Effects of polymorphisms in the growth hormone and insulin-like growth factor axis on intrauterine and postnatal growth

Raja Narendar Rao Padidela

UCL Institute of Child Health

University College London

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Declaration

I, Raja Narendar Rao Padidela, 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.

Signed …… Date …….

Abstract

CONTEXT:

Intrauterine and postnatal growth influences future risks for metabolic syndrome. Body size and blood pressure (BP) are polygenic traits. The role for genetic variations in growth hormone (GH)-insulin-like growth factor (IGF) axis genes on intrauterine and early childhood growth and blood pressure, as well as gene loci identified through Genome Wide Association Studies (GWAS), are unclear.

OBJECTIVE:

To determine whether common variations in the genes of the GH-IGF axis associate with antenatal growth and birth size and play a role in the determination of body size and blood pressure at 3 years of age

STUDY DESIGN:

Pregnant women from white European families were recruited by the University College London Fetal Growth Study (n = 774). Fetal growth was measured by ultrasonography at each trimester. Postnatal growth data were collected prospectively at 6 months, 1 year, 2 years and 3 years of age. BP was measured at 3 years of age. Genotyping was performed by a combination of restriction fragment length polymorphism analysis, KBioscience Competitive Allele specific PCR genotyping System (KASP) analysis and multiplex polymerase chain reaction (PCR).

RESULTS:

The *GHR* exon 3 deletion genotype was significantly associated with birth weight and placental weight. *IGF1* SNPs did not demonstrate significant consistent longitudinal association with parameters investigated. *IGF2* SNPs were significantly associated with intrauterine growth (rs680), birth weight (rs680), placental weight (rs680) and BP (rs3842759) at 3 years of age. Several SNPs in genes found to be associated with adult BMI and BP from GWAS were significantly associated with early childhood size (*MTCH2, SH2B3*), body composition (*SH2B1, TMEM18*) and BP (*FTO*).

CONCLUSION:

These data suggest that several polymorphisms in the GH-IGF axis and in GWAS-identified genes for adult BMI and BP are significantly associated with intrauterine and early childhood size and BP at 3 years of age.

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Abbreviations

AC	Abdominal circumference
AGA	Appropriate for gestational age
ALS	Acid labile subunit
ANOVA	One-Way Analysis of Variance
BMI	Body mass index
BP	Blood pressure
BPD	Biparietal diameter
CRL	Crown-rump length
CV	Coefficient of variance
DNA	Deoxyribonucleic acid
EDD	Expected date of delivery
EDTA	Ethylenediaminetetraacetic acid
FL	Femoral length
GA	Gestational age
GH	Growth hormone
GHBP	Growth hormone binding protein
GHD	Growth hormone deficiency
GHR	Growth hormone receptor
GHRH	Growth hormone-releasing hormone
GWAS	Genome-wide association studies
HC	Head circumference
hGH-N	Pituitary growth hormone
hGH-V	Placental growth hormone
HWE	Hardy-Weinberg equilibrium
IGF	Insulin-like growth factor
IGF1R	Insulin-like growth factor 1 receptor
IGFBP	Insulin-like growth factor binding protein
ISS	Idiopathic short stature
LGA	Large for gestational age
M-6-P	Insulin-like growth factor/mannose-6-phosphate
KASP	KBiosciences Allele-specific genotypic system
MAF	Minor allele frequency
MAS	Mid-arm circumference
MLR	Multiple linear regression
mRNA	Messenger ribonucleic acid

MSLR	Multiple stepwise linear regression
NCBI	National Centre for Biotechnology Information
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase chain reaction
rhGH	Recombinant human growth hormone
QSF	Quadriceps skin-fold thickness
SGA	Small for gestational age
SDS	Standard deviation score
SNP	Single nucleotide polymorphism
SSF	Sub-scapular skin-fold thickness
TSF	Triceps skin-fold thickness
Tukey's HSD	Tukey's honest significant differences
UCL-FGS	University College London Fetal growth study
χ^2	Chi-square test
µl	microlitre

Chapter 1

Introduction

1.1 Context of the research

Individuals who display poor growth in utero and postnatally are at an increased risk for the metabolic syndrome which ultimately leads to the premature development of cardiovascular disease (Barker, 2007). The mechanism remains unclear but alterations in several endocrine axes have been noted, with effects on childhood and adult growth (Clayton et al., 2007) and puberty (Hernandez and Mericq, 2008); resetting of the insulin-like growth factor (IGF) and insulin systems with propensity for relative insensitivity (Iniguez et al., 2006); mild hyperthyrotropinaemia in the absence of overt hypothyroidism, and increased fetal/neonatal glucocorticoid exposure (Boguszewski et al., 1997). There is also an increased risk of premature adrenarche and the polycystic ovarian syndrome (PCOS) (Chernausek, 2012).

Insulin-like growth factors (IGFs) are important hormones for fetal growth while growth hormone (GH) and the GH-IGF axis are required for postnatal growth. Deficiency of IGFs are associated with intrauterine growth restriction and severe short stature (Woods et al., 1996). GH deficiency causes short stature (Alatzoglou and Dattani, 2010). The GH and IGF system also influences adiposity and body composition, which has bearing on the genesis of metabolic syndrome, obesity and hypertension (Veldhuis et al., 2005).

Size is a polygenic trait with variations in multiple genes influencing size at birth and later in life. Such variations might account for the fetal onset of adult diseases. Since GH and the GH-IGF axis contributes significantly towards growth, genetic variations in these genes have the potential to influence size more than any other known variations.

In this research, we have investigated the common genetic variations in IGFs and the GH receptor (GHR) which could influence size and blood pressure (BP) and have potential for contributing towards the “fetal onset of adult disease” hypothesis.

Recently, genome wide association studies (GWAS) have identified various other genetic variations that influence body mass index (BMI) and BP. We have investigated the role of some of these genes in influencing size at birth, early postnatal growth and BP.

1.2 Overview of the growth hormone and insulin-like growth factor axis

Growth hormone (GH) is secreted by somatotroph cells, which are located predominantly in the lateral wings of the anterior pituitary gland and account for 35% to 50% of pituitary cells. The human GH gene locus is located on the long arm of chromosome 17 (17q22-24) spanning approximately 66 kb and consisting of a cluster of five highly conserved genes. The *hGH-N* gene is selectively transcribed in somatotrophs and codes for a 22 kilodalton (191amino acid) GH protein. Pituitary somatotrophs also secrete a 20-kd GH that accounts for 10% of pituitary GH secretion and arises by alternative splicing of *hGH-N*. During pregnancy, the *hGH-V* gene, which is expressed in the placental syncytiotrophoblast, encodes for placental GH, which is the predominant GH in the maternal circulation.

GH is secreted by somatotrophs in pulses with 70% of daily GH secretion occurring with the first episode of slow-wave sleep (Van et al., 1998). GH pulsatility is regulated by GH-releasing hormone (GHRH) and somatostatin secreted by the hypothalamus. GH and insulin-like growth factor-I (IGF-I) exert a negative feedback on GH secretion. Thyroxine is essential for *hGH-N* gene transcription. The sex steroids, and in particular oestradiol, enhances pituitary GH content and GH secretion. Ghrelin and GH-releasing peptides stimulate GH release synergistically with GHRH although it is unclear whether they play a role in physiological GH secretion (Kojima et al., 1999).

In the circulation, GH binds to high- and low-affinity GH-binding proteins (GHBP) (Baumann, 1991). High-affinity GHBP is the predominant binding protein, which may dampen acute oscillations in serum GH concentration, associated with pulsatile pituitary GH secretion, and prolong plasma GH half-life by decreasing renal clearance.

GH action is mediated through the GH-receptor (GHR). Binding of GH dimerises GHR triggering a phosphorylation cascade involving the JAK/STAT pathway (Carter-Su et al., 1996).

The predominant action of GH is on longitudinal bone growth. GH stimulates bone growth by the synthesis of IGF-I, the main GH-dependent growth factor (Le et al., 2001). IGF-I is a 70 amino acid peptide with a molecular weight of 7.5 kDa and a structure which is 70% homologous to IGF-II and 50% homologous to

proinsulin (Daughaday and Rotwein, 1989). IGF-I is synthesised in the liver and circulates bound to six binding proteins, insulin-like growth factor binding proteins (IGFBPs) 1-6, which prolong its half-life. IGFBP-3 is the predominant binding protein, which binds with 80% of the circulating IGF-I. IGF-I circulates in a ternary complex consisting of one molecule each of IGF-I, IGFBP3 and an acid-labile subunit (ALS). The hepatic production of all three components of the 150 kDa complex is regulated by growth hormone (Rosenfeld et al., 1994). Most IGF-1 is secreted by the liver and is transported to other tissues, acting as an endocrine hormone (Le et al., 2001). The ternary complex prolongs the half-life of both IGFBP-3 and IGF. The half-life of unbound IGFBP-3 is between 30 and 90 min and the half-life of free IGF-I is less than 10 min, while the half-life of the ternary complex is approx 12 hours (Hasegawa et al., 1995). The binding of IGFs to IGFBP-3 and ALS maintains IGFs in the intravascular space for steady delivery of IGF-I in contrast to the pulsatile levels of GH (Baxter, 1994).

GH also stimulates IGF-I production in extra hepatic tissues, such as cartilaginous cells, where it acts like a paracrine hormone (Nilsson et al., 1986). Paracrine IGF-I produced in extrahepatic tissues appears critical for growth because growth persists even when hepatic IGF-I is deleted in mice (Yakar et al., 1999).

GH also stimulates production of IGF-II from the liver. IGF-II, along with IGF-I, plays an important role in fetal growth. It is also important in placental growth, where high concentrations of IGF-II exist (Collett-Solberg and Cohen, 2000). IGF-II is not an important hormone for postnatal growth and its role in adults is still unclear. *IGF2* is a maternally imprinted gene and paternally expressed (O'Dell and Day, 1998).

The biological functions of IGF-I and IGF-II are mediated by a family of transmembrane receptors, which includes the insulin, type 1 IGF and type 2 IGF/mannose-6-phosphate (M-6-P) receptors (LeRoith et al., 1995). The type I IGF receptor (IGF1R) is a heterotetramer with a structure similar to the insulin receptor (Collett-Solberg and Cohen, 2000). IGF1R is a tyrosine kinase receptor that mediates most of the effects of IGF-I and IGF-II. The M-6-P receptor is structurally different from the IGF1R and insulin receptors and its physiologic functions are still unclear. Most of studies demonstrate that this receptor functions mainly as a scavenger, facilitating the degradation of IGF-II (Collett-

Solberg and Cohen, 2000). Figure 1.1 gives an overview of the GH and IGF-I axis.

Besides its main role in linear growth, GH has a role in metabolism. GH is lipolytic, diabetogenic and anabolic. The metabolic effects of GH involves adipocyte differentiation and stimulation of lipolysis in the adipose tissue resulting in an increased flux of free fatty acids (FFAs) into the circulation (Chen et al., 2001). The effects of GH on carbohydrate metabolism are more complicated and may be mediated indirectly via the antagonism of insulin action (Vijayakumar et al., 2010). Furthermore, GH has a net anabolic effect on protein metabolism by stimulating protein synthesis while inhibiting proteolysis (Mauras et al., 2000).

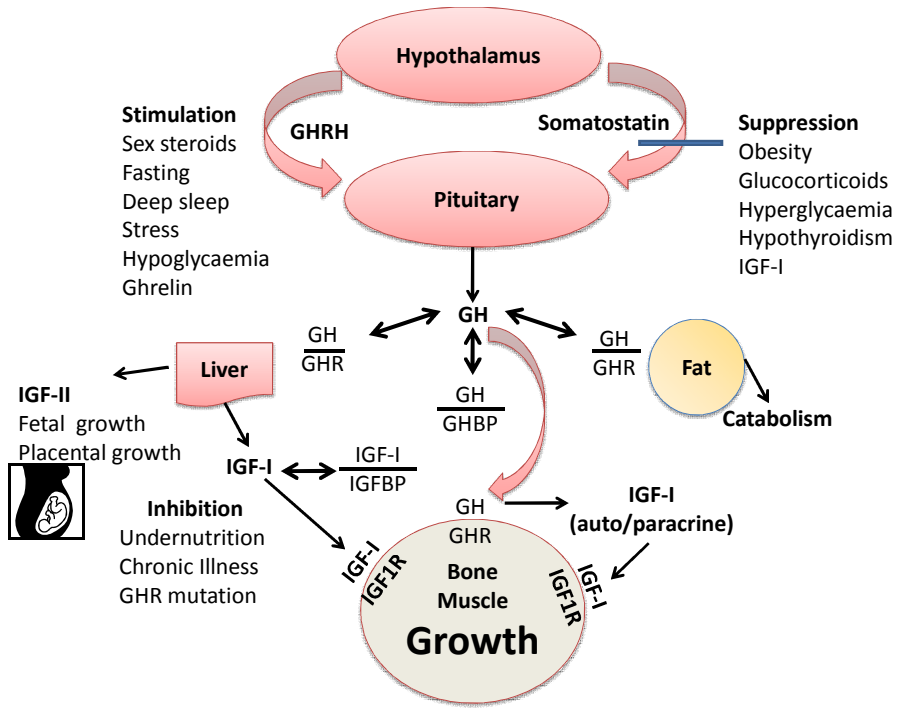


Figure 1.2 Schematic diagram of the GH–IGF-I axis showing the release, circulation and actions of GH and IGF-I.

GH, growth hormone; GHR, growth hormone receptor; GHRH, growth hormone–releasing hormone; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; IGF1R, IGF-I receptor; Modified from (Rosenbloom, 1999)

1.3 Intrauterine growth

1.3.1 Introduction

Fetal growth is finely balanced to meet the demands of the growing fetus for survival and mother's capability to deliver. The period between conception to delivery can profoundly influence future health of an individual. The rate of growth is greater during this period than at any other time in life. Studying factors that affect human birth weight is of value for several reasons. It has multiple implications for perinatal health. Extremes of birth weight are more likely to experience perinatal morbidity, require more healthcare resources and leads to selection for an optimum birth weight. The environment in which the fetus grows is not only critical for its survival but also for its long-term health (Murphy et al., 2006). Overall, gestational age-adjusted birth weight is inversely related to all-cause mortality in adulthood, with a 6% decrease in mortality per kilogram of birth weight within the normal birth weight range (McElroy et al., 2012).

In the early 20th century weight criterion of less than 2500g was considered as a definition for prematurity. However not all small infants are premature (McBurney, 1947). The World Health Organisation in 1961 produced a formal definition of prematurity delinking it from birth weight and recognising the concept of small for gestational age and intrauterine growth restriction (Battaglia and Lubchenco, 1967). Despite improvements in obstetric care the incidence of premature delivery and intrauterine growth restriction remains unchanged (Beck et al., 2010). Premature and growth restricted infants are at a greater risk of physical and neurological complications. There is also increasing evidence of long term outcome on adult health of an adverse intrauterine environment. Intrauterine growth and early postnatal growth can influence the risks for developing future cardiovascular and metabolic diseases (Barker et al., 1993a; Frankel et al., 1996; Ravelli et al., 1998). The association between size at birth and adult disease have been explained by alterations in fetal nutrition and endocrine status, which permanently change the structure, physiology and metabolism of the fetus and predispose individuals to adult disease (Barker et al., 2002).

Fetal growth is a product of complex multidirectional interactions of maternal environment; maternal complications of pregnancy; maternal, fetal and placental hormones and fetal and parental genetics. Of these factors, maternal

complications of pregnancy such as preeclamsia and placental insufficiency produce the most profound restriction on fetal growth in otherwise normal infants. Fetal chromosomal and genetic abnormalities and maternal persistent and excessive use of drugs are other factors, which can lead to significant fetal growth restriction associated with dysmorphology and adverse neurology. The numerous factors associated with impaired fetal growth are listed in Table 1.1. Genetic and endocrine factors are the main focus of this thesis and will be discussed in greater detail below.

Table 1.1. Factors associated with intrauterine growth restriction Source (Bryan and Hindmarsh, 2006).

Fetal Problems	
Multiple birth	
Fetal malformation	
Chromosomal anomalies	
Inborn errors of metabolism	
Intrauterine infections	
Medical Complications	
Pre-eclampsia	Systemic lupus erythematosus
Acute or chronic hypertension	Antiphospholipid syndrome
Antepartum haemorrhage	Anaemia
Severe chronic disease	Malignancy
Severe chronic infections	Abnormalities of the uterus
	Uterine fibroids
Maternal Social Conditions	
Malnutrition	Drug use:
Low pregnancy body mass index	- smoking
Low maternal weight gain	- alcohol
Delivery at age <16 or >35 years	- illicit drugs
Low socioeconomic status	
Environmental Problems	
High altitude	
Toxic substances	
Abnormalities of the Placenta	
Reduced blood flow	
Reduced area for exchange	
- infarcts	
- haematomas	
- partial abruption	

1.3.2 Genetic factors influencing fetal growth

In any species, parental size determines the size of the offspring. Genetic factors play an important role in the aetiology of infant's birth weight. Ounsted et al were the first to suggest significant association between birth weight of offspring and maternal birth weight, raising a possibility of fetal growth inherited through maternal genetics (Ounsted et al., 1988).

Intergenerational studies and studies on offspring of twins have described familial correlations in birth weight and fetal growth (Clausson et al., 2000). Magnus et al found that 50 percent of the variability in birth weight could be explained by fetal genes (Magnus, 1984b; Magnus, 1984a) whereas Clausson et al estimated heritability to be around 40 percent (Clausson et al., 2000). Accumulation of large data sets from birth records permit analyses to separate genetic and environmental factors controlling variation in the complex phenotype, birth weight (Beaty, 2007). In a large Norwegian study parent-offspring data were obtained from the Medical Birth Registry of Norway from 1967 to 2004 (Lunde et al., 2007). For analysis of birth weight and crown-heel length, 101,748 families were included. About 50 percent of the variation in the birth size was explained by what was modelled as a genetic factor. Maternal genetic factors explained 19-22 percent of natural variation and fetal genes about 31 percent of the normal variation in birth weight and birth length and 27 percent of the normal variation in head circumference (Lunde et al., 2007).

Most of the genetic variability studies on birth weight have focussed on term infants. It is, therefore, not possible to delineate the influence of genetic and environmental factors on fetal growth at various phases of intrauterine growth. In addition, the genetic influence on fetal growth may change over gestational age or different covariates may be involved in different phases of gestational age. It is likely that at term environmental factors may form a large part of the covariance matrix as compared to the first trimester. Twin studies are a powerful method of assessing genetic variability on birth weight as they reduce the need to control for confounding factors. They however have limitations in assessing genetic variability for size at late gestational age as the intrauterine growth of twins deviates from the growth of singletons (Soucie et al., 2006). The East Flanders Prospective Twin Survey obtained perinatal data from 4232 live-born twin pairs (Gielen et al., 2008). This cohort had one of the highest birth weights and

placental weights of twin studies (Gielen et al., 2006). In this study heritability of birth weight decreased during gestation from 25-42 weeks, from 52 percent at 25 weeks to 30 percent at 42 weeks.

Paternal and fetal genes in combination also influence fetal growth. Disentangling the influence of their relative contributions in a polygenic trait is difficult. The correlation between father and child in birth weight is less confounded by non-genetic effects than the mother-child correlation. The influence of paternal genes on fetal growth is provided by correlation of paternal birth weight with offspring birth weight, which remains after correction for maternal birth weight (Little, 1987; Magnus et al., 2001). Evidence suggests that the effect of maternal genes on infant birth weight may be greater than that of paternally derived genes (Klebanoff et al., 1998; Nahum and Stanislaw, 2003). Parental genes may have a differential influence on infant size. In 567 white Caucasian singleton, non-diabetic, full term pregnancies recruited from Exeter, UK, all measurements of fetal skeletal growth including crown-rump, knee-heel and head circumference were associated with paternal height while maternal BMI correlated with birth weight and birth length (Knight et al., 2005). In a study performed to investigate both maternal and paternal contributions in the familial aggregation of small for gestational age, the risk of a small for gestational age (SGA) offspring was 4.7 times greater for mothers and 3.5 times greater for fathers who were small for gestational age, compared with average for gestational age counterparts (Jaquet et al., 2005). The risk of a small for gestational age offspring was 16.3 times greater when both parents were SGA. These results indicate that SGA in both mother and father significantly influences the risk of their offspring being small for gestational age, strongly suggesting a genetic component in the familial aggregation of birth weight (Jaquet et al., 2005).

In an attempt to disentangle parental genetic influence and intrauterine influence on the fetal growth, Rice and Thapar used a novel approach to examine the contributions of maternally provided genetic influences, intrauterine influences and paternally provided genetic influences on infant birth weight. They examined children who differ in their genetic relatedness to mother and father because of assisted reproductive technology (homologous IVF, IVF with sperm donation and IVF with egg donation) (Rice and Thapar, 2010). They studied 423 singleton full term offspring, of whom 262 were conceived via homologous IVF (both parents

related), 66 via sperm donation (mother only related) and 95 via egg donation (father only related) (Rice and Thapar, 2010). Both maternal and paternal genes significantly contributed towards infant's birth weight. Maternal height correlated with fetal growth and gestational age (in donor egg) presumably by putting a physical constraint on the growth of the fetus (Rice and Thapar, 2010). Genetic relatedness was the main contributing factor between measures of parental weight and offspring birth weight as correlations were only significant when the parent was related to the child (Rice and Thapar, 2010).

To conclude observational studies based on twin siblings, intergenerational studies and on infants born after reproductive technology confirm a strong familial correlation in birth weight and fetal growth. The genetic contribution to fetal growth varies from 30-70% in different studies.

1.3.3 Endocrine factors influencing fetal growth

Adequate maternal supply of oxygen and nutrients to the fetus is of paramount importance for fetal growth. Adequate substrate accretion is dependent on hormonal regulation. Factors that control nutrient transfer are not fully characterised, but GH and IGF-1 acting on the maternal and the fetal sides of the placenta have been shown to alter diffusion capacity and lactate uptake in sheep (Gluckman, 1995). Hormones of maternal, fetal and placental origin influence fetal growth. Simple and clear evidence of the hormonal influences on fetal growth is seen in infants of diabetic mothers. Increased glucose transfer to the fetus leads to beta-cell hyperplasia, increased insulin secretion and fetal overgrowth with increased adiposity. In a study of 4,000 normal pregnancies from Cambridge, a continuous relationship was observed between maternal glucose and the birth weight of the offspring (Ong et al., 2008). Subtle variation in maternal glucose concentration can also effect the birth size, as is seen in mothers and infants with defects in the glucokinase gene. A maternal heterozygous glucokinase mutation in combination with non-mutation-carrying offspring results in heavier birth weight, while heterozygous mutation in the offspring leads to a decrease in birth weight (Spyer et al., 2009).

1.3.4 Growth hormone and fetal growth

Pituitary growth hormone is an important determinant of postnatal growth but is not important for early fetal growth and may have a small measurable impact on late third trimester growth as demonstrated by birth lengths of 0.8-1.7 SD below the mean in infants with congenital hypopituitarism (Gluckman et al., 1992).

The placenta produces a variant of growth hormone, placental growth hormone (hGH-V), which suppresses maternal pituitary growth hormone secretion. The role for hGH-V in fetal growth is not clear but maternal concentrations of this are low in growth restricted pregnancies (Bajoria et al., 2001). Severe growth restriction has been described in an infant with a *hGH-V* gene deletion (Rygaard et al., 1998). hGH-V might contribute towards uteroplacental circulation as lower concentrations have been found to be associated with increased arterial resistance in growth restricted infants (Schiessl et al., 2007). The expression profile of placental *hGH* genes in placenta is altered in pregnancies accompanied by SGA and LGA compared with AGA newborns, and thus, it may directly affect the circulating fetal and maternal placental GH and placental lactogen levels (Mannik et al., 2010).

Unlike the minor role played by growth hormone, the insulin-like growth factors (IGF) play a major role in fetal growth and development (Randhawa and Cohen, 2005). Depending on the stage of fetal development, the concentration of IGFs and binding proteins are closely regulated within each tissue (Allan et al., 2001). They are significantly influenced by insulin concentrations and fetal nutrition (Chard, 1994). Experiments performed in animals and case reports of humans with defects in the *IGF* genes indicate that IGFs perform a central role in fetal growth (Woods et al., 1996).

1.3.5 The IGF system and fetal growth

The IGF system includes two ligands, IGF-1 and IGF-II; two receptors; and six binding proteins, IGFBPs (Jones and Clemmons, 1995). Signal transduction for IGF-I and IGF-II occurs through the IGF type 1 receptor which leads to differentiation, mitogenic and anti-apoptotic effects, with a net effect which leads to tissue growth. The type 2 IGF receptor does not take part in signal transduction. It controls the extracellular concentration of IGF-II by mediating its endocytosis and subsequent degradation in lysosomes (Jones and Clemmons,

1995). The role of IGFBPs are complex. About 70-80% of IGFs form part of the ternary complex containing IGF-I or IGF-II, IGFBP-3 (or IGFBP-5) and an Acid labile subunit (ALS); the remaining 20-25% are associated with one of the IGFBPs in a binary complex, with less than 1% of the IGFs present in free form (Gicquel and Le, 2006).

