C* and 29% of samples showed abnormal imprinting of *IGF2.* This

suggested that some miscarriages might be associated with or even caused by abnormal imprinting.

Two approaches were then used to study genetic factors associated with RM. The first involved a genetic association study with *ANXA5*, which encodes a placental anti-coagulant protein. A distinct haplotype (M2) containing four nucleotide substitutions in its promoter was genotyped in 500 White European couples with RM and were compared to 250 normal pregnancy control trios. Carriers of the M2 haplotype were found to exhibit a higher RM risk than non-carriers, which was in agreement with previous studies. However, this was only true for the patients who suffered with early miscarriages, before the 12th week of gestation.

Next-generation exome sequencing was used to investigate a single family where the patient had experienced a total of 29 miscarriages and no successful pregnancies. WES was used to search for a rare genetic variant potentially causative of the RM phenotype. Two candidate genes were identified, *FKBP4* and *SERPINB2,* which were then further investigated by Sanger sequencing in cohorts of Asian RM patients (n=100) and White European RM patients (n=120). In *FKBP4*, which was a very interesting candidate since knockout *Fkbp4* mice have previously been shown to be infertile, three novel variants (p.A16E, p.Q381L, p.R399Q) and one very rare SNP (p.N125S) were identified in four Asian patients. Two of these variants are located in the FK506 binding domain of the protein, and two are in the tubulin-binding domain. All four were predicted to be damaging by different programs designed to predict the functional impact of amino acid substitutions. Future studies to investigate these potential mutations will involve laboratory-based functional analysis of each variant such as protein-protein interaction studies and pull-down assays.

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APPENDICES

Appendix A - Imprinted genes highly expressed in the placenta

Locus	Gene	Origin*	Mouse KO Phenotypes	Human growth phenotypes
6q24	*PLAGL1*	P	FGR, bone malformation, high neonatal lethality	TNDM (pUPD6, pDup6q24, ICR hypomethylation)
6q25	*IGF2R*	M/biallelic	Fetal and placental overgrowth, organ and skeletal abnormalities	CVS expression positively correlated to BW and CRL
7p12	*GRB10*	M/P	Fetal and placental overgrowth	Implicated in SRS (mDup7p11.2)
7q21.3	*PEG10*	P	Embryonic lethal due to placental malformation	Hypermethylation at ICR and reduced expression in LBW cord blood. Upregulated in FGR placenta.
7q32.2	*MEST*	P	Fetal and placental growth restriction, high postnatal lethality, abnormal maternal behaviour	Implicated in SRS (mUPD 7q31-qter)
11p15	*H19*	M	Fetal and placental overgrowth	ICR hypomethylation and CNV in SRS
	IGF2	P	Fetal and placental growth restriction	CVS expression positively correlated to BW and CRL BWS, Wilm's tumor
	CDKN1C	M	Gestational fetal and placental overgrowth	Mutated in BWS and SRS patients
	SLC22A18	M		Implicated in breast and lung cancers
	PHLDA2	M	Placental overgrowth	Highly expressed in LBW and FGR placenta. Promoter variant associated with BW
14q32	*DLK1*	P	Pre- and postnatal growth restriction, high perinatal lethality, obese postnatally	Associated with T1D, UPD14 syndromes,T1D SNP correlated to BW
	MEG3	M	Postnatal lethal (mat del), pre- and postnatal growth restriction, high perinatal lethality (pat del)	Associated with T1D, Reduced expression in FGR placenta
19q13.4	*PEG3*	P	Placental and fetal growth restriction, abnormal maternal behaviour	

Origin* = parental origin of the expressed allele, M = maternally expressed, P = paternally expressed, Dup = duplication, mat del = maternally inherited deletion, pat del = paternally inherited deletion, TNDM = Transient neonatal diabetes mellitus, T1D = type 1 diabetes

Appendix B - Demographic characteristics of the CVS cohort

Lab ID	Parity	Gender	GA (days)	BW (g)	BW centile	MA (years)	Ethnicity	MW (Kg)	Height (cm)	BMI ($kg/m^{2)}$	Smoking	IGF2	IGF2R	IGF2/ IGF2R	IGF1R	IGF2/ IGF1R	IGF1R/ IGF2R	CLASS
34	Multip	Female	269	2384	2.1	44.6	White	56	162	21.34	no	3.0476	0.1182	25.7777	N/A	N/A	N/A	<10th
119	Nullip	Female	259	3920	99.7	18.1	White	64	167.6	22.64	no	3.7742	0.0742	50.8982	0.2145	17.5917	2.8933	>90th
124	Multip	Male	272	3620	81.1	39.7	White	59	166	21.43	no	3.5323	0.0724	48.7811	0.3222	10.9619	4.4501	10-90th
142	Multip	Female	289	4058	83.7	36	White	64	158	25.44	no	2.1165	0.0909	23.2949	0.3635	5.8221	4.0011	10-90th
161	Multip	Male	273	3632	80	34.5	White	74	157.5	29.63	no	4.2256	0.0987	42.8028	0.4486	9.4190	4.5443	10-90th
164	Multip	Male	293	3180	11.2	31.3	White	59	167.6	21	no	4.7296	0.0952	49.6828	0.4657	10.1561	4.8919	10-90th
193	Multip	Female	273	3482	68.1	33.2	Oriental	60	152	25.97	no	4.0817	0.0679	60.0844	0.2327	17.5441	3.4248	10-90th
205	Nullip	Male	274	2497	2.1	41.9	Asian	51	154.9	21.26	no	4.0383	0.1294	31.1976	N/A	N/A	N/A	<10th
274	Multip	Male	272	3575	77.9	39.7	White	65	160	25.58	no	2.9697	0.1068	27.8009	0.2997	9.9101	2.8053	10-90th
279	Multip	Male	269	2355	1.8	33.4	White	66	162	25.15	no	3.9636	0.1120	35.3781	0.4391	9.0268	3.9192	<10th
292	Multip	Male	278	4001	92.3	40.8	White	59	168	20.9	no	3.2295	0.1005	32.1499	N/A	N/A	N/A	>90th
333	Nullip	Male	270	3340	62.4	38.8	White	57	156	23.42	no	3.9609	0.0659	60.0869	0.3109	12.7400	4.7164	10-90th
358	Multip	Female	295	3660	43.3	43.6	White	85	154.9	35.43	no	6.3346	0.1252	50.6081	0.4504	14.0638	3.5985	10-90th
363	Nullip	Male	296	4230	86.8	33.6	White	68	164	25.28	no	4.2662	0.1057	40.3712	0.4200	10.1568	3.9748	10-90th
397	Multip	Female	281	3859	81.8	28	White	67	164	24.91	no	5.8983	0.1146	51.4703	0.4752	12.4122	4.1468	10-90th
409	Multip	Female	280	3377	42.1	36.7	White	63	172.7	21.12	no	4.4341	0.0979	45.3003	0.3235	13.7047	3.3055	10-90th
513	Multip	Female	289	3670	52.9	27.7	Black	63	162.6	23.83	no	5.4675	0.1119	48.8506	0.3247	16.8384	2.9011	10-90th
529	Multip	Male	266	3510	85.1	38.6	White	64	157.5	25.8	no	8.2091	0.1307	62.8314	0.3706	22.1535	2.8362	10-90th
605	Multip	Female	273	2639	5.3	35.5	White	56	160	21.68	Yes	2.4064	0.0961	25.0412	0.3077	7.8202	3.2021	<10th
611	Multip	Male	292	3940	72	31.9	White	64	163	23.94	no	5.1944	0.1125	46.1594	0.3281	15.8316	2.9156	10-90th
699	Nullip	Female	273	2780	10.2	41.2	White	69	162	26.29	no	5.6531	0.1413	39.9952	0.3872	14.5985	2.7397	10-90th
733	Multip	Male	280	4767	99.9	38.1	White	88	168	31.18	no	2.6283	0.0914	28.7657	0.2604	10.0939	2.8498	>90th
768	Nullip	Female	285	3632	56.3	36.7	White	66	162	25.15	no	2.3531	0.1115	21.1011	0.2521	9.3355	2.2603	10-90th
771	Nullip	Male	277	3600	69.5	38.2	White	70	167.6	24.92	no	5.5179	0.1254	44.0062	0.4052	13.6179	3.2315	10-90th
807	Multip	Female	290	2950	4.9	40.2	White	88	167	31.55	no	4.0941	0.1019	40.1973	0.2129	19.2295	2.0904	<10th

Lab ID	Parity	Gender	GA (days)	BW (g)	BW centile	MA (years)	Ethnicity	MW (Kg)	Height (cm)	BMI ($kg/m^{2)}$	Smoking	IGF2	IGF2R	IGF2/ IGF2R	IGF1R	IGF2/ IGF1R	IGF1R/ IGF2R	CLASS
825	Nullip	Male	286	3689	59.8	41.6	White	110	180.3	33.84	no	4.9634	0.1214	40.9002	0.4022	12.3394	3.3146	10-90th
894	Multip	Female	276	3796	85.3	40.5	Black	70	167	25.1	no	4.8646	0.0631	77.0368	0.2345	20.7483	3.7129	10-90th
947	Multip	Male	271	4150	98.9	39	White	107	177.8	33.83	no	3.7180	0.1343	27.6765	0.1813	20.5021	1.3499	>90th
975	Multip	Male	275	3859	89.8	39.5	White	65	162.6	24.59	Yes	9.5582	0.0705	135.6521	0.1931	49.5100	2.7399	10-90th
983	Nullip	Male	285	4556	99.1	39.3	White	68	165	24.98	no	3.6286	0.0909	39.9282	0.2597	13.9748	2.8572	>90th
1002	Nullip	Male	287	3660	55.3	38.7	White	54	162.6	20.42	no	5.9873	0.0892	67.1563	0.2960	20.2283	3.3199	10-90th
1096	Multip	Male	259	3288	85	39.6	White	70	162	26.67	no	6.6135	0.1117	59.2105	0.3867	17.1026	3.4621	10-90th
1143	Multip	Male	268	3973	98.1	37.3	White	78	165.1	28.62	no	7.5503	0.0362	208.6736	0.3552	21.2560	9.8172	>90th
1146	Multip	Female	269	3172	48.4	41.8	White	80	162.6	30.26	no	9.5563	0.1717	55.6590	0.4280	22.3284	2.4927	10-90th
1161	Multip	Male	277	3778	82.8	32.2	White	92	164	34.21	no	4.4976	0.0970	46.3811	0.4406	10.2088	4.5432	10-90th
1194	Multip	Male	273	3400	60.5	39.3	White	61	172.7	20.39	Yes	11.4596	0.1169	98.0563	0.3094	37.0356	2.6476	10-90th
1233	Multip	Male	276	4597	99.9	30.9	White	67	162.6	25.23	no	3.9723	0.1051	37.7823	0.3234	12.2834	3.0759	>90th
1301	Nullip	Female	287	3155	14.6	34.5	White	90	147	41.65	no	7.7957	0.1886	41.3257	0.4088	19.0686	2.1672	10-90th
1353	Nullip	Female	282	4160	94.2	36.9	White	63	167	22.59	no	3.8148	0.0970	39.3400	0.1818	20.9847	1.8747	>90th
1364	Multip	Male	278	3810	83.2	27.3	White	71	153	30.33	no	4.0014	0.1245	32.1321	0.2198	18.2036	1.7651	10-90th
1368	Multip	Male	284	3496	45.4	36.5	White	75	157	30.43	Yes	7.3220	0.1118	65.4937	0.2394	30.5797	2.1417	10-90th
1387	Multip	Female	282	3433	43.4	35.5	White	59	160	23.05	no	7.5732	0.1344	56.3468	0.2425	31.2340	1.8040	10-90th
1438	Multip	Female	272	3377	60.8	27.8	White	64	170.2	21.96	Yes	7.3756	0.0927	79.5877	0.3204	23.0190	3.4575	10-90th
1461	Nullip	Male	277	2412	0.9	42.3	White	65	154.9	27.09	no	2.9037	0.0935	31.0474	0.2412	12.0371	2.5793	<10th
1487	Multip	Female	273	3350	55.7	39.9	White	84	173	28.07	no	10.0578	0.1022	98.4380	0.2646	38.0177	2.5893	10-90th
1525	Multip	Female	279	4230	97.2	37.2	White	74	160	28.91	no	3.7899	0.1050	36.0784	0.2403	15.7703	2.2877	>90th
1541	Multip	Female	285	3600	53.3	34.2	White	63	160	24.58	no	3.6972	0.0881	41.9829	0.2723	13.5785	3.0919	10-90th
1567	Multip	Female	274	3575	73.8	40.5	White	64	160	24.84	no	11.4570	0.0866	132.3364	0.2367	48.3962	2.7344	10-90th
1582	Multip	Male	282	3600	59.2	38.6	White	60	167.6	21.36	no	9.9951	0.1093	91.4543	0.2743	36.4384	2.5098	10-90th
1590	Multip	Male	272	3550	76	40.9	White	72	175.3	23.35	no	12.0021	0.0996	120.5172	0.3105	38.6559	3.1177	10-90th
1664	Multip	Female	265	3405	80.2	40.5	White	74	180.3	22.76	no	4.7263	0.0565	83.6192	0.1886	25.0548	3.3374	10-90th
1679	Nullip	Female	284	3377	34.5	41.7	White	70	170.2	24.3	no	6.9710	0.1288	54.1076	0.2263	30.8028	1.7566	10-90th

Lab ID	Parity	Gender	GA (days)	BW (g)	BW centile	MA (years)	Ethnicity	MW (Kg)	Height (cm)	BMI ($kg/m^{2)}$	Smoking	IGF2	IGF2R	IGF2/ IGF2R	IGF1R	IGF2/ IGF1R	IGF1R/ IGF2R	CLASS
1752	Multip	Male	283	3433	41.4	35.3	White	61	170.2	20.99	no	4.4045	0.0857	51.3880	0.2246	19.6116	2.6203	10-90th
1769	Nullip	Female	284	3916	81.1	37.3	White	59	170.2	20.37	no	4.1891	0.1090	38.4282	0.2240	18.7025	2.0547	10-90th
1777	Multip	Female	269	2886	22.3	31	White	70	154	29.52	no	6.6088	0.1095	60.3615	0.4255	15.5319	3.8863	10-90th
1778	Multip	Male	278	3800	82.6	40.1	White	60	170	20.76	no	6.3741	0.1081	58.9643	0.2211	28.8306	2.0452	10-90th
1811	Multip	Female	271	3887	94.7	43.1	White	54	167.6	19.22	no	7.1323	0.1226	58.1651	0.3405	20.9467	2.7768	>90th
1877	Multip	Female	278	3462	54.7	31.1	White	63	165.1	23.11	no	9.5049	0.1166	81.4872	0.2652	35.8339	2.2740	10-90th
1885	Multip	Male	285	3320	28.1	45.7	White	52	154.9	21.76	no	8.0268	0.0765	104.9727	0.2144	37.4460	2.8033	10-90th
1912	Nullip	Male	283	3235	24.5	41.5	White	62	165	22.77	no	6.4167	0.0478	134.2479	0.2536	25.2998	5.3063	10-90th
1913	Multip	Female	285	3820	72.7	41.8	White	75	173	25.06	no	6.2581	0.0697	89.7446	0.2489	25.1440	3.5692	10-90th
1928	Multip	Female	283	3717	67.7	40.3	White	70	167.6	24.92	no	8.3172	0.0996	83.4731	0.1957	42.4902	1.9645	10-90th
1935	Multip	Male	276	3008	19	37.6	White	65	167.6	23.28	no	4.9513	0.0493	100.3984	0.2589	19.1215	5.2506	10-90th
1937	Multip	Female	279	3510	57	41.6	White	70	170.2	24.13	no	8.1568	0.0841	96.9685	0.2606	31.2971	3.0983	10-90th
1957	Multip	Male	273	3140	35.3	42.5	White	54	156	22.19	no	5.1270	0.0601	85.2379	0.3944	12.9988	6.5574	10-90th
1970	Multip	Male	285	3405	35.3	44.6	White	64	175.3	20.7	no	5.2037	0.0974	53.4337	0.3986	13.0535	4.0934	10-90th
1988	Multip	Male	278	3263	35.7	42.5	White	65	157.5	26.2	Yes	6.2364	0.0819	76.1029	0.2598	24.0062	3.1701	10-90th
2003	Nullip	Male	289	3263	18.9	26.7	White	55	157.5	21.97	no	7.0073	0.0718	97.5836	0.1889	37.0920	2.6309	10-90th
2014	Nullip	Female	273	3206	41.5	40.3	White	49	154	20.66	no	5.4832	0.0831	66.0151	0.3401	16.1204	4.0951	10-90th
2042	Multip	Male	280	3260	31.6	39.2	White	62	160	24.22	no	4.8797	0.0705	69.2363	0.3779	12.9140	5.3614	10-90th
2077	Multip	Female	265	2542	8.3	20.9	Black	63	160	24.61	no	4.2418	0.0882	48.1177	0.2766	15.3371	3.1373	<10th
2261	Multip	Female	285	4029	86.4	32.3	White	52	163	19.57	no	4.5895	0.1157	39.6785	0.2164	21.2067	1.8710	10-90th
2454	Multip	Male	293	3664	46.3	40.4	White	53	152.4	22.82	no	2.3972	0.0340	70.5564	0.2357	10.1708	6.9372	10-90th
2457	Nullip	Male	284	3700	64.4	31.4	Black	57	165.1	20.98	no	4.7138	0.0656	71.8765	0.2972	15.8588	4.5323	10-90th
2512	Nullip	Male	282	2865	6.4	30.5	Asian	57	161.5	21.85	no	3.9744	0.0426	93.2993	0.2553	15.5656	5.9939	<10th
2522	Multip	Male	259	3206	79.2	30.3	White	45	157.5	17.94	no	6.1977	0.0506	122.5112	0.1007	61.5392	1.9908	10-90th
2567	Multip	Male	291	3746	56.8	36.4	White	55	160	21.64	no	2.4939	0.0381	65.3876	0.2787	8.9484	7.3072	10-90th
2570	Nullip	Female	290	3508	36.4	25.4	Black	90	163	33.87	Yes	5.6612	0.0574	98.6679	0.2578	21.9586	4.4934	10-90th
2588	Multip	Female	273	2740	8.5	41.6	White	66	167	23.67	no	4.1215	0.0962	42.8292	0.3131	13.1633	3.2537	<10th

Lab ID	Parity	Gender	GA (days)	BW (g)	BW centile	MA (years)	Ethnicity	MW (Kg)	Height (cm)	BMI ($kg/m^{2)}$	Smoking	IGF2	IGF2R	IGF2/ IGF2R	IGF1R	IGF2/ IGF1R	IGF1R/ IGF2R	CLASS
2593	Nullip	Female	287	2710	1.8	39.4	White	65	160	25.39	no	4.7851	0.1352	35.3863	0.2370	20.1928	1.7524	<10th
2670	Multip	Female	259	2781	37.1	42.4	White	57	165.5	20.81	Yes	5.0216	0.0556	90.2987	0.1978	25.3868	3.5569	10-90th
2697	Multip	Female	276	3405	53.8	36.3	White	59	154.9	24.63	no	3.6690	0.0472	77.6776	0.1261	29.0995	2.6694	10-90th
2702	Multip	Male	276	4001	94	33.7	White	70	165.1	25.64	no	3.1498	0.1259	25.0188	0.2170	14.5181	1.7233	>90th
2713	Nullip	Female	273	2856	14	35.6	N/A	65	162	24.77	Yes	5.7443	0.0536	107.1711	0.2412	23.8143	4.5003	10-90th
2715	Multip	Female	268	2912	26.4	29.1	White	51	157	20.69	no	2.0476	0.0368	55.6987	0.3143	6.5148	8.5496	10-90th
2717	Nullip	Male	272	3831	92	29.9	White	90	162.6	34	no	4.6217	0.0556	83.0726	0.1763	26.2186	3.1685	>90th
2770	Nullip	Female	275	2875	12.5	33.6	White	72	165.1	26.41	no	3.5189	0.0600	58.6207	0.2535	13.8835	4.2223	10-90th
2781	Nullip	Male	265	2979	39.9	38.5	White	83	160	32.42	no	3.6425	0.0641	56.7862	0.1810	20.1199	2.8224	10-90th
2805	Nullip	Male	273	3632	80	46	White	55	157.5	22.01	no	4.1331	0.0574	71.9741	0.2269	18.2181	3.9507	10-90th
2821	Multip	Female	282	3660	64.6	41.9	White	78	177.8	24.67	no	3.1851	0.0591	53.8692	0.2119	15.0311	3.5839	10-90th
2842	Multip	Male	261	2752	28.8	41.1	White	70	160	27.34	no	3.8622	0.0972	39.7322	0.1410	27.3868	1.4508	10-90th
2848	Multip	Male	286	3840	72.7	37.1	White	68	170.2	23.47	no	4.9169	0.0754	65.2059	0.2816	17.4612	3.7343	10-90th
2852	Nullip	Female	288	3433	32.9	29.9	White	73	170.2	25.2	no	5.0951	0.1426	35.7320	0.2871	17.7493	2.0132	10-90th
2869	Nullip	Male	286	3348	28.9	36.7	White	52	152.4	22.48	no	3.9502	0.0665	59.4287	0.2294	17.2211	3.4509	10-90th
2870	Nullip	Male	276	3086	24.6	35.7	White	80	163	30.11	no	3.9343	0.0885	44.4543	0.1949	20.1871	2.2021	10-90th
2882	Multip	Male	282	4000	88.3	35.4	White	51	158	20.43	no	4.5656	0.1287	35.4724	0.3129	14.5932	2.4307	10-90th
2888	Nullip	Female	293	3604	40.8	32.5	White	75	165.1	27.51	Yes	3.9915	0.1591	25.0881	0.2674	14.9297	1.6804	10-90th
2893	Multip	Female	268	3176	51.6	36.9	White	62	163	23.34	no	4.1994	0.0845	49.6925	0.2550	16.4651	3.0181	10-90th
2932	Multip	Female	279	2790	5.9	37.2	Black	60	156	24.65	no	4.0278	0.1051	38.3257	0.2562	15.7196	2.4381	<10th
2956	Multip	Male	295	4140	82.7	32.1	White	60	160	23.44	no	2.9295	0.1090	26.8737	0.2526	11.5961	2.3175	10-90th
2981	Nullip	Female	265	2611	11.4	21.4	White	55	172.7	18.27	no	3.7209	0.1156	32.1882	0.3503	10.6205	3.0308	10-90th
2994	Nullip	Male	281	3645	65.3	32.5	White	57	175.3	18.55	no	3.7376	0.1271	29.4175	0.2682	13.9350	2.1111	10-90th
3047	Multip	Female	282	3547	54.2	33.5	White	68	173	22.72	Yes	3.1080	0.1419	21.9060	0.1643	18.9218	1.1577	10-90th
3051	Multip	Male	277	3800	84.1	38.6	Asian	66	161	25.46	no	4.3202	0.1320	32.7391	0.2257	19.1412	1.7104	10-90th
3063	Nullip	Female	281	3240	28.1	31.1	White	60	164	22.31	no	3.0618	0.1017	30.1139	0.2750	11.1355	2.7043	10-90th
3100	Nullip	Female	261	2746	28.3	37.1	White	65	160	25.39	no	5.1837	0.1008	51.4164	0.2881	17.9901	2.8580	10-90th

Lab ID	Parity	Gender	GA (days)	BW (g)	BW centile	MA (years)	Ethnicity	MW (Kg)	Height (cm)	BMI ($kg/m^{2)}$	Smoking	IGF2	IGF2R	IGF2/ IGF2R	IGF1R	IGF2/ IGF1R	IGF1R/ IGF2R	CLASS
3208	Multip	Female	266	3250	64.5	42.6	White	54	162.6	20.46	no	3.9029	0.1504	25.9490	0.1837	21.2429	1.2215	10-90th
3604	Multip	Male	285	3322	28.3	35.7	White	87	165	31.96	no	3.8299	0.1635	23.4211	0.2233	17.1511	1.3656	10-90th
3623	Multip	Male	286	3689	59.8	47	White	54	156	22.19	no	4.0378	0.1380	29.2686	0.3000	13.4601	2.1745	10-90th
3630	Multip	Male	269	3515	79.6	39.1	White	55	160	21.48	Yes	6.8110	0.1302	52.3179	0.3363	20.2501	2.5836	10-90th
3682	Nullip	Male	267	3604	88.6	38	Asian	90	164	33.46	no	4.7284	0.1635	28.9282	0.3243	14.5820	1.9838	10-90th
3729	Multip	Female	298	3220	10.2	40.6	White	65	158	26.04	no	4.1068	0.0838	48.9799	0.3089	13.2962	3.6837	10-90th
3812	Nullip	Male	264	3036	48.6	38.6	White	59	162.6	22.16	no	4.3765	0.1141	38.3501	0.4125	10.6106	3.6143	10-90th
3820	Multip	Male	287	3859	72.7	40.9	White	68	155	28.3	no	4.3202	0.0855	50.5582	0.1589	27.1886	1.8595	10-90th
3839	Multip	Female	289	3832	67.4	40.3	White	59	162	22.48	no	2.6164	0.1001	26.1441	0.2080	12.5774	2.0787	10-90th
3840	Multip	Male	293	5092	99.9	36.8	White	75	175	24.49	no	3.9859	0.1273	31.3205	0.3224	12.3646	2.5331	>90th
3930	Multip	Female	282	3650	63.7	39.2	Asian	54	158	21.63	no	3.6229	0.0805	44.9936	0.2423	14.9548	3.0086	10-90th
3932	Nullip	Female	285	3405	35.3	37.8	White	64	175.3	20.83	no	3.8181	0.0410	93.1295	0.1985	19.2327	4.8422	10-90th
3933	Nullip	Female	264	2700	18.2	31.9	White	53	157	21.5	no	4.6542	0.1046	44.4988	0.3240	14.3637	3.0980	10-90th
3948	Nullip	Male	269	3040	35.4	29.2	White	52	160	20.31	no	4.1622	0.0986	42.2253	0.2705	15.3885	2.7440	10-90th
3957	Multip	Female	266	3200	59.6	41.9	Oriental	53	160	20.7	no	6.3129	0.1137	55.5382	0.2289	27.5755	2.0140	10-90th
4160	Nullip	Male	278	3590	66.6	24.9	White	62	167.6	22.07	no	3.8949	0.1135	34.3153	0.1178	33.0641	1.0378	10-90th
4185	Multip	Male	272	4040	97.3	42.6	White	75	161	28.93	no	4.0462	0.0911	44.4298	0.2583	15.6635	2.8365	>90th
4204	Multip	Male	264	2620	13.2	40.4	N/A	49	147	22.68	no	3.7798	0.1075	35.1554	0.3292	11.4821	3.0618	10-90th
4210	Multip	Female	291	4313	93.2	40.1	White	66	175.3	21.41	no	4.4921	0.0876	51.2555	0.2991	15.0173	3.4131	>90th
4225	Multip	Male	267	3462	80	31.6	White	66	165.1	24.14	no	3.0220	0.1225	24.6689	0.2928	10.3203	2.3903	10-90th
4234	Multip	Male	288	3377	28.3	38.9	White	54	158	21.63	no	7.3944	0.1572	47.0303	0.3066	24.1178	1.9500	10-90th
4235	Nullip	Female	275	2746	7.1	34.9	Asian	59	169	20.66	no	3.7002	0.0748	49.4900	0.2663	13.8966	3.5613	<10th
4253	Multip	Female	273	3160	37.2	35.1	White	52	160	20.31	no	7.0855	0.1249	56.7471	0.1123	63.0749	0.8997	10-90th
4275	Multip	Female	271	3598	81.5	39.8	White	60	157.5	24.19	no	5.7282	0.0993	57.6980	0.0834	68.6658	0.8403	10-90th
4312	Multip	Male	273	3320	52.7	35.9	White	60	152.4	25.83	no	6.1006	0.1032	59.1410	0.2791	21.8544	2.7061	10-90th
4336	Multip	Male	282	3642	63	40.8	White	66	169.5	22.97	no	3.7052	0.0615	60.2505	0.1674	22.1327	2.7222	10-90th
4338	Nullip	Female	293	3547	35.8	38.3	White	60	158	24.03	no	6.4808	0.0970	66.8073	0.1943	33.3620	2.0025	10-90th

Lab ID	Parity	Gender	GA (days)	BW (g)	BW centile	MA (years)	Ethnicity	MW (Kg)	Height (cm)	BMI ($kg/m^{2)}$	Smoking	IGF2	IGF2R	IGF2/ IGF2R	IGF1R	IGF2/ IGF1R	IGF1R/ IGF2R	CLASS
4347	Multip	Female	285	4070	88.4	29.2	White	62	163	23.34	no	4.5965	0.1140	40.3201	0.2011	22.8605	1.7638	10-90th
4430	Multip	Female	285	3400	34.8	38.8	White	83	170.2	28.51	no	4.8618	0.1314	37.0098	0.2649	18.3553	2.0163	10-90th
4450	Nullip	Female	281	3150	21.3	33	White	59	170.2	20.37	no	4.2207	0.0941	44.8645	0.2133	19.7886	2.2672	10-90th
4454	Nullip	Male	263	3462	87.6	25.1	White	67	162.6	25.42	no	5.4406	0.1390	39.1333	0.3213	16.9330	2.3111	10-90th
4590	Multip	Male	278	3645	71.3	25.9	White	57	162.6	21.63	no	5.4691	0.1765	30.9793	0.3383	16.1660	1.9163	10-90th
4609	Multip	Male	284	4040	88	37.9	Oriental	62	165	22.77	no	3.8876	0.1044	37.2532	0.3381	11.4976	3.2401	10-90th
4640	Multip	Male	283	4114	92	37.3	White	83	170.2	28.51	no	5.0188	0.1125	44.5982	0.3205	15.6615	2.8476	>90th
4719	Multip	Male	279	4040	92.8	34.7	White	74	161	28.55	no	4.7571	0.1096	43.3969	0.2580	18.4368	2.3538	>90th
4722	Multip	Female	274	2870	13.5	40.6	White	58	165	21.3	no	4.2268	0.0609	69.4297	0.2818	14.9982	4.6292	10-90th
4775	Nullip	Female	296	1840	0	31.1	N/A	62	162	23.62	no	2.8260	0.1233	22.9238	0.1198	23.5906	0.9717	<10th
4787	Multip	Female	276	3150	29.8	36.1	White	75	164	27.89	no	4.6621	0.0695	67.0416	0.3005	15.5164	4.3207	10-90th
4800	Multip	Female	287	4370	96.5	42.3	White	57	162.6	21.63	no	3.3832	0.0671	50.4464	0.1339	25.2684	1.9964	>90th
4801	Multip	Female	279	2878	8.8	28.9	Black	59	162	22.48	Yes	4.8211	0.0852	56.5814	0.3750	12.8572	4.4007	<10th
4828	Nullip	Male	271	4005	97.2	36.3	White	63	158	25.24	no	2.6075	0.1044	24.9831	0.0955	27.2910	0.9154	>90th
4833	Multip	Female	276	3120	27.3	28.2	Black	52	157	21.1	no	4.9308	0.0983	50.1691	0.3057	16.1288	3.1105	10-90th
4859	Multip	Female	289	3646	50.6	35.8	Black	102	166	37.02	no	3.9628	0.0334	118.5138	0.1714	23.1160	5.1269	10-90th
4872	Multip	Male	278	3316	40.6	39.3	White	60	157	24.34	no	3.7728	0.0640	58.9229	0.2803	13.4610	4.3773	10-90th
4900	Multip	Male	287	3973	80.8	45	White	52	160	20.23	no	4.5420	0.0834	54.4645	0.1710	26.5558	2.0509	10-90th
4914	Nullip	Female	292	3194	12.5	39.6	White	63	166	22.86	no	5.1139	0.0301	170.0574	0.2150	23.7898	7.1483	10-90th
4990	Nullip	Male	281	2853	6.6	34.6	White	66	166	23.95	no	4.5177	0.0497	90.8177	0.3380	13.3656	6.7949	<10th
4994	Multip	Male	286	3490	41.2	42.1	White	54	162.6	20.42	no	4.1273	0.0251	164.6536	0.1629	25.3333	6.4995	10-90th
5022	Multip	Male	294	4060	78.5	28	White	86	163	32.37	no	5.1467	0.1478	34.8117	0.2252	22.8553	1.5231	10-90th
5025	Nullip	Male	283	3402	38.6	36.4	White	62	170	21.45	no	4.4213	0.0489	90.4747	0.1684	26.2554	3.4459	10-90th
5114	Multip	Female	284	2923	7	36.5	White	65	162.6	24.4	no	5.1203	0.0420	122.0060	0.1229	41.6603	2.9286	<10th
5121	Multip	Male	289	4060	83.8	33.9	White	67	170.2	23.03	no	3.5103	0.1148	30.5695	0.1115	31.4941	0.9706	10-90th
5127	Multip	Male	265	3200	62.4	31.8	Asian	53	158	21.23	no	3.3350	0.0285	116.9949	0.0956	34.8864	3.3536	10-90th
5148	Multip	Female	284	3632	58.2	35.2	White	59	165.1	21.65	Yes	3.7573	0.0437	85.9515	0.1593	23.5881	3.6439	10-90th