1.3.6 IGF expression during fetal development

IGF-I and IGF-II concentrations are higher in pregnant women than non-pregnant women reaching peak concentrations by the third trimester (Hernandez-Valencia et al., 2001). Maternal IGF-I production is stimulated by placental GH and human placental lactogen which are synthesized by placental syncytiotrophoblasts and released into the maternal circulation (Gude et al., 2004). Fetal and maternal growth factor concentrations are directly associated with fetal size in normal pregnancies (Chard, 1994; Reece et al., 1994).

Fetal IGF-I and IGF-II are expressed from the earliest pre-implantation stage to the final phase of tissue maturation before birth (Rotwein et al., 1987). From as early as the first trimester, receptors for IGFs have been identified in the human fetus which would allow IGF-I and IGF-II to exert growth-promoting effects on fetal tissue (Sara et al., 1983).

IGF-II expression is more extensive in fetal tissues than IGF-I from mid to late gestation in rodents and humans (Randhawa and Cohen, 2005). IGFs in the fetus act mostly in an autocrine fashion, but endocrine-systemic actions may occur during the second half of gestation (Gicquel and Le, 2006). Even though the serum IGF-II concentration is much higher in later gestation, no correlations between serum IGF-II and birth weight have been found. In contrast, fetal and cord serum IGF-I concentrations have been found to correlate with birth weight (Fowden, 2003). In fetuses with intrauterine growth restriction IGF-I concentrations are decreased in utero and at birth while it is increased in neonates born large for gestational age (Nayak and Giudice, 2003). Administration of IGF-I to normal fetal sheep and monkeys in late gestation has little effect on fetal body weight, although it increases the weight of individual fetal tissues (Lok et al., 1996). However, when poor placental perfusion compromises fetal growth, fetal IGF-I treatment increases body weight in sheep and rabbits (Fowden, 2003). Both IGF-I and IGF-II, therefore, appear to stimulate tissue

accretion (Fowden and Forhead, 2009b). Since plasma IGF-I concentrations are more responsive to glucose and oxygen than IGF-II in the fetus, IGF-I may act as a nutrient sensor that ensures fetal growth is commensurate with the nutrient supply, while IGF-II provides the constitutive drive to fetal mass accumulation (Fowden and Forhead, 2009a).

There is a shift from IGF-II predominance to IGF-I predominance after birth. IGF production then becomes growth hormone and nutrition dependent. This resets the regulation of postnatal growth to ensure appropriate growth in the new nutritional environment (Fowden and Forhead, 2009a; Gicquel and Le, 2006).

1.3.7 Effects of IGFs on fetal growth and development

Targeted deletions of components of the IGF axis in mice have revealed that the IGF ligand and IGF receptors are the most important group of molecules in the regulation of fetal growth (Baker et al., 1993).

The birth weight of *Igf1* or *Igf2* null mice is reduced by 40%, with *Igf2* null mice exhibiting smaller placentas (Baker et al., 1993; DeChiara et al., 1990). Combined knockout of *Igf1* and *Igf2* demonstrate an additive growth retardation of 80% and were non-viable. *Igf1r* null mice show a 55% decrease in birth weight (Ludwig et al., 1996). In addition, *Igf2* and *Igf1r* double knockouts exhibit greater growth retardation than single *Igf1r* knockouts, suggesting that IGF-II functions via another receptor during fetal growth (Randhawa and Cohen, 2005). IGF-II was determined to act via the insulin receptor (INSR) in early development prior to detectable *IGF1R* gene expression (Butler and LeRoith, 2001).

The IGFs exert their growth effect by acting on cell proliferation, differentiation, maintenance, regeneration and anti-apoptosis pathways (Fowden, 2003). Besides stimulating general somatic growth in utero, IGFs also have specific developmental effects on individual tissues. During late gestation they stimulate cell differentiation in fetal muscle, bone, brain and adrenal cells (Fowden and Forhead, 2009a). IGF-I has anabolic effects on fetal metabolism. Administration of IGF-I to fetal sheep and rats enhances fetal glucose utilisation, reduces amino acid concentrations, protein catabolism and the oxidative use of amino acid carbon with the net result of increasing protein synthesis in the whole body and in individual tissues such as liver and skeletal muscle (Bloomfield et al., 2002; Shen et al., 2003).

Observations in monozygotic twins, who are genetically identical in addition to sharing a common uterine environment, have demonstrated a differential role of IGFs in fetal growth. In twin to twin transfusion where growth of one of the twins is compromised, IGF-I concentrations are similar while concentrations of IGF-II are significantly lower and IGFBP-I are significantly higher in the growth compromised twin (Bajoria et al., 2001). Similarly, another study of monozygotic twins with discordant growth found lower IGF-II, similar IGF-I, and increased total IGFBP-1 in the growth-restricted twin compared with the normally grown co-twin (Westwood et al., 2001). These studies suggest that in fetal serum IGF-I concentrations are primarily determined by genetic influences, whereas IGF-II and IGFBP-1 concentrations are determined both by maternal environment and genetic factors (Murphy et al., 2006).

1.3.8 Effects of IGFs on placental growth and development

IGF-I and IGF-II are produced by the placenta, where they act as growth regulators (Fant et al., 1986). At all gestational ages the mRNA abundance of placental IGF-II is greater than that of IGF-I and is found throughout the chorionic villi, chorionic plate, basal plate, and fetal membranes (Han et al., 1996). Across a group of normal and diabetic pregnancies, placental IGF-II mRNA was positively correlated with placental weight (Liu et al., 1996).

The influence of the IGFs on fetal growth may be mediated through changes in placental growth. In rodents, IGF-II acts by paracrine and endocrine mechanisms to stimulate growth of all zones of the rodent placenta (Bloomfield et al., 2002). Deletion of the *Igf2* gene in the placenta reduced placental weight by 30–50% near term (Fowden and Forhead, 2009b). Mice lacking only the placental-specific transcript of IGF-II have small placentas (Constancia et al., 2002). Increasing IGF-II exposure by imprint relaxation or deletion of the *Igf2r* gene causes placentomegaly with placental weight more than doubling (Fowden and Forhead, 2009b).

IGF-II action is mediated through its binding to the type 1 IGF receptor. Mice containing null mutations in both the *IGF2* and *IGF1R* genes were more severely growth restricted than those in which only the receptor had been ablated (Baker et al., 1993). Placental weight is reduced in IGF-II-deficient mice suggesting that not all of IGF-II's effects are mediated through IGF1R. In the human placenta

actions of IGF-II could be mediated through the type 2 IGF receptor and the insulin receptor, both of which are expressed by trophoblasts (Desoye et al., 1994; Rebourcet et al., 1998).

In addition to regulating placental size, IGF-II influences placental morphology. In the human placenta the outer syncytiotrophoblast layer forms an immune barrier and transporting epithelium between the maternal and fetal circulation (Harris and Westwood, 2012). The syncytiotrophoblast is terminally differentiated and is continually renewed and expanded by an underlying cytotrophoblast progenitor layer. There is a correlation between IGF-II expression in the cytotrophoblast layer and its proliferative activity in forming the syncytiotrophoblast layer (Thomsen et al., 1997). IGF-II is known to provide a survival signal in many cell systems. In the maternal–fetal interface it plays a role in this context since it can protect both first trimester (Forbes et al., 2008) and term (Harris and Westwood, 2012) cytotrophoblasts from apoptosis. Successful implantation and placental development depend on adequate extravillous trophoblast invasion of the maternal endometrium. IGF-II mRNA is abundantly expressed in the trophoblastic columns of the anchoring villi implicating IGF-II as a mediator of this process (Han et al., 1987). In the complete *Igf2* null placenta, there is a 50% reduction in the average number of cells in the labyrinthine and junctional zones in late gestation (Lopez et al., 1996). The *Igf2* gene, therefore, appears to regulate the number, size and type of cells present in the placenta (Fowden and Forhead, 2009b).

IGF-II also influences fetal growth by influencing nutrient transfer across the placenta. IGF-II stimulates both glucose and amino acid uptake by cultured human trophoblasts (Karl, 1995). In the guinea pig, maternal administration of IGF–II has been shown to increase placental transport of nutrients to the fetus resulting in enhanced fetal growth (Sferruzzi-Perri et al., 2006). Increased maternal IGF-II abundance in early pregnancy promotes fetal growth and viability near term by increasing placental structural and functional capacity, whereas IGF-I appears to divert nutrients from the mother to the conceptus (Sferruzzi-Perri et al., 2006). This suggests major and complementary roles of IGF-I and IGF-II in placental and fetal growth (Bloomfield et al., 2002).

To conclude, the IGFs influence growth of fetal tissues directly and indirectly by increasing the size of placenta and through changes in the placental capacity to

deliver nutrients to the fetus. Altering and adjusting the relative proportions of different nutrients supplied to the fetus is as important as the absolute quantity of nutrients available in programming intrauterine development.

1.3.9 Insulin and fetal growth

Insulin is an important hormone influencing fetal growth. Its deficiency causes growth restriction. The reduction in fetal growth is symmetrical with the majority of fetal tissues affected (Fowden et al., 1989). Fetal hyperinsulinaemia leads to an increase in fetal weight which is greatest in species, like the human, that normally have a high body fat content at birth (Fowden, 1989). In contrast to tissue growth, tissue differentiation during late gestation appears to proceed normally in both hypo- and hyperinsulinaemic fetuses (Fowden, 1989). Insulin promotes growth by its action on tissue accretion rather than tissue differentiation.

Insulin stimulates fetal growth, in part, by its anabolic effects on glucose and amino acid metabolism by increasing the cellular uptake of these metabolites and enhancing the rates of protein synthesis and glucose utilisation (Shen et al., 2003). The glucose taken up by the fetal tissues is used for both oxidative and non-oxidative processes as carbon dioxide production and muscle glycogen deposition are both reduced in pancreatectomised sheep fetuses (Fowden et al., 1989).

1.3.10 Role of imprinted genes on fetal growth

Human fetal growth is delicately balanced by genomic imprinting. The paternal allele promotes fetal growth while the maternal allele suppresses growth to prevent overgrowth of fetus, which has the potential of compromising maternal health during delivery and her ability to have successful future offspring (Moore and Haig, 1991). Imprinting refers to the differential expression of genes from the maternal and paternal alleles (Miozzo and Simoni, 2002). In humans, many imprinted genes are expressed in the fetus and placenta where they control resource utilisation (Constancia et al., 2004). Paternally expressed (maternally imprinted) genes increase the transfer of resources (nutrients) to the fetus, thereby promoting growth, while maternally expressed (paternally imprinted) genes reduce nutrient transfer to the fetus, thereby conserving maternal resources for her survival and for future offspring (Constancia et al., 2004).

The *IGF2* gene is paternally expressed, whereas the *IGF2 receptor* gene is maternally expressed. Mutations of these genes are associated with disorders of fetal growth, such as the fetal and postnatal overgrowth observed in Beckwith-Wiedemann syndrome, as a result of overexpression of IGF-II (Murrell et al., 2004). Altered expression of imprinted genes can exert its effect by altering placental nutrient exchange thereby indirectly controlling fetal growth and development (Sibley et al., 2004). Genomic imprinting is an example of the delicate balance that is human fetal growth regulation. The needs of the mother must be protected, while also allowing the fetus to grow to its full potential. The mother and fetus interact via endocrine signals from the placenta, which control the complex process of fetal growth. Environmental influences that alter any aspect of placental function, such as blood flow, nutrient transporter expression, glucocorticoid metabolism, or hormone production, play a significant role in reducing fetal growth, with consequences for long-term health.

1.3.11 Genetic variation and size at birth

The impetus to identify the common genetic regulators of size at birth has increased with the observation that size at birth is an important predictor of adult disease risk (Hales and Barker, 2001).

Several twin studies have shown that serum IGF-I concentrations are highly heritable, especially at a young age (Ester and Hokken-Koelega, 2008). Heritability of serum IGF-I concentrations ranges from 77% to 93% for girls and boys in cord blood and from 54% to 66% in free serum IGF-I concentrations of 6–18-year-old schoolgirls (Li et al., 2005; Verhaeghe et al., 1996). These could be true associations or simply a reflection that IGF-I and IGF-II concentrations are related to the size of the subjects.

A series of animal knockout studies performed by Efstratiadis et al have demonstrated the role of the *IGF1, IGF2* and *insulin* genes and their receptors in fetal growth and size at birth (Baker et al., 1993). Infants born with rare defects of the *IGF1* gene are small at birth, with a reduction in head size (Bonapace et al., 2003; Woods et al., 1996). Mutation of the IGF type 1 receptor (*IGF1R*) gene also causes intrauterine growth restriction (Abuzzahab et al., 2003). Copy number variations of *IGF1R* can also influence fetal and postnatal growth (Okubo et al., 2003). Variable rates of imprinting and expression of the *IGF2R* gene have been

reported in relation to size at birth (Wutz et al., 1998). Overexpression of the normally imprinted fetal insulin-like growth factor II (IGF2) has been implicated in the pathogenesis of the cancer-predisposing Beckwith-Wiedemann syndrome (BWS) (Morison et al., 1996). Relaxation of imprinting through biallelic expression of H19 and downregulation of *IGF2* causes Russell-Silver syndrome, a congenital disorder characterized by severe intrauterine growth restriction, dysmorphic facial features and body asymmetry (Gicquel et al., 2005).

Cord blood concentrations of IGF-I, IGF-II, and insulin are positively related to size at birth (Ong et al., 2002). Thus, common polymorphisms in genes regulating expression of the genes encoding IGF-I, IGF-II, insulin, and their respective receptors could relate to size at birth (Dunger et al., 2007).

In candidate gene approaches, which are hypothesis driven studies, the association between polymorphisms in growth factor genes and birth size has been investigated. Polymorphisms of a common *IGF1* promoter CA repeat, have been reported to be associated with size at birth (Johnston et al., 2003; Vaessen et al., 2002). The role of the promoter region of the IGF-I gene polymorphism and the concentration of plasma IGF-I and body composition parameters in Japanese infants has been studied and results suggest a genetic influence of this variant on prenatal growth and plasma IGF-I concentrations (Kinoshita et al., 2007). In SGA infants and those with lowest birth weight, small head size has been found to be associated with normal sequence variant rs35767 in the *IGF1* gene (Ester and Hokken-Koelega, 2008).

Genetic variations in *IGF2* and *IGF2* receptor (*IGF2R*) genes have also been found to be associated with birth weight. Polymorphism rs680 in the *IGF2* gene is associated with birth weight in Japanese children (Kaku et al., 2007), but not Brazilians or Belgians (Gomes et al., 2005; Heude et al., 2007), and with adult male BMI among some British cohorts (Gaunt et al., 2001). Multiple sequence variants have been identified in *IGF2R* and *IGF2* that are associated with birth weight (Adkins et al., 2010).

The growth hormone receptor gene, *GHR* contains an unusual variant where there is skipping of exon 3, which is inherited in a Mendelian fashion (Pantel et al., 2000). The *GHR exon 3-deletion* polymorphism has been found to be associated with birth size and placental weight (Audi et al., 2006; Padidela et al., 2012; Sorensen et al., 2010).

Unlike the candidate gene approach for association studies, genome wide association studies (GWAS) are hypothesis free. Advances in technology and data management in rapidly screening hundreds of thousands of variants across the human genome have provided a powerful tool in finding genetic variants for common polygenic traits. In a meta-analysis of six GWAS studies of 10,623 Europeans from pregnancy/birth cohorts, normal sequence variant rs900400 near LEKR1 and CCNL1 (P = 2 x 10(-35)) and rs9883204 in ADCY5 (P = 7 x 10(-15)) were robustly associated with birth weight (Freathy et al., 2010). The common sequence variants in ADCY5 were recently implicated in regulation of glucose levels and susceptibility to type 2 diabetes (Dupuis et al., 2010), providing evidence that the well-described association between lower birth weight and subsequent type 2 diabetes and hypertension might have a shared genetic component (Barker et al., 1993b; Jarvelin et al., 2004). The normal sequence variant rs900400 near LEKR1 and CCNL1 was also found to be associated with birth weight in preterm infants, indicating the potential importance of this marker on birth weight regardless of gestational age (Ryckman et al., 2012).

1.3.12 Summary

Fetal growth is the product of complex multidirectional interactions. The maternal and fetal communications are established through the placenta. Maternal development before pregnancy is influenced by the maternal genome and environmental interaction. Several factors such as maternal health, nutritional status, smoking and hypoxia influence the maternal pregnancy state. Placental morphology and hormones produced by the placenta influence maternal metabolism and behaviour, uterine artery blood flow and nutrient intake, which promotes placental development and growth. Adequate placental growth is assisted by trophoblast invasion and the ensuing increase in blood flow. Increased placental growth enhances hormone production allowing signalling between the mother and fetus and transporters to transfer nutrients and waste between the mother and fetus. Adequate placental function promotes fetal growth, which is further influenced by the fetal genome and maternal constraint. Dysfunction in any of these multidirectional pathways can lead to variations in fetal growth, which has adverse consequences both in the short and long term

1.4 Postnatal growth

After birth infants grow rapidly but at a sharply decelerating rate (Freeman et al., 1995a). Postnatal growth is regulated by a combination of environmental and genetic factors. Low birth weight with accelerated growth in the postnatal period is associated with many diseases in adulthood, including hypertension (Jarvelin et al., 2004), type 2 diabetes (Hales et al., 1991) and ischemic heart disease (Leon et al., 1998).

1.4.1 Heritability of postnatal growth

Heritability studies of postnatal anthropometric measures in infancy and childhood have been performed to estimate genetic influences on growth during this critical period. In one of the earliest population based cross sectional studies of 166 twin pairs, heritability for body weight increased from 28% at 14 days to 64% at one year with corresponding values for body length 16% and 48% respectively (Levine et al., 1987). In the recently performed Quebec Newborn Twin Study (QNTS), a population-based birth cohort of 672 twin pairs, influence of family environment disappeared by 5 months and genetic effects were high (approximately 90%) for both sexes at 5 months and 5 years of age (Dubois et al., 2007).

Unlike cross-sectional studies that compare heritability estimates of anthropometric measures for different age groups, longitudinal studies provide an optimal description of the actual growth process that has long-term negative effects. In the Fels Longitudinal Study of 501 infants, heritability estimates of 66% (0–6 mo), 55% (0–12 mo), and 82% (0–24 mo) were shown for weight change by using pedigree data (Demerath et al., 2007). A large study of 4649 Dutch twin pairs between birth and 2.5 years of age showed heritability estimates of weight velocity at the age of 1 y of 57% (girls) and 63% (boys) (van et al., 2004).

Heritability of infant growth was analyzed in 522 infants from the East Flanders Prospective Twin Survey for age windows of 0–1, 1–6, 6–12, and 12–24 months (Touwslager et al., 2011). The heritability of growth was high at 94% from 1 to 6 mo, 85% from 6 to 12 mo, and 86% in the 12-24-mo growth period (Touwslager et al., 2011). No genetic contribution to growth in the 0-1month period was found. However, during this period the genetic contribution to growth was low in the average birth weight range but higher at both extremes of birth weight

(Touwslager et al., 2011). United Kingdom Data from Gemini, a population sample of 2402 UK families with twins revealed heritability of weight at birth and three months was low (38%), but it was higher at six months (62%) (Johnson et al., 2011).

1.4.2 Nutritional factors influencing growth

Nutrition may be the principal regulator of growth during infancy with minimal contribution from growth hormone (Karlberg et al., 1987). The importance of nutrition as a major factor contributing towards infant growth was initially supported by near normal growth of growth hormone deficient (GHD) infants during infancy (Karlberg and bertsson-Wikland, 1988). Studies on a larger cohort of children with GHD has however demonstrated an important role of the GH-IGF axis in infantile growth (Pena-Almazan et al., 2001). Data from humans and transgenic animal models suggest that the hormones and receptors within the GH–IGF axis also play their part in this early phase of growth (Lupu et al., 2001). Nevertheless, it is during this period that alterations in dietary intake are likely to have the greatest impact on growth. Early onset of obesity in an otherwise normal infant is more likely to lead to tall stature than obesity developing later in childhood (Cameron et al., 2003).

1.4.3 Endocrine control of postnatal growth

Growth hormone (GH) and insulin-like growth factors (IGFs) are principal hormones influencing growth in the postnatal period. Other hormones such as thyroid hormones, adrenal androgens, sex steroids, glucocorticoids, leptin and insulin also influence growth, but their contribution is mainly through interaction with the GH-IGF axis.

GH secretion occurs in discrete pulses every 3-4 hours. GH secretion is influenced by constant infusion of growth hormone-releasing hormone (GHRH) from the hypothalamus, ghrelin and somatostatin (SS) (Strobl and Thomas, 1994). GHRH and ghrelin act in synergy to increase the amplitude of the pulse. Withdrawal of SS determines the timing of the pulses.

GH exerts its effect through the growth hormone receptor (GHR), which is mainly expressed in the liver (Carter-Su et al., 1996). Most of the growth promoting

action of GH is mediated by production of IGFs through the signal transduction and activation of transcription (STAT) pathway.

IGFs have a structural homology to proinsulin. IGF-1 and IGF-2 are important for fetal growth, however only IGF-1 has been found to be important for postnatal growth. GH controls the secretion of only IGF-1. IGFs are present in circulation in concentrations 1000 times that of insulin, and to prevent its insulin-like action they are bound to insulin-like growth factor binding proteins (IGFBPs) (Jones and Clemmons, 1995). IGFBPs extend the half-life of the IGFs within the vascular space. Two distinct receptors of IGFs are known; type 1, which mediates mitogenic actions of IGF-1 and IGF-2 and type-2, which mainly binds to IGF-2. The physiological consequence of this latter interaction are not known.

The major physiological role of GH during growth and development is to promote longitudinal bone growth. During embryonic development, IGF-I and IGF-II are key determinants of growth, acting independently of GH (Woods et al., 1996). GH and IGF-I play a critical role in determining longitudinal skeletal growth throughout childhood and puberty (Guler et al., 1988; Isaksson et al., 1987), and children with GH deficiency (GHD) and insensitivity display short stature.

A classic study of the direct action of GH on skeletal growth was demonstrated by unilateral infusion of GH into the tibial artery of the GH-deficient male rat that stimulated ipsilateral longitudinal bone growth (Isaksson et al., 1982). GH drives a number of local bone effects such as skeletal IGF-I synthesis; proliferation of prechondrocytes; hypertrophy of osteoblasts; bone remodelling; and net mineralisation (Ohlsson et al., 1998). Studies have also found an independent role for IGF-I and IGF-II exerting proliferative effects on both human osteoblast cells and human marrow stromal cells. Combined stimulation with GH, IGF-I and IGF-II exhibits synergism in enhancing the proliferative response and promoting skeletal growth (Langdahl et al., 1998). Local production of IGF-I and IGF-II modulates both osteoblast-osteoclast interactions and osteoclast formation and plays an important role in bone remodelling (Hill et al., 1995).

1.4.4 Role of GH and the GH-IGF axis on body mass index

Growth hormone (GH) and the GH-IGF axis are important for skeletal growth in humans. They also play an important role in metabolism of carbohydrate and metabolism and distribution of fat. IGF-1 and growth hormone (GH) interact with insulin to modulate its control of carbohydrate and fat metabolism. Growth hormone deficient subjects have an increase in fat mass compared to lean body mass. They also have visceral deposition of fat, which is detrimental to cardiovascular health (Veldhuis et al., 2005). GH decreases body fat mass by stimulating lipolysis and reciprocal antagonism of the lipogenetic actions of insulin (Veldhuis et al., 2005), whereas IGF-I exerts lipogenic effects similar to those of insulin and inhibits GH actions by negative feedback (Le et al., 2001). In obese individuals GH secretion is markedly blunted (Scacchi et al., 1999), and there are strong relationships between obesity-related variables in epidemiological studies and parameters of GH secretion (Iranmanesh et al., 1991). Excess GH causes insulin resistance and hyperglycaemia, whereas IGF1 has insulin-like effects that reduce blood glucose concentrations (LeRoith and Yakar, 2007).

Decreased GH concentration in obesity is secondary to reduced secretion and increased elimination rates (Iranmanesh et al., 1991). Increased adiposity suppresses GH secretory burst activity, decreases the stimulatory effects of secretagogues, expands the GH distribution volume and increases the metabolic clearance of GH (Martha, Jr. et al., 1992). Increased elimination of GH is related to intrabdominal fat rather than subcutaneous fat, the mechanism for which is not clear (Pijl et al., 2001).

A role for an effect of GH on adipose tissue is further demonstrated by the replacement of GH in GH deficient children and adults. GH replacement therapy in the adult initially elevates (days to weeks) and then suppresses (months to years) insulin and leptin concentrations (Veldhuis et al., 2005). The delayed decline in insulin and leptin concentrations parallels a gradual reduction in visceral fat and total adiposity induced by exogenous GH (Holloway et al., 1994; Ogle et al., 1994). GH is involved in the regulation of lipid and lipoprotein metabolism as GH replacement therapy of adult GHD patients is associated with beneficial changes in lipid and lipoprotein profiles (Russell-Jones et al., 1994).

1.4.5 Role of GH and the GH-IGF axis on blood pressure

Growth hormone (GH) and insulin-like growth factors (IGFs) have favourable effects in the reduction of abdominal/visceral obesity, an improved insulin sensitivity, and favourable effects on lipoprotein metabolism and diastolic blood pressure (Johannsson et al., 1997). Hypertension is an important complication of acromegaly. The prevalence of hypertension in acromegalic patients is about 35%, ranging from 18 to 60% in different clinical series, and the incidence is higher than in the general population (Bondanelli et al., 2001).

Observations in patients with acromegaly and *in vitro* studies give a clear contribution of GH and the GH-IGF axis in controlling an individual's blood pressure. The *IGF1* gene is expressed in endothelial and vascular smooth muscle cells and IGF-I is an important mitogen for these cells (Kamide et al., 2000). IGF-I has been found to stimulate local angiontensinogen production in cultured vascular smooth muscle cells, which may have a role in progression of the vascular hypertrophy and atherosclerosis in patients with hypertension through activation of the tissue renin-angiotensin-aldosterone mechanism (Kamide et al., 2000). Increased expression of IGF-I and GH-receptor mRNA in blood vessels and heart has been noted following increased systemic pressure overload and volume overload, suggesting that GH and the GH-IGF-I axis may be important links in mediating structurally adaptive growth responses in the blood vessel wall (Wickman et al., 1997) and in the heart (Guron et al., 1996). GH itself induces structural changes in the arterial wall through an increase in the diameter and collagen content as a significant increase in the intima-media thickness of common carotid arteries has been observed in patients with acromegaly (Colao et al., 2001). In contrast, however, in normotensive populations, higher IGF-I levels are associated with lower atherosclerosis risk and cardiovascular mortality (Higashi et al., 2010). IGF-I is potentially protective by enhancing nitric oxide-dependent endothelial cell function and promoting plaque stabilisation by increasing vascular smooth muscle cell survival (Conti et al., 2004).

Recent animal studies indicate that IGF-I exerts both pleiotropic anti-oxidant effects and anti-inflammatory effects, which together reduce atherosclerotic burden and has an important role in contributing towards maintenance of BP in an individual (Higashi et al., 2010).

IGF-I concentration in the lower end of the normal range increases the risk of myocardial infarction and heart failure (Ezzat et al., 2008). This could be secondary to low IGF-I concentration resulting in insulin resistance and accelerated atherosclerosis (Clemmons, 2007). Serum IGF-I declines more rapidly in African than in white individuals, raising a possibility that lower IGF-I levels may be a cause for increase risk of premature atherosclerosis in African populations (Schutte et al., 2010).

1.4.6 Summary

Growth is a polygenic trait and postnatal growth is a result of complex multidirectional interaction between environmental factors and endocrine factors. The endocrine factors contribute significantly towards growth, which are also influenced by genetic factors. Heritability of growth has been estimated to range from 60-80% in various longitudinal studies. The GH and IGF systems are the main contributors towards growth and body composition, as genetic and acquired abnormalities in them cause profound growth restriction. Structural variations in the genes coding for GH and the IGF system are anticipated to influence and contribute toward heritability of growth, body composition and blood pressure.

1.5 Obesity as a polygenic trait

Obesity is one of the biggest health challenges we face. Cardiovascular and metabolic complications associated with obesity and cost of managing these complications puts a huge burden on our health care resources. The incidence of obesity in children is progressively increasing. The National Child Measurement Programme: England 2008/2009 has shown that in school children in year 6, around 18% of children are obese and approximately 14% are overweight. Statistics in obesity, physical activity and diet: England, 2010, has shown that in adults this figure increases to 66% of men and 57% of women being overweight (including obese). The secular trend in the prevalence of obesity is secondary to changing lifestyle and rapid globalisation, which is fuelling a global pandemic of obesity. Migrant studies provide evidence for a critical role of environmental factors contributing to the recent prevalence in obesity. Pima Indians living in the United States have higher Body Mass Index compared to those living in Mexico (Kopelman, 2000). Similarly Africans in the United States and Asian Indians in the United Kingdom have higher body mass index compared to native populations (Balakrishnan et al., 2008; Jebb et al., 2004). Obesity is a polygenic trait with a genetic component playing an important role. In the current obesogenic environment, some have escaped obesity, underlining the multifactorial nature of this disorder. Obesity is influenced by a combination of multiple genetic and environmental factors. In the current climate, those who are genetically susceptible are more prone to develop obesity and its related complications. Obesity is also a heritable trait with inter-individual variations explained by inherited factors. Data from twin, adoption and family studies have demonstrated that obesity is highly heritable with genetic factors contributing towards 40-70% of inter-individual variations (Hebebrand and Hinney, 2009; Maes et al., 1997).

The intrapair correlation for body mass index among 25,000 pairs of monozygotic twins born in Sweden between 1886 and 1958 reared apart was calculated as 0.70 for men and 0.66 for women demonstrating the most direct estimate of heritability for this trait (Stunkard et al., 1990). In a similar study in a UK twin's cohort a correlation for genetic influence of 0.61 was observed (Price and Gottesman, 1991). These data were further strengthened by meta-analyses of Japanese, Finnish and American twin studies (Allison et al., 1996). Studies on

twins by manipulating the energy intake and physical activity in a controlled manner has further demonstrated that genetic variations influences responses to fluctuations in environmental factors (Bouchard et al., 1996).

Studies on adopted children provide strong evidence for polygenic traits. Data from a Danish adoption register based on 5000 individuals showed that strong correlations exit between adoptees and their biological parents with regards to body fat distribution and body mass index but none between adoptee and adoptive parents (Stunkard et al., 1986).

Obesity is a net product of increased energy intake, decreased energy expenditure or increased conversion of energy to fat. The role for each component's contribution towards weight gain is difficult to define and measure, especially the energy intake. Where energy intake studies have been performed in twins, results have shown a strong genetic link towards preference for type of nutrients consumed (Wade et al., 1981). Energy expenditure studies are easier to perform, as robust reproducible techniques exist such as doubly-labelled water. Studies have shown that 40 percent variation in resting energy expenditure, influence of low to moderate exercise and thermic effect of food can be explained by genetic influence (Bouchard and Tremblay, 1990).

1.5.1 Genetic variations and obesity

In the last two decades, the search for the genetic variations responsible for obesity has intensified. Initially the candidate gene approach was the most common method applied by researchers. Candidate gene studies are a hypothesis-driven approach, which are based on our current understanding of the pathophysiology and biology of obesity. Genes have been identified which have been implicated in obesity related traits, obese animal models, or by studying extreme/monogenic forms of obesity in humans, and genetic variations in these genes are tested for association in the population (Li and Loos, 2008). By 2005 around 127 candidate gene association studies on obesity had been performed and were listed in the human obesity gene map database (Rankinen et al., 2006). In the last decade genetic methods were costly and cumbersome and hence only few variants in every gene were studied, most typically the ones thought to be of functional significance. With the vast data available from databases such as dbSNP and the international HapMap project, a much more detailed overview of

SNPs and their linkage patterns within a gene is available to researchers. With the decrease in the cost of genotyping and with the knowledge available from scientific databases, carefully selected tagSNPs and their haplotypes within a gene have been studied to identify regions of a gene responsible for the obesity trait. The results are interpreted with the knowledge that the casual variant would be in linkage disequilibrium with either one of the tagSNPs or captured by the haplotypes (Loos, 2009). Results of casual variants within a gene lead to physiological experiments to identify the functional consequences of the identified gene variants (Li and Loos, 2008).

Multiple theories have been proposed for explaining the genetic basis for the increase in the prevalence of obesity e.g. the thrifty gene hypothesis, the fetal programming hypothesis, the sedentary lifestyle hypothesis and the ethnic shift hypothesis (Walley et al., 2009). Since obesity is a complex multifactorial interaction between genetics and environmental factors, acceptable consensus on a single hypothesis is lacking.

Many genetic variants in candidate genes have been found to be positively associated with obesity related traits (Rankinen et al., 2006). However, the potential effects of many of these variants have not been successfully replicated in successive studies casting doubts on the significance of the findings. Most of the candidate gene approaches were performed in a sample size, which was insufficiently powered where positive results do not prove and negative results do not disprove association. Recent candidate gene approach studies have been performed in larger sample sizes and are more robust in picking up association. Obesity is a multifactorial disorder and it is anticipated that multiple genetic variants contribute towards the variation seen in the population with each genetic variant accounting for a small percentage of genetic variance. A large sample size is therefore required to identify this small variance with each genetic variant. Larger sample studies and meta-analyses of smaller studies have now given some consistent findings in candidate genes being investigated.

Mutations in the *MC4R* gene (melanocortin 4 receptor) are the most common cause of monogenic obesity with about 5% of severely obese children carrying a mutation in this gene (Farooqi et al., 2003). *MC4R* encodes a G-protein-linked receptor that is widely expressed in the central nervous system and plays a key role in the regulation of food intake and energy homeostasis (Huszar et al.,

1997). Its role in common forms of obesity remained elusive. The candidate gene approach was targeted towards the *MC4R* gene to investigate association of susceptibility to common obesity. With large sample studies and with meta-analyses a consistent association has now been found between genetic variation in the *MC4R* gene and common obesity. Two large meta-analyses studies have confirmed an inverse association of the V103I variant and risk of obesity in different populations (Stutzmann et al., 2007; Young et al., 2007). Within MC4R, a variant I251L, has also been found to have strong protective effect on BMI and risk of adult and childhood obesity. A recent meta-analyses of case control studies has found a nearly 50% reduced risk for obesity among I251L allele carriers (Stutzmann et al., 2007).

Other genes identified by candidate gene approach are the adrenergic β3 receptor (*ADRB3*) gene, the prohormone convertase 1 (*PCSK1*) gene, the brain-derived neurotrophic factor (*BDNF*) gene, and the endocannabinoid receptor 1 (*CNR1*) gene (Walley et al., 2009).

As the growth hormone (GH) and insulin-like growth factor(IGF) system contributes towards adiposity and body composition (Veldhuis et al., 2005), variations in the GH-IGF system genes have also been found to contribute towards genetic variance in obesity. For example, in a study of 1474 healthy white Caucasian men aged 45-65y, *IGF2* SNP rs680 AA homozygotes showed a mean body weight 4 kg lower than GG homozygotes (77.6 ± 10.9 kg vs 81.6 ± 11.5 kg, P = 0.003) with heterozygotes (GA) intermediate (80.1 ± 11.9 kg) (O'Dell et al., 1997). The *IGF2* rs680 AA genotype was therefore associated with lower mean body weight but higher serum IGF-II concentrations than the GG genotype. GG homozygotes incur a 1.67-fold risk of pathological BMI (> 30 kg/m^2) compared with AA homozygotes (O'Dell et al., 1997).

In a subsequent study the group identified three further SNPs in the *IGF2* gene associated with BMI (Gaunt et al., 2001). The total proportion of BMI variance explained by these studies was 2.25%, strongly suggesting that *IGF2* genetic variation is a significant determinant of body weight in middle-aged males (Gaunt et al., 2001). These results have been replicated in separate smaller cohorts (Rodriguez et al., 2006). The results however have not been consistent as in a separate cohort of 2797 women and 2203 men, aged 39-79y, the *IGF2* SNP rs680 was not associated with BMI but was positively associated with adult

height (Heude et al., 2007).

Genetic variations in the *IGF1* gene have also been found to be associated with BMI. In a study of white males and females (n = 925/836) and black males and females (533/705) aged 70-79. The IGF1 promoter polymorphism rs35767 was predictive of differences in body composition, primarily in black females (Kostek et al., 2010).

Growth hormone receptor (*GHR*) gene polymorphisms have also been found to be associated with body composition, blood pressure and IGF1 concentration (Mong et al., 2010). In a study of 981 randomly selected Hong Kong Chinese adolescents there were significant associations between the *GHR* gene SNP rs4410646 and body composition (P = 0.0044) and blood pressure (P = 0.00017). There was also an association between the *GHR* SNP rs7703713 and the IGF-I concentration (P = 0.0033). The GA and AA carriers of rs7703713 had higher serum IGF-I and higher serum IGFBP-3 concentration (P = 0.00069 and 0.025 respectively) (Mong et al., 2010).

In GWAS, the SNP rs9926289, in the *FTO* gene has been found to be significantly associated with obesity (Fawcett and Barroso, 2010). In cross-sectional data from 3882 subjects aged 20-79 years a statistically significant relationship between the *FTO* SNP and IGF-I concentration was found suggesting that in obese subjects the GH-IGF-I axis is a mediator for the relationship between *FTO* and BMI (Rosskopf et al., 2011).

The interpretation of candidate gene studies however requires caution because of the possibilities of problems such as population stratification, small sample size and publication bias. Exact pathophysiological mechanisms linking candidate gene SNPs with obesity remains elusive.

GWAS therefore has several advantages over the candidate gene approach. They are performed on large sample sizes with large *p* values for statistical significance. GWAS are also unconstrained by prior hypothesis and scans the whole genome to identify SNPs which could be related to a polygenic trait. Genome-wide association studies (GWAS) screen the whole genome at higher resolution levels than linkage studies and are therefore able to narrow down the associated locus more accurately. GWAS performed in obesity have revealed multiple genes that have association with BMI. Meta-analysis of the GWAS have

identified new loci which have highlighted neuronal influence on body weight regulation (Willer et al., 2009).

SNPs within the fat mass and obesity-associated (*FTO*) gene became the first to be associated reproducibly with human body mass. Of all the loci identified, the genetic variation in *FTO* has the largest effect on susceptibility to obesity (Fawcett and Barroso, 2010). *FTO* encodes for a Fe(II)- and 2-oxoglutarate-dependent oxygenase involved in DNA demethylation (Fawcett and Barroso, 2010). *FTO* mRNA is most abundant in the hypothalamic regions, which control energy homeostasis and the FTO protein shows wide expression patterns in central as well as peripheral regions (Stratigopoulos et al., 2008). Null mice to *Fto* have reduced fat mass, and mice with over expression of *Fto* demonstrate increased energy intake and increased adiposity (Church et al., 2010).

Even though several studies have replicated the association of *FTO* with weight/BMI, the mechanisms by which *FTO* affect obesity are still not fully understood (Fawcett and Barroso, 2010). Potentially the *FTO* gene variation could exert its influence on BMI through the GH-IGF1 axis (Mong et al., 2010).

1.5.2 Summary

Obesity is influenced by a complex multidirectional interaction between the environment, genetic predisposition, and human behaviour**.** Heritability of BMI is expected to be around 40-70%. Current research on the genetic basis for obesity has identified only <2% of genetic variance for BMI. The role of variation in identified genes represent a polarized size class and still do not explain their influence in the continuum across the size spectrum in the general population.

1.6 Blood pressure as a polygenic trait

High blood pressure (BP) and hypertension are major global health challenges. It is estimated that one third of adults are affected and both contribute about 50% of the risk for ischemic heart disease, end stage renal disease and stroke (Lawes et al., 2008). Even increases in systolic and diastolic BP within the normative ranges have a graded influence on cardiovascular associated mortality (Lewington et al., 2002). Environmental factors including decreased physical activities, elevated body mass index, dietary sodium intake and alcohol excess, are known to increase BP. However, familial aggregation suggests a substantial heritable component to BP (Havlik et al., 1979). These aggregations have been demonstrated in paediatric populations where environmental influences are not very prominent (Fossali et al., 1990). Genetic factors account for 30-60% of variance in BP (Mongeau, 1989).

Soon after the measurement of arterial BP became routine practice, heritability was noted. A stronger correlation between the BP of monozygotic than of dizygotic twins was demonstrated (MIALL and OLDHAM, 1963). In a prospective Dutch study of 1577 subjects (682 males, 895 females) consisting of 580 monozygotic twins, 664 dizygotic twins and 333 of their siblings, genetic variance for systolic BP was 48-60% while for diastolic BP it was 34-70%. In prospective measurement of BP in these subjects, stable tracking of BP over time was demonstrated (Hottenga et al., 2005). Stable tracking of BP has been replicated in other cohorts (Rice et al., 2000). A systematic review and meta-regression analysis of 50 cohort studies (January 1970 and July 2006) demonstrated strong evidence for BP tracking from childhood into adulthood (Chen and Wang, 2008). Childhood BP is associated with BP in later life, and hence early intervention is important. Genetic and environmental assessments are best performed in infancy and early childhood as adult lifestyle events can independently influence the BP.

1.6.1 Genetic variation and blood pressure

Efforts in determining the genetic basis for heritability of BP have been slow because arterial BP is a complex polygenic trait that is influenced by multiple variants, gene–gene interactions (epistasis) and the environment (Lalouel, 2003).

Candidate gene studies were a preferred approach in initial efforts to identify the genetic basis for variance in BP. The selection of candidate genes was based on encoded proteins that are important for BP regulation and on the genes that lead to Mendelian forms of hypertension. From 1996 to 2004, more than 105 studies of this type were published, focusing on 26 candidate genes (Agarwal et al., 2005). Even though variations in some of the candidate genes were identified to be associated with BP variability (Tikhonoff et al., 2003), none could be consistently replicated in different cohorts.

As GH and GH-IGF axis have a role in control of blood pressure, genetic variations in their genes have been explored. In a German study of 66 pairs of dizygotic twin subjects and their parents in a sib-pair analysis to look for linkage of selected candidate genes to the quantitative trait BP, a quantitative trait loci for BP was found near the *IGF1* gene (Nagy et al., 1999). In the Dutch Generation *R* study of 538 subjects, a variant type of the IGF-I promoter region was associated with lower blood pressure but not with left heart dimensions at the age of 2 years (van, V et al., 2008).

In a study of 111 hypertensive patients, 155 stroke patients and 121 healthy adults, genetic variations in the growth hormone gene promoter (*GH1)* were significantly associated with stroke and hypertension (Horan et al., 2006). An association between *GHRd3* genotype and hypertension was observed among stroke patients, particularly females (Horan et al., 2006).

In a Rotterdam Study of 3484 normotensive subjects, 1648 hypertensive and 462 untreated hypertensive subjects association with a polymorphism of the cytosine-adenosine repeat 192-bp (wild-type) allele in the promoter region of the *IGF1* gene was explored (Schut et al., 2003). Mean systolic and diastolic blood pressure did not differ between genotypes. However, in hypertensive subjects, carotid intima-media thickness (IMT) was significantly increased in noncarriers of the 192-bp allele compared with heterozygous or homozygous carriers (P=0.04) (Schut et al., 2003). Pulse wave velocity (PWV) was also significantly higher in hypertensive subjects who were non-carriers of the 192-bp allele compared with heterozygous or homozygous carriers (P=0.02) (Schut et al., 2003). In the same cohort, genetic variation in 192-bp allele in the promoter region of the *IGF1* gene was associated with an increased risk of heart failure in subjects aged 55 to 75 years (Bleumink et al., 2004).

Despite these findings, others have not replicated these results (Allen et al., 2002) and in a study in the UK of 640 individuals, the opposite association was found between the 192 bp allele and IGF-1 concentrations (Frayling et al., 2002). The failure of candidate gene approaches in identifying the genetic basis of variance in BP suggests that the choice of candidate genes might be inappropriate or individual effects are small or vary between different populations (Cowley, Jr., 2006). Alternatively, the genes in which heritable variations that effect blood pressure occur might be involved in events that take place either upstream of the points of action of the selected candidates (for example, in the synthetic pathways of these molecules) or in downstream signalling events (Cowley, Jr., 2006). Candidate-gene studies rely on a prior hypotheses about disease mechanisms, so that the discovery of genetic variants in previously unknown pathways is precluded (Cowley, Jr., 2006). Candidate-gene studies also often lack sufficient power to identify variants that underlie complex traits in humans as exemplified by the studies noted above.

Compared to the candidate gene approach and linkage analysis, GWAS is hypothesis free and involve large cohort sizes. GWAS uses hundreds of thousands of single nucleotide polymorphism (SNP) markers spanning the genome. In 34,433 subjects of European ancestry eight new loci of SNP were found to be significantly associated with blood pressure (Newton-Cheh et al., 2009). Some of the loci were in the near vicinity of the proteins which may have a role in blood pressure regulation (Newton-Cheh et al., 2009). Some of these loci have been indentified in cohorts of different ancestry (Kato et al., 2011).

1.6.2 Summary

BP is influenced by a complex multidirectional interaction between the environment and genetic predisposition. Heritability of BP is expected to be around 40-60%. Current research on the genetic basis for BP have identified only <5% of genetic variance for BP. The role of variation in identified genes may represent polarised size class and still does not explain their influence in the continuum across the spectrum in the general population.

1.7 Fetal origin of adult cardiovascular diseases and type 2 diabetes

Epidemiological studies have suggested that patterns of intrauterine and postnatal growth correlate with cardiovascular diseases and diabetes in later life (Barker et al., 1993a; Leon et al., 1998). These observations were first noted by Baker and his colleagues in two cohorts of 1586 men born at a Sheffield maternity hospital during 1907-1925 and 5654 men born in Hertfordshire during 1911-1930. In the Sheffield cohort low birth weight and low ponderal index was associated with higher risk of cardiovascular disease (Barker et al., 1993c). In the Hertfordshire cohort lower weight at one year was associated with higher risk of cardiovascular diseases (Barker et al., 1989). These studies have been confirmed by numerous other cohort studies. In the Aberdeen children of the 1950s prospective study of 10,803 individual, birth weight was inversely associated with coronary heart disease and stroke in a population born at a time when environmental circumstances, as indexed by low infant mortality rates, were relatively advantageous for infants (Osler et al., 2009). Systematic review of seventeen published studies which includes 144,795 singletons has demonstrated that with a 1 kg higher birth weight risk of ischemic heart disease decreases by 10–20% (Huxley et al., 2007).

An inverse association of birth weight with adult hypertension has also been observed. In the Reykjavik Study of 4601 Icelandic men and women, low birth weight was associated with an increase in incidence of hypertension. This risk was more prominent in women than in men (Gunnarsdottir et al., 2002).

Low birth weight, particularly when associated with postnatal catch-up growth, appears to predict cardiovascular diseases in adults. In a Danish cohort of 9,143 individuals followed between age of 25-52 years, risk of coronary heart disease was highest in those with low birth weight and overweight in adulthood (Osler et al., 2009).

Similarly, the association of birth weight with type 2 diabetes has been investigated in epidemiological data collected over the last 15 years. Meta-analyses of these studies have demonstrated that a graded, inverse and independent relationship exists between birth weight and diabetes (Whincup et al., 2008). These associations remained following adjustment for adult body size and were not confounded by adult socioeconomic status. Overall, there was a

reduction in type 2 diabetes risk of about one-fifth per 1-kg increase in birth weight (Whincup et al., 2008).

Along with birth weight pattern, postnatal growth has also been found to influence development of type 2 diabetes in adulthood. In a French study of 91,453 women, birth weight and body size at 8 years, at menarche, and in young adulthood (20–25 years) were inversely associated with the risk of diabetes, independent of adult BMI during follow-up. In mid-adulthood (35–40 years), the association was reversed, with an increase in risk related to a larger body size. An increase in body size from childhood to mid-adulthood amplified the risk of diabetes (de Lauzon-Guillain et al., 2010). These findings have been confirmed in other studies conducted in different countries underlining the universal nature of this phenomenon (Bavdekar et al., 1999; Leunissen et al., 2009; Levitt et al., 2005; Mericq et al., 2005).

Association of birth weight and weight gain in early childhood with adult obesity and body composition has also been extensively investigated in epidemiological studies. Smaller birth size leads to decreased adult height and weight however it is associated with more subcutaneous and abdominal fat and less lean body mass in adulthood, a harbinger for adult type 2 diabetes and cardiovascular diseases (Loos et al., 2001). Reduced lean mass and increased fat mass have been demonstrated by body composition studies using dual-energy X-ray absorptiometry (DXA). Gale et al in their study of 143 men and women aged 70-75 years used DXA to confirm the association of low birth weight with increased fat mass; this was more prominent in those with rapid post natal growth (Gale et al., 2001). Studies measuring body composition using DXA in 9-10 year olds have also associated higher ponderal index at birth with an increase in lean body mass and reduced fat mass to lean mass ratio (Rogers et al., 2006). In a much larger study of DXA measurements and birth weights in 2228 dizygotic and 842 monozygotic female twins aged between 18 and 80 years, Skidmore et al found that the absolute amounts of fat and lean tissue in adult life were determined in the fetal period to some degree and that twins with a higher birth weight also had a more favourable body composition in adulthood. This higher ratio of lean to fat tissue was determined through the shared common environment, which indicated that although smaller infants had a lower absolute fat mass at birth, it was accompanied by a much larger deficit in lean mass, which led to a propensity to

accumulate fat throughout the life cycle (Skidmore et al., 2009).

It is clear from the epidemiological studies that fetal growth and postnatal growth influences body weight and body composition. The mechanism for decreased muscle mass can be explained by previous studies which have confirmed that muscle cell number in the body is determined before birth and postnatal increase in muscle mass through the life course can only be achieved by hypertrophy (Skidmore et al., 2009). In contrast adipocytes can undergo both hyperplasia and hypertrophy (Hausman et al., 2001). In this situation any increase in body weight increases the fat mass to lean mass ratio.

Despite convincing evidence of the fetal origin of adult diseases, the pathophysiological mechanism or mechanisms of this phenomenon is unclear. The adverse fetal environment contributing towards the low birth weight also changes the body composition of the fetus with decreased adiposity, muscle mass, vascular structure and size of internal organs including pancreas. The pattern of postnatal weight gain and alteration in body composition with increase in fat mass to lean mass ratio predisposes to an increased risk of cardiovascular diseases. The exact mechanisms of central/visceral adiposity leading to type 2 diabetes mellitus and metabolic syndrome is still unclear. It is increasingly recognised that adipocytes act as an endocrine organ with a role in lipid and glucose metabolism through secretion of adiponectin. An excess of adipocytes leads to infiltration of macrophages and release of cytokines involved in insulin resistance and metabolic syndrome (Hajer et al., 2008).