Lab ID	Parity	Gender	GA (days)	BW (g)	BW centile	MA (years)	Ethnicity	MW (Kg)	Height (cm)	BMI ($kg/m^{2)}$	Smoking	IGF2	IGF2R	IGF2/ IGF2R	IGF1R	IGF2/ IGF1R	IGF1R/ IGF2R	CLASS
5163	Nullip	Male	289	3150	12.6	28.4	White	64	170.2	21.96	no	3.2897	0.0431	76.3413	0.1182	27.8321	2.7429	10-90th
5181	Multip	Female	272	3090	33	40.4	White	76	152.4	32.85	no	4.3884	0.0578	75.9501	0.1215	36.1130	2.1031	10-90th
5189	Nullip	Male	288	3212	16.8	20.8	White	61	167	21.87	no	3.9128	0.0301	130.1199	0.1101	35.5300	3.6623	10-90th
5209	Multip	Female	280	3774	77.6	26.8	White	96	160	37.42	no	3.5216	0.0304	115.6642	0.2699	13.0468	8.8653	10-90th
5213	Nullip	Female	273	3064	28.6	27.7	White	74	155	30.8	no	4.1686	0.0452	92.1657	0.1072	38.8702	2.3711	10-90th
5270	Multip	Male	283	3660	62.7	41.8	White	59	163	22.21	no	3.0488	0.0450	67.6848	0.1317	23.1495	2.9238	10-90th
5300	Multip	Male	282	4143	93.7	35.6	White	95	177.8	30.15	no	3.1997	0.0983	32.5457	0.1614	19.8223	1.6419	>90th
5308	Nullip	Male	281	3730	72.5	27.2	White	83	170	28.58	Yes	4.3742	0.0283	154.6520	0.0966	45.2667	3.4165	10-90th
5384	Multip	Male	273	3200	41	30	Oriental	55	160	21.48	no	6.3888	0.0532	120.0269	0.2681	23.8306	5.0367	10-90th
5549	Nullip	Male	295	4200	86	31.5	White	70	167.6	24.88	no	2.5119	0.1043	24.0920	0.3890	6.4571	3.7311	10-90th
5557	Nullip	Male	269	2680	10	41.4	White	64	156	26.3	no	3.4583	0.1255	27.5626	0.3294	10.4992	2.6252	10-90th
5566	Multip	Male	288	4050	84.3	40.2	White	108	175.3	35.18	no	2.9011	0.1027	28.2419	0.3141	9.2365	3.0576	10-90th
6161	Multip	Male	291	4080	82.9	43.3	White	72	175.3	23.33	no	2.4189	0.1270	19.0420	0.2734	8.8468	2.1524	10-90th
6252	Multip	Male	290	4682	99.2	23.1	White	112	170.2	38.7	no	6.2848	0.0863	72.8515	0.2590	24.2663	3.0022	>90th
6350	Multip	Male	280	4090	93.6	37.3	White	52	164	19.33	no	1.7041	0.0878	19.4006	0.2680	6.3576	3.0516	>90th
6429	Multip	Female	293	4114	82.9	37.6	White	71	172.7	23.81	no	3.2791	0.0978	33.5255	0.2817	11.6421	2.8797	10-90th
6611	Multip	Male	276	2752	6.6	26.2	White	45	154.9	18.75	no	3.4851	0.0770	45.2345	0.2033	17.1425	2.6387	<10th
6674	Multip	Female	262	2500	9.8	32.8	White	47	157.5	18.95	Yes	3.8315	0.1075	35.6468	0.2567	14.9247	2.3884	<10th
6683	Multip	Male	286	4370	96.8	30.4	White	71	167.6	25.2	no	3.7635	0.1128	33.3628	0.2541	14.8133	2.2522	>90th
6762	Multip	Male	294	4034	76.7	26	Asian	55	160	21.48	no	2.2559	0.1107	20.3844	0.2330	9.6802	2.1058	10-90th
6781	Multip	Female	281	4341	98	43.7	White	67	165	24.61	no	3.2795	0.0860	38.1465	0.2021	16.2239	2.3513	>90th
6792	Multip	Male	279	4140	95.5	35.7	White	60	165	22.04	no	2.5241	0.0774	32.6058	0.2042	12.3602	2.6380	>90th
6797	Multip	Male	293	4256	90	31.8	White	104	165.1	38.15	no	N/A	0.1078	N/A	0.2520	N/A	2.3385	>90th
6821	Multip	Male	287	4076	86.7	29.2	White	69	171	23.6	no	2.6274	0.1073	24.4832	0.1872	14.0358	1.7443	10-90th
8827	Multip	Female	279	2550	1.6	29.2	White	52	157.5	20.96	no	1.8667	0.0634	29.4255	0.1869	9.9855	2.9468	<10th
8953	Multip	Male	294	4498	96.4	34.7	White	48	160	18.75	no	5.1042	0.1225	41.6817	0.3071	16.6219	2.5076	>90th
9293	Multip	Female	270	2602	6.2	37.8	White	66	170.2	22.78	no	3.8672	0.0976	39.6155	0.3405	11.3566	3.4883	<10th

Lab ID	Parity	Gender	GA (days)	BW (g)	BW centile	MA (years)	Ethnicity	MW (Kg)	Height (cm)	BMI ($kg/m^{2)}$	Smoking	IGF2	IGF2R	IGF2/ IGF2R	IGF1R	IGF2/ IGF1R	IGF1R/ IGF2R	CLASS
9530	Multip	Male	271	2494	3.1	38.8	White	66	164	24.54	no	4.4485	0.0869	51.2187	0.3118	14.2685	3.5896	<10th
9721	Nullip	Female	265	2174	1	40.9	White	61	170	21.11	no	2.8698	0.0799	35.9110	0.1889	15.1943	2.3634	<10th
9874	Nullip	Male	289	2412	0.2	36.6	White	53	154.9	22.13	no	4.1629	0.0725	57.4040	0.2204	18.8870	3.0393	<10th
36458	Multip	Female	273	2639	5.3	34.2	White	81	175.3	26.36	Yes	4.1668	0.1502	27.7361	0.2769	15.0495	1.8430	<10th
71132	Nullip	Female	274	2710	6.7	17.2	Black	55	166	19.96	no	4.7604	0.1105	43.0680	0.3161	15.0617	2.8594	<10th
71740	Nullip	Male	290	4586	98.5	35.5	White	71	171	24.28	no	4.1403	0.1068	38.7526	0.1940	21.3425	1.8157	>90th
74016	Multip	Female	287	4370	96.5	41.4	White	54	162.6	20.42	no	4.4185	0.1204	36.7121	0.3364	13.1355	2.7949	>90th
74169	Multip	Female	260	2740	30.3	37.3	White	56	154.9	23.34	no	2.8981	0.0964	30.0529	0.2837	10.2165	2.9416	10-90th
74788	Nullip	Female	271	2116	0.2	27.3	Oriental	54	165.1	19.81	no	1.6119	0.0698	23.1070	0.2384	6.7606	3.4179	<10th
77253	Multip	Female	277	4256	98.3	41	White	67	157.5	26.89	no	4.2318	0.0669	63.2143	0.1633	25.9120	2.4396	>90th
77797	Multip	Male	291	4820	99.6	32.3	White	79	162.6	29.88	no	8.1644	0.1414	57.7520	0.2980	27.3938	2.1082	>90th
83552	Multip	Female	289	2667	1.1	23.7	White	54	155	22.48	no	3.5012	0.1400	25.0025	0.3465	10.1030	2.4748	<10th
A0178	Nullip	Female	288	4320	95	36.3	White	74	167.6	26.17	no	4.3326	0.0870	49.7768	0.2442	17.7428	2.8055	>90th
A0700	Nullip	Male	293	2355	0.1	40.1	White	60	169	21.01	Yes	3.0125	0.0722	41.7403	0.2755	10.9357	3.8169	<10th
A0712	Multip	Female	263	4313	100	37.6	White	95	177.8	30.05	no	5.5059	0.0948	58.0836	0.2940	18.7272	3.1016	>90th
A0747	Multip	Female	267	2412	3.3	37.2	White	56	162.6	21.16	no	3.3959	0.0904	37.5488	0.2204	15.4086	2.4369	<10th
A0801	Nullip	Female	287	2752	2.3	40	White	60	162.6	22.69	Yes	2.7056	0.0899	30.0978	0.3892	6.9519	4.3294	<10th
A0989	Multip	Female	289	4360	95.4	40	White	64	162.6	24.21	no	5.3485	0.0994	53.8286	0.2908	18.3923	2.9267	>90th
A1013	Multip	Female	264	2667	16	41.8	White	79	170.2	27.27	no	4.4651	0.1042	42.8476	0.3777	11.8223	3.6243	10-90th
A1125	Nullip	Male	285	4300	95.9	35.9	White	63	171	21.55	no	4.4565	0.0979	45.5397	0.2686	16.5940	2.7443	>90th
A1251	Multip	Female	285	4512	98.8	37	White	75	162.6	28.37	no	4.8959	0.1183	41.3731	0.2757	17.7590	2.3297	>90th
A1253	Multip	Female	292	4380	94.6	35.2	White	68	162.6	25.72	no	3.7456	0.1032	36.3024	0.2184	17.1490	2.1169	>90th
A1466	Nullip	Male	279	2580	1.9	29.5	Asian	55	160	21.48	no	2.7935	0.0839	33.3060	0.2524	11.0663	3.0097	<10th
A1467	Multip	Male	271	2600	5.4	27.1	White	84	172.7	28.16	no	3.3562	0.1029	32.6060	0.2950	11.3784	2.8656	<10th
A1694	Multip	Female	286	4455	98	41	White	78	175.3	25.38	no	4.4002	0.0982	44.8212	0.3278	13.4249	3.3387	>90th
A1786	Nullip	Male	275	2506	2	40.5	White	40	159	15.82	no	2.6939	0.0673	40.0466	0.2819	9.5571	4.1903	<10th
A1816	Nullip	Female	291	2724	1.3	37.8	White	50	160	19.53	no	3.0864	0.0798	38.6731	0.2155	14.3219	2.7003	<10th

Lab ID	Parity	Gender	GA (days)	BW (g)	BW centile	MA (years)	Ethnicity	MW (Kg)	Height (cm)	BMI ($kg/m^{2)}$	Smoking	IGF2	IGF2R	IGF2/ IGF2R	IGF1R	IGF2/ IGF1R	IGF1R/ IGF2R	CLASS
A2096	Nullip	Female	288	2750	2	42.5	Asian	65	160	25.39	no	2.3595	0.0977	24.1620	0.3679	6.4129	3.7677	<10th
A2098	Nullip	Female	289	4654	99.1	43.4	White	77	170.2	26.58	no	3.1304	0.1037	30.1867	N/A	N/A	N/A	>90th
A2099	Multip	Male	269	4540	100	42.5	White	83	160	32.42	Yes	3.2235	0.0883	36.4998	0.1931	16.6915	2.1867	>90th
A2325	Nullip	Male	298	4682	98.2	34.5	White	66	163	24.84	no	5.1648	0.1283	40.2448	0.2865	18.0293	2.2322	>90th
A2345	Nullip	Male	278	4313	98.5	35.4	White	74	167.6	26.34	Yes	3.7176	0.1342	27.6970	0.2551	14.5754	1.9003	>90th
A2394	Multip	Female	270	2667	8.4	29.4	White	55	157.5	22.17	Yes	3.1569	0.0969	32.5889	0.2749	11.4836	2.8379	<10th
A2491	Multip	Male	282	4272	96.7	29.4	White	63	152.4	27.13	no	4.4044	0.1347	32.6871	0.2996	14.7002	2.2236	>90th
A2611	Multip	Male	289	4427	96.8	38.3	White	88	160	34.38	no	5.3131	0.1221	43.5228	0.3003	17.6944	2.4597	>90th
A2742	Multip	Male	293	5022	99.9	26.8	White	68	167.6	24.21	no	2.6671	0.1059	25.1733	0.2051	13.0066	1.9354	>90th
A2744	Nullip	Female	265	2710	17.1	41.1	White	65	154.9	27.09	no	2.3195	0.0774	29.9625	0.3113	7.4502	4.0217	10-90th
A3331	Multip	Male	281	4360	98.3	31.4	White	75	162.6	28.37	no	3.7001	0.1273	29.0598	0.1996	18.5373	1.5676	>90th
A3412	Multip	Female	287	4341	95.9	41.3	White	75	175.3	24.41	no	4.6198	0.1129	40.9027	0.3225	14.3255	2.8552	>90th
A3618	Nullip	Male	265	2752	20	28.9	Oriental	67	168	23.74	no	3.3577	0.0921	36.4407	0.2686	12.5025	2.9147	10-90th
A3633	Nullip	Male	290	4380	95.5	39.9	White	95	167.6	33.82	no	3.5643	0.0977	36.4816	0.3546	10.0510	3.6296	>90th
A3637	Nullip	Female	279	2582	1.9	35	White	52	147.3	23.97	no	2.2260	0.0766	29.0481	N/A	N/A	N/A	<10th
A3731	Nullip	Female	259	2150	2.1	41.2	Oriental	60	165.1	21.98	no	2.7607	0.1073	25.7323	0.2759	10.0058	2.5717	<10th
A3741	Multip	Male	281	4586	99.6	36.9	White	105	177.8	33.21	no	3.2537	0.1006	32.3566	0.3137	10.3705	3.1201	>90th
A3946	Multip	Female	277	4341	99	39.8	White	101	172.7	33.86	no	4.3168	0.1130	38.2090	0.3578	12.0665	3.1665	>90th
A3956	Nullip	Male	287	4500	98.3	38.5	White	97	170.2	33.49	no	5.6616	0.1664	34.0191	0.3630	15.5983	2.1809	>90th
A4139	Nullip	Male	289	4940	99.9	32	White	57	165.1	20.91	no	6.8767	0.1061	64.8191	0.2281	30.1472	2.1501	>90th
A4240	Multip	Female	283	2470	0.6	30.6	White	55	152.4	23.68	no	2.3353	0.1005	23.2307	0.3293	7.0915	3.2759	<10th
A4241	Nullip	Male	288	4400	96.6	36.3	White	91	180.3	27.99	Yes	4.4577	0.0957	46.5853	0.2786	16.0001	2.9116	>90th
A4545	Nullip	Female	282	2526	1	24.6	White	96	171	32.83	no	1.6318	0.0727	22.4382	0.2101	7.7650	2.8897	<10th
A4876	Nullip	Male	288	4914	99.9	31.1	White	74	175.3	24.08	no	6.5278	0.1277	51.1325	0.3071	21.2540	2.4058	>90th
A5328	Multip	Male	275	4385	99.5	39.5	White	89	167.6	31.68	no	5.2113	0.1020	51.0841	0.2986	17.4532	2.9269	>90th
A5590	Multip	Male	273	4398	99.7	37.3	White	86	167.6	30.54	no	5.8641	0.1034	56.6982	0.2482	23.6230	2.4001	>90th
A5647	Multip	Male	271	2696	8.6	41	White	98	167.6	34.89	Yes	1.7922	0.0652	27.4927	0.2416	7.4194	3.7055	<10th

Lab ID	Parity	Gender	GA (days)	BW (g)	BW centile	MA (years)	Ethnicity	MW (Kg)	Height (cm)	BMI ($kg/m^{2)}$	Smoking	IGF2	IGF2R	IGF2/ IGF2R	IGF1R	IGF2/ IGF1R	IGF1R/ IGF2R	CLASS
A5655	Multip	Male	280	4400	98.8	34	White	72	165.1	26.41	no	3.7962	0.1013	37.4606	0.2444	15.5300	2.4121	>90th
A5775	Multip	Female	279	2530	1.4	44.3	White	65	170	22.49	no	2.3171	0.0741	31.2659	0.2568	9.0234	3.4650	<10th
A5795	Nullip	Male	290	4345	94.6	35.3	White	70	172.7	23.47	no	5.9438	0.1048	56.7349	0.2690	22.0928	2.5680	>90th
A5886	Multip	Male	273	4800	100	33.5	Oriental	52	155	21.64	no	4.3882	0.0887	49.4838	0.2193	20.0101	2.4729	>90th
A5920	Nullip	Male	274	4341	99.4	17.7	White	75	177.8	23.69	no	7.5266	0.1114	67.5859	0.2036	36.9611	1.8286	>90th
A6021	Multip	Male	279	4290	98	38.9	White	66	172	22.14	no	7.1938	0.1374	52.3383	0.3039	23.6716	2.2110	>90th
A6302	Multip	Female	278	2611	2.6	31.4	White	57	152.4	24.54	no	3.0216	0.1025	29.4823	0.2898	10.4259	2.8278	<10th
A6435	Nullip	Female	273	2130	0.2	25.8	White	55	157.5	22.17	no	3.8766	N/A	N/A	0.2990	12.9644	N/A	<10th
A7006	Multip	Female	286	2740	2.3	40.2	White	82	173.8	27.15	no	2.6296	0.1213	21.6733	0.3150	8.3489	2.5959	<10th
A7044	Nullip	Male	300	4330	89.1	35.8	Black	82	164	30.49	no	6.4334	0.1268	50.7246	0.2979	21.5931	2.3491	10-90th
A7272	Nullip	Female	275	2724	6.4	19.6	Black	82	168	29.05	no	4.0098	0.1166	34.3917	0.3844	10.4320	3.2968	<10th
A7380	Multip	Male	286	4483	98.3	30.8	White	44	154.9	18.34	no	5.2126	0.1485	35.1046	0.3180	16.3928	2.1415	>90th
A7549	Nullip	Female	269	2354	1.7	33.3	White	54	165.1	19.81	no	3.2881	0.1212	27.1292	0.3326	9.8848	2.7445	<10th
A7732	Multip	Male	290	4852	99.7	40.8	White	74	170.2	25.55	no	3.6056	0.0755	47.7338	0.1479	24.3810	1.9578	>90th
A7767	Nullip	Male	281	2724	3.5	46	White	57	157.5	23.06	no	4.4249	0.1134	39.0226	0.3192	13.8622	2.8150	<10th
A8186	Multip	Male	284	4300	96.4	35.7	White	80	172	27.04	no	5.7246	0.1286	44.4996	0.3427	16.7035	2.6641	>90th
A8314	Multip	Male	284	2724	2.6	26	White	60	160	23.44	no	4.2365	0.1061	39.9115	0.3286	12.8939	3.0954	<10th
A8315	Multip	Male	283	4300	96.8	38.1	White	64	162.6	24.21	no	5.2470	0.1403	37.4065	0.1868	28.0826	1.3320	>90th
A9235	Nullip	Male	261	2164	1.7	24	Black	70	144.8	33.3	no	3.1133	0.1023	30.4322	0.2926	10.6416	2.8597	<10th

APPENDIX C – *ANXA5* haplotypes of RM and control cohorts

Early miscarriage patients

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	APLS	FVL	LAC	ANXA5 Female	ANXA5 Male
1	17	4	3	0	1	NEG	WT	EQUIV ABN ABN	M1	WT
2	130	3	3	0	0	NEG	WT	NEG	WT	WT
3	141	5	4	0	1	NEG	WT	NEG EQUIV	WT	WT
4	181	4	4	0	0	NEG	WT	ABN EQUIV NEG	M2	M2*
5	185	4	3	0	1	NEG POS	WT	ABN EQUIV NEG	M1	WT
6	227	7	6	0	1	NEG	WT	NEG	WT	WT
7	247	6	6	0	0	NEG	WT	EQUIV	WT	M1*
8	699	4	4	0	N/A	NEG	WT	NEG	WT	WT
9	951	N/A	E	0	N/A	POS	N/A	NEG	M1/M2	WT
10	1074	3	3	0	0	POS	HET	NEG	WT	M2
11	1081	4	3	0	1	POS	WT	NEG	M2	WT
12	1098	3	3	0	0	POS	WT	ABN EQUIV POS	WT	WT
13	1225	3	3	0	0	NEG POS	WT	NEG	M1/M2	M1
14	1255	N/A	E	0	N/A	POS	WT	NEG	WT	WT
15	1291	N/A	E	0	N/A	NEG POS	WT	NEG	M1	WT
16	1299	3	3	0	0	NEG	WT	NEG	WT	WT
17	1303	N/A	E	0		POS	WT	NEG	WT	M1
18	1306	5	5	0	0	NEG	WT	POS NEG POS	WT	WT
19	1329	4	4	0	0	NEG POS	WT	NEG	WT	M2
20	1349	5	4	0	1	NEG POS	WT	NEG	WT	WT
21	1353	5	4	0	1	POS	WT	NEG	M2	WT
22	1364	3	3	0	0	POS	WT	NEG EQUIV	WT	WT
23	1365	6	5	0	1	POS	WT	NEG	M2	M2
24	1367	4	4	0	0	NEG POS	WT	NEG	M1	WT
25	1401	3	3	0	0	N/A	WT	N/A	WT	M1
26	1434	4	4	0	0	POS	WT	NEG	WT	WT
27	1435	4	3	0	1	POS	WT	POS NEG EQUIV	M2	WT
28	1445	3	3	0	0	POS	WT	EQUIV CLO EQUIV	WT	WT
29	1447	3	3	0	0	POS	WT	NEG	WT	WT
30	1448	3	3	0	0	NEG POS	WT	NEG	WT	M2
31	1459	3	3	0	0	NEG	WT	NEG	M1	M2
32	1482	6	6	0	0	NEG	WT	ABN NEG NEG	WT	WT
33	1485	3	3	0	0	POS	WT	NEG NEG EQUIV	WT	WT
34	1492	3	3	0	0	POS	WT	ABN	WT	WT
35	1494	N/A	E	0	N/A	POS	WT	NEG	WT	WT
36	1496	3	3	0	0	NEG	WT	POS	WT	WT
37	1505	4	3	0	1	NEG POS	WT	NEG	M1	WT
38	1507	3	3	0	0	POS	WT	ABN EQUIV	M1	WT
39	1510	3	3	0	0	NEG	WT	NEG	M2	M1/M2
40	1514	5	4	0	0	POS	WT	NEG	WT	M2
41	1515	3	3	0	3	POS	WT	NEG	M2	M2

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	APLS	FVL	LAC	ANXA5 Female	ANXA5 Male
42	1516	7	4	0	2	NEG POS	WT	EQUIV NEG	M2	WT
43	1517	6	4	0	1	POS	WT	NEG	M1	WT
44	1518	6	5	0	2	NEG POS	WT	NEG	WT	WT
45	1523	6	4	0	2	NEG	WT	NEG	WT	WT
46	1541	N/A	E	0	N/A	POS	WT	NEG	WT	WT
47	1556	4	3	0	1	NEG	WT	NEG	WT	M1/M2
48	1558	3	3	0	0	NEG	HET	NEG	M2	M1
49	1559	6	6	0	0	NEG	WT	NEG EQUIV NEG	M2	M2
50	1570	4	3	0	1	NEG	WT	NEG	WT	WT
51	1572	3	3	0	0	NEG	WT	POS	WT	WT
52	1576	5	4	0	1	POS	WT	NEG	M2*	WT
53	1577	3	3	0	0	POS	WT	NEG	WT	WT
54	1580	6	5	0	1	POS	WT	ABN ABN NEG	WT	M2
55	1583	3	3	0	0	NEG	WT	NEG	M2	M1
56	1584	4	4	0	0	POS	WT	NEG POS	M2	N/A
57	1596	8	7	0	1	NEG	WT	EQUIV	WT	WT
58	1597	N/A	E	0	N/A	NEG	WT	NEG	WT	M2*
59	1600	10	10	0	0	NEG	WT	EQUIV EQUIV NEG	WT	M2
60	1601	3	3	0	0	POS	HET	NEG	WT	M2
61	1604	4	4	0	0	NEG POS	WT	NEG	WT	WT
62	1606	3	3	0	0	POS	WT	NEG	WT	M1
63	1615	N/A	E	0	N/A	POS	WT	NEG	M1	M2
64	1616	3	3	0	0	POS	WT	NEG	WT	M2
65	1621	5	4	0	1	POS	WT	NEG	M2	M2
66	1625	11	10	0	1	NEG POS	WT	NEG	M2	WT
67	1631	3	3	0	0	NEG	WT	NEG	WT	WT
68	1651	4	3	0	1	POS	WT	ABN ABN	WT	M2*
69	1663	5	4	0	1	POS	WT	NEG	WT	M1
70	1680	9	8	0	1	NEG POS	WT	ABN NEG	WT	WT
71	1681	3	3	0	0	NEG	WT	ABN NEG	WT	M2
72	1719	4	3	0	1	NEG POS	WT	NEG	WT	M2
73	1733	5	4	0	1	POS	WT	NEG	WT	WT
74	1791	6	5	0	1	NEG POS	WT	NEG ABN	M1	M2*
75	1797	3	3	0	0	POS	WT	NEG	WT	WT
76	1812	4	3	0	1	POS	WT	POS	WT	M2
77	1822	3	3	0	0	NEG POS	WT	NEG	M1	M2
78	1857	3	3	0	0	NEG POS	WT	NEG	WT	WT
79	1864	4	3	0	1	POS	N/A	NEG NEG POS	M2	M1/M2
80	1876	4	4	0	0	POS	WT	ABN NEG NEG	M2	WT
81	1892	6	6	0	0	POS	WT	NEG	M2	M2
82	1899	4	3	0	1	POS	WT	NEG	M1	M1
83	1900	3	3	0	0	POS	HET	NEG	WT	WT
84	1904	6	6	0	0	POS	WT	NEG	WT	M2
85	1923	5	5	0	0	N/A	WT	N/A	M2	M1

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	APLS	FVL	LAC	ANXA5 Female	ANXA5 Male
86	1957	4	3	0	0	POS	HET	NEG	WT	M2
87	1959	3	3	0	0	POS	WT	NEG EQUIV NEG	WT	WT
88	1962	5	5	0	0	POS	WT	NEG	WT	WT
89	1996	3	3	0	0	POS	WT	NEG	M2	WT
90	2002	4	3	0	0	POS	WT	NEG ABN	WT	M1
91	2009	3	3	0	0	POS	WT	NEG EQUIV	M1	WT
92	2015	4	3	0	0	POS	WT	POS	N/A	N/A
93	2017	8	8	0	0	NEG POS	WT	NEG	WT	WT
94	2019	3	3	0	0	POS	HET	POS	WT	WT
95	2356	N/A	E	0	N/A	POS	WT	ABN EQUIV	WT	WT
96	2516	N/A	E	0	N/A	NEG	WT	NEG	WT	WT
97	2740	N/A	E	0	N/A	NEG	WT	NEG	M2	WT
98	2796	N/A	E	0	N/A	NEG	WT	NEG	WT	WT
99	2918	N/A	E	0	N/A	NEG	WT	NEG	WT	WT
100	5427	4	4	0	0	NEG	WT	NEG	WT	WT
101	5435	5	5	0	0	NEG	WT	NEG	M2	WT
102	5436	5	5	0	0	NEG	WT	NEG	M2	WT
103	5437	7	5	0	2	POS	WT	NEG	M2	M2
104	5442	6	5	0	1	N/A	N/A	NEG	M2	M1
105	5445	4	4	0	0	NEG	N/A	NEG	M1	WT
106	5446	3	3	0	0	POS	WT	NEG POS	M2	M1
107	5450	4	3	0	1	NEG	WT	NEG	M1	WT
108	5453	6	5	0	1	NEG	WT	NEG	WT	WT
109	5461	6	5	0	1	NEG	WT	NEG POS NEG	WT	M1
110	5466	5	5	0	0	NEG	HET	NEG	M1*	M2
111	5469	10	9	0	1	NEG	N/A	NEG	WT	N/A
112	5474	5	5	0	0	NEG	WT	NEG POS NEG	WT	WT
113	5478	3	3	0	0	NEG	WT	NEG	WT	WT
114	5486	7	7	0	0	NEG	WT	NEG	M2	WT
115	5493	4	4	0	0	POS	WT	NEG NEG ABN	M1	WT
116	5497	5	5	0	0	NEG	WT	NEG	WT	WT
117	5498	5	5	0	0	NEG	WT	NEG	WT	WT
118	5500	3	3	0	0	NEG	WT	NEG	WT	WT
119	5501	3	3	0	0	NEG POS	WT	NEG	WT	WT
120	5502	4	3	0	1	NEG	WT	POS	N/A	M2
121	5504	5	5	0	0	NEG	WT	NEG	WT	WT
122	5507	5	4	0	1	NEG	N/A	NEG	M1/M2	WT
123	5512	3	3	0	0	NEG	WT	N/A	WT	M2
124	5514	9	8	0	1	POS	WT	NEG	M2	M1/M2
125	5515	5	3	0	2	NEG	WT	NEG	WT	M2
126	5524	3	3	0	0	NEG	WT	NEG	WT	M2
127	5528	7	5	0	2	NEG POS	WT	NEG	WT	WT
128	5530	3	3	0	0	NEG	WT	NEG	M1	WT
129	5538	4	4	0	0	NEG POS	WT	NEG	WT	M2