1.8 Genetic variations

1.8.1 Single nucleotide polymorphisms

Within the cells deoxyribonucleic acid (DNA) is organised into long structures called chromosomes. Humans have 23 pairs of chromosomes; each individual has 22 pairs of autosomes, and one pair of sex chromosomes (XY, male; XX, female). Therefore, all autosomal genes should be represented by two alleles, one inherited from the mother and the other from the father.

DNA is a polymer of nucleotides, which are organised in a double helix structure with two strands running antiparallel to each other. Strands bond to each other by strong hydrogen bonds connecting the nucleotides. Nucleotides are made up of nucleobases, a 2-deoxyribose sugar and a phosphate. Nucleobases that form the backbone of DNA are classified into purines and pyrimidines. Purines are adenine (A) and guanine (G) while pyrimidines are cytosine (C) and thymine (T). In the double helix, nucleotide A binds with T and G with C.

The human genome consists of 2.85 billion base pairs. Of these 2.85 billion base pairs only 20-25,000 coding genes exist (http://hapmap.ncbi.nlm.nih.gov/) with a surprising amount of non coding elements. Of the whole genome 0.1% of DNA varies between individuals which amounts to a 2-3 million base pair difference (Jorde and Wooding, 2004). These base pair differences are known as single nucleotide polymorphisms (SNP). They occur approximately once every 100 to 300 bases in the human genome. SNPs are DNA sequence variations that occur when a single nucleotide (A,T,C,or G) in the genome sequence is altered. For example a SNP might change the DNA sequence A**A**GGCTAA to A**G**GGCTAA. Any single nucleotide has a potential to change into any of the other three nucleotides, but most SNPs consistently have one base pair difference across the species. Common SNPs have a minor allele frequency of about 5%. SNPs within the coding region which can lead to change in the structure and function of the coding proteins are designated as genetic mutations. These single gene genetic mutations cause one of about 1,700 rare Mendelian genetic diseases known in humans. Other rare SNPs in the coding regions may be variants without an obvious pathological role (“rare allelic variants”), or may alter the nucleotide sequence whilst leaving the protein code unaltered (“synonymous changes”). The frequency of SNPs seen in human populations follows the shape of power-law

distribution with rarer SNPs accounting for most overall variations Figure 1.8.1 (Gibbs, 2005). Individuals can be homozygous for one SNP (e.g. AA or GG) or heterozygous for this variant (e.g. AG) (see section 3.5.1).

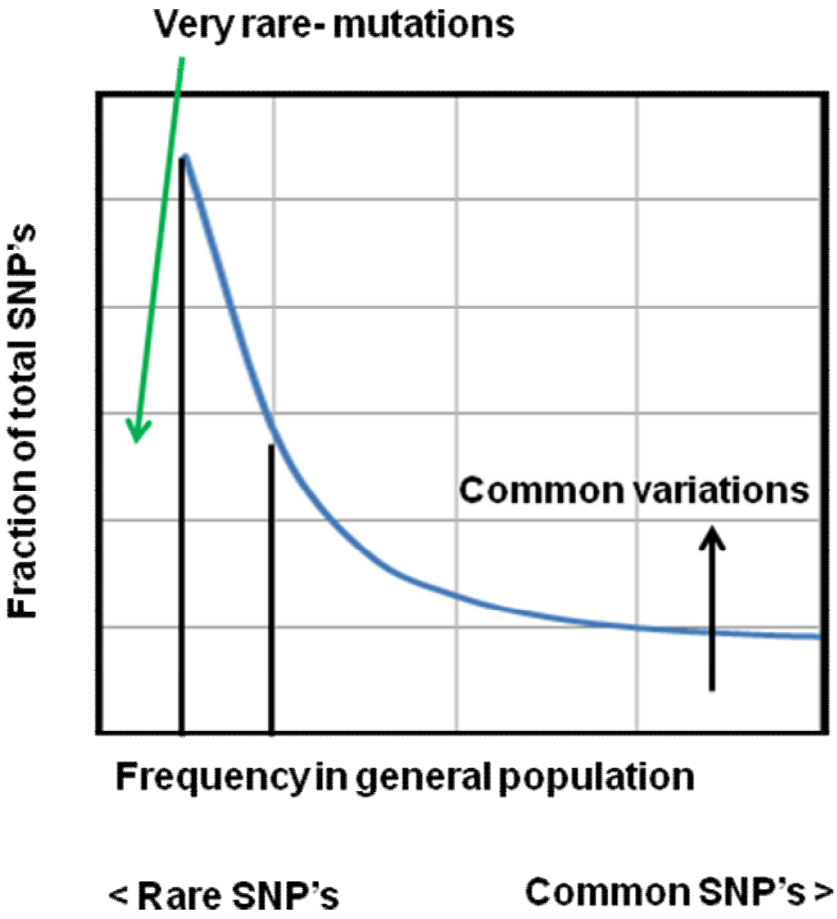


Figure-1.8.1 Genetic variations in humans.

Variation is measured by SNPs. Common SNPs appear on the right side of the graph and they may contribute towards common variations such as height, weight and predispositions to common diseases such as type 2 diabetes.

Most SNPs occur in the non-coding and non-regulatory region of the gene. If they occur in the coding and regulatory regions, they may have potential to be directly involved in disease or influence polygenic trait and variation seen between individuals. SNPs can be located outside a know gene and are known as non genic SNPs. Genic SNPs are located within a gene and based on its location it can be of following five types (Cargill et al., 1999):

1. **Intronic SNPs** are present in the non-coding region between exons. They are transcribed into the pre-messenger RNA (mRNA) but are not translated as they are removed during formation of mature mRNA, but could potentially alter RNA splicing and stability
2. SNPs located in the **5 prime untranslated region** (5'UTR) of the DNA that are transcribed into mRNA but not translated by the ribosome into protein. This includes the region of the mRNA between the transcription start site and the ATG codon for translation initiation.
3. SNPs located in the **3 prime untranslated region** (3'UTR) of the DNA. This includes the region of the mRNA after the stop codon and around the poly-A tail.
4. **Synonymous SNPs:** SNPs which are located within the coding region and are translated by the ribosome but nucleotide substitutions do not change the amino acid sequence of the protein.
5. **Non-synonymous SNPs:** These SNPs are located in the coding region and nucleotide substitutions result in a change to the amino acid sequence of the protein.

1.8.2 Structural variations in the human genome

Besides the SNPs, the coding of human genome project has revealed a huge amount of structural variants (Bentley et al., 2008; Levy et al., 2007; Wheeler et al., 2008). These include gene insertions, deletions, inversions, copy number variants, duplications and translocations. Recent studies have defined approximately 5% of the human genome as structurally variant in the normal population (Sharp et al., 2006).This inherent nature of the human genome lays a platform for genetic variation and determining these genetic variations between individuals within the population has opened up the possibility to associate genotype with phenotype.

Weight, body mass index and blood pressure are polygenic traits, which are controlled by environmental factors and multiple genes. Multiple candidate gene approaches and GWAS have been performed which have provided compelling statistical associations for a total of over 300 different loci in the human genome (Frazer et al., 2009). Most of the polygenic traits including weight, height, body mass index etc have been addressed in GWAS. However, it is important to

realise that in general only common allelic variations (>5%) are represented during SNP analysis for the GWAS studies and rare variants that might influence phenotype are not usually represented. In addition genetic variance alone probably cannot account for disease susceptibility without the addition of pre- and postnatal environmental and/or behavioural factors (Andreasen and Andersen, 2009).

1.8.3 Linkage, linkage disequilibrium and tag single nucleotide polymorphism

Single nucleotide polymorphisms (SNPs) are stably inherited, highly abundant, and distributed throughout the genome (Takeuchi et al., 2005). These variations contribute towards diversity within and among populations.

During prophase 1 of meiosis chromosome recombination occurs leading to new combinations of genes that differ from the combination within the parental chromosomes. This mechanism prevents all alleles located in same chromosome being linked to each other in subsequent generations. Some alleles that are located close to each other on the same chromosome escape chromosomal recombination. These tend to stay together during meiosis, and are thus genetically *linked.* In simple terms linkage is the association of two or more loci within the chromosome with limited recombination between them. They are frequently inherited together and assessment of one allele can reflect inheritance of multiple alleles linked together.

Linkage disequilibrium (LD) is the non-random association of alleles at two or more loci not necessarily on the same chromosome. LD patterns in human populations reflect the block-like structure with SNPs within a high LD region transmitted together from generation to generation (Gabriel et al., 2002). These high LD regions are termed “haplotype blocks”. As set of tag SNPs can serve as proxies for other SNPs based on degrees of correlation between their alleles (Johnson et al., 2001).

Instead of genotyping each SNP of a gene, the use of tagging SNPs within a haplotype greatly reduces the genotyping burden in association studies as a fewer number of tag SNPs need to be genotyped while maintaining the same information and power as if one had genotyped a much larger number of random SNPs (Hinds et al., 2005). Large genome wide association studies (GWAS) are

based on genotyping selected tag SNPs within a haplotype thereby covering the whole genome. The HapMap project has delineated the haplotype block structure of the human genome and provides data for the identification of tag SNPs across multiple populations (Hinds et al., 2005).

1.8.4 Summary

Humans demonstrate wide variation in their phenotypic characters. These differences stem from permutations and combinations of the genome we inherit from our ancestors. Epidemiological studies have confirmed that more than 50% variation in weight, body mass index and blood pressure can be explained by genetics with environment and behaviour playing a key role as well. Complete mapping of the Human Genome has revealed a huge variation in the form of SNPs, gene insertions, deletions, inversions, copy number variants, duplications and translocations. Some variations in phenotype can be now explained by variations seen in the human genome. Complete sequencing of the whole genome along with identification of variations has laid down a platform for identification of genetic variations which account for some of the variations in common polygenic traits.

Chapter 2

Study Aims

The University College London Fetal Growth Study (UCL-FGS) consists of a cohort of healthy pregnant mothers followed through pregnancy to delivery and their infants up to 3 years of age. Antenatal growth parameters were obtained through pregnancy and anthropometric and BP measurements for offspring were performed until 3 years of age. DNA was extracted from blood samples of the parents and from the cord blood and the placenta for the infants. DNA samples were genotyped for common variations in *IGF* genes, the growth hormone receptor exon 3 deletion and genes found to be significantly associated with body mass index and blood pressure in genome wide association studies (GWAS).

This study aims to explore the relationships between antenatal growth, birth size, postnatal anthropometry and BP until 3 years of age with common variation in IGF genes, GHR exon 3 deletions and common variation in selected genes associated with BMI and BP in GWAS.

2.1 Hypotheses

The hypotheses to be tested are that common variations in the genes of the GH-IGF axis associate with antenatal growth and birth size and play a role in the determination of body size and blood pressure at 3 years of age.

Chapter 3

Materials and Methods

3.1 Characteristics of the University College London Hospital Fetal Growth Study Cohort

3.1.1 Recruitment for the Study

3.1.1.1 Inclusion/exclusion Criteria

The UCL Fetal Growth Study was established to determine whether the Barker observations are relevant to a modern British population. Differences in birth weight between ethnic and racial groups are well recognised (Wang et al., 1994). As the original Barker cohort was white Caucasians, the UCL Fetal Growth Study recruited subjects of white Caucasian ancestry. This group of individuals accounted for 60% of the pregnant women attending the Obstetric Department at University College London Hospital (UCLH). To date the pregnancy accurately using ultrasound, pregnant women booking at $\leq$20 weeks gestation with a singleton pregnancy were included in the study. Pregnant women with medical disorders during pregnancy, which could have adversely influenced fetal growth, were excluded.

3.1.1.2 Recruitment in the antenatal clinic and consent

Ethical approval for the study was obtained from the Joint Ethics Committee of UCL Hospitals and University College London. All white Caucasian pregnant women who fulfilled the inclusion criteria were approached for the study. Written informed consent was obtained from all the participants at the booking visit, and again after birth for inclusion of the baby in the study and also for follow up to 3 years of age.

3.1.1.3 Demographic data recorded at booking

A detailed obstetric, medical, personal and family history was taken from pregnant women recruited into the study. Obstetric history sought information on age, the date of last menstrual period, parity, past obstetric outcome, birth weights of previous siblings, and a history of medical and surgical problems in the past and in previous pregnancies. The family history of the mother and her partner was taken. Any history of alcohol intake, cigarette smoking and drug intake (recreational and non-recreational) was obtained. Timing of folic acid

intake, preconceptually or in the first trimester, was noted. To categorise women according to socioeconomic group information was sought in relation to the age full-time education was completed, marital status, occupation, and partner's occupation. The classification of the Office of Population Census and Statistics was used to define this (Standard Occupational Classification 1991).

3.1.2 Maternal anthropometric measurements at booking

A permanently secured wall stadiometer (Holton Limited, Crymych, U.K) was used to measure height which was recorded in centimetres (cm) to the nearest 0.1 cm. Weight without footwear and wearing only light clothing was recorded using Seca scales (CMS Weighing Equipment Limited, London, U.K.). Body mass index was calculated using the formula weight (kg) / height $(m)^2$. Blood pressure was measured in the right arm after women had rested for 20 minutes. The sphygmomanometer was placed at the same level as the participant's heart. The definition used for hypertension was a blood pressure of greater than 140/90 mmHg.

3.1.3 Assessment of fetal growth by ultrasound

An ultrasound scan was used to accurately assess fetal growth. At booking ultrasonography was performed to confirm viability of the fetus, ensure singleton pregnancy and to date the pregnancy. In those women who booked between 6 and 12 weeks of gestational age (GA), the crown-rump length (CRL) was measured in millimetres (mm). Between 12-14 weeks of GA, depending on position and activity of the fetus, CRL or biparietal diameter (BPD) was used. After 14 weeks GA BPD was measured to the nearest mm. These measurements were entered into The King's Database computer software package (The King's Database, London, UK) to assist dating of the pregnancies. Expected date of delivery (EDD) was calculated from the first day of the last menstrual period when a difference of 7 days or less was recorded between ultrasound measurement and post menstrual age. Ultrasound measurements were used to calculate the EDD when differences of more than 7 days were recorded between the ultrasound measurement and postmenstrual age.

A second trimester ultrasound scan was mainly performed between 18-20 weeks of gestational age to check for the presence of fetal anomalies. BPD, head circumference, abdominal circumference and femoral length were also measured

to the nearest mm to assess fetal growth. Centiles for these parameters were calculated from well-established fetal growth charts (Altman and Chitty, 1994; Chitty et al., 1994a; Chitty et al., 1994b; Chitty et al., 1994c).

A third trimester scan was performed as part of the study even though it was not routine practice for clinical care. BPD, head circumference, abdominal circumference and femoral length were obtained for fetal growth assessment. The scans were ideally performed between 30-34 weeks of gestational age but for practical reasons any scan performed between 28-36 weeks of gestational age was included for assessing third trimester fetal growth. All measurements were adjusted for gestational age based on fetal growth centile charts (Altman and Chitty, 1994; Chitty et al., 1994a; Chitty et al., 1994b; Chitty et al., 1994c).

3.1.3 Anthropometric measurements of the newborn and children until 3 years if age

British growth reference centile charts were used for birth weight, length and head circumference (Cole et al., 1995; Cole, 1995; Freeman et al., 1995b). Birth weight was measured using Marsden scales (Marsden, London, UK). The baby was measured naked within 60 minutes of birth. Weight was recorded within the nearest 5 grams.

Birth length was measured within 48 hours of birth using a stadiometer (Kiddimeter, Child Growth Foundation). Length was recorded to the nearest 0.1 cm. Three separate measurements were taken and the mean value was recorded.

The head circumference was measured within 48 hours of birth using a standard Holtian tape measure (Holtian, Crymych, UK). Occipito-frontal circumference was recorded in cm, to the nearest 0.1 cm. The mean value from three separate measurements was recorded.

For all the anthropometric measurements at birth, three separate measurements were taken and the mean value was recorded. The coefficient of variation of the measurement error for anthropometric measurements was 0.16% based on 10 infants each measured five times by three observers.

Longitudinal anthropometric measurements were undertaken at 6 months, 1 year, 2 years and 3 years of age. Weight was measured using electronic scales (Seca, Precision for health, UK) and recorded to nearest 0.1 kg. Length was

measured until 2 years of age using a stable measuring board (Seca, Precision for health, UK) and height was measured at 3 years of age using a stadiometer (Seca, Precision for health, UK) and recorded to the nearest 0.1cm. The coefficient of variation of measurement error for length was 0.2% on the basis of data of 15 infants each measured four times on the same day and that at 3 years of age was 0.1%. BMI was calculated using the formula mass(kg)/(length/height (m))^2. All measurements were converted into SDS using 1990 British growth references (Freeman et al., 1995a).

The mid-arm circumference was measured at the halfway point between the acromial notch of the shoulder joint and the olecranon at the elbow. A Holtian tape measure (Holtian, Crymych, UK) was used for measuring mid-arm circumference in cm to the nearest 0.1cm. Three separate measurements were taken and the mean value was recorded. Triceps, sub-scapular and quadriceps skin-fold were measured using skin-fold callipers (Holtain Limited, Crymych, UK). Three separate measurements were made and the mean was recorded.

BP at 3 years was measured in a standard room temperature environment of 19-22˚C using a Omron M6 BP monitor (Omron Healthcare UK, Milton Keynes, UK). Appropriate-sized paediatric BP cuffs (extending completely around the arm, with a bladder width covering at least two-thirds of the upper arm) were used for the measurements. Means of three BP recordings were taken at 1-minute intervals, after 5-minute rest periods in between. The coefficient of variance of measurement error for 19 children, aged 3-5, for systolic and diastolic BP were 2.3% and 3.9% respectively.

3.1.5 Endocrine analysis of the IGF axis from cord blood

After delivery of the baby and prior to completion of the third stage of labour, blood was taken from the umbilical cord. In a small number of cases where there was either rapid completion of the third stage of labour or difficult postpartum bleeding, the placenta was delivered and blood samples subsequently taken from the cord. The policy in the unit was to collect arterial cord blood for pH analysis in as many cases as possible, which was analysed immediately after collection. Venous blood was taken for assessment of the IGF axis. The sample was immediately stored at 4°C, centrifuged within 24 hours of sampling, and subsequently stored at -70°C.

IGF-I assay

A commercial radioimmunoassay (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA) was used to measure IGF-I. The within assay CVs were 4.6% and 3.3% at 61.0 and 292.5 ng/ml respectively. The between assay CV were 15.5% and 11.3% at 88.6 and 240.4 ng/ml. The minimum detection limit of the assay was 6 ng/ml.

IGF-II and IGFBP-III assays

IGF-II and IGFBP-III were measured by commercial coated tube immunoradiometric assays (Diagnostic Science Laboratories, Webster, TX, USA). For IGF-2 the within assay CVs were 6.5%, 3.4% and 4.7% at 245, 409 and 1432 ng/ml respectively, the between assay CVs were 14.5% and 7.2% at 273 and 785 ng/ml respectively. The minimum detection limit of the assay was 12 ng/ml. For IGFBP-3 the within assay CVs were 3.9%, 3.2% and 1.8% at 7.35, 27.53 and 82.72 µg/l respectively and the between assay CVs were 7.6% and 4.2% at 5.43 and 27.15 µg/l respectively. The minimum detection limit of the assay was 0.5 µg/l.

Growth Hormone

GH was measured by a commercially available immunoradiometric assay (Hybritech Europe, Liége, Belgium). For growth hormone the within assay CVs were 10.6%, 4.9%, 5.2% 4.9% and 5.0% at 1.4, 3.5, 14.4, 26.4 and 99.4 mU/l respectively and the between assay CVs were 15.4%, 9.7%, 8.0% and 6.3% at 4.1, 8.1, 13.6 and 35.8 mU/l respectively. The minimum detection limit of the assay was 0.5 mU/l.

3.2 Characteristics of the Baby Biobank Cohort

The Moore Baby Biobank Cohort recruited white Caucasian subjects. To date the pregnancy accurately using ultrasound, pregnant women booked at ≤20 weeks gestation were included in the study. Only singleton pregnancies were included. Though the cohort consisted of subgroups of pregnant women with complications of pregnancy, for the purpose of this research pregnant woman with medical disorders of pregnancy, which could adversely influence fetal growth, were excluded from the study.

Ethics approval for the study was obtained from The Hammersmith and Queen Charlotte's and Chelsea Hospitals' Trust Research Ethics Committee

(registration no. 2001/6029). All white Caucasian pregnant women who fulfilled the criteria were approached for the study. Written informed consent was obtained from all the participants.

Maternal weight, height, age, parity, baby's gender, birth weight, head circumference, placental weight, past and present medical history, mode of and indication for delivery, pregnancy complications, smoking, diet and alcohol consumption, and partner's medical history were collected from the mother's notes from all families participating in the study (Apostolidou et al., 2007).

After delivery, a small sample of placenta was collected. Only normal placenta was used, with samples dissected near the umbilical cord insertion point, snap frozen in liquid nitrogen, and stored at -80°C until use for DNA preparation. Ten millilitres of blood samples were collected in EDTA tubes from each parent for DNA extraction. The samples were collected under the guidelines of the Hammersmith and Queen Charlotte's and Chelsea Hospitals' Trust Research Ethics Committee.

3.3 DNA extraction from blood and placenta

DNA from blood was extracted using a Gentra® Puregene® Blood Kit (QIAGEN, Crawley, UK) based on the instructions provided in their manual. In brief, cells were lysed with an anionic detergent in the presence of a DNA stabiliser. The DNA stabiliser limits the activity of intracellular DNases and also DNases found elsewhere in the environment. RNA was then removed by treatment with an RNA digesting enzyme. Other contaminants, such as proteins, were removed by salt precipitation. Finally, the genomic DNA was recovered by precipitation with alcohol, washed, and dissolved in hydration solution (1 mM EDTA, 10 mM Tris·Cl pH7.5). The DNA was stored at –20°C.

DNA from the placenta was extracted by the KBioscience laboratories (KBioscience, Unit 7, Maple Park, Hoddesdon, Herts, England), using an in-house silica based system for tissue extraction. In brief, DNA was bound to silica in the presence of guanidinium isothiocyanate. With subsequent washing with ethanol/isopropanol and final elution with ethanol, DNA was separated from the silica pellet.

3.4 Genetic techniques used to genotype genetic variation

Several techniques have been developed to determine SNPs and genomic variants. This section describes the techniques I have used to determine SNPs in the IGF-axis genes, growth hormone receptor exon 3 retention/deletion polymorphism and genes associated with BMI and BP from original published GWAS studies.

3.4.1 Polymerase chain reaction

The polymerase chain reaction (PCR) was first developed in the early 1980's by Karry Mullis (Mullis and Faloona, 1987). PCR is a technique of enzymatically amplifying a copy of a section of DNA by many orders of magnitude, generating millions of copies of the DNA in that genomic region. The PCR process involves multiple cycles and in each cycle original quantities of DNA are approximately doubled leading to an exponential increase in the quantities of the original DNA sequence present ("the amplicon"). PCR is widely used for gene sequencing, cloning, genetic engineering, diagnosis of microbiological infections and in forensic laboratories.

An outline of the principle methods typically involved in PCR is described below (Markham, 1993):

1. A small sample of DNA (0.5-1µl of 10ng/ µl) is placed in a tube.
2. Two oligonucleotides, also known as primers, are added into the tube. These have sequences matching two sequences of the DNA that flank the region of interest. Oligonucleotide sequences are complementary to the three prime (3′) ends of the sense and antisense strand of the DNA region to be amplified.
3. A mixture is added containing: a) a thermostable DNA polymerase, *Taq* polymerase that works at an optimal temperature of around 70˚C; b) buffer solution to optimise activity and stability of the DNA polymerase; c) deoxynucleotide triphosphates (dNTPs; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesises a new DNA strand and d) monovalent and divalent cations.
4. *Denaturing step*: The mixture is heated to between 90-100˚C for 20-30 seconds. This process causes disruption of hydrogen bonds between complementary DNA bases, dissociating the DNA into two single strands.

5. *Annealing step*: The solution is allowed to cool to 50-65˚C and the single strands bind to the oligonucleotides, which are in excess.
6. *Extension/elongation step*: The oligonucleotide now acts as a primer for the DNA polymerase which catalyses the extension of a new double stranded molecule. The temperature used for this step of the reaction is between 70-80˚C, which is optimal for the activity of *Taq* polymerase. *Taq* DNA polymerase was isolated from the thermophilic organism, *Thermus aquaticus,* which can tolerate high temperatures. *Taq* polymerase attaches new residues only to the 3′ end of the primers. This simple consideration dictates the design of synthetic oligonucleotide primers from the 5′ to the 3’ end.
7. The cycle is repeated through a number of cycles (typically 30-40), with the amount of DNA being amplified approximately doubling with each cycle.
8. *Final Extension*: This final step is performed between 70-75˚C to facilitate full extension of any remaining single stranded DNA.

A successful PCR generates millions of copies of the DNA fragment being amplified. Amplified DNA fragments can be visualised by agarose gel electrophoresis. The technique separates PCR products based largely on size. A successful PCR should generate DNA fragments of known sizes, which can be compared by simultaneously running a DNA “ladder” containing defined molecular weight fragments corresponding to known base pairs lengths.