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	APLS	FVL	LAC	ANXA5 Female	ANXA5 Male
130	5541	4	3	0	1	NEG	WT	NEG	WT	WT
131	5545	3	3	0	0	NEG POS	WT	NEG	N/A	N/A
132	5575	7	4	0	3	NEG	N/A	NEG	WT	WT
133	5586	4	4	0	0	NEG POS	WT	NEG	WT	WT
134	5602	5	3	0	2	NEG	WT	NEG	WT	WT
135	5609	5	4	0	1	NEG	N/A	NEG	WT	WT
136	5617	6	5	0	1	NEG POS	WT	NEG	M2	WT
137	5621	4	4	0	0	NEG	WT	POS NEG	M1	WT
138	5630	5	4	0	1	NEG	WT	N/A	M2*	WT
139	5637	8	8	0	0	NEG	WT	POS	M1	M2
140	5638	8	7	0	1	NEG	WT	NEG	WT	M1/M2
141	5641	4	4	0	0	NEG	WT	NEG	M2	WT
142	5642	5	4	0	1	NEG	WT	NEG	M1	WT
143	5648	7	7	0	0	NEG	WT	NEG	M2	M2
144	5658	3	3	0	0	NEG	WT	NEG	M2*	WT
145	5685	4	3	0	1	NEG	WT	NEG	WT	WT
146	5689	5	5	0	0	N/A	N/A	N/A	WT	WT
147	5699	5	5	0	0	NEG	WT	NEG	M1	M2
148	5717	3	3	0	0	N/A	N/A	N/A	WT	WT
149	5718	4	3	0	1	NEG	WT	NEG	WT	WT
150	5723	4	3	0	1	NEG	WT	NEG	M1	WT
151	5728	5	4	0	1	NEG	WT	NEG NEG POS	WT	WT
152	5729	5	3	0	2	NEG	WT	NEG	WT	M2
153	5738	3	3	0	0	NEG	N/A	NEG	WT	WT
154	5739	5	5	0	0	NEG	WT	NEG	M2	WT
155	5741	4	4	0	0	NEG	WT	POS	WT	WT
156	5742	7	7	0	0	NEG	WT	NEG	WT	M1
157	5755	5	3	0	2	NEG	WT	POS NEG NEG	M1*	WT
158	5775	4	4	0	0	NEG	WT	POS NEG	WT	M1
159	5788	4	3	0	1	N/A	N/A	N/A	WT	WT
160	5789	3	3	0	0	NEG	HET	NEG	WT	WT
161	5790	4	3	0	1	NEG	WT	NEG	M2*	WT
162	5792	5	4	0	1	POS	WT	NEG	M1	M2
163	5797	3	3	0	0	NEG	WT	POS	WT	WT
164	5799	3	3	0	0	NEG	WT	NEG	M2	WT
165	5804	4	4	0	0	NEG	WT	N/A	WT	M2
166	5812	3	3	0	0	NEG	WT	POS NEG	M1	WT
167	5816	6	5	0	1	NEG	WT	NEG	M2	M2
168	5822	7	7	0	0	NEG	WT	NEG	WT	WT
169	5837	4	4	0	0	NEG POS	WT	NEG	WT	M2
170	5840	4	3	0	1	POS	WT	NEG	WT	WT
171	5841	4	3	0	1	N/A	N/A	N/A	WT	WT
172	5847	5	4	0	1	NEG	WT	NEG	M1	WT
173	5848	3	3	0	0	NEG	WT	N/A	WT	WT

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	APLS	FVL	LAC	ANXA5 Female	ANXA5 Male
174	5849	4	3	0	1	N/A	N/A	N/A	M2	WT
175	5858	6	5	0	1	NEG	WT	NEG	M2	WT
176	5860	5	5	0	0	NEG	HET	NEG	M1	WT
177	5864	4	4	0	0	NEG	WT	NEG	WT	M2
178	5869	9	7	0	2	NEG	WT	NEG	WT	M2
179	5871	4	3	0	1	NEG	WT	NEG	WT	WT
180	5875	4	4	0	0	NEG	WT	NEG	M2	WT
181	5878	5	4	0	1	NEG	WT	NEG	M2	WT
182	5879	3	3	0	0	NEG	WT	NEG	WT	WT
183	5882	5	5	0	0	NEG	WT	NEG	M1	WT
184	5885	4	3	0	1	NEG	WT	NEG	WT	M1/M2
185	5889	11	10	0	1	NEG	N/A	NEG	M1	WT
186	5896	6	6	0	0	NEG	WT	NEG	WT	WT
187	5907	6	5	0	1	NEG	WT	N/A	WT	M1
188	5909	6	4	0	2	NEG	WT	NEG	M2	M2
189	5925	4	4	0	0	NEG	WT	NEG POS NEG	M2	M1/M2
190	5928	5	5	0	0	NEG	WT	POS NEG	N/A	WT
191	5937	8	7	0	1	NEG	WT	NEG	WT	WT
192	5938	3	3	0	0	NEG	WT	NEG	M2	M2
193	5939	3	3	0	0	NEG	WT	POS NEG NEG	WT	WT
194	5940	11	9	0	2	NEG	N/A	NEG	WT	WT
195	5941	4	4	0	0	NEG	WT	POS NEG NEG	WT	M2
196	5948	4	4	0	0	NEG	WT	NEG	WT	WT
197	5949	3	3	0	0	NEG POS	WT	NEG	M2	M2
198	5950	6	3	0	3	NEG	WT	POS NEG POS	M1	WT
199	5953	5	5	0	0	NEG	WT	NEG POS NEG	WT	WT
200	5954	4	4	0	0	NEG POS	WT	NEG	M1	WT
201	5960	4	4	0	0	NEG	WT	NEG	M2	M2
202	5961	5	5	0	0	NEG	WT	NEG	M2	M1/M2
203	5967	5	4	0	1	POS	WT	NEG	M2*	WT
204	5977	3	3	0	0	POS	WT	NEG	M2	M2
205	5979	6	5	0	1	NEG	WT	NEG	M1	WT
206	5985	5	4	0	1	NEG	WT	NEG	M2	WT
207	5987	5	5	0	0	NEG	WT	POS	WT	WT
208	5988	8	7	0	1	NEG	WT	NEG	WT	WT
209	5989	4	4	0	0	NEG	WT	NEG	WT	M2
210	5991	4	4	0	0	NEG	WT	NEG	M1/M2	WT
211	5998	5	4	0	1	NEG POS	WT	NEG	WT	WT
212	5999	4	4	0	0	NEG	WT	NEG	WT	WT
213	6002	6	5	0	1	NEG	WT	NEG	WT	M1/M2
214	6003	3	3	0	0	NEG	WT	NEG	WT	M2
215	6004	3	3	0	0	NEG	WT	NEG	WT	M1
216	6007	9	9	0	0	NEG	WT	POS	M2	M1
217	6008	14	14	0	0	NEG POS	WT	POS NEG NEG	WT	WT

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	APLS	FVL	LAC	ANXA5 Female	ANXA5 Male
218	6009	5	5	0	0	NEG POS	WT	NEG	M1	WT
219	6011	4	4	0	0	NEG	WT	NEG	WT	WT
220	6014	3	3	0	0	NEG	WT	NEG	WT	M2
221	6016	4	3	0	1	NEG	WT	NEG	M1	WT
22	6020	3	3	0	0	POS	WT	POS NEG NEG	M1	WT
223	6030	6	4	0	2	NEG POS	WT	NEG	WT	WT
224	6034	8	8	0	0	NEG	N/A	NEG	M1	WT
225	6035	7	6	0	1	NEG	WT	NEG	WT	WT
226	6036	3	3	0	0	NEG	WT	NEG	WT	M1
227	6037	4	3	0	1	NEG	WT	NEG	WT	WT
228	6038	3	3	0	0	NEG	WT	NEG	WT	WT
229	6039	6	4	0	2	NEG	WT	NEG	WT	N/A
230	6042	6	6	0	0	NEG	WT	NEG	WT	WT
231	6043	6	5	0	1	NEG POS	N/A	NEG	WT	WT
232	6050	6	5	0	1	NEG	WT	NEG	M1	WT
233	6051	8	7	0	1	NEG	WT	NEG	WT	M2
234	6061	3	3	0	0	NEG	WT	NEG	WT	WT
235	6063	4	4	0	0	NEG	N/A	NEG	M2	WT
236	6066	3	3	0	0	NEG POS	WT	NEG	WT	M2
237	6068	4	4	0	0	NEG	WT	NEG	WT	WT
238	6077	3	3	0	0	NEG	WT	NEG	M1/M2	M2
239	6081	3	3	0	0	NEG	WT	NEG	M1	WT
240	6083	6	6	0	0	NEG	WT	NEG	WT	WT
241	6084	6	6	0	0	NEG	WT	NEG	WT	M1/M2
242	6086	6	5	0	1	POS	WT	NEG	M2	WT
243	6087	4	4	0	0	NEG	WT	NEG	WT	WT
244	6089	5	4	0	1	NEG POS	N/A	NEG	WT	WT
245	6096	3	3	0	0	NEG	WT	NEG	M1*	M1/M2
246	6097	4	3	0	1	NEG	N/A	NEG	M2	M1
247	6101	3	3	0	0	NEG	WT	N/A	WT	WT
248	6102	4	3	0	1	NEG	WT	NEG	WT	WT
249	6106	6	5	0	1	NEG	N/A	NEG	M1	WT
250	6111	3	3	0	0	N/A	N/A	N/A	WT	WT
251	6116	3	3	0	0	NEG	WT	NEG	WT	M1
252	6119	4	3	0	1	NEG	WT	N/A	WT	WT
253	6123	4	3	0	1	NEG POS	WT	NEG	WT	WT
254	6124	6	6	0	0	NEG	WT	POS NEG NEG	WT	WT
255	6127	4	3	0	1	NEG POS	WT	NEG POS	WT	WT
256	6138	4	4	0	0	NEG POS	WT	NEG	M2	WT
257	6139	5	5	0	0	NEG	WT	NEG	WT	M2
258	6140	3	3	0	0	NEG	WT	NEG	WT	WT
259	6141	3	3	0	0	NEG	WT	NEG	WT	WT
260	6147	6	5	0	1	NEG	WT	NEG	WT	M2
261	6149	4	4	0	0	NEG POS	WT	POS	WT	WT

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	APLS	FVL	LAC	ANXA5 Female	ANXA5 Male
262	6160	4	3	0	1	NEG	WT	NEG	M1	M2
263	6163	4	3	0	1	NEG	WT	NEG	M1	M2
264	6166	7	6	0	1	NEG	WT	NEG	WT	M2*
265	6171	4	3	0	1	NEG	N/A	POS NEG	WT	WT
266	6183	3	3	0	0	NEG POS	HET	NEG	WT	WT
267	6184	4	3	0	1	NEG	WT	NEG	WT	WT
268	6188	4	4	0	0	NEG	WT	NEG	WT	N/A
269	6191	4	3	0	1	NEG	WT	NEG	M1	M2
270	6193	3	3	0	0	NEG	WT	NEG	WT	WT
271	6201	4	4	0	0	NEG	WT	NEG	WT	M2
272	6204	3	3	0	0	NEG	WT	NEG	M1	WT
273	6206	6	5	0	1	NEG	WT	NEG	M2	WT
274	6208	5	4	0	1	NEG	WT	NEG	WT	WT
275	6219	4	3	0	1	NEG	WT	NEG	WT	M1
276	6220	4	3	0	1	NEG	WT	NEG	WT	WT
277	6224	5	5	0	0	NEG	WT	NEG	WT	WT
278	6231	5	4	0	1	NEG	WT	NEG	M2	M2*
279	6370	4	4	0	0	NEG	WT	NEG	M2	WT
280	6373	5	5	0	0	NEG	WT	NEG POS NEG	WT	WT
281	6376	5	5	0	0	NEG	WT	NEG	M2	M2
282	6378	8	7	0	1	NEG	WT	NEG	M2	WT
283	6381	3	3	0	0	NEG	WT	NEG	WT	WT
284	6383	5	5	0	0	NEG	WT	NEG	M2	WT
285	6385	6	6	0	0	NEG POS	WT	N/A	WT	M1
286	6387	5	5	0	0	NEG	WT	NEG	M2	WT
287	6390	3	3	0	0	NEG	WT	NEG	M1	M1
288	6391	5	5	0	0	NEG	WT	NEG	M2	M2
289	6396	4	4	0	0	NEG	WT	NEG	M2	N/A
290	6399	3	3	0	0	NEG	WT	NEG	M2	M2
291	6404	4	4	0	0	NEG	WT	POS	WT	M1/M2
292	6410	3	3	0	0	NEG	N/A	NEG	M1/M2	WT
293	6417	8	7	0	1	NEG	WT	NEG	WT	WT
294	6423	5	4	0	1	NEG	WT	NEG	WT	M1
295	6426	6	6	0	0	NEG	WT	NEG	WT	WT
296	6428	4	4	0	0	NEG	WT	NEG	WT	WT
297	6430	3	3	0	0	NEG	WT	NEG	M1	WT
298	6434	4	3	0	1	NEG	WT	NEG	WT	WT
299	6438	3	3	0	0	NEG	N/A	NEG	WT	WT

Late miscarriage patients

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	APLS	FVL	LAC	ANXA5 Female	ANXA5 Male
1	222	6	5	1	0	NEG	WT	EQUIV NEG POS	WT	WT
2	235	N/A	N/A	L	N/A	NEG	WT	EQUIV EQUIV NEG	WT	WT
3	315	N/A	N/A	L	N/A	POS	WT	EQUIV EQUIV	WT	WT
4	324	3	1	1	1	NEG POS	HOMO	NEG	M1	WT
5	344	N/A	N/A	L	N/A	NEG	WT	NEG	WT	WT
6	524	N/A	N/A	L	N/A	N/A	WT	NEG	M2	M1
7	620	2	N/A	2	0	NEG POS	WT	NEG NEG ABN	N/A	N/A
8	626	N/A	N/A	L	N/A	POS	WT	POS	M1*	M2
9	628	N/A	N/A	L	N/A	POS	HET	NEG EQUIV EQUIV	WT	M1
10	631	3	2	1	0	POS	WT	NEG	WT	M2
11	636	N/A	N/A	L	N/A	NEG POS	WT	NEG	WT	WT
12	641	7	3	4	0	NEG	WT	NEG POS ABN	M1	M2*
13	664	1	N/A	1	0	NEG	HET	NEG	WT	WT
14	681	N/A	N/A	L	N/A	NEG	WT	NEG	M2	WT
15	682	N/A	N/A	L	N/A	NEG	WT	NEG ABN	M1	WT
16	706	4	2	1	1	N/A	WT	N/A	M2	WT
17	718	3	1	1	1	N/A	WT	NEG	M1/M2	WT
18	735	7	4	2	1	NEG	WT	NEG ABN	M1	WT
19	748	2	1	1	0	NEG	WT	ABN NEG	M1/M2	WT
20	785	N/A	N/A	L	N/A	NEG	WT	NEG	WT	M2
21	818	N/A	N/A	L	N/A	POS	WT	POS	M1	WT
22	845	N/A	N/A	L	N/A	NEG	WT	NEG EQUIV	WT	WT
23	848	3	N/A	3	0	NEG	WT	NEG	M2*	M1
24	876	6	4	1	1	NEG	WT	NEG	WT	WT
25	896	1	N/A	1	0	NEG POS	WT	NEG	M2	M2
26	987	N/A	N/A	L	N/A	NEG POS	WT	NEG	WT	WT
27	1001	9	8	1	0	POS	HET	EQUIV	M1	WT
28	1022	5	2	2	1	NEG POS	WT	ABN POS	WT	WT
29	1023	4	2	2	0	NEG POS	WT	NEG	WT	WT
30	1079	N/A	N/A	L	N/A	NEG	HET	NEG	WT	WT
31	1097	N/A	N/A	L	N/A	NEG	WT	EQUIV NEG NEG	WT	WT
32	1195	3	2	1	0	POS	WT	NEG	M2	WT
33	1210	N/A	N/A	L	N/A	POS	WT	POS NEG POS	WT	WT
34	1227	N/A	N/A	L	N/A	POS	WT	EQUIV NEG	M1	WT
35	1379	N/A	N/A	L	N/A	POS	WT	ABN POS	WT	M1
36	1389	N/A	N/A	L	N/A	POS	WT	POS	WT	M2
37	1409	N/A	N/A	L	N/A	NEG	HET	NEG	M2	M1
38	1410	5	3	1	1	N/A	WT	N/A	WT	WT
39	1440	2	N/A	2	0	POS	WT	NEG	M1	WT
40	1458	N/A	N/A	L	N/A	N/A	N/A	N/A	WT	WT
41	1478	2	1	1	0	NEG POS	WT	EQUIV NEG	WT	WT
42	1479	N/A	N/A	L	N/A	NEG POS	WT	ABN POS ABN	WT	WT

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	APLS	FVL	LAC	ANXA5 Female	ANXA5 Male
43	1480	N/A	N/A	L	N/A	NEG	WT	NEG	M1	WT
44	1481	N/A	N/A	L	N/A	NEG	WT	ABN EQUIV	WT	M1
45	1484	5	1	3	1	POS	WT	POS	M1	WT
46	1497	N/A	N/A	L	N/A	POS	WT	NEG	M1	M2
47	1509	3	1	2	0	NEG	WT	NEG	M1	WT
48	1519	3	1	2	0	NEG	WT	NEG	WT	WT
49	1530	N/A	N/A	L	N/A	POS	WT	EQUIV NEG	WT	WT
50	1532	N/A	N/A	L	N/A	NEG	WT	NEG	WT	WT
51	1551	N/A	N/A	L	N/A	NEG	WT	EQUIV NEG	WT	M1
52	1591	2	N/A	2	0	POS	WT	NEG	WT	WT
53	1602	N/A	N/A	L	N/A	NEG POS	WT	ABN	WT	WT
54	1623	2	1	1	0	POS	WT	NEG	WT	WT
55	1624	N/A	N/A	L	N/A	POS	WT	NEG. ABN	WT	M1
56	1626	N/A	N/A	L	N/A	NEG	WT	NEG EQUIV NEG	WT	M2
57	1645	N/A	N/A	L	N/A	NEG POS	WT	EQUIV	WT	WT
58	1648	N/A	N/A	L	N/A	NEG POS	WT	NEG	M1	WT
59	1667	N/A	N/A	L	N/A	NEG POS	WT	NEG NEG ABN	WT	WT
60	1670	5	3	1	1	POS	WT	EQUIV POS	WT	M2
61	1679	4	3	1	0	NEG	WT	NEG	WT	M2
62	1684	N/A	N/A	L	N/A	POS	WT	POS	WT	WT
63	1701	N/A	N/A	L	N/A	NEG POS	WT	NEG ABN	WT	WT
64	1716	N/A	N/A	L	N/A	POS	WT	POS NEG POS	M1	M1
65	1725	5	2	1	2	POS	WT	ABN NEG	WT	WT
66	1747	N/A	N/A	L	N/A	POS	WT	NEG	M2*	WT
67	1753	N/A	N/A	L	N/A	POS	WT	POS NEG POS	WT	WT
68	1755	4	2	1	1	NEG	WT	NEG	WT	WT
69	1800	5	2	2	1	NEG POS	WT	POS	WT	M2
70	1816	4	2	1	1	POS	WT	ABN NEG	M2	WT
71	1826	N/A	N/A	L	N/A	POS	WT	POS	M1*	M1
72	1827	6	2	2	2	N/A	N/A	EQUIV EQUIV POS	WT	M2
73	1833	N/A	N/A	L	N/A	POS	N/A	NEG	WT	WT
74	1880	4	2	1	1	POS	WT	NEG	M1	M2*
75	1893	N/A	N/A	L	N/A	POS	WT	POS ABN	WT	M1
76	1915	4	3	1	0	POS	WT	NEG	M2	WT
77	1916	N/A	N/A	N/A	N/A	POS	WT	POS NEG NEG	WT	WT
78	1928	4	2	2	0	POS	N/A	NEG	WT	WT
79	1942	4	2	2	0	NEG	WT	NEG EQUIV	M2	M1
80	1949	3	2	1	0	POS	WT	NEG	WT	M1/M2
81	1954	N/A	N/A	L	N/A	NEG	WT	ABN ABN	WT	M2
82	1963	7	5	1	1	POS	WT	NEG NEG EQUIV	M2	M2
83	1970	N/A	N/A	L	N/A	POS	WT	ABN	WT	WT
84	2031	N/A	N/A	L	N/A	POS	WT	EQUIV POS NEG	M1	WT
85	2095	N/A	N/A	L	N/A	NEG POS	WT	NEG	WT	M1
86	2098	N/A	N/A	L	N/A	POS	WT	NEG	WT	M1

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	APLS	FVL	LAC	ANXA5 Female	ANXA5 Male
87	2099	N/A	N/A	L	N/A	POS	WT	ABN	WT	WT
88	2164	N/A	N/A	L	N/A	NEG POS	WT	ABN POS	WT	WT
89	2174	N/A	N/A	L	N/A	N/A	N/A	N/A	WT	WT
90	2209	N/A	N/A	L	N/A	POS	WT	NEG	M2	WT
91	2234	N/A	N/A	L	N/A	NEG POS	HET	NEG ABN	WT	WT
92	2283	N/A	N/A	L	N/A	POS	HET	NEG	M2	M2
93	2439	N/A	N/A	L	N/A	POS	WT	NEG	WT	WT
94	2512	N/A	N/A	L	N/A	NEG POS	WT	NEG	WT	WT
95	2856	N/A	N/A	L	N/A	NEG	WT	NEG	WT	M1
96	3213	N/A	N/A	L	N/A	N/A	WT	N/A	M1	WT
97	3261	N/A	N/A	L	N/A	N/A	N/A	N/A	M1*	WT
98	3401	N/A	N/A	L	N/A	POS	WT	POS NEG	M1	M1
99	4552	2	1	1	0	NEG	WT	NEG	M2	WT
100	4565	N/A	N/A	N/A	1SB	POS	N/A	POS	WT	WT
101	4568	4	3	1	0	NEG	WT	NEG	WT	M1
102	4573	4	3	1	0	NEG	WT	NEG	M1	WT
103	4883	N/A	N/A	N/A	1SB	NEG	N/A	NEG	WT	M1*
104	3504	N/A	N/A	L	N/A	NEG	HET	NEG	WT	N/A
105	3747	N/A	E	L	0	NEG	WT	NEG ABN NEG	WT	M2
106	3761	2	0	2	0	NEG	WT	NEG	N/A	WT
107	3792	N/A	E	L	N/A	NEG	WT	EQUIV NEG POS	WT	WT
108	3793	N/A	E	L	N/A	NEG	WT	EQUIV EQUIV	M1	M2
109	3837	N/A	N/A	L	N/A	NEG	N/A	NEG	WT	M1
110	3839	N/A	E	L	N/A	NEG	WT	POS	WT	M2
111	3840	N/A	E	L	N/A	NEG	WT	NEG	M1	N/A
112	3878	N/A	E	L	N/A	POS	WT	NEG	WT	M1
113	3885	2	1	1	0	POS	WT	ABN EQUIV EQUIV	WT	M2*
114	3971	4	2	2	0	NEG	WT	NEG	WT	M2
115	3988	3	2	1	0	NEG	WT	ABN NEG NEG	WT	M1
116	3995	6	4	2	0	NEG	WT	NEG	M2	M2
117	4002	3	1	2	0	NEG	WT	EQUIV POS	M2	WT
118	4003	8	2	5	1	NEG	WT	EQUIV	WT	M2
119	4019	3	2	1	0	NEG	WT	NEG	WT	WT
120	4070	2	1	1	0	NEG	WT	NEG	WT	WT
121	4119	4	3	1	0	NEG	WT	ABN	WT	WT
122	4120	2	1	1	0	NEG POS	WT	N/A	M2	N/A
123	4180	3	2	1	0	NEG	WT	ABN NEG NEG	M2	WT
124	4328	10	7	1	2	NEG	WT	NEG	WT	WT
125	4330	3	1	1	1	NEG	WT	NEG	M2	WT
126	4375	4	3	1	0	NEG POS	WT	NEG	WT	M2
127	4398	4	2	1	1	NEG POS	WT	NEG	WT	M1
128	4411	5	3	1	1	NEG	HET	NEG	WT	M1
129	4444	7	5	1	1	NEG	WT	NEG	WT	WT
130	4445	4	3	1	0	NEG	WT	EQUIV EQUIV	WT	WT

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	APLS	FVL	LAC	ANXA5 Female	ANXA5 Male
131	4456	5	3	1	1	NEG	WT	NEG	M1	M2
132	4511	4	3	1	0	NEG	WT	NEG	M2	WT
133	4518	4	3	1	0	NEG	WT	NEG	WT	WT
134	4525	5	0	4	1	NEG	WT	NEG EQUIV POS	M1	M1
135	4528	8	5	2	1	NEG	WT	NEG	WT	M1
136	4535	4	2	1	1	NEG	WT	NEG	M1	M2
137	4596	3	2	1	0	NEG	N/A	EQUIV	WT	WT
138	4655	3	2	1	0	NEG	WT	ABN NEG NEG	M1	WT
139	4675	4	1	2	1	NEG	WT	N/A	WT	WT
140	4682	3	0	2	1	NEG	WT	POS	WT	WT
141	4693	6	4	1	1SB	POS	WT	POS	M1	WT
142	4717	6	4	1	1	NEG	WT	NEG	WT	WT
143	4719	7	5	1	1	NEG	WT	NEG	WT	WT
144	4720	6	4	1	1	NEG	WT	NEG	WT	WT
145	4725	4	3	1	0	NEG POS	WT	N/A	WT	WT
146	4761	3	2	1	0	NEG	WT	POS NEG NEG	WT	M2
147	4796	5	3	1	1	NEG	N/A	NEG	M1	WT
148	4823	6	4	2	0	N/A	N/A	N/A	WT	WT
149	4855	5	2	1	2	NEG	WT	NEG	M1	WT
150	4917	5	0	3	2	NEG	WT	NEG	M2	WT
151	4951	6	4	1	1	POS	WT	NEG	M2	M1
152	4982	13	6	5	2	NEG	N/A	NEG	WT	WT
153	4996	5	2	2	1	N/A	N/A	N/A	M2	M2
154	5014	4	1	2	1	NEG	WT	NEG	M2	WT
155	5159	4	1	2	1	NEG	WT	NEG	WT	M2
156	5182	5	3	1	1	NEG	WT	NEG	M1	WT
157	5214	8	5	1	2	NEG	WT	NEG	M1	WT
158	5248	4	3	1	0	NEG	WT	NEG	M2	N/A
159	5332	3	2	1	0	NEG	WT	NEG	WT	WT
160	5422	4	1	2	1	NEG	WT	NEG	WT	M2
161	5424	7	5	1	1	NEG	WT	NEG	M1	WT
162	5475	4	1	1	2	NEG	WT	NEG	WT	M1
163	5479	12	5	3	4	NEG	WT	NEG	M1	WT
164	5480	4	2	1	1	NEG	WT	NEG	WT	WT
165	5516	4	2	1	1	NEG	WT	NEG	M2	WT
166	5531	6	5	1	0	NEG	WT	NEG NEG POS NEG	WT	WT
167	5612	3	1	2	0	NEG	WT	NEG	WT	M2
168	5631	5	0	3	2	N/A	N/A	N/A	M2	WT
169	5645	N/A	N/A	1	N/A	NEG	WT	POS NEG	M2	M2
170	5646	8	5	2	1	NEG	WT	NEG	WT	WT
171	5740	3	2	1	0	NEG	WT	NEG	M1	WT
172	5749	5	2	1	2	NEG	HET	NEG	WT	M2
173	5753	6	4	1	1	NEG	WT	NEG	WT	M1
174	5811	2	1	1	0	N/A	WT	N/A	M1	M1

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	APLS	FVL	LAC	ANXA5 Female	ANXA5 Male
175	5846	3	0	3	0	NEG	WT	NEG	WT	WT
176	5890	12	10	1	1	NEG	WT	POS NEG POS	WT	WT
177	5969	4	2	1	1	NEG	WT	NEG	M2	M1
178	6044	5	1	3	1	NEG POS		NEG	WT	M2
179	6076	2	0	1	1	NEG	WT	NEG	WT	N/A
180	6090	3	2	0	1SB	NEG	WT	NEG	WT	WT
181	6095	5	4	1	0	NEG POS	WT	NEG	WT	WT
182	6144	6	4	1	1	NEG	WT	NEG	M2	WT
183	6177	7	4	1	2	NEG	WT	NEG	M2	M1
184	6182	1	0	1	0	POS	WT	NEG	WT	WT
185	6205	3	2	1	0	NEG	WT	NEG	WT	WT
186	6218	4	3	1	0	NEG	WT	NEG	WT	WT
187	6221	4	3	1	0	NEG	WT	NEG	WT	WT
188	6413	3	2	1	0	NEG	WT	NEG	M1	M2
189	6437	10	2	5	3	NEG	N/A	NEG	WT	WT

Moore control cohort

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	ANXA5 Female	ANXA5 Male	ANXA5 Placenta	Gender
1	1	2	0	0	2	M2	WT	M2	M
2	3	1	0	0	1	WT	M2	M2	F
3	5	2	0	0	2	WT	M2	M2	M
4	7	1	0	0	1	WT	WT	WT	F
5	8	1	0	0	1	WT	WT	WT	M
6	9	2	0	0	2	WT	M1	M1	M
7	10	1	0	0	1	WT	WT	WT	F
8	11	1	0	0	1	M1	M2	WT	F
9	14	1	0	0	1	WT	M1	M1	M
10	16	1	0	0	1	WT	WT	WT	M
11	18	1	0	0	1	WT	WT	WT	F
12	19	1	0	0	1	WT	WT	WT	M
13	20	1	0	0	1	WT	WT	WT	M
14	21	1	0	0	1	WT	WT	WT	F
15	22	2	0	0	2	M1*	M1	M1	M
16	24	3	0	0	3	M1	WT	M1	M
17	25	1	0	0	1	WT	M1	M1	M
18	26	1	0	0	1	M2	M2	M2*	F
19	38	1	0	0	1	M1	M1	M1	M
20	39	1	0	0	1	M2	WT	M2	F
21	40	1	0	0	1	WT	WT	WT	M
22	43	1	0	0	1	WT	WT	WT	M
23	47	1	0	0	1	WT	WT	WT	F
24	48	1	0	0	1	WT	M1	M1	F
25	49	1	0	0	1	WT	WT	WT	M
26	54	1	0	0	1	WT	M2	M2	M
27	57	1	0	0	1	M2	WT	M2	M
28	58	2	0	0	2	WT	M2	WT	F
29	64	2	0	0	2	M2	M2	M2	M
30	66	1	0	0	1	WT	M1	M1	F
31	67	2	0	0	2	WT	WT	WT	M
32	74	1	0	0	1	WT	M2	WT	M
33	79	1	0	0	1	WT	M1	M1	F
34	81	1	0	0	1	WT	M2	WT	F
35	82	2	0	0	2	WT	WT	WT	M
36	83	1	0	0	1	WT	M1	M1	F
37	86	2	0	0	2	WT	WT	WT	F
38	89	3	0	0	3	WT	WT	WT	M
39	90	1	0	0	1	WT	WT	WT	M
40	92	1	0	0	1	WT	WT	WT	F
41	93	1	0	0	1	WT	WT	WT	F
42	94	2	0	0	2	M1	M2	M1/M2	M
43	95	2	0	0	2	M2	WT	WT	M
44	96	1	0	0	1	WT	WT	WT	M
45	97	1	0	0	1	WT	WT	WT	F
46	100	1	0	0	1	WT	WT	WT	M
47	102	1	0	0	1	WT	M2	M2	M

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	ANXA5 Female	ANXA5 Male	ANXA5 Placenta	Gender
48	106	2	0	0	2	WT	M1	M1	F
49	108	2	0	0	2	WT	M1/M2	M2	M
50	109	1	0	0	1	WT	WT	WT	F
51	110	1	0	0	1	WT	WT	WT	M
52	111	3	0	0	3	WT	M1	WT	F
53	113	2	0	0	2	WT	M1	WT	M
54	115	2	0	0	2	WT	WT	WT	M
55	117	2	0	0	2	WT	WT	WT	M
56	118	3	0	0	3	WT	WT	WT	F
57	119	1	0	0	1	M1	WT	M1	M
58	122	1	0	0	1	WT	WT	WT	F
59	123	3	0	0	3	WT	M2	M2	M
60	126	1	0	0	1	WT	WT	WT	F
61	127	3	0	0	3	M1*	WT	M1	M
62	129	2	0	0	2	WT	WT	WT	M
63	131	1	0	0	1	M1*	M1	M1*	M
64	134	1	0	0	1	WT	M2	WT	F
65	143	1	0	0	1	WT	WT	WT	M
66	145	1	0	0	1	WT	WT	WT	F
67	149	2	0	0	2	M2	M1	M1/M2	M
68	154	2	0	0	2	WT	WT	WT	F
69	156	1	0	0	1	WT	M1	WT	M
70	157	2	0	0	2	M1	WT	WT	M
71	158	2	0	0	2	M2*	WT	M2	F
72	161	3	0	0	3	M2	WT	WT	M
73	162	3	0	0	3	M1*	WT	M1	F
74	167	1	0	0	1	WT	WT	WT	M
75	169	2	0	0	2	M1	WT	M1	M
76	170	2	0	0	2	M1	WT	M1	M
77	176	1	0	0	1	WT	WT	WT	F
78	178	2	0	0	2	WT	WT	WT	F
79	179	2	0	0	2	M2	WT	M2	F
80	185	1	0	0	1	M2	WT	M2	M
81	186	2	0	0	2	WT	WT	WT	F
82	191	2	0	0	2	WT	WT	WT	F
83	192	2	0	0	2	WT	WT	WT	M
84	194	2	0	0	2	WT	M1	M1	M
85	197	1	0	0	1	WT	M1	M1	M
86	198	1	0	0	1	WT	M1	M1	M
87	199	2	0	0	2	M1	M1	WT	F
88	200	2	0	0	2	WT	M1	M1	M
89	201	1	0	0	1	WT	WT	WT	M
90	202	1	0	0	1	WT	WT	WT	M
91	205	3	0	0	3	WT	WT	WT	F
92	211	3	0	0	3	WT	WT	WT	F
93	222	2	0	0	2	WT	WT	WT	M
94	223	2	0	0	2	WT	M2	WT	F
95	224	1	0	0	1	WT	WT	WT	M
96	228	3	0	0	3	WT	WT	WT	F
97	240	1	0	0	1	WT	WT	WT	F
98	243	1	0	0	1	M1	WT	M1	F
99	245	1	0	0	1	WT	WT	WT	M