Primer design

Primers are small synthetic oligonucleotides, which serve as a starting point for DNA synthesis in the PCR reaction. Effective primer design is necessary to optimise the PCR reaction and requires a balance of specificity and efficiency (Dieffenbach et al., 1993). Primers with poor specificity will produce multiple unrelated or non-specific products, which will form multiple ethidium bromide-stained bands on agarose gel electrophoresis. Primers with poor efficiency will not produce sufficient copies of the desired DNA product, which may produce a faint ethidium bromide-stained band on agarose gel electrophoresis. Amplification with the *Taq* DNA polymerase greatly increases the specificity of

the reaction (Saiki et al., 1988), so that many PCR amplicons can be detected as a single ethidium bromide-stained band on an electrophoresis gel.

The optimal length for primers is at least 20 base pairs with a mixture of different nucleotides (Nybo, 2009). Primer design programmes are now available to help in this regard (for example, Primer3Web).

3.4.2 Restriction fragment length polymorphism (RFLP)

Restriction fragment length is one of the simplest and earliest methods developed to detect single nucleotide polymorphisms, and relies on the DNA cutting properties of restriction enzymes. Restriction enzymes are produced by bacteria to protect themselves from bacteriophage viruses. These enzymes cut the DNA of viruses at specific sites, protecting them from the invading virus. Bacteria with four or five independent restriction enzymes could be virtually impregnable by viruses. Danna and Nathans were the first to demonstrate the use of a restriction enzyme called “endonuclease R,” to produce specific fragments of simian virus 40 (SV40) DNA (Danna and Nathans, 1971). The authors showed that these fragments could be cleanly separated from one another by electrophoresis on a polyacrylamide gel (Danna and Nathans, 1971). The most commonly available and used restriction enzymes recognise specific DNA sequences and give rise to very specific DNA cleavage.

An example of the action of a restriction enzyme (in the case *Apa* I) in detecting a single nucleotide polymorphism is shown in Figure 3.4.2

Restriction fragment length polymorphism was used to detect the rs680 polymorphism of the *IGF2* gene, also known as the ‘*Apa* I polymorphism’. The protocol used to amplify this region and for RFLP analysis to detect this variant is described below.

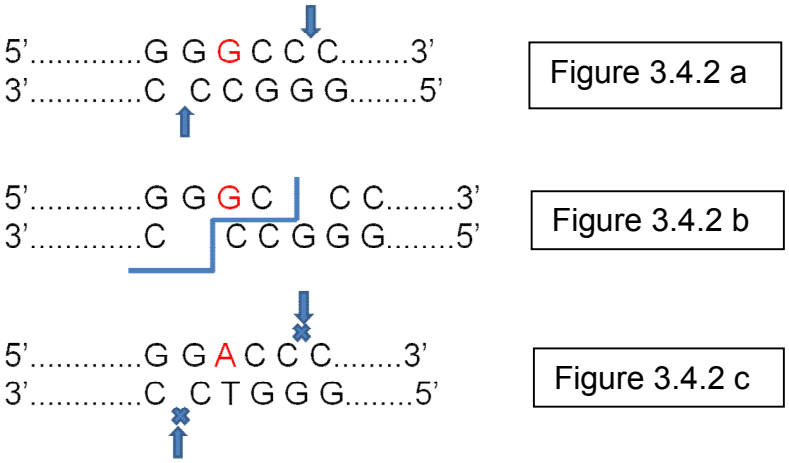


Figure 3.4.2 Mechanism of action of restriction enzyme.

The *Apa* I restriction enzyme recognises a specific DNA sequence and cleaves the DNA at the sites marked by bold arrows (Figure 3.4.2 a & b). On agarose gel electrophoresis, the native DNA will split into two bands (fragments) secondary to enzyme cleavage (Figure 3.4.3 b). A change in the DNA sequence secondary to a mutation or SNP will render the restriction enzyme ineffective in cleaving the DNA (Figure 3.4.2 c). This presence of single nucleotide polymorphism G to A transversion will lead to no cleavage and presence of single band on agarose gel electrophoresis (Figure 3.4.3 b).

The initial polymerase chain reaction was performed as per the protocol described below.

1. Primer design. The primers were designed as described before (Kaku et al., 2007). Forward and reverse primers were constructed to flank the SNP site, rs680 of the *IGF2* gene.
2. Samples of DNA (1µl of 10ng/ µl) were placed in individual wells of a 96 well plate.
3. A mastermix was prepared by adding 18 µl of MegaMix (Microzone Limited, Haywards Heath, UK) to 0.5 µl (0.8 nM) each of forward and reverse primers. MegaMix is a commercially available mixture containing a thermostable DNA polymerase, *Taq* polymerase; buffer solution for optimal activity and stability of the DNA polymerase; deoxynucleotide triphosphates (dNTPs; nucleotides containing triphosphate groups) and monovalent and divalent cations.
4. Mastermix solution (19 µl) was added into the individual wells of 96 well plate containing DNA samples. Plates were covered by an adhesive PCR film (ABgene, Thermo Scientific, Cambridge, UK). Plates were centrifuged at 3000 rpm for 2 minutes (Sorvall Legend RT, Sorvall, Germany). PCR was

performed on a Mastercycler (Eppendof Scientific, Stevenage, UK) using the following conditions:

4.1. *Denaturing step*. The plate was heated to 94˚C for 1 minute.

4.2. *Annealing step*. The plate was cooled to 60˚C.

4.3. *Extension/elongation step*. The plate was heated to 72˚C for 1 min for optimal activity of the enzyme *Taq* polymerase.

4.4. The above steps were repeated for 35 cycles.

4.5. *Final Extension*. This final step was performed at 72˚C to facilitate full extension of any remaining single stranded DNA.

The 96 well DNA plate was centrifuged and thermal seal was removed. Restriction enzyme digestion was performed on the samples according the protocol described below.

1. An enzyme “mastermix” was prepared for *Apa* I (New England Biolabs, Hitchin, UK). The mixture (per sample) was made up of enzyme 0.3µl (50units/µl), buffer 0.5 µl, bovine serum albumin 2.5 µl (1 µg/µl) and purified water 1.7 µl (total volume, 5 µl per sample).
2. The enzyme mixture was placed in the 96 well plate with 5 µl aliquoted per well of PCR product. A thermal seal was applied and plates were centrifuged at 3000rpm for 2 minutes (Sorvall Legend RT, Sorvall, Germany).
3. Plates were incubated in a water bath at 37˚C for 16 hours for the restriction enzyme digestion to occur.
4. After 16 hours the 20 µl reaction samples were loaded into individual wells of a ethidium-stained agarose gel and gel electrophoresis was performed for characterisation of the PCR products.
5. Agarose gels were prepared by mixing 1.8 g of agarose (1.2%) in 150ml of water and heating the solution in a microwave oven to melt the agarose. The solution was cooled, then 3 µl of ethidium bromide was carefully added to it and mixed in a fume cupboard. The agarose was poured into a gel-tank with appropriately sized comb and set. Once cold the gel was immersed in TAE buffer in the gel-tank. Samples were prepared by adding 2.7 µl loading buffer (containing Orange G) to each sample and carefully loading each sample to one of the wells. DNA ladder (10 µl) was loaded into a separate well. Gel electrophoresis was performed (85 volts for 40

minutes) to separate the bands. Visualization was performed on ChemiDoc imaging system (Bio-Rad Laboratories Ltd, UK).

Figure 3.4.3a below shows the position of the rs680 polymorphism, which is the site of action of the *Apa* I restriction enzyme. Figure 3.4.3b shows an example of the *Apa* I agarose gel electrophoresis of products of restriction enzyme digestion.

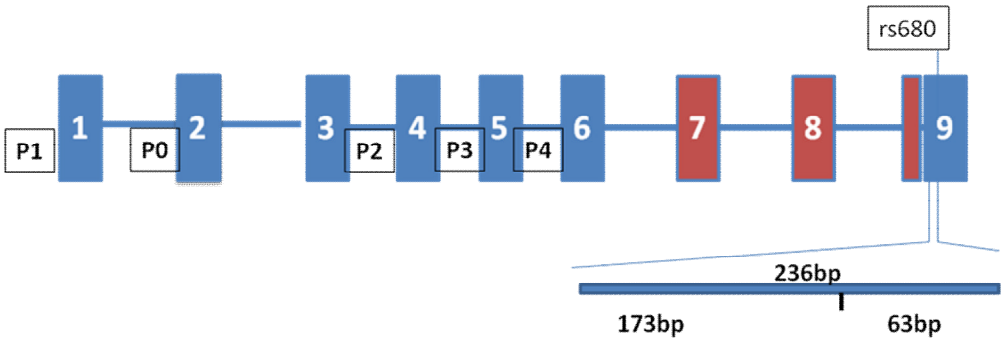


Figure 3.4.3a Schematic diagram of the IGF2 gene, indicating position of the sequence amplified by PCR and *Apa*I restriction enzyme to detect SNP rs680.

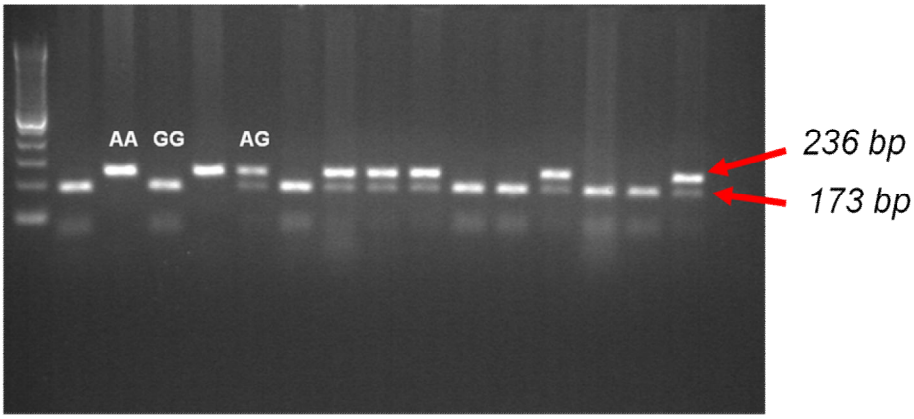


Figure 3.4.3b Electrophoresis products of rs680 polymorphism.
Unrestricted product AA has a size of 236bp while complete digestion of the GG variant generates bands of 173bp and 63bp.The heterozygous AG variant produces fragments of all three sizes.

3.4.3 Limitations of restriction fragment length polymorphism analysis

Although RFLP is a very useful technique, there are several limitations to its use. The technique of restriction fragment length polymorphism is time consuming and labour intensive. Typically, only one SNP can be assessed on a limited number of samples. Restriction enzyme digestion is time dependent restricted by the time it takes to digest the DNA efficiently. Restriction enzymes are also very sensitive to temperature changes and repeated thawing decreases their activity. Methylated regions of DNA are generally resistant to the actions of the restriction enzymes. Therefore, even though the numbers of restriction enzymes have increased since they were first discovered, there are still limitations in using this approach to cover the increasing number of SNPs which are being recognised by the HapMap project.

Technology for SNP genotyping has now advanced with newer approaches. Some of these newer assays require prior amplification of the genomic target, whereas others are sensitive enough to work directly on genomic DNA or cDNA (Twyman, 2004). Low, medium and high throughput methods have evolved in genotyping SNPs. Platforms are available for high throughput methods which can detect many thousands of SNPs and multiple samples (for example, Affymetrix or Illumina platforms). These platforms are used for large genome wide studies. Low to medium throughput technologies such as TaqMan® (Applied Biosystems) use a competitive allele specific real time PCR detection system for SNP genotyping and these approaches have been replacing restriction fragment length enzyme digestion and agarose gel electrophoresis for SNP genotyping. This project therefore utilised the KBiosciences Competitive Allele-Specific PCR genotyping system (KASP) which is being extensively used worldwide for SNP genotyping (Alfred et al., 2012; Reiling et al., 2011; Voorhuis et al., 2011; Zammit et al., 2011). The basic principle and protocol is described in the section below.

3.4.4 KBioscience Competitive Allele -Specific PCR genotyping System (KASP)

KASP is a fluorescent genotyping technology. The system consists of a SNP specific assay (a combination of three unlabelled primers) and a universal reaction mix which contains Taq polymerase enzyme and a universal fluorescent reporting system. The oligonucleotides which are used for the system are-

1) Two allele-specific primers (one for each SNP allele). Each primer contains a unique unlabelled tail sequence at the 5' end.
2) One common reverse primer.
3) Two 5' fluorescent labelled oligonucleotides, one labelled with FAM (6-carboxyfluorescein) and other with CAL fluorescent orange 560. These oligonucleotide sequences are designed to interact with the sequences of the tails of the allele-specific primers. Hence FAM and CAL fluorescent orange 560 will be very specific for the allele primer it binds to.
4) Two oligonucleotides with quenchers bound at the 3' ends. These quencher oligonucleotides are complementary to the fluorescent oligonucleotides and are therefore complementary to the tails of the allele-specific primers. These quencher oligonucleotides bind to the fluorescent oligonucleotide and quench the signals until the fluorescent oligonucleotides binds with the sequences of the tails of the allele specific primers.

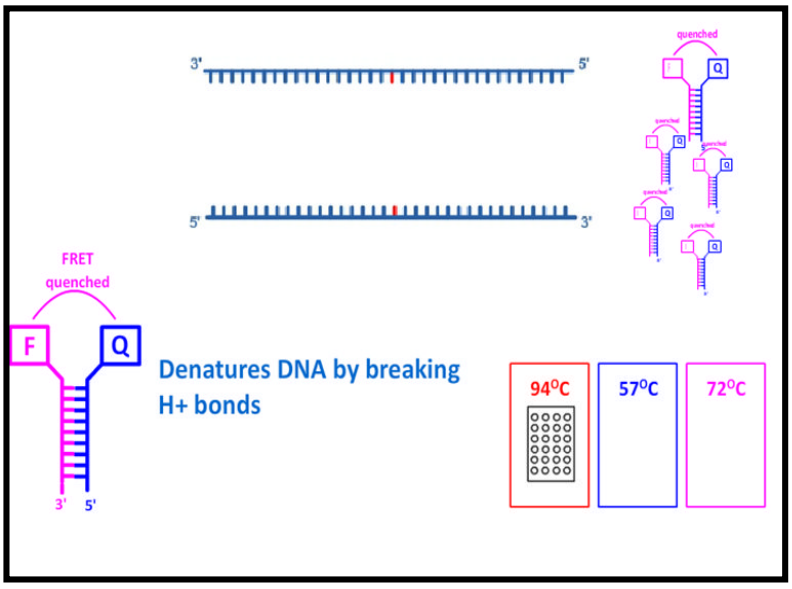


Figure 3.4.4 A Initial reaction of PCR with separation of DNA strands at 94˚C.

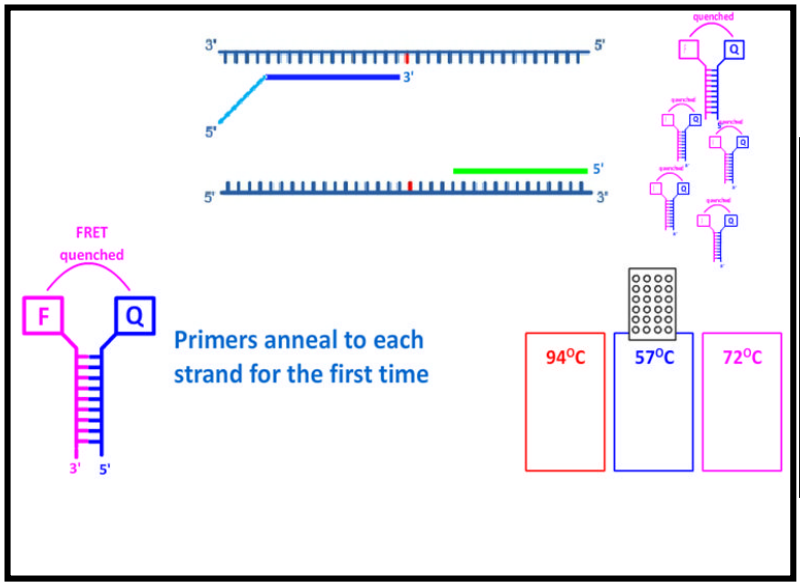


Figure 3.4.4 B- Allele specific primer binds to its complementary region directly upstream of the SNP with the 3' end of the primer positioned at the SNP nucleotide. Common reverse primer binds to its complementary region.

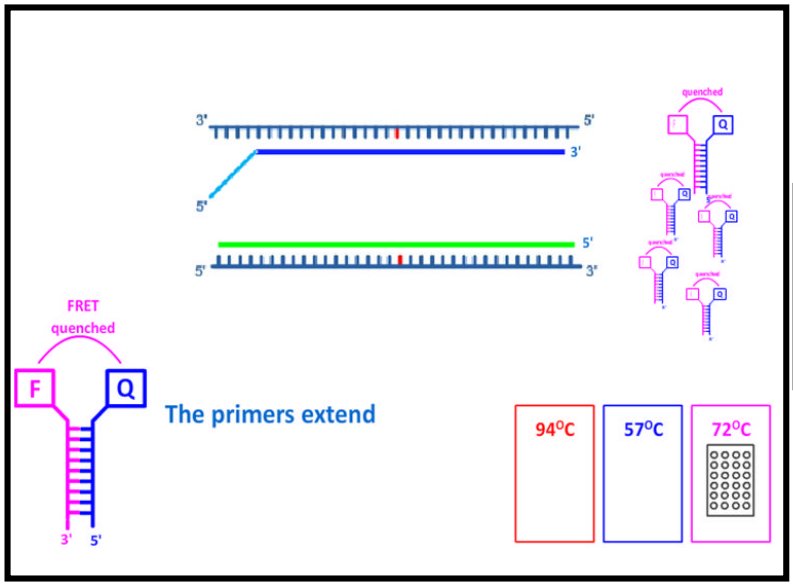


Figure 3.4.4 C- Allele specific primer and common reverse primer extend in the presence of Taq polymerase enzyme.

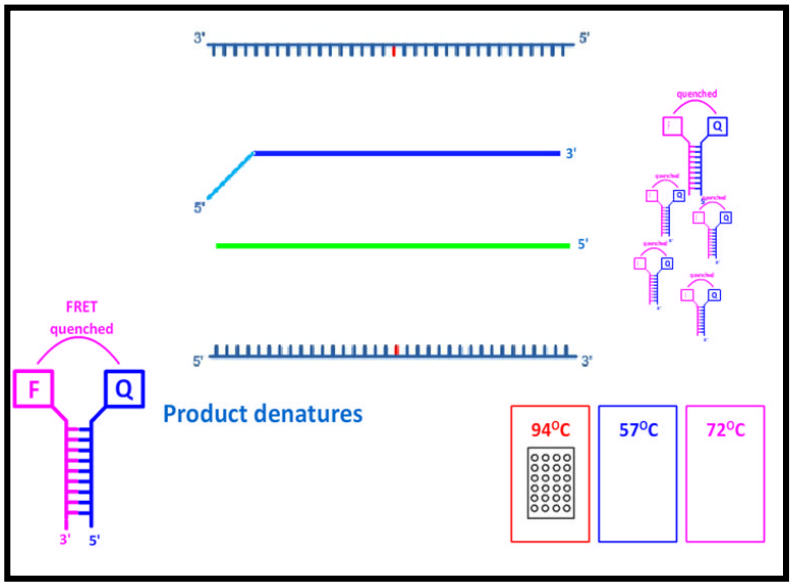


Figure 3.4.4 D- New DNA strands are separated at 94˚C.

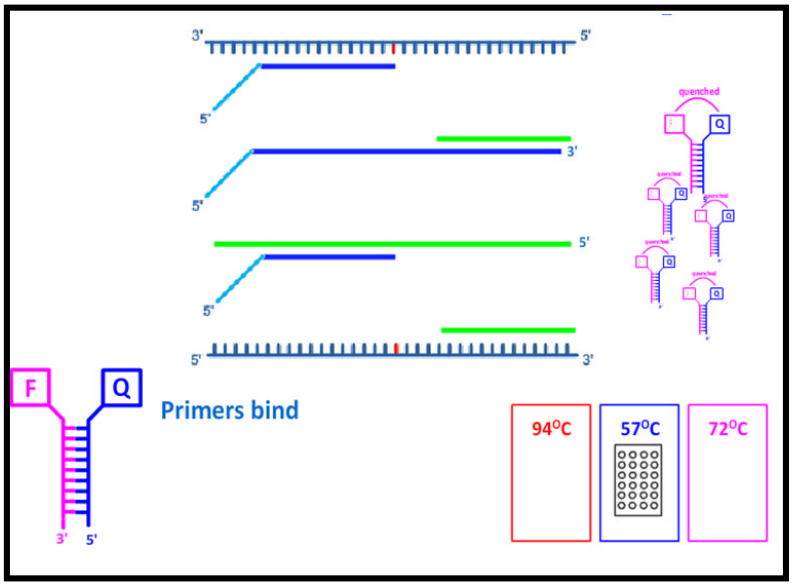


Figure 3.4.4.E- New DNA strands are formed by primers annealing and extending.

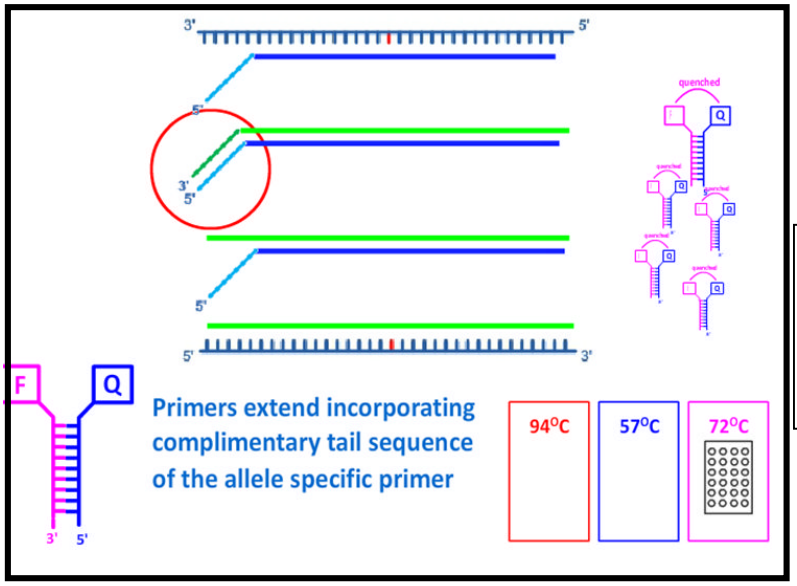


Figure 3.4.4 F- Primers extend into the complementary tail sequence of the allele specific primers.

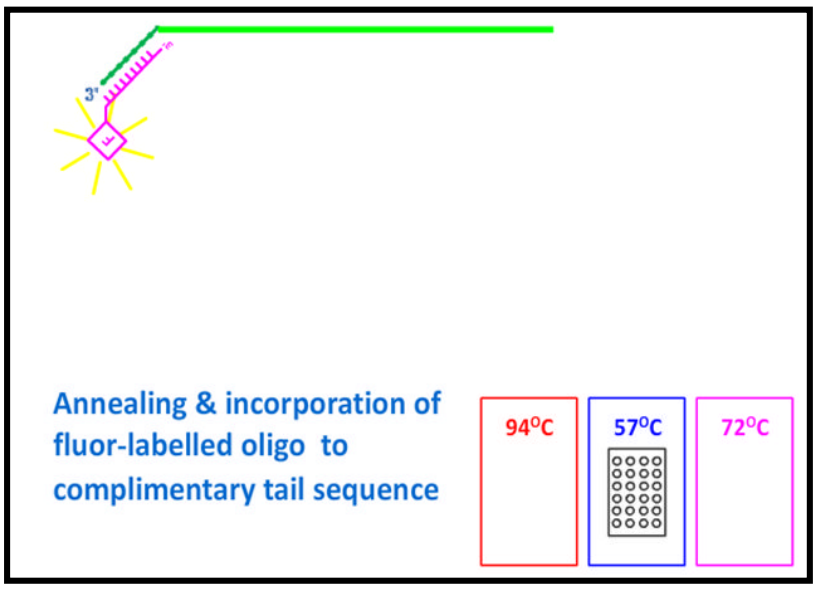


Figure 3.4.4 G- As PCR proceeds further, one of the fluorescent labeled oligonucleotides corresponding tothe amplified allele also gets incorporated into the template and hence is no longer bound to the quencher complement.

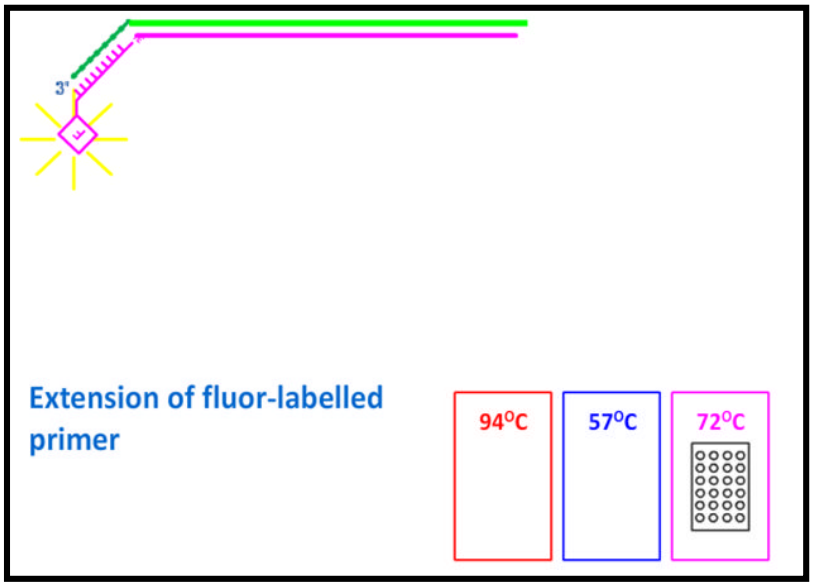


Figure 3.4.4 H- Fluorescent labelled primer extends and emits fluorescent signal.