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	ANXA5 Female	ANXA5 Male	ANXA5 Placenta	Gender
100	249	3	0	0	3	M1	WT	WT	M
101	250	4	0	0	4	WT	WT	WT	M
102	251	3	0	0	3	WT	WT	WT	F
103	252	2	0	0	2	M1	WT	M1	F
104	253	2	0	0	2	WT	M2	WT	F
105	257	1	0	0	1	M2	WT	M2	M
106	260	1	0	0	1	WT	M1	M1	M
107	262	1	0	0	1	WT	WT	WT	M
108	264	1	0	0	1	M1	WT	M1	M
109	268	3	0	0	3	WT	WT	WT	F
110	269	2	0	0	2	WT	WT	WT	F
111	273	1	0	0	1	WT	M1	WT	F
112	274	1	0	0	1	M2	M1	M1	M
113	275	1	0	0	1	M1	M1	M1	F
114	276	2	0	0	2	WT	M1	WT	F
115	283	1	0	0	1	WT	WT	WT	M
116	287	2	0	0	2	M1*	WT	M1	F
117	288	1	0	0	1	WT	M1	WT	M
118	289	1	0	0	1	WT	M2	M2	M
119	290	1	0	0	1	M2	M2	M2*	M
120	291	1	0	0	1	WT	WT	WT	M
121	294	1	0	0	1	M2	WT	M2	F
122	295	1	0	0	1	WT	WT	WT	M
123	304	1	0	0	1	WT	WT	WT	F
124	306	1	0	0	1	WT	WT	WT	M
125	307	3	0	0	3	M2	WT	M2	M
126	308	1	0	0	1	WT	WT	WT	F
127	N03	1	0	0	1	M1	WT	M1	F
128	N07	1	0	0	1	WT	WT	WT	M
129	N13	2	0	0	2	WT	M1	M1	F
130	N15	1	0	0	1	WT	WT	WT	F
131	N18	4	0	0	4	WT	WT	WT	M
132	N25	2	0	0	2	WT	WT	WT	F
133	N26	3	0	0	3	M2	WT	M2	M
134	N29	2	0	0	2	M2	M2	M2	F
135	N30	1	0	0	1	WT	WT	WT	M
136	N32	1	0	0	1	M2	WT	M2	F
137	N33	3	0	0	3	M2	WT	M2	M
138	N43	2	0	0	2	M1	M1	WT	F
139	N47	1	0	0	1	WT	M2	M2	M
140	N50	3	0	0	3	M2*	WT	M2	F
141	N51	2	0	0	2	WT	WT	WT	M
142	N58	1	0	0	1	WT	WT	M1	F
143	N59	2	0	0	2	M2	WT	M2	F
144	N61	1	0	0	1	WT	M1*	M1	M
145	N65	2	0	0	2	M2	M2	M2	F
146	N69	2	0	0	2	M1/M2	WT	M2	M
147	N71	2	0	0	2	M1	M1	M1	F
148	N75	1	0	0	1	WT	WT	WT	M
149	b00250	2	0	0	2	WT	M2	WT	M
150	b00251	1	0	0	1	WT	WT	WT	M
151	b00293	1	0	0	1	WT	WT	WT	F

No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	ANXA5 Female	ANXA5 Male	ANXA5 Placenta	Gender
152	b00296	1	0	0	1	WT	M1	WT	M
153	b00302	3	0	0	3	M1	WT	WT	F
154	b00371	1	0	0	1	M1	M2	M1	M
155	b00375	1	0	0	1	M1	WT	M1	F
156	b00376	1	0	0	1	WT	WT	WT	M
157	b00391	1	0	0	1	WT	WT	WT	M
158	b00392	2	0	0	2	WT	WT	WT	M
159	b00399	2	0	0	2	M2*	M2	M2*	M
160	b00400	1	0	0	1	M1	M1	M1	F
161	b00401	1	0	0	1	WT	M2	WT	F
162	b00402	3	0	0	3	WT	WT	WT	M
163	b00480	2	0	0	2	WT	WT	WT	F
164	b00482	1	0	0	1	WT	WT	WT	M
165	b00483	1	0	0	1	WT	WT	WT	F
166	b00486	1	0	0	1	WT	M2	M2	F
167	b00563	1	0	0	1	M2*	WT	M2	F
168	b00571	2	0	0	2	WT	WT	WT	M
169	b00573	2	0	0	2	M2	WT	M2	M
170	b00578	1	0	0	1	WT	M2	M2	F
171	b00581	1	0	0	1	M2	WT	M2	F
172	b00582	2	0	0	2	WT	M2	M2	F
173	b00589	1	0	0	1	WT	WT	WT	M
174	b00590	1	0	0	1	M2	M2	M2*	M
175	b00593	2	0	0	2	M2	WT	WT	M
176	b00701	1	0	0	1	M1	WT	M1	F
177	b00708	1	0	0	1	WT	M1	WT	M
178	b00742	1	0	0	1	WT	WT	WT	F
179	b00794	1	0	0	1	WT	WT	WT	M
180	b00802	1	0	0	1	M1	WT	M1	F
181	b00806	2	0	0	2	WT	WT	WT	F
182	b00808	1	0	0	1	WT	WT	WT	M
183	b00844	1	0	0	1	WT	M2	WT	M
184	b00847	2	0	0	2	M2	WT	M2	M
185	b00850	2	0	0	2	WT	WT	WT	M
186	b00913	3	0	0	3	WT	WT	WT	F
187	b00948	2	0	0	2	WT	M2	WT	M
188	b00952	3	0	0	3	WT	WT	WT	F
189	b00955	2	0	0	2	M1	WT	M1	F
190	b00996	1	0	0	1	M2	WT	WT	F
191	b01006	1	0	0	1	M2	M2	WT	F
192	b01010	1	0	0	1	M2	WT	M2	M
193	b01015	2	0	0	2	M1	WT	WT	F
194	b01016	3	0	0	3	M2	WT	M2	F
195	b01038	2	0	0	2	WT	WT	WT	F
196	b01040	2	0	0	2	WT	M2*	M2	F
197	b01043	1	0	0	1	M1	WT	WT	F
198	b01044	1	0	0	1	M2	WT	M2	M
199	b01047	1	0	0	1	WT	WT	WT	F
200	b01050	1	0	0	1	WT	M1	M1	F
201	b01082	1	0	0	1	M1	M1	WT	F

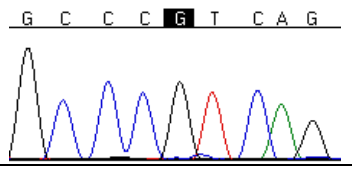
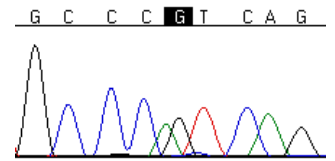
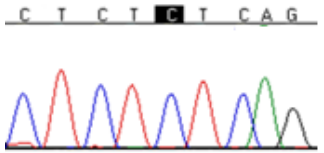
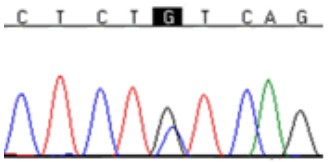
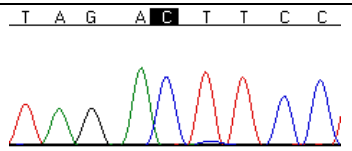
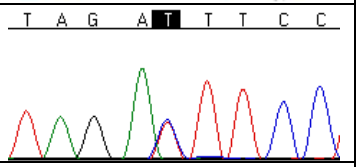
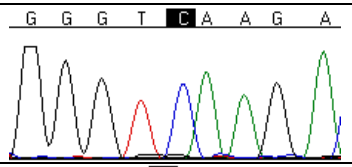
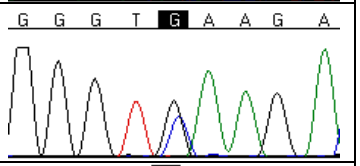
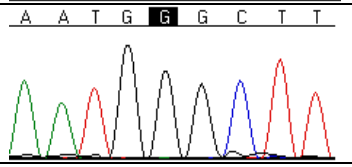
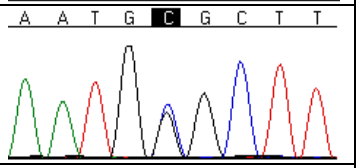
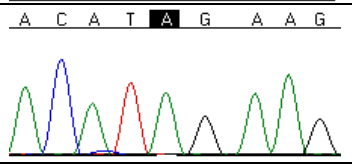
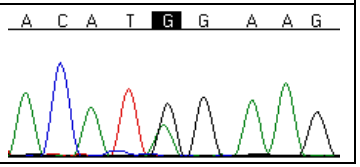
No	Lab ID	Pregnancy	Early Miscarriage	Late Miscarriage	Live Birth	ANXA5 Female	ANXA5 Male	ANXA5 Placenta	Gender
202	b01083	2	0	0	2	WT	WT	WT	M
203	b01084	1	0	0	1	WT	WT	WT	M
204	b01085	1	0	0	1	M2	M2	M2*	F
205	b01086	1	0	0	1	WT	WT	WT	M
206	b01090	1	0	0	1	WT	M1*	M1	M
207	b01134	1	0	0	1	WT	WT	WT	F
208	b01135	2	0	0	2	WT	WT	WT	M
209	b01144	1	0	0	1	M2	WT	WT	M
210	b01180	3	0	0	3	M1	M1	WT	F
211	b01181	2	0	0	2	WT	WT	WT	M
212	b01236	2	0	0	2	WT	WT	WT	F
213	b01237	1	0	0	1	M2	M1/M2	M2	F
214	b01238	1	0	0	1	M2	M2	M2	M
215	b01240	1	0	0	1	M2	WT	M2	F
216	b01241	1	0	0	1	M1	M2	M1	M
217	b01301	2	0	0	2	M1	WT	WT	M
218	b01302	1	0	0	1	WT	WT	WT	M
219	b01321	1	0	0	1	WT	WT	WT	F
220	b01328	1	0	0	1	WT	WT	WT	F
221	b01348	1	0	0	1	WT	M2	WT	F
222	b01354	1	0	0	1	WT	WT	WT	F
223	b01388	1	0	0	1	WT	M2	WT	M
224	b01394	3	0	0	3	M1	WT	M1	M
225	b01423	1	0	0	1	M1	M2	WT	F
226	b01434	2	0	0	2	WT	M2*	M2	F
227	b01451	1	0	0	1	WT	WT	WT	F
228	b01452	1	0	0	1	M1	WT	WT	F
229	b01460	2	0	0	2	WT	WT	WT	F
230	b01487	1	0	0	1	WT	WT	WT	F
231	b01513	1	0	0	1	WT	WT	WT	M
232	b01532	1	0	0	1	M1	WT	M1	F
233	b01533	3	0	0	3	WT	WT	WT	M
234	b01536	1	0	0	1	WT	M1	M1	F
235	b01539	1	0	0	1	WT	WT	WT	F
236	b01540	1	0	0	1	WT	WT	WT	F
237	b01541	2	0	0	2	WT	M1	WT	M
238	b01544	1	0	0	1	WT	WT	WT	M
239	b01545	1	0	0	1	M2	WT	M2	F
240	b01550	1	0	0	1	WT	WT	WT	F
241	b01553	1	0	0	1	M2	WT	M2	M

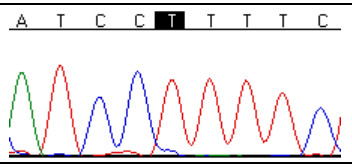
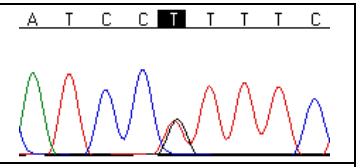
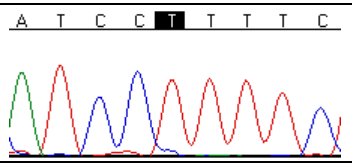
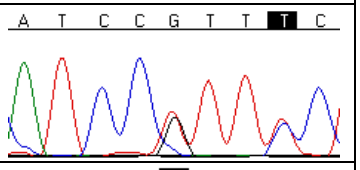
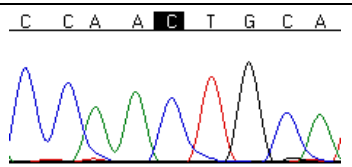
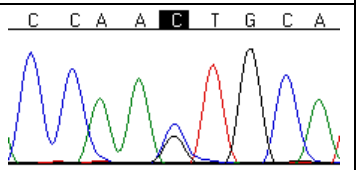
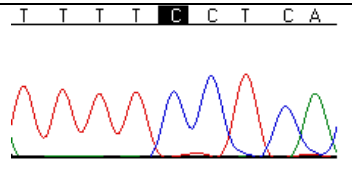
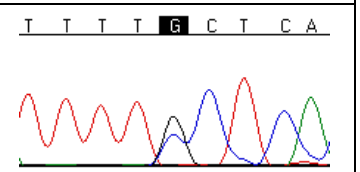
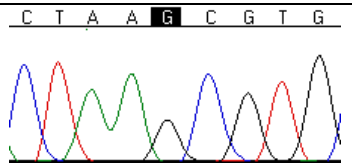
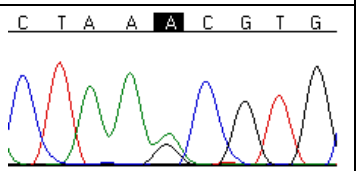
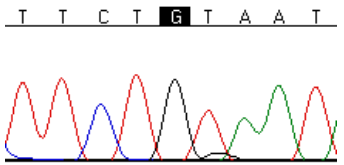
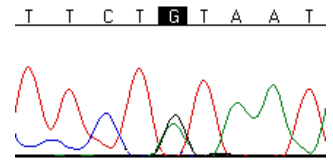
Appendix D - A list of genes prioritized using Ingenuity

A list of variants and genes were prioritized using the software program Ingenuity. This table lists all the genes that passed the "Genetic Analysis" filter. Indicated in red are the genes we chose to further investigate.