Figures 3.4.4 A-H KBioscience Competitive Allele specific PCR genotyping System (KASP).

In the initial stage of the PCR, the appropriate allele specific primer binds to its complementary region directly upstream of the SNP with the 3' end of the primer positioned at the SNP nucleotide (Figure 3.4.4 a). The common reverse primer also binds and the PCR proceeds with allele specific primer becoming incorporated into the template (Figure 3.4.4 b & c). During this phase the fluorescent labeled oligonulcoetides remain bound to their quencher bound complementary oligonucleotide and no fluorescent signal is released (Figure 3.4.4 c-f). As PCR proceeds further, one of the fluorescent labeled oligonucleotide corresponding to amplified allele also gets incorporated into the template and hence is no longer bound to the quencher complement (Figure 3.4.4 g). Since the fluorescent is no longer quenched, fluorescent signal is generated and detected. If the genotype at a given SNP is homozygous only one signal is generated. If the individual is heterozygous, mixed fluorescent signal is generated.

3.4.5 Multiplex Polymerase Chain Reaction

The growth hormone receptor exon 3 retention (*GHR* fl)/deletion (*GHR* d3) polymorphism was determined by multiplex polymerase chain reaction. This technique is a variant of the polymerase chain reaction in which two or more loci are simultaneously amplified in the same reaction. It was first described in 1988 by Chamberlain et al where they used the technique for deletion screening of the Duchenne muscular dystrophy gene *DMD* (Chamberlain et al., 1988). This method is now widely used in many areas of DNA testing, including analyses of deletions, mutations and polymorphisms, or quantitative assays and reverse transcription PCR (Henegariu et al., 1997).

Multiplex PCR used for the *GHR* genotype was based on the technique described by Pantel et al (Pantel et al., 2000). The technique was modified to obtain strong reliable bands on agarose gel electrophoresis as described by Henegariu et al in their step by step protocol for the multiplex PCR.

An outline of the procedure used for growth hormone receptor genotyping is below:

1) Primer design. Three primers (designated G1, G2 and G3) were used for the PCR. The G1 and G2 primer pair allowed amplification of the *GHR* d3 allele while the G1 and G3 primer pair allowed amplification of the *GHR* fl allele. Figure 3.4.5 shows the role of these primers in amplifying these genotypic polymorphisms of exon 3 (Pantel et al., 2000). Primer design for G1, G2 and G3 was based on the GenBank accession number AF155912. Primer sequences are: G1 (5'-TGTGCTGGTCTGTTGGTCTG-3'),
   G2 (5'-AGTCGTTCCTGGGACAGAGA-3'), and
   G3 (5'-CCTGGATTAACACTTTGCAGACTC-3').
2) Samples of DNA (1 µl of 10 ng/ µl) were placed in individual wells of a 96 well plate.
3) A mastermix was prepared by adding primers G1 (1 µl, 0.75 nM), G2 (0.5 µl, 0.5 nM), G3 (1 µl, 0.8 nM) and 16.5 µl of MegaMix (Microzone Limited, Haywards Heath, UK) per sample, as described in section 3.4.2.
4) Mastermix solution (19 µl) was added into the individual wells of 96 well plate containing DNA samples. Plates were covered by an adhesive PCR film (ABgene, Thermo Scientific, Cambridge, UK). Plates were centrifuged at 3000rpm for 2 minutes (Sorvall Legend RT, Sorvall, Germany). PCR

was performed on a Mastercycler (Eppendof Scientific, Stevenage, UK) using the following conditions:

a. *Denaturing step*. The sample was heated to 94˚C for 30 seconds. This causes disruption of hydrogen bonds between complementary DNA bases, dissociating the DNA into two single strands.
b. *Annealing step*. The sample was cooled to 58˚C and the single strands bind to the oligonucleotides, which are in excess.
c. *Extension/elongation step*. The oligonucleotide now acts as a primer for the DNA polymerase and is extended to form a new double stranded molecule. This reaction was performed at 72˚C for 2 minutes. A longer extension time than usual was used as the PCR amplicon is longer than the *IGF2* amplification described before.
d. The above steps were repeated for 35 cycles.
e. *Final extension*. This final extension step was performed at 72˚C to facilitate full extension of any remaining single stranded DNA.

5) PCR products were characterised by agarose gel electrophoresis and visualized on a ChemiDoc imaging system (Bio-Rad Laboratories Ltd, UK) as described in section 3.4.2. Figure 3.4.6 shows the PCR products obtained after gel electrophoresis. Single 935 base pair (bp) bands correspond to samples with a homozygous full length (fl) genotype while single 532bp bands demonstrate a homozygous exon 3 deleted (d3) phenotype. A heterozygous phenotype is demonstrated by two separate bands of 935bp and 532bp

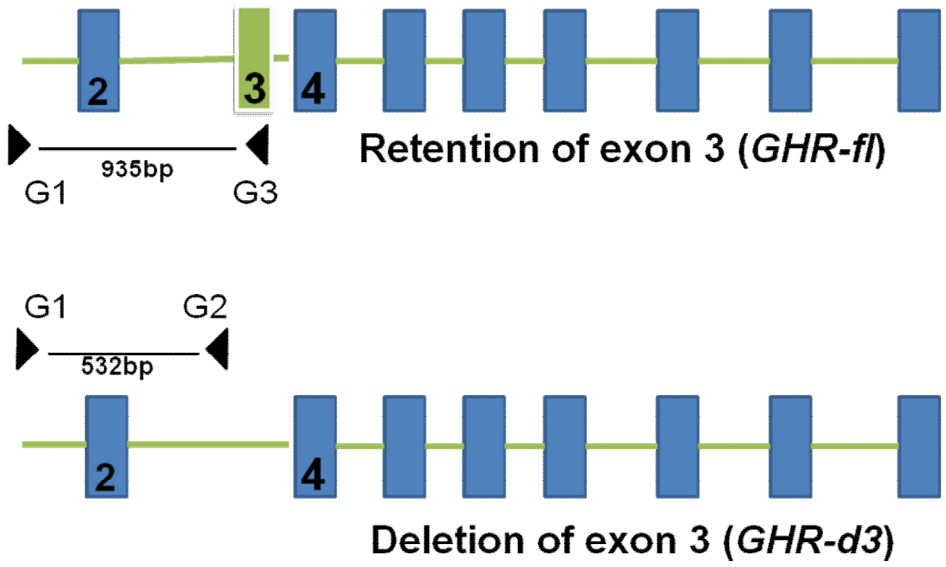


Figure 3.4.5 Genotyping assay at the *GHR*-exon3 locus.

Figure shows human *GHR* fl region including exon 3 (green box) and *GHR*d3 lacking exon 3. The position and orientation of primers G1, G2, and G3 used in the multiplex PCR assay are indicated by arrowheads.

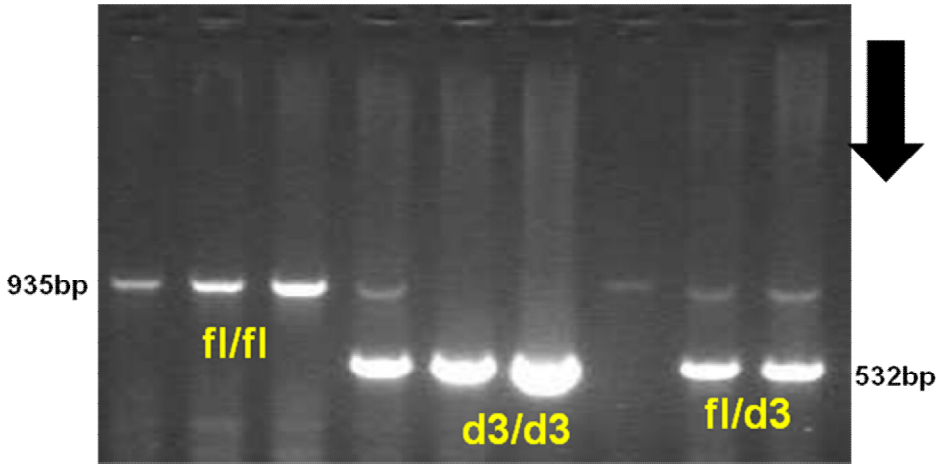


Figure 3.4.6 Electrophoresis products of *GHR*-exon 3 polymorphism following multiplex PCR.

Under specific experimental conditions (i.e. denaturation 94°C, 30s; annealing 58°C, 30s; and elongation 72°C, 2 min), primers G1 and G2 allowed the amplification of *GHR* d3 alleles only, where as primers G1 and G3 amplify *GHR* fl alleles. The homozygous *GHR* fl, homozygous *GHR* d3 and heterozygous *GHR* fl/*GHR* d3 genotypes are denoted by fl/fl, d3/d3 and fl/d3, respectively.

3.5 Statistical Analysis

Data were analysed using Statistical Package for Social Sciences (SPSS) version 16 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

In population genetics and association studies, the principle of Hardy-Weinberg equilibrium (HWE) is an important concept. This is described below.

3.5.1 Hardy-Weinberg Principle

In association studies it is a common practice to check whether observed genotypes conform to HWE expectations (Crow, 1988). These expectations appear to hold for most human populations, and deviations from HWE at particular markers may suggest problems with genotyping or population structure or, in samples of affected individuals, an association between the marker and disease susceptibility (Wigginton et al., 2005).

The HWE characterises the frequency distributions of genotype in populations that are not evolving, and is thus the fundamental null model for population genetics (Ryckman and Williams, 2008). The principle was independently described by Hardy and Weinberg in 1908 (Hardy, 1908; Mayo, 2008). The principle states that in a large random mating population with no selection, mutation, or migration, the allele frequencies and the genotype frequencies are constant from generation to generation and that, furthermore, there is a simple relationship between the allele frequencies and the genotype frequencies (Guo and Thompson, 1992).

If the frequencies of an allele in a population with two alleles at a locus are a and b, then the expected genotype frequencies are a^2, $2ab$, and b^2 (Crow, 1999). Once a population is in HWE, this frequency distribution will not change from generation to generation. For example, if the frequency of allele A in the population is a and the frequency of allele B in the population is b, then the frequency of genotype $AA = a^2$, the frequency of genotype $AB = 2ab$, and the frequency of genotype $BB = b^2$ (Mayo, 2008). If there are only two alleles at a locus, then $a + b$, by mathematical necessity, equals one. The Hardy-Weinberg genotype frequencies, $a^2 + 2ab + b^2$, represent the binomial expansion of $(a + b)^2$, and also sum to one (which is a must for the frequencies of all genotypes in any population, whether it is in HWE or not) (Crow, 1999).

The HWE applies only when some assumptions about the population are taken into consideration (Mayo, 2008). These assumptions include that the locus being studied is not influencing natural selection (i.e. probabilities of survival and reproduction are not consistently different with any of the alleles) and that there is no origin of new alleles through mutation or migration. If other assumptions hold, non-random mating will not change allele frequencies from one generation to the next but it can cause natural selection, which can cause evolutionary change (Mayo, 2008).

Deviations from HWE can indicate inbreeding, population stratification, and even problems in genotyping (Wigginton et al., 2005).

In the current study, SNP genotype distribution for HWE between the observed and expected frequencies of homozygous and heterozygous alleles was assessed using by Chi-square tests.

3.5.2 Assessment of data for normal distribution

One of the assumptions of parametric tests such as One-Way Analysis of Variance (ANOVA) is that the measurement variables are normally distributed. When data are plotted as a frequency histogram, the histogram should resemble a bell-shaped “normal” curve. Extreme deviation of the data to the right or left will lead to distortion of the normal curve. There is a chance of false positive results occurring if ANOVA is performed with the assumption of normal distribution of the data. Fortunately, ANOVA is not very sensitive to moderate deviations from normality; simulation studies, using a variety of non-normal distributions, have shown that the false positive rate is not affected very much by this violation of the assumption (Harwell et al., 1992; Lix et al., 1996).

Normality of the measured variable can be assessed by visual inspection of the frequency histogram, probability plots (P-P plots) and quantile plots (Q-Q plots).

Basic objective assessment of normality in SPSS and GraphPad Prism are provided by skew and kurtosis. Both skew and kurtosis should be zero for a perfectly normally distributed variable. For most purposes, a value between -2 to 2 is accepted to be within the normal range.

Formal tests of normality of measured variables can be undertaken by a range of tests which include the Kolmogrov-Smirnov test, K-S Lilliefors Test, the Shapiro-Wilk test, the Jarque-Bera LM test and the D’Agostino-Pearson K2 omnibus test.

All the formal normality tests suffer from the likelihood of rejecting a variable that deviates only slightly from normality when the sample size is large.

With a large sample size, such as the one in our study (n>1000 for most variables), we have assessed normality of our measured variables using the subjective assessment of frequency histogram, P-P and Q-Q plots and objective Skew and Kurtosis tests.

3.5.3 Student's *t* test

Student's *t* test can be used to determine if the averages of two samples are significantly different. The null hypothesis for the test is that there is no difference between the variables of the two samples tested. A probability value of less than 0.05 rejects the null hypothesis suggesting that the two groups are different in their measured variables.

In an independent samples t-test, the test statistic is computed by dividing the difference between the sample means by the standard error of the difference (Bewick et al., 2004). As the standard error of the difference is an estimate of the variability within each group, in Student's t test the difference (or variability) between the samples is compared with the variability within the samples (Bewick et al., 2004).

In the UCL Fetal Growth Study cohort we have suitable DNA samples available from 774 infants on which genotyping was performed. Students t test was used to compare the difference between the measured variables of the genotyped group and the total cohort (n=1674) to ensure there was no clear selection bias in the large subgroup studied.

3.5.4 One-way analysis of variance (ANOVA)

One way analysis of variance (ANOVA) is used to compare differences of means of measured variables for more than two groups. ANOVA compares group means by analysing comparisons of variance estimates. It generalises the Student's *t* test to more than 2 groups providing a statistical test of whether or not the means of several groups are all equal. In Student's t test the standard error of the differences is used to compare the variability in the samples. In ANOVA, variance is used rather than standard error of difference, to compare variability between the measured variables of more than two groups (Bewick et al., 2004).

The underlying assumptions for one-way analysis of variance are that the

observations are independent and randomly selected from normally distributed populations with equal variances. It is not necessary to have equal sample sizes (Bewick et al., 2004).

The null hypothesis for ANOVA is that all population means are equal; the alternative hypothesis is that at least one mean is different.

After significant effects have been formed from ANOVA, results of ANOVA will inform if the means of variable differ significantly across the measured factor, but does not inform which pairs of the factor levels are significantly different from each other (Bewick et al., 2004). Tukey's honest significance test conducts pairwise comparisons.

3.5.5 Tukey's honest significant differences (HSD) *post hoc* test

Tukey's HSD *post hoc* test is a one-step multiple comparison procedure and statistical test used in combination with an ANOVA to find out which mean is significantly different from other ones. In simple terms, ANOVA gives a measure of overall differences of variance between three or more groups while Tukey's honest significance test gives a split up of differences of variance between every other pair of groups. It applies simultaneously to the set of all pairwise comparisons and identifies where the difference between two means is greater than the standard error would be expected to allow.

3.5.6 Chi-Square test

The Chi-square test is used to detect statistical differences between observed and the expected values. For example, the frequency of each genotype of a SNP in the population is expected to follow Hardy Weinberg equilibrium (HWE), but in any study of genotype of a population group, the frequency distribution of the SNP genotype will vary. The Chi-square test is used to check if the observed frequency distribution of the SNP in the tested population varies from the expected frequency distribution of the SNP derived from HWE. Chi-square is the sum of the squared difference between observed and the expected data (or the deviation), divided by the expected data in all possible categories. The null hypothesis in Chi-square test states that there is no difference between the frequencies of observed values from the expected values. The Chi-square test can only be used for discontinuous variables.

3.5.7 Multiple stepwise linear regression

Regression analysis is a statistical method that determines the strength of the relationship between a dependent variable and a series of other changing variables known as independent variables. Regression analysis attempts to find the best straight-line relationship to explain how the variation in a dependable variable (or outcome) depends on the variation in a predictor (independent or explanatory) variable. Regression analysis helps in understanding how the value of a dependent variable (or outcome) changes when any one of the independent variables is changed, while the other independent variables are held fixed (Marill, 2004a).

Based on an analysis of the available data or sample, the technique also can be used to draw inferences about a larger population or data set, or to make predictions about future data (Marill, 2004a).

The two basic types of regression are linear regression and multiple linear regression (MLR). Linear regression uses one independent variable to explain and/or predict the outcome of a dependent variable (Marill, 2004a), while multiple regression uses two or more independent variables to predict the outcome (Marill, 2004b).

MLR is a statistical method of finding a mathematical relationship between a group of random variables. The statistical model attempts to create a relationship between groups of random variables in the form of a straight line that best approximates all of the individual data points.

The advantages of the MLR approach are that this method assists in a more accurate and precise understanding of the association of each individual factor with the outcome (Marill, 2004b). It also yields an understanding of the association of all of the factors as a whole with the outcome, and the associations between the various predictor variables themselves (Marill, 2004b).

MLR makes few assumptions. MLR is used for determining linear relationships between the predictor and criterion variable only when the relationship follows a straight line. The criterion variable should be on a continuous scale such as intervals and ratios. The predictor variable should be measured on ratios, intervals or on an ordinal scale. MLR requires large numbers of observations and certainly they should exceed the number of predictor variables which are being

used for regression. As a rough guide, at least five times more participants are required as a predictor variable with more acceptable ratio of 10:1 to 40:1.

Relationships between the predictor and criterion variable are measured by R values. R^2 indicates how good a prediction of the criterion variable we can make by knowing the predictor variable. Adjusted R^2 takes into account the number of variables in the model and number of participants present in the model.

In stepwise regression analysis, the independent variable that is believed to be the strongest predictor and correlates to the dependent variable is measured first. In a systematic process, additional dependent variables are added to the equation and the degree to which each predict the dependend variable is calculated.

3.5.8 Power Calculation

Standard deviation and *P* values are important contributors towards disproving null hypotheses. Both of these have been shown to depend strongly on the size of the study sample in question. A large sample size generally avoids generating type I error claiming an effect when there is actually no effect. Large sample sizes generally result in narrower confidence intervals and smaller *P* values (Whitley and Ball, 2002). The sample size for meaningful statistical results is therefore of importance.

The power calculation for a statistical test is needed in order to reduce the likelihood of committing a type II error, which is avoiding the statistical test to fail and to reject a false null hypothesis (Lerman, 1996). Power is the probability of rejecting the null hypothesis when the null hypothesis is false; that is, the probability of saying there is no difference when a difference actually exists (Case and Ambrosius, 2007). With an increase in the power of the statistical test the chances of the type II error decreases (Lerman, 1996). An underpowered study does not have a sufficiently large sample size to answer the research question of interest while, an overpowered study has too large a sample size and wastes resources (Case and Ambrosius, 2007). Recruiting an excessive number of participants may be unethical, particularly in a randomized control trial where an unnecessary doubling of the sample size may result in twice as many patients receiving placebo or potentially inferior care, as is necessary to establish the worth of the new therapy under consideration (Whitley and Ball, 2002).

Power calculation in the current research project was performed using Altman's Nomogram (Figure 5) for calculating sample size or power (Whitley and Ball, 2002). The acceptable target differences for each measured variable was determined. Standardised difference was calculated using the formula:

$$\text{Standardised Difference} = \frac{\text{Target difference}}{\text{Standard deviation}}$$

To calculate the power of the sample to detect variables at percentage level of significance, standardised difference with sample size was plotted on the Altman's nomogram (Figure 3.4.7). For most of the variables we equated at 90% statistical power at the p=0.01 level of significance.

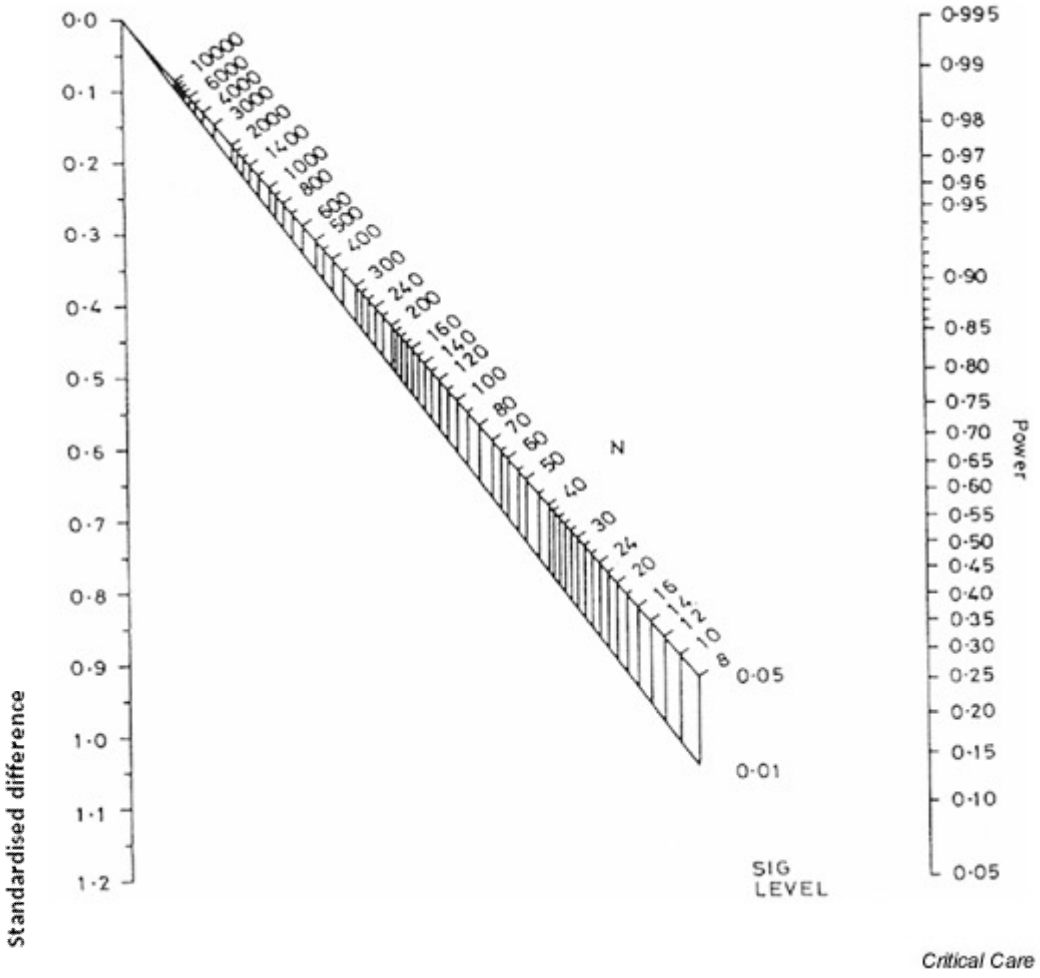


Figure 3.4.7 Nomogram for calculating sample size or power. Y-axis on the left represents standardised difference; Y-axis on the right represents power of the sample (Whitley and Ball, 2002).

Chapter 4
Results and Discussion

4.1 Demography of maternal and neonatal population

Patient Characteristics

All white Caucasian women attending UCL Hospital who fulfilled the recruitment criteria over a period between 1/4/1996 to 30/6/1997 were approached. There were 1790 eligible women for inclusion to the study, and 1650 (92%) agreed to participate (Hindmarsh et al., 2008). No demographic differences were found between those who refused to join the study and those who were recruited. As the recruitment rate was so high, this group can be deemed to be representative of the general white Caucasian population attending the hospital.

Of the 1650 women who booked, 1484 delivered a live infant, of whom 70 were preterm deliveries and 196 developed complications of pregnancy. Of the 166 who did not complete the study, 127 had moved away or been lost to follow-up, 28 had either a miscarriage or termination of pregnancy, and 11 withdrew. The maternal characteristics and demographic factors of the study group of 1484 women, who delivered a live infant, are described in Table 4.1.1. The clinical and anthropometric details of the women are shown in Table 4.1.2. In the UCL FGS cohort, Student's *t*-test was used to compare the genotyped group (n = 774) and total cohort (n = 1650) mean values to ensure there was no selection bias in those samples used for genotype analysis Table 4.1.3.

Intrauterine growth

The anthropometric measures derived from ultrasound examination at 20 and 30 wk of gestation are shown in Table 4.1.4. There were significant sex differences in abdominal circumference, biparietal diameter, and head circumference, but not femur length (Hindmarsh et al., 2002).

Table 4.1.1. Characteristics of pregnant women recruited in UCL-FGS.

	N=1650 No. (%)
Mean maternal age (SD)	30.8 (5.6)
Mean parity (SD)	0.8 (1.2)
Marital status	
Married	917 (55.6)
Single	691 (41.9)
Divorced	17 (1.0)
Widowed	2 (0.1)
Other	23 (1.4)
Cigarette smoking	314 (19.1)
Never	1188 (72)
Ex-smoker	148 (9)
<10 per day	160 (9.7)
10-20 per day	120 (7.3)
>20 per day	34 (2.1)
Alcohol consumption	570 (34.5)
Socioeconomic group (SEG)	
SEG 1	224 (13.6)
SEG 2	688 (41.7)
SEG 3	355 (21.5)
SEG 4	206 (12.5)
SEG 5	177 (10.7)

Table 4.1.2 Clinical and anthropometric details of UCL cohort of 1650 women (Hindmarsh et al., 2000).

Characteristic	**Mean (SD)***
Age (yr)	30.8 (5.6)
Height (cm)	165.5 (6.8)
Weight (kg)	64 (11.4)
Body Mass Index (kg/m^2)	23.7 (4.2)
Parity (%)	
0	870 (52.7)
1	490 (29.7)
2	164 (9.9)
3+	126 (7.6)
Gestation age at booking (wk)	12.9 (2.6)
Gestation age at delivery (wk)	39.4 (1.7)
*Except parity, which is number of women with percentage in parenthesis	

Table 4.1.3 Comparison of maternal and infant anthropometric and demographic data in the genotyped group and the total cohort in the UCL-FGS group.

	Total Cohort UCL-FGS, n=1484	Genotyped group n=774	P
Maternal Parameters			
Maternal age (y)	30.93 (5.8)	30.73 (5.5)	0.5
Maternal height (cm)	164.6 (6.9)	164.5 (6.7)	0.7
Maternal weight (kg)	63.9 (11.9)	64.0 (10.8)	0.83
Parity (%) **0** **1** **2** **3+**	 51.5 27.6 10.2 9.7	 52.3 29.1 7.5 6.2	0.4
Cigarette smoking % **None** **Stopped** **<10** **10-20** **>20**	 72.3 7.8 10.4 7.3 1.9	 74.2 8.2 8.2 8.3 1.7	0.42
Infant Parameters			
Gestation (wks)	39.4	39.5	0.5
Birth weight SDS	0.12	0.03	0.06
Birth length SDS	-0.12	-0.13	0.9
Head circumference SDS	-0.005	-0.05	0.4

Table 4.1.4. Anthropometric measures of fetal growth derived from ultrasound examination at 20 and 30 weeks of gestation (Hindmarsh et al., 2002).

	Male (M)	Female (F)	*p*
Age at Measurement **20 wk (750M:705F)** **30 wk (682M:652F)**	 20.3 (1.0) 32.2 (1.2)	 20.2 (1.0) 32.3 (1.3)	 0.5 0.04
Abdominal circumference (mm) **20 wk (748M:702F)** **30 wk (682M:652F)**	 157.8 (13.3) 288.5 (17.5)	 155.2 (12.7) 285.7 (17.9)	 <0.001 <0.001
Biparietal diameter (mm) **20 wk (748M:704F)** **30 wk (604M:568F)**	 50.1 (3.4) 85.2 (4.0)	 48.9 (3.4) 83.9 (4.2)	 <0.001 <0.001
Head circumference (mm) **20 wk (749M:704F)** **30 wk (604M:568F)**	 179.7 (12.3) 302.6 (13.3)	 176.0 (11.7) 297.8 (14.3)	 <0.001 <0.001
Femur length (mm) **20 wk (739M:690F)** **30 wk (681M:652F)**	 32.7 (3.0) 62.1 (3.1)	 32.6 (2.7) 62.1 (3.2)	 0.3 0.8

Data shown with mean with SD in parenthesis. Results adjusted to mean age at measurement. M, male; F, female

Size at birth

The anthropometric measures at birth in the 1484 infants born are detailed in table 4.1.5. Birth weight SDS (0.08, SD 1.0) and head circumference SDS (−0.03, SD 1.1) were not significantly different from the UK reference values, and length SDS (−0.1, SD 1.2) only marginally so, indicating that this population was representative, in growth terms, of the UK population (Freeman et al., 1995a). Mean placental weight was 666 g (SD 135). There were significant sex differences in birth weight, length, and head circumference with males heavier, longer, leaner (greater mid-arm circumference but smaller skinfolds), and having larger heads than females (Hindmarsh et al., 2002).

Table 4.1.5. Anthropometric measure at birth of 1484 infants from UCL-FGS live births.

	Males (n=767)	**Females (n=718)**	**Combined (n=1484)**	***p***
Weight (kg) **Weight SDS**	3.47 (0.54) 0.06 (1.0)	3.32 (0.52) 0.1 (1.0)	3.40 (0.54) 0.08 (1.0)	<0.001 0.38
Length (cm) **Length SDS**	50.4 (2.6) -0.1 (1.2)	49.5 (2.4) -0.1 (1.1)	50.0 (2.5) -0.1 (1.2)	<0.001 0.69
Head circumference (cm) **Head circumference SDS**	34.8 (1.5) -0.06 (1.2)	34.2(1.8) 0.0 (1.0)	34.5 (1.7) -0.03 (1.1)	<0.001 0.12
Mid-arm circumference (cm)	10.5 (1.0)	10.3 (1.1)	10.4 (1.0)	0.02
Triceps skinfold (mm)	5.7 (1.6)	5.8 (1.5)	5.8 (1.5)	0.3
Subscapular skinfold (mm)	5.2 (1.5)	5.4 (1.6)	5.3 (1.6)	0.03
Quadriceps skinfold (mm)	7.2 (1.9)	7.5 (1.9)	7.4 (1.9)	0.001
Placental weight (g)	673 (135)	658 (135)	666 (135)	0.17
Data shown as mean with SD in parenthesis				

Anthropometric measurements at 6 months of age

Anthropometric measurements of infants at 6 months of age are shown in table 4.1.6. There was no significant difference in weight, length and head circumference between males and females. BMI and mid-arm circumference of females was significantly less than the males.

Table 4.1.6: Anthropometric measurement of UCL-FGS infants at 6 months of age (n= 1057).

	Males (n=553)	Females (n=504)	Combined (n=1057)	*p*
Weight SDS	0.15 (1.1)	0.06 (1.1)	0.1 (1.0)	0.20
Length SDS	0.3 (1.0)	0.3 (1.3)	0.3 (1.1)	0.25
Head circumference SDS	-0.2 (1.0)	-0.2 (1.9)	-0.2 (1.5)	0.9
BMI SDS	0.17 (1.0)	-0.15 (1)	0.0 (1.0)	<0.0001
Mid-arm circumference (cm)	14.2(1.1)	13.8 (1.2)	14.0 (1.2)	<0.0001
Triceps skinfold (mm)	12.5 (2.6)	12.1 (2.4)	12.3 (2.5)	0.06
Subscapular skinfold (mm)	8.1 (1.9)	8.1 (1.9)	8.0 (1.9)	0.9
Quadriceps skinfold (mm)	21.0 (3.7)	21.2 (3.6)	21.0 (3.6)	0.68
Data shown as mean with SD in parenthesis				

Anthropometric measurement at 1 year of age

Anthropometric measurements of infants at 1 year of age are shown in table 4.1.7. There was no significant difference in weight, length and head circumference. BMI of females was significantly less than the males. Females also had significantly lower mid-arm circumference and higher quadriceps skinfold. Males were heavier (high BMI) and leaner (greater mid-arm circumference but smaller skinfolds).

Table 4.1.7. Anthropometric measurement of UCL-FGS infants at one year of age (n=979).

	Males (n=516)	Females (n=463)	Combined (n=979)	*p*
Weight SDS	0.13 (1.0)	0.16 (1.1)	0.14 (1.0)	0.62
Length SDS	0.4 (1.0)	0.4 (1.1)	0.4 (1.0)	0.96
Head circumference SDS	-0.5 (1.5)	-0.4 (1.0)	-0.4 (1.3)	0.09
BMI SDS	0.1 (1.0)	-0.12 (1.0)	0.0 (1.0)	<0.0001
Mid-arm circumference cm	15.4 (1.2)	15.1 (1.2)	15.1 (1.2)	<0.0001
Triceps skinfold (mm)	12.3 (2.4)	12.4 (2.5)	12.3 (2.5)	0.61
Sub-scapular skinfold (mm)	8.3 (2.0)	8.3 (1.9)	8.3 (2.0)	0.5
Quadriceps skinfold (mm)	19.2 (3.7)	19.9 (3.8)	19.5 (3.7)	0.004
Data shown as mean with SD in parenthesis				

Anthropometric measurement at two years of age

Anthropometric measurements of infants at two years of age are shown in table 4.1.8. There was no significant difference in weight, length and head circumference. BMI of females was significantly less than the males. Females also had significantly lower mid-arm circumference and higher quadriceps skinfold. Males were heavier (high BMI) and leaner (greater mid-arm circumference but smaller skinfolds).

Table 4.1.8. Anthropometric measurement of UCL-FGS cohort at two years of age (n=720).

	Males (n=379)	Females (n=341)	Combined (n=720)	*p*
Weight SDS	0.4 (1.0)	0.34 (1.0)	0.38 (1.0)	0.2
Length SDS	0.2 (1.0)	0.1 (1.1)	0.15 (1.0)	0.06
Head circumference SDS	-0.5 (1.0)	-0.5 (1.1)	-0.5 (1.0)	0.95
BMI SDS	0.14 (1.1)	-0.10 (1.0)	0.03 (1.1)	0.002
Mid-arm circumference cm	15.7 (1.4)	15.6 (1.3)	15.7 (1.3)	0.002
Triceps skinfold (mm)	12.4 (2.3)	12.5 (2.2)	12.4 (2.3)	0.78
Subscapular skinfold (mm)	7.1 (1.7)	7.2 (1.7)	7.2 (1.7)	0.66
Quadriceps skinfold (mm)	16.7 (3.6)	17.5 (3.3)	17.0 (3.5)	0.009
Data shown as mean with SD in parenthesis				

Anthropometric measurement at three years of age

Anthropometric and BP measurements of infants at three years of age are shown in table 4.1.9. There was significant difference in weight, length, head circumference and quadriceps skinfold. There were no significant differences in BMI and in the systolic and diastolic BP of males and females.

Table 4.1.9. Anthropometric and BP measurement of USL-FGS cohort at three years of age (n=590).

	Males (n=306)	Females (n=284)	Combined (n=590)	*p*
Weight SDS	0.1 (0.9)	-0.14 (1.0)	-0.1 (1.0)	0.004
Length SDS	0.3 (1.0)	0.1 (1.0)	0.2 (1.0)	0.01
Head circumference cm	50.8 (1.5)	49.8 (3.4)	50.3 (2.6)	0.0002
BMI SDS	0.08 (1.0)	-0.05 (1.1)	0.01 (1.0)	0.07
Mid-arm circumference SDS	16.3 (1.6)	16.1 (1.2)	16.2 (1.5)	0.14
Triceps skinfold (mm)	13.0 (5.6)	14.0 (6.5)	13.5 (6.1)	0.09
Subscapular skinfold (mm)	7.1 (4.6)	7.1 (2.0)	7.1 (3.6)	0.67
Quadriceps skinfold (mm)	17 (5.6)	18.6 (3.4)	17.8 (4.8)	0.008
Systolic BP (mmHg)	92 (11)	92 (11)	92 (11)	0.9
Diastolic BP (mmHg)	58 (9)	59 (10)	58 (9)	0.2
Data shown as mean with SD in parenthesis				

4.2 Genetic variations in the growth hormone receptor gene retention/deleted for exon three and fetal and postnatal growth

4.2.1 Introduction

The human growth hormone receptor (GHR) gene (MIM: *600946) is located on chromosome 5 (5p13.1-p12) and consists of nine coding exons numbered from 2-10 (Godowski et al., 1989; Pantel et al., 2000). Exon 2 codes for a signal peptide, the extracellular domain is coded by exons 3-7, the transmembrane domain by exon 8 and the cytoplasmic domain by exons 9 and 10 (Godowski et al., 1989; Pantel et al., 2000).

In humans, the *GHR* transcripts exist in two isoforms, retention (*GHR* fl) or deletion (*GHR* d3) of exon 3. *GHR* exon 3 skipping results from a homologous recombination of two highly homologous 251 bp repeat retroviral sequences flanking this exon in humans that mimics an alternative splicing event (Pantel et al., 2000). In the full-length version of *GHR* (*GHR* fl) the region flanked by these repeats is about 2.7 kb and contains repeat 1 of 251bp and exon 3 followed by repeat 2 of 251bp. In contrast the exon 3 deleted version (*GHR* d3) retains only repeat 2 but lacks repeat 1 and exon 3 (Figure 4.2.1). Repeat 1 and 2 are identical except for three SNPs in positions 14, 245 and 246 (Pantel et al., 2000).

Historically the exon 3 deletion was first reported as a pathological condition in two patients with growth hormone insensitivity (Godowski et al., 1989). In earlier studies a tissue specific distribution of the two isoforms was demonstrated with *GHR* d3 mainly expressed in the placenta (Urbanek et al., 1992). Subsequent studies however confirmed that these *GHR* isoforms were specific for individuals (Wickelgren et al., 1995) inherited as a Mendelian trait (Stallings-Mann et al., 1996).

Growth hormone action is mediated through the growth hormone receptor. GHR belongs to a class 1 cytokine receptor super-family with a single-pass transmembrane domain (the polypeptide crosses the lipid bilayer only once) without intrinsic tyrosine kinase activity (Waters et al., 2006).

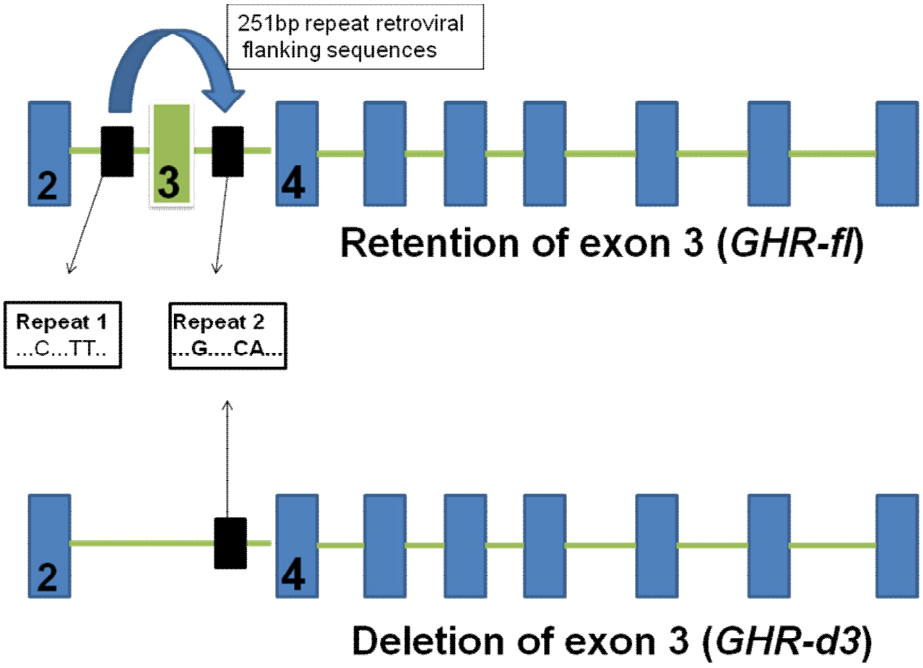


Figure 4.2.1 Illustration of *GHR* with retention and deletion of exon 3. Two retroviral repeat sequences flank the exon 3.

GHR exists as a dimer with ligand-independent dimerisation occurring in the endoplasmic reticulum before transport to the cell membrane (Gent et al., 2002). GH binding to a constitutive receptor dimer results in relative rotation of receptor subunits in the homodimer, producing realignment of Janus Kinase 2 (JAK2s) bound to the membrane-proximal sequence below the cell membrane (Waters et al., 2006). Appropriately aligned JAK2s are then able to activate each other by transphosphorylation, initiating signalling cascades (Brown et al., 2005; Waters et al., 2006). Signal transducer and activator of transcription 5B (STAT5b) is one of molecules in the signalling cascade activated by JAK2s. Postnatal growth is largely controlled by IGF-I, generated by STAT5a/b activation.

During infancy and childhood, pituitary GH and IGF-I influence growth by virtue of GH binding to the GHR. During pregnancy, expression of pituitary GH in the mother is suppressed; and placental growth hormone (hGH-V), a GH variant expressed by the placenta, becomes the predominant GH in the mother (Handwerger and Freemark, 2000). hGH-V influences placental and fetal growth by binding to the maternal and placental GHR. Maternal concentrations of hGH-V have been found to be low in growth restricted pregnancies (Bajoria et al., 2001). Severe growth restriction has been described in an infant with *hGH-V* gene deletion (Rygaard et al., 1998) but the relevance of this to the general population

is unclear.

Inherent variation in the *GHR* with insertion/deletion of an exon could explain some of the variability seen in antenatal and postnatal growth and response to recombinant human GH (r-hGH) treatment in children with growth disorders (Padidela et al., 2012).

Dos Santos et al were the first to suggest an influence of the *GHR* exon 3 deletion on the response to treatment with hGH in idiopathic short stature (ISS) and small for gestational age (SGA) children (Dos et al., 2004). They found that the *GHR* d3 isoform was associated with a 1.7 to 2 times greater growth acceleration induced by growth hormone than the *GHR* fl isoform ($P < 0.0001$) in ISS and SGA cohorts (Dos et al., 2004). No difference of *GHR* d3 frequency was found in short children compared to control adults of normal height, suggesting that this polymorphism is not primarily related to the genesis of short stature in individuals (Dos et al., 2004). This lack of effect was postulated to be secondary to compensation by endogenous pituitary growth hormone secretion, which masks the effects of the *GHR* polymorphism on basal growth rate (Dos et al., 2004).

A number of studies have addressed the possible role of the *GHR* exon 3 polymorphism on the response to rhGH treatment on diverse clinical conditions which includes GHD, SGA, ISS and Turner syndrome (Wassenaar et al., 2009). The results of these investigations have been inconsistent. Discrepancies in the results could be secondary to the limited number of patients recruited in these studies (Beavis, 1998). A recent meta-analyses of all these studies indicates *GHR* d3 to be associated with an increased response to rhGH albeit a small increase of 0.5cm in the first year of treatment (Wassenaar et al., 2009).

At present, little is known about the role of the *GHR* exon 3 polymorphism on antenatal and early postnatal growth with small sample sizes limiting interpretation (Audi et al., 2006; Jensen et al., 2007; Sorensen et al., 2010). We have evaluated the role of the *GHR* exon 3 isoform on antenatal growth, birth size, placental weight and postnatal growth up to 3 years of age in two large prospectively recruited white European cohorts.

4.2.2 Materials and methods

Study cohort

Subjects for this study were recruited from two separate white European cohorts, the University College London Hospital Fetal Growth Study (UCL-FGS) (*n* = 1484) and the Moore cohort (*n* = 310). Characteristics' of both the cohorts have been described in detail in Chapter 3.

Ethical approval for performing genetic studies was obtained from the University College London Hospitals and Queen Charlotte Hospital for Women and Hammersmith Hospital Ethics Committees. Written informed consent was obtained from all the participants on entrance into the study and then again for the participation of the infant in the study after birth. Suitable DNA samples for this study were available in 1048 infants, 774 from UCL-FGS and 274 from Moore cohort. Inclusion criteria in both cohorts were white European families presenting for the first prenatal visit before 20 weeks of pregnancy and ultrasound examination demonstrating a structurally normal single fetus. Pregnancies with antenatal complications and adverse fetal findings were excluded from the study.

Maternal weight, height, age, parity, baby's gender, birth weight, head circumference, placental weight, past and present medical history, mode and indication for delivery, pregnancy complications, smoking, diet and alcohol consumption, and partner's medical history were collected from the mother's notes from all families participating in the study. All measurements at birth were corrected for gestational age and sex by converting them into standard deviation scores (SDS).

Analysis of GH receptor isoforms

In the UCL-FGS cohort, genomic DNA was extracted from cord blood (*n* = 449) and from placenta (*n* = 325) where suitable cord blood sample was not available. In the Moore cohort, genomic DNA samples were obtained from placental tissue (*n* = 274).

For genotyping of the *GHR* exon 3 locus, a simple multiplex PCR assay was used (see methods section 3.4.5.)

Statistics

All data were assessed for normal distribution by exploring the data for skewness and kurtosis. Infants were defined as small for gestational age (SGA) if birth weight was below the 10th centile for the gestational age and large for gestational age (LGA) by birth weights above 90th centile for gestational age. An increase in weight SDS of over 0.6 in the first year of life was defined as postnatal catch-up growth, while catch-down growth was defined as a decrease in weight SDS of 0·6 over the first year of life (Dunger et al., 1998). In the UCL FGS cohort, Student's *t*-test was used to compare the total cohort (*n* = 1484) and GHR genotyped group (*n* = 774) mean values to ensure there was no selection bias in those individuals participating in the genotype analysis (Table 4.1.3).

Chi-square tests were used to compare frequency distributions of the *GHR* genotypes and to confirm if they were in Hardy–Weinberg equilibrium. Differences between the mean anthropometric measures in the *GHR exon 3* genotypes were determined by one-way Analysis of Variance (ANOVA), with the Tukey's honest significant differences (HSD) *post hoc* test. Multiple stepwise linear regression was used to determine factors influencing placental weight and birth weight SDS.

A sample size of 1048 was estimated to have a power of 90% to identify a 50 g difference in placental weight and a 100 g difference in birth weight at the 5% level of significance between the SGA and LGA groups.

4.2.3 Results

General

The UCL-FGS and Moore cohorts in combination consisted of a total of 1048 singletons with equal number of male and female infants. The UCL-FGS and Moore cohorts were similar in maternal and infant characteristics (Table 4.2.1).

Table 4.2.1. Maternal and infant anthropometric and demographic data from the UCL-FGS and the Moore cohort.

	UCL-FGS n=774	**Moore cohort n=274**
Maternal Parameters		
Maternal height (cm)	164.6 (6.90)	165.8 (6.25)
Maternal weight (kg)	63.9 (11.9)	66.8 (11.9)
Infant Parameters		
Gestation (wks)	39.4 (1.05)	39.1 (1.00)
Placental weight (g)	699.2 (136.8)	708.8 (137.7)
Birth weight SDS	0.12 (0.98)	0.17 (0.93)
Birth length SDS	-0.12 (0.75)	-
Head circumference SDS	-0.005 (0.98)	-0.01 (1.0)

Data shown as mean with SD in parentheses. UCL-FGS, University College London Hospital Fetal Growth Study; SDS, standard deviation scores.

In the UCL-FGS, there was no differences in the data between those who underwent GHR analysis (*n* = 774) and those remaining participants who did not (Table 4.1.3). There was also no difference in the demographic data between the UCL-FGS and the Moore cohort (Table 4.2.1). All the data had a normal distribution and hence log transformation was not required. Table 4.2.2 shows distribution of cigarette smoking across the *GHR* genotypes with χ^2 demonstrating no significantly different distribution. Table 4.2.3 shows distribution of parity across the *GHR* genotypes with χ^2 demonstrating no significant difference in distribution. The frequency distribution of the *GHR exon 3* genotype was as follows: fl/fl 48%, fl/d3 45% and d3/d3 7% (Table 4.2.4). Table 4.2.4 shows association of *GHR* genotype with anthropometric measures of antenatal growth (UCL-FGS) and birth parameters (UCL-FGS and Moore cohort).

Table 4.2.2. Frequency distribution of cigarette smoking across the *GHR* genotypes with χ^2 demonstrating no significantly different distribution.

	GHR genotype			
	fl/fl	fl/d3	d3/d3	Total
Smokers	47%	45%	8%	570
Non smokers	47%	49%	4%	206

χ^2=4.35; degree of freedom=5; P=0.113.

Table 4.2.3. Frequency distribution of parity across the *GHR* genotype with χ^2 demonstrating no significantly different distribution.

Parity	*GHR genotype*			
	fl/fl	fl/d3	d3/d3	Total
1	46%	45%	9%	239
2	40%	55%	5%	73
3	42%	47%	11%	36
4	63%	37%	0%	8
5	20%	80%	0%	5
6	50%	50%	0%	2
7	0%	100%	0%	1
8	0%	100%	0%	1
9	100%	0%	0%	1
Total				**776**

χ^2=13.3; degree of freedom=37; P=0.77.

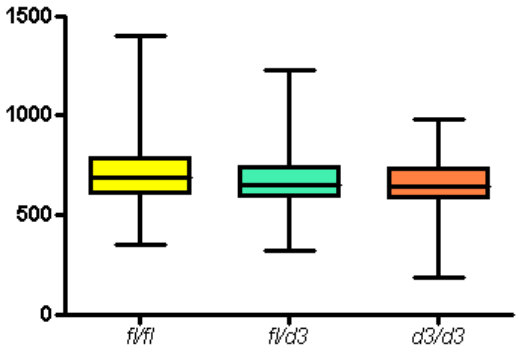
TABLE 4.2.4. Association of infant *GHR* exon 3 genotype with antenatal growth (n=774), placental weight and anthropometric measurements at birth (n=1048).