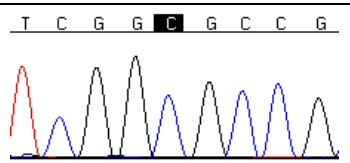
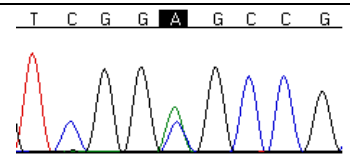
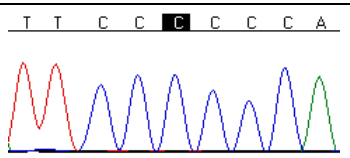
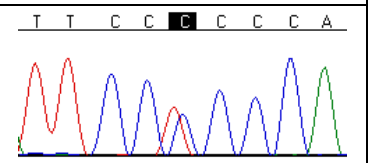
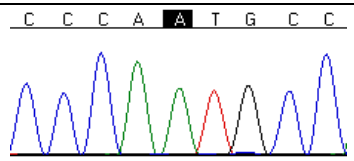
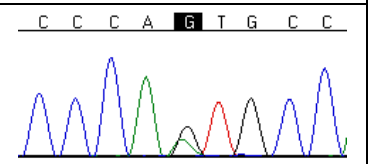
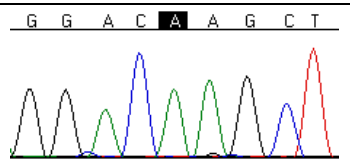
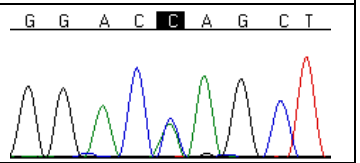
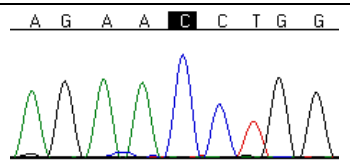
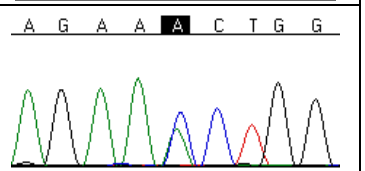
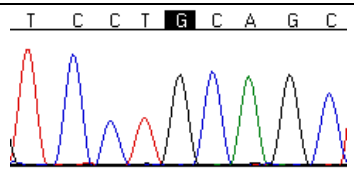
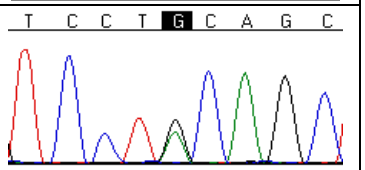
Chr...	Position	Gene Region	Gene Symbol	Protein Variant	Case Samples	Control Samples	Translation Impact	SIFT Functio...	Regulatory Site	Regulator	Variant Findings	dbSNP ID
1	22186113	Exonic	HSPG2	T1747P		– – –	missense	Damaging			1	199860743
1	22211078	Exonic	HSPG2	T533P		– – –	missense	Damaging				
1	204159612	Exonic	KISS1	X139fs*		– – –	frameshift				1	71745629
1	207963638	Exonic, Intronic	CD46	T360fs*, T375fs*		– – –	frameshift					
2	152296545	Exonic	RIF1	N517K		– – –	missense	Damaging				
2	179422184	Exonic	TTN	V20204I, V2032		– – –	missense					
2	179612216	Exonic, Intronic	TTN	I4971V		– – –	missense					
3	10088408	Splice Site	FANCD2			– – –			Splice Site Loss			
4	3037213	Exonic	GRK4	L425P, L457P		– – –	missense	Damaging				34022679
6	16327921	Exonic	ATXN1	Q207H		– – –	missense	Tolerated			1	201030692
6	32548628	Exonic	HLA-DRB1	R220W		– – –	missense	Damaging				34624872
6	32549531	Exonic	HLA-DRB1	Y152C		– – –	missense	Damaging				112796209
7	128843396	Exonic	SMO	R168H		– – –	missense	Damaging				61746143
9	96941126	microRNA	let-7			– – –			microRNA			
10	105792717	Exonic	COL17A1	A1435V		– – –	missense	Damaging				146841330
11	6411936	Exonic	SMPD1	36_37insALA		– – –	in-frame					
11	70130104	Intronic, microF	mir-548, PPFIA			– – –			microRNA			
11	92715335	Exonic	MTNR1B	R316C		– – –	missense	Damaging				192246153
12	2904352	Exonic	FKBP4	A16E		– – –	missense	Damaging				
12	6777070	Exonic	ZNF384	398_399delQQ,		– – –	in-frame					
12	51740413	Exonic	CELA1	L4fs*		– – –	frameshift					
13	20763264	Exonic	GJB2	V153I		– – –	missense	Tolerated			3	111033186
13	20763341	Exonic	GJB2	R127H		– – –	missense	Tolerated			12	111033196
13	110435294	Exonic	IRS2	1034_1036delPF		– – –	in-frame					
16	1306817	Exonic	TPSD1	A92T		– – –	missense	Tolerated			1	3993983
16	2807889	Exonic	SRRM2	P153L		– – –	missense					
17	5036249	Exonic	USP6	M80I		– – –	missense	Damaging				140991770
17	37564065	Exonic	MED1	S1470F		– – –	missense	Damaging				
17	38975265	Exonic, Promo	KRT10, TMEM	S508fs*		– – –	frameshift		Promoter Loss	ELK1, ETS1, SP		
17	38975266	Exonic, Promo	KRT10, TMEM	S508fs*		– – –	frameshift		Promoter Loss	ELK1, ETS1, SP		
18	61564364	Exonic	SERPINB2	L110V		– – –	missense	Damaging				
18	61597386	Exonic	SERPINB10	Q200E		– – –	missense	Activating				144898270
19	9045821	Exonic	MUC16	T11937I		– – –	missense					61744216
19	42596335	Exonic, Intronic	POU2F2	L413P, L429P		– – –	missense	Tolerated			1	
20	42169613	Exonic	L3MBTL1	L722V, L790V		– – –	missense	Damaging				61736753
20	46279858	Exonic	NCOA3	1253_1255delQ		– – –	in-frame					
X	31893367	5'UTR, Exonic	DMD	P1002S, P1005		– – –	missense	Activating				
X	48900727	Exonic	TFE3	R9P		– – –	missense	Damaging				
X	66765159	Exonic	AR	59delQ		– – –	in-frame					
X	105937255	Exonic	RNF128	S8fs*		– – –	frameshift					
X	153588181	Exonic	FLNA	P1300S		– – –	missense	Damaging				

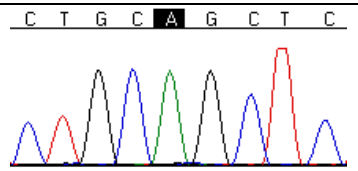
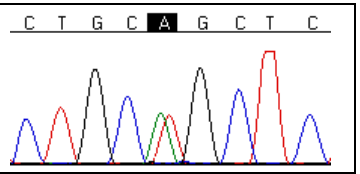
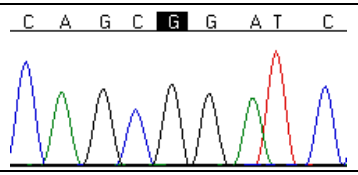
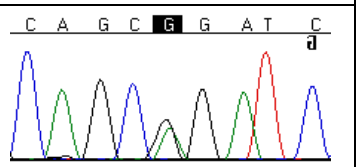
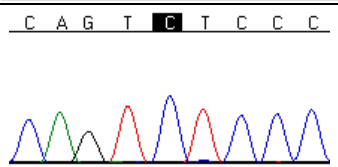
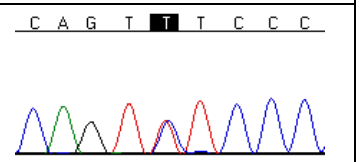
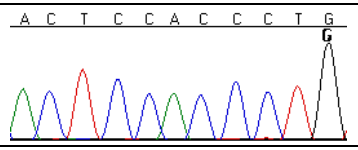
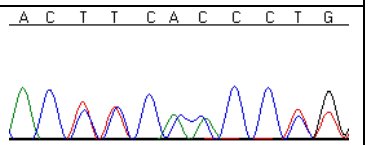
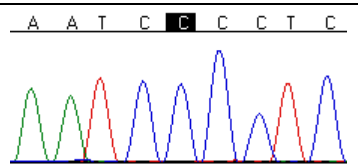
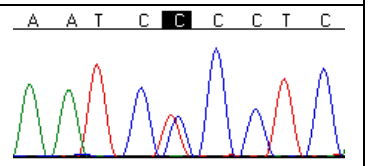
APPENDIX E – Variants found in RM patients and controls in gene *SERPINB2*

No	Region	Coordinates	Subst	Asians RM	White Europ. RM	Bangladeshi controls	Protein change	SNP type and SNP frequency	WT chromatogram	HET chromatogram
1	5'UTR	18:61554966	G>A	13 het 2 homo	2 het	11 het		rs2288288 0.035 in Europ. 0.256 in Asians		
2	Exon 4	18:61564364	C>G	Patient (exome)	NONE	NONE	L110V	Missense NOVEL		
3	Exon 5	18:61565014	C>T	2 het	1 het	NONE	D157D	Synonymous rs6106 793 in 13006 alleles		
4	Exon 5	18:61565062	C>G	17 het 3 homo	14 het	15 het 2 homo	V173V	Synonymous rs6105 575 in 13006 alleles		
5	Exon 6	18:61569091	G>C	1 het	NONE	2 het	G218A	Missense rs143202684 10 in 13006 alleles		
6	Exon 7	18:61569697	A>G	1 het	NONE	2 het	I246M	Missense rs138446596 20 in 13006 alleles		

7	Exon 8	18:61570470	T>G	22 het 4 homo	30 het 6 homo	37 het 3 homo	P393P	Synonymous rs6102 2648 in 13006 alleles		
8	Exon 8	18:61570473	T>C	1 het	NONE	NONE	F394F	Synonymous NOVEL		
9	Exon 8	18:61570503	C>G	31 het 8 homo	30 het 6 homo	35 het 15 homo	N404K	Missense rs6103 3501 in 13006 alleles		
10	Exon 8	18:61570529	C>G	31 het 8 homo	32 het 6 homo	35 het 10 homo	S413C	Missense rs6104 3493 in 13006 alleles		
11	3'UTR	18:61570546	G>A	3 het	NONE	1 het		NOVEL		
12	3'UTR	18:61570982	G>A	4 homo	1 het 5 homo	1 het 13 homo		rs12102 0.336 in Europ. 0.488 in Asians		

APPENDIX F – Variants found in RM patients and controls in gene *FKBP4*

No	Region	Coordinates	Subst	Asians RM	White Europ. RM	Bangladeshi controls	Protein change	SNP type and SNP frequency	WT chromatogram	HET chromatogram
1	Exon 1	12:2904352	C>A	Patient (exome)	NONE	NONE	A16E	Missense NOVEL		
2	Exon 3	12:2907013	C>A	1 het	NONE	1 het	P123P	Synonymous NOVEL		
3	Exon 3	12:2907018	A>G	1 het	NONE	NONE	N125S	Missense rs145385559 2 in 13006 alleles		
4	Exon 5	12:2908280	A>C	3 het	1 het	1 het	K181Q	Missense Reported SNP but rs unknown 5 in 7632 alleles		
5	Exon 5	12:2908330	C>A	1 het	8 het	NONE	N197K	Missense rs56196860 289 in 13006 alleles		
6	Exon 9	12:2910390	G>A	1 het	NONE	NONE	L380L	Synonymous Reported SNP but rs unknown 1 in 13006 alleles		

7	Exon 9	12:2910392	A>T	1 het	NONE	NONE	Q381L	Missense NOVEL		
8	Exon 9	12:2910446	G>A	1 het	NONE	NONE	R399Q	Missense NOVEL		
9	3'UTR	12:2912469	C>T	1 het	NONE	NONE		rs1803818 203 in 12806 alleles		
10	3'UTR	12:2912477	C indel	2 het	NONE	NONE		NOVEL		
11	3'UTR	12:2913025	C>T	1 het	NONE	NONE		rs116573718 3 in 118 alleles		

APPENDIX G – Publications

CHAPTER 2 – *IGF2* expression in CVS

Charalambos Demetriou, Sayeda Abu-Amero, Anna C. Thomas, Miho Ishida, Reena Aggarwal, Lara Al-Olabi, Lydia J. Leon, Jaime L. Stafford, Argyro Syngelaki, Donald Peebles, Kypros Nicolaides, Lesley Regan, Philip Stanier and Gudrun E. Moore (2014) Paternally Expressed, Imprinted Insulin-Like Growth Factor-2 in Chorionic Villi Correlates Significantly with Birth Weight. **Published Research Article** in PLoS ONE 9(1): e85454.

Charalambos Demetriou, Sayeda Abu-Amero, Anna C. Thomas, Miho Ishida, Reena Aggarwal, Lara Al-Olabi, Lydia J. Leon, Jaime L. Stafford, Argyro Syngelaki, Donald Peebles, Kypros Nicolaides, Lesley Regan, Philip Stanier and Gudrun E. Moore (2014) Paternally Expressed, Imprinted Insulin-Like Growth Factor-2 in Chorionic Villi Correlates Significantly with Birth Weight. **Oral presentation** at the Mammalian Genetics and Development workshop, London, 2013. (Published in Genet Res (2014) **95**: 183)

Charalambos Demetriou, Sayeda Abu-Amero, Anna C. Thomas, Miho Ishida, Reena Aggarwal, Lara Al-Olabi, Lydia J. Leon, Jaime L. Stafford, Argyro Syngelaki, Donald Peebles, Kypros Nicolaides, Lesley Regan, Philip Stanier and Gudrun E. Moore (2014) Paternally Expressed, Imprinted Insulin-Like Growth Factor-2 in Chorionic Villi Correlates Significantly with Birth Weight. **Poster presentation** at the American Society of Human Genetics (ASHG) meeting in San Francisco, 2012.

Gudrun E. Moore, Miho Ishida, **Charalambos Demetriou**, Lara Al-Olabi, Lydia J. Leon, Anna C. Thomas, Sayeda Abu-Amero, Jennifer M. Frost, Jaime L. Stafford, Yao Chaoqun, Andy Duncan, Rachel Baigel, Marina Brimioulle, Isabel Iglesias-Platas, Sophia Apostolidou, Reena Aggarwal, John C. Whittaker, Argyro Syngelaki, Kypros H. Nicolaides, Lesley Regan, David Monk, Philip Stanier (2015) The Role and Interaction of Imprinted Genes in Human Fetal Growth. **Published Review article in press** in Philos Trans R Soc Lond B Biol Sci. 5;370(1663). pii: 20140074.

Sayeda Abu-Amero, Anna C. Thomas, Shawnelle White, Katherine Rogers, Ana Maria Perez Miranda, Nita Solanky, Lydia J. Leon, **Charalambos Demetriou**, Xiayi Ke, Sam Stanier, Ben Stanier, Harry Costello, Samrawit Tzehaie, Lara Al-Olabi, Catherine Williamson, Mark Johnson, Lesley Regan, and Gudrun E. Moore (2014) The Baby Bio Bank-A Legacy for Researchers Worldwide into Common Complications of Pregnancy. **Published Review Article** in Journal of General Practice (2):158 doi:10.4172/2329-9126.1000158

CHAPTER 3 – Imprinting analysis of miscarried products of conception

Charalambos Demetriou, Miho Ishida, Anna C. Thomas, Shawnelle White, Philip Stanier, Lesley Regan and Gudrun E. Moore. Imprinting analysis of miscarried products of conception. **Oral Presentation** at the Mammalian Genetics and Development Workshop, London, 2014.

CHAPTER 4 – Role of Annexin A5 (*ANXA5*) in recurrent miscarriage

Charalambos Demetriou, Sayeda Abu-Amero, Shawnelle White, Arseni Markoff, Philip Stanier, Gudrun E. Moore and Lesley Regan. Investigations of the Annexin A5 M2 haplotype

in 500 White-European recurrent miscarriage couples. **Research Article (submitted for publication)**.

Charalambos Demetriou, Sayeda Abu-Amero, Shawnelle White, Arseni Markoff, Philip Stanier, Gudrun E. Moore and Lesley Regan. Investigations of the Annexin A5 M2 haplotype in 500 White-European recurrent miscarriage couples. **Poster Presentation** at the British Society for Genetic Medicine (BSGM) meeting in Liverpool, 2014.

CHAPTER 5 - Whole-exome sequencing in a recurrent miscarriage family

Charalambos Demetriou, Nuria Seto-Salvia, Anna C. Thomas, Estelle Chanudet, Hywel Williams, Rojeen Shahni, Maria Bitner-Glindzicz, Philip Stanier, Lesley Regan and Gudrun E. Moore. Whole exome sequencing in a patient with multiple miscarriages identifies novel candidate genes. **Poster Presentation** at the American Society for Human Genetics (ASHG) meeting in San Diego, 2014.