	***GHR* exon 3 genotype**				
Anthropometric measurements	***GHR* fl/fl (48%)**	***GHR* fl/d3 (45%)**	***GHR* d3/d3 (7%)**	***df***	***P* Value**
First Trimester (12-14 weeks of gestational age)					
Crown rump length (cm)	5.37 (1.74)	5.28 (1.77)	5.8 (1.79)	604 (2)	0.22
Bi parietal diameter (cm)	2.4 (0.4)	2.3 (0.46)	2.4 (0.47)	294 (2)	0.51
Second Trimester (18-20 weeks of gestational age)					
Femur length (cm)	3.3 (0.3)	3.3 (0.3)	3.2 (0.2)	750 (2)	0.97
Abdominal circumference (cm)	15.7 (1.3)	15.7 (1.3)	15.6 (1.0)	759 (2)	0.66
Bi parietal diameter (cm)	4.95 (0.32)	4.95 (0.42)	4.90 (0.22)	760 (2)	0.61
Third Trimester (30-34 weeks of gestational age)					
Femur length (cm)	6.2 (0.3)	6.2 (0.3)	6.1 (0.4)	700 (2)	0.46
Abdominal circumference (cm)	28.7 (1.8)	28.6 (1.7)	28.4 (2.1)	700 (2)	0.70
Bi parietal Diameter (cm)	8.5 (0.43)	8.4 (0.38)	8.3 (0.50)	615 (2)	0.08
Birth					
Placental weight (g)	697 (142)	665 (131)	656 (136)	990 (2)	**0.001**
Birth weight SDS	0.22 (1.0)	0.14 (0.95)	0.05 (0.90)	1045 (2)	**0.037**
Birth length SDS (UCL-FGS, n=774)	-0.04 (1.17)	-0.18 (1.14)	-0.21 (1.08)	774 (2)	0.2
Head circumference SDS	0.05 (1.13)	-0.05 (1.09)	-0.04 (0.95)	1019 (2)	0.42

Data shown as mean with SD in parentheses, P value calculated by One Way Analysis of Variance (ANOVA). P values <0.05 are underlined. df= degree of freedom within group with between group degree of freedom shown in parentheses.

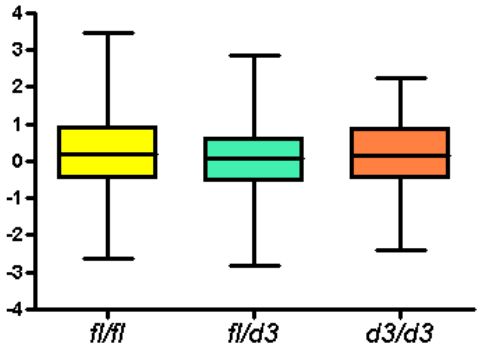
In the UCL-FGS, *GHR* genotype was not associated with anthropometric measures of antenatal growth. *GHR* exon 3 genotype was significantly associated with placental weight (one-way ANOVA $F = 7{\cdot}557$; $P = 0{\cdot}001$, df = 992) and birth weight SDS (one-way ANOVA $F = 3{\cdot}3$; $P = 0{\cdot}037$, df = 1047). In infants, placental weight and birth weight SDS were higher in the *GHR* fl/fl genotype group and lower in the *GHR* d3/d3 genotype (Table 4.2.4, Figure 4.2.2 and Figure 4.2.3). *GHR* genotype was not significantly associated with birth length SDS (one-way ANOVA $F = 1{\cdot}6$; $P = 0{\cdot}2$, df = 776) or head circumference SDS (one-way ANOVA $F = 0{\cdot}865$; $P = 0{\cdot}42$, df = 1021).

Table 4.2.5 shows the association of *GHR* genotype with postnatal growth at 6 months (n=566), 1 year (n=522), 2 years (n=440) and 3 years (n=342) of age.



X axis- placental weight in grams.
Y axis- *GHR* genotypes.

Figure 4.2.2. Association of *GHR* genotypes with placental weight.
ANOVA with post hoc test, Significant association of *GHR* isoforms with placental weight SDS p=0.037



X axis- birth weight SDS.
Y axis- *GHR* genotypes.

Figure 4.2.3. Association of *GHR* genotypes with birth weight.
ANOVA with post hoc test, Significant association of *GHR* isoforms with birth weight SDS p=0.001

Table 4.2.5. Association of infant *GHR* genotype with growth parameters from 6 months to three years of age. P value calculated by one-way analysis of variance (ANOVA).

Age	Wt SDS	Lt SDS	BMI SDS	HC SDS	MAC	TSF	SSSF	QSF	SBP	DBP
6 months	0.90	0.74	0.65	0.45	0.31	0.56	0.04	0.19	-	-
12 months	0.90	0.90	0.83	0.25	0.59	0.80	0.77	0.80	-	-
2 years	0.24	0.58	0.18	0.46	0.33	0.05	0.16	0.47	-	-
3 years	0.90	0.95	0.64	-	0.93	0.65	0.004	0.87	0.91	0.09

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF-triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; SBP-systolic blood pressure; DBP-Diastolic blood pressure.

There was a greater proportion of d3/d3 (14%) and lower proportion of fl/fl (36%) in the SGA group at birth (χ^2 = 11·2, *P* = 0·003, df = 2). In the LGA category the fl/fl genotype was overrepresented (60%) and fl/d3 and d3/d3 genotypes were underrepresented (χ^2 = 6·1, *P* = 0·047, df = 2) (Figure 4.2.4)

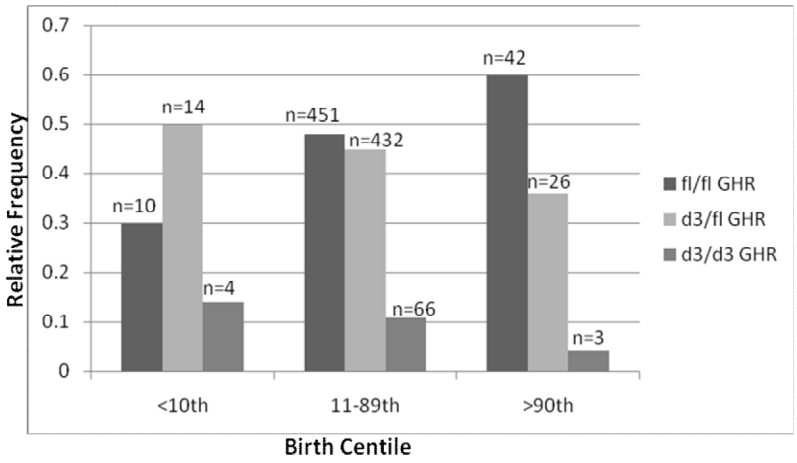


Figure 4.2.4. Frequency of *GHR exon 3* genotypes based on birth weight centile.
Chi square p value; <10th centile -0.003, >90th Centile -0.047, Degrees of freedom=2.

In multiple regression analysis (adjusted R^2 35%), *GHR* genotype, booking weight, parity and gestational age of delivery were associated with placental weight while maternal age and cigarette smoking did not influence placental weight (Table 4.2.6). In multiple regression analysis, birth weight SDS was not influenced by *GHR* genotype (Table 4.2.7).

TABLE 4.2.6. Multiple stepwise linear regression analysis of factors influencing placental weight with *GHR* genotype as one of the covariants.

Covariate	Coefficient	t-ratio	*p* value
GHR genotype	-0.13	-3.482	0.001
Booking Weight	0.12	3.15	0.002
Gestational age at delivery	0.28	7.55	0.0001
Parity	0.14	3.73	0.0001

Degree of freedom for regression= 7, residuals=664. Statistically insignificant variables excluded from the model are cigarette smoking and maternal age.

TABLE 4.2.7. Multiple stepwise linear regression analysis of factors influencing birth weight with *GHR* genotype as one of the covariants.

Covariate	Coefficient	t-ratio	*p* value
GHR genotype	-0.011	-0.18	0.858
Booking Weight	0.124	1.96	0.001
Gestational age at delivery	0.28	7.55	0.0001
Parity	0.198	3.267	0.001
Cigarette smoking	-0.088	-1.34	0.182
Maternal age	0.019	0.255	0.799

Degree of freedom for regression= 8, residuals=664. Statistically insignificant variables excluded from the model are cigarette smoking, maternal age and *GHR* genotype.

4.2.4 Discussion

In this study there was a significant association between *GHR* exon 3 genotype and placental and birth weight in a cohort of white Caucasian European pregnant women and their offspring. This genotype appears to influence intrauterine growth in late gestation, as our study demonstrated no clear effect on ultrasonographic measures of antenatal growth up to the third trimester of pregnancy (30–32 week scan). Further, the effect does not seem to be maintained into postnatal life, as no long-term effect on size could be determined nor on the catch-up and catch-down growth process.

Adequate placentation and placental growth are essential for fetal growth. There is a strong correlation between placental weight and fetal growth with most low birth infants having a small placenta (Barker et al., 1990; Godfrey et al., 1996). This implies that placental growth is linked to fetal growth. The placenta expresses GHR, which can bind to the increasing concentrations of placental growth hormone (hGH-V) encoded by chorionic somatomammotropin hormone 1 (CSH1), (MIM: *150200) during pregnancy. hGH-V is secreted by syncytiotrophoblasts and its concentration progressively rises in maternal plasma from 10–12 weeks of gestation to term (Scippo et al., 1993). hGH-V is also expressed in invasive extravillous trophoblasts suggesting that the physiological role of hGH-V, in addition to endocrine effects in the mother, might also include a direct influence on placental development via an autocrine or paracrine mechanism (Lacroix et al., 2002). hGH-V is expressed in the placenta (Frankenne et al., 1992) and could potentially influence autocrine or paracrine effects of placental or fetal GH on placental growth (Jensen et al., 2007). Circumstantial evidence for this comes from the observation that deletion of the *PGH* gene in the fetus is associated with marked fetal growth restriction and a small placenta (Chowen et al., 1996) and that the placental weight of *GHR* fl/fl group in our study was heavier than that of the *GHR* d3/d3 group.

Our data would suggest that the size of the effect of the *GHR* exon 3 isoform on birth weight is less than that upon the placenta but given the interaction of placental weight and birth size it is not easy to dissect this further. In the multiple linear regression model, placental weight is influenced by *GHR* genotype, while birth weight SDS is not, suggesting that any *GHR* genotype effect on birth weight could be mediated through the placenta.

Several reports have been published recently that investigate an association of *GHR* exon 3 isoforms and SGA infants and postnatal growth. In a Danish study, Jensen *et al.* investigated the role of *GHR* exon 3 genotype in 115 healthy adolescents who were divided into those born small for gestational age (SGA) and appropriate for gestational age with or without intrauterine growth restriction. They demonstrated an influence of the *GHR* exon 3 deleted genotype on fetal growth velocity in SGA infants and increased postnatal growth in both SGA and AGA infants (Jensen et al., 2007). There was a high prevalence (88%) of *GHR* exon 3 deleted isoforms in the group born SGA with verified intrauterine growth restriction. The *GHR* exon 3 deleted allele was associated with a decreased third-trimester fetal growth velocity (P = 0.05) in SGA subjects. In the entire cohort, carriers of the *GHR* d3 allele had a significantly increased height at adolescence (-0.10 vs. 0.34 SD score; P = 0.017) and change in height from birth to adolescence compared with carriers of the full-length *GHR* allele (0.57 vs. -0.02 SD score; P = 0.005) (Jensen et al., 2007). Furthermore in a cross-sectional study of 618 healthy boys Sorensen *et al.* have shown a lower birth weight and birth length in infants with the *GHR* d3/d3 genotype similar to the findings in our study (Sorensen et al., 2010).

However, the results of *GHR* exon 3 genotyping in the SGA population have not been consistent. A Spanish study investigated the role of *GHR* exon 3 isoforms in 289 adult subjects of normal stature born AGA and 247 short SGA children and adolescents of both sexes and found the *GHR* fl/fl genotype to be as frequent in SGA patients than in the normal stature population born SGA (Audi et al., 2006).

Unlike other studies, we could not demonstrate any effect of the *GHR* exon 3 genotype on prenatal growth up to 32 weeks of gestational age and postnatal growth. This probably reflects the fact that our cohort represents a continuum across the birth size spectrum in the general population and does not represent polarised size class groups. In addition, the larger sample size in our study reduces the likelihood of false-positive findings.

Of note, large GWAS have not found any influence of the *GHR* locus on height or metabolic parameters (Lettre, 2011). This could simply mean that *GHR* variation does not significantly influence growth or the SNP that tags *GHR* exon 3 in the GWAS does not recapitulate all the genomic variability at the locus, including the

d3 status, other copy number variations, or additional rare variants (Bougneres, 2010).

In conclusion, we found that the *GHR* exon 3 polymorphism genotype is associated with placental weight and birth weight with carriers of the *GHR* fl/fl allele having a heavier placenta and higher birth weight. Placental weight could be potentially influenced by an autocrine/paracrine action of hGH-V or other products of the chorio-somatomammotropic gene on the *GHR* polymorphism present in the placenta.

4.3 Genetic variations in the *IGF1* gene and fetal and postnatal growth

4.3.1 Introduction

Insulin-like growth factor 1 (*IGF1*; OMIM*147440, NM_000618, gene map locus: 12q22-q24.1) is an important hormone for fetal and postnatal growth. Fetal IGF-I is expressed from the earliest pre-implantation stage right through to the final phase of tissue maturation before birth (Rotwein et al., 1987). From as early as the first trimester, receptors for IGFs have been identified in the human fetus which would allow IGF-I to exert growth-promoting effects on fetal tissue (Sara et al., 1983). Plasma IGF-I concentrations are responsive to glucose and oxygen concentrations in the fetus, IGF-I therefore acts as a nutrient sensor that ensures fetal growth is commensurate with the nutrient supply (Fowden and Forhead, 2009a).

Targeted deletion of *IGF1* and *IGF1R* genes in mice leads to a 40% and 55% reductions in birth weight, respectively. Infants born with rare defects of the *IGF1* gene are small at birth, with a particular reduction in head size (Bonapace et al., 2003; Woods et al., 1996). Mutation of the type 1 IGF receptor (*IGF1R*) gene also causes intrauterine and postnatal growth retardation (Abuzzahab et al., 2003). Studies in mice and observations in humans support the concept that the IGF system is an important mediator of embryonic, fetal and postnatal growth.

Common polymorphisms in genes regulating expression of the gene encoding IGF-I could relate to size at birth (Dunger et al., 2007). Several studies have addressed this. For example, polymorphisms in *IGF1* gene have been investigated in relation to birth size. Polymorphisms of a common *IGF1* promoter CA repeat, have been reported to be associated with size at birth (Johnston et al., 2003; Vaessen et al., 2002). In addition, polymorphism in the promoter region associates with plasma IGF-I concentration and body composition parameters in Japanese infants (Kinoshita et al., 2007). In SGA infants and those with the lowest birth weight, small head size has been found to be associated with the rs35767 SNP in the *IGF1* gene (Ester and Hokken-Koelega, 2008). These results have however not been consistently replicated in other studies (Vella et al., 2008).

Although the role of genetic variations in the *IGF1* gene on fetal growth have been investigated on birth size in AGA and SGA infants, the role of these genetic variations in different phases of fetal and postnatal growth have not been studied in detail. We have therefore investigated common genetic variations in the *IGF1* gene and their relationship to fetal growth, birth size, postnatal growth and BP at 3 years of age.

4.3.2 Material and Methods

Subjects for this study were recruited from the University College London Hospital Fetal Growth Study (UCL-FGS). Suitable DNA samples for this study were available in 774 infants. Details of the cohort are provided in Chapter 3.

Choice of SNPs

The NCBI SNP database (dbSNP, http://www.ncbi.nlm.nih.gov/snp) lists 1740 SNPs in the human *IGF1* gene locus. Of these 21 are present in the coding region (16 nonsynonymous and 5 synonymous). For the SNPs to influence size and to provide sufficient power for sample size we have investigated SNPs with minor allele frequency (MAF) of greater than 5%. The choice of SNPs were also based on selected tag SNPs which are in linkage disequilibrium on the *IGF1* gene from genotype data in the CEU panel (Americans of European ancestry) of the phase II HapMap Project (Lettre et al., 2007). Previously 22 SNPs in the *IGF1* gene were identified with this approach (Lettre et al., 2007). For genotyping we have selected nine SNPs in the *IGF1* gene locus which have homozygous MAF of >5%. The SNPs chosen are described in table 5.1 and shown in figure 4.3.1. One of the selected SNPs, rs35767, is in linkage disequilibrium with the 192 bp CA-repeat in the *IGF1* promoter region (Ester et al., 2009b). The *IGF1* promoter 192 bp CA-repeat (also known as *IGF1* 737/738) is one of the most commonly investigated polymorphisms in the *IGF1* gene and has been found to be associated with low birth weight, low adult height, low serum IGF-1 concentrations and a higher risk for diabetes mellitus and myocardial infarction, although conflicting results have been reported (Ester et al., 2009b).

Genotype methods

Genotyping for SNPs on the *IGF1* gene was performed by KBioscience Competitive Allele Specific PCR genotyping System (KASP) analysis. The details of the method adopted have been described in Chapter 3.4.4.

Statistics

All data were assessed for normal distribution by exploring the data for skewness and kurtosis. Infants were defined as small for gestational age (SGA) and large for gestational age (LGA) if birth weights were below the 10th centile or above the 90th centile, respectively, for gestational age. Postnatal catch-up or catch-down growth was defined as an increase or decrease in weight SDS of 0·6 over the first year of life (Dunger et al., 1998).

Chi-square tests were used to compare frequency distributions of the *IGF1* genotypes. One-way Analysis of Variance (ANOVA), with the Tukey's honest significant differences (HSD) *post hoc* test, was used to determine differences between the mean anthropometric measures found with the different *IGF1* genotypes.

A sample size of 774 was estimated to have a power of 90% to evaluate a 35 g difference in placental weight and a 75 g difference in birth weight at the 1% level of significance between the SGA and LGA groups

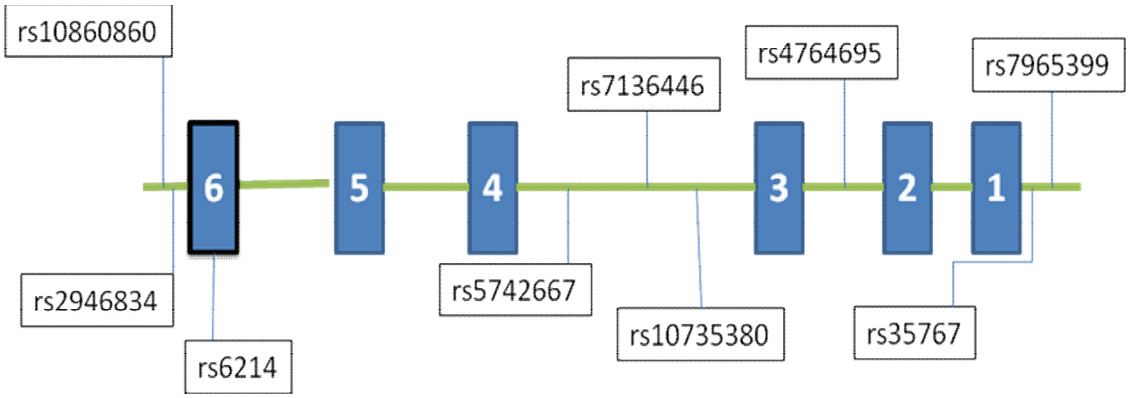


Figure 4.3.1. Schematic scale diagram of the human *IGF1* gene with position of the SNPs genotyped. Although not to scale, the relative spacing of exons are represented.

4.3.3 Results

The genotype frequency for the nine SNPs tested and χ^2 test for Hardy-Weinberg equilibrium are provided in Table 4.3.1. All the tested SNPs were in Hardy Weinberg equilibrium.

Table 4.3.1 Genotype frequencies of SNPs in the *IGF1* gene and Hardy Weinberg's equilibrium test. P value calculate by χ^2 test.

SNP	N	1/1 (%)	1/2(%)	2/2(%)	P value for H-W
rs6214 1=G;2=A	774	33	50	17	0.81
rs35767 1=C;2=T	728	71	27	2	0.83
rs2946834 1=C;2=T	771	56	33	11	0.80
rs4764695 1=A;2=G	768	23	60	17	0.75
rs5742667 1=C;2=T	774	54	32	14	0.09
rs7136446 1=T;2=C	774	39	35	26	0.92
rs7965399 1=T;2=C	773	92	7	1	0.31
rs10735380 1=A;2=G	774	54	32	14	0.6
rs10860860 1=A;2=T	767	47	29	24	0.60

Antenatal growth

Table 4.3.2 provides data of association of the *IGF1* genotype with antenatal growth parameters measured by ultrasonography. Some growth parameters showed statistically significant association with the *IGF1* genotypes. None of the growth parameters however demonstrated consistent significant longitudinal association across the three trimesters (Table 4.3.2).

Table 4.3.2. Association of infant *IGF1* genotype with antenatal growth (*n* = 774). P value calculated by one-way analysis of variance (ANOVA).

IGF1 SNP	First Trimester (12-14 wk GA)		Second Trimester (18-20 wk GA)			Third trimester (30-34 wk GA)		
SNPs	**CRL**	**BPD**	**FL**	**AC**	**BPD**	**FL**	**AC**	**BPD**
rs6214	0.27	0.30	0.87	0.60	0.68	0.94	0.99	0.29
rs35767	0.35	0.79	0.97	0.79	0.54	0.63	0.38	0.35
rs2946834	0.84	0.59	0.84	0.99	0.84	0.38	0.99	0.26
rs4764695	0.91	0.73	**0.01**	**0.01**	0.36	0.13	0.61	0.85
rs5742667	0.48	092	0.26	0.38	0.12	0.12	0.11	0.85
rs7136446	**0.004**	0.99	0.06	0.27	0.06	0.09	0.42	0.50
rs7965399	0.66	0.77	0.80	0.59	0.70	0.98	**0.01**	0.41
rs10735380	0.30	**0.03**	0.84	0.89	0.09	0.89	0.84	0.29
rs10860860	0.62	0.12	0.47	0.75	0.43	0.51	0.76	0.14

SNP- single nucleotide polymorphism; CRL- crown rump length; BPD-biparietal diameter; FL-femoral length; AC- abdominal circumference; P values <0.05 are underlined.

Anthropometric parameters at birth

Table 4.3.3 provides data of the association of *IGF1* genotype with growth parameters at birth. There were no significant associations of the *IGF1* genotypes with birth weight SDS, birth length SDS and placental weight. The *IGF1* SNP rs10735380 was significantly associated with head circumference SDS (AA: 0.07 ± 1.1, AG: -0.15 ± 1.1, GG: 0.001 ± 1.1; df=762; F=2.95 p = 0.05) at birth and with cord serum IGF-I concentrations (AA: 68.5 ± 2.4, AG: 68.4 ± 2.7, GG: 77.8 ± 2.9; df=716; F=5.4 p = 0.005). *IGF1* SNPs rs7965399 was associated with cord serum GH (TT: 30.1 ± 2.4, TC: 39.0 ± 3.2, TT: 40.1 ± 1.5; df=707; F=3.7; p = 0.025), rs10860860 with the cord serum IGFBP-III (AA: 965.8 ± 27.9, AT: 918.5 ± 26.1, TT: 910.2 ± 26.7; df=706; F=3.2 p = 0.043) and cord serum insulin concentrations (AA: 7.7 ± 6.7, AT: 8.0 ± 8.6, TT: 6.2 ± 5.2; df=688; F=3.6 p = 0.028).

The proportion of genotypic distribution in SGA, AGA and LGA infants and association of genotypes in catch-up and catch-down growth are presented in Table 4.3.4. Some of the *IGF1* genotypes were present in significantly different proportions in the SGA infants (rs4764695, rs7965399, rs10735380 and rs10860860), AGA infants (rs10735380 and rs10860860) and in LGA infants (rs35767, rs10735380 and rs10860860). *IGF1* SNPs rs4764695 and rs10735380 were significantly associated with catch-up growth.

Table 4.3.3. Association of infant *IGF1* genotype with growth parameters at birth (*n* = 774). P value calculated by one-way analysis of variance (ANOVA).

SNP	Bw SDS	Bl SDS	HC SDS	Pw	CIgf1	CIgf2	CGh	CIgfBp3	CIns
rs6214	0.91	0.29	0.68	0.13	0.48	0.48	0.34	0.74	0.74
rs35767	0.43	0.73	0.13	0.99	0.50	0.96	0.52	0.42	0.29
rs2946834	0.49	0.88	0.08	0.28	0.25	0.99	0.70	0.83	0.24
rs4764695	0.68	0.12	0.77	0.19	0.21	0.60	0.40	0.60	0.49
rs5742667	0.87	0.49	0.59	0.27	0.47	0.27	0.85	0.73	0.80
rs7136446	0.67	0.46	0.61	0.40	0.76	0.45	0.06	0.37	0.70
rs7965399	0.35	0.64	0.63	0.24	0.23	0.44	**0.03**	0.53	0.94
rs10735380	0.41	0.16	**0.05**	0.09	**0.005**	0.29	0.61	0.68	0.50
rs10860860	0.32	0.60	0.27	0.30	0.27	0.32	0.32	**0.04**	**0.03**

SNP- single nucleotide polymorphism; BW- birth weight; Bl- birth length; HC- head circumference; Pw- placental weight; CIgf1- cord insulin-like growth factor-1, CIgf2- cord insulin-like growth factor 2; CGh- cord growth hormone; CIgfBp3- cord insulin-like growth factor 3; CIns- cord insulin; P values <0.05 are underlined.

Table 4.3.4. Association of infant *IGF1* genotype with SGA, AGA and LGA at birth and with catch-up and catch-down growth in the first year of age. P value calculated by χ^2 test.

SNP	SGA	AGA	LGA	Catch-up	Catch-down
rs6214	0.84	1.0	0.92	0.45	0.81
rs35767	0.59	0.97	**0.0001**	0.34	0.4
rs2946834	0.94	0.75	0.18	0.44	0.57
rs4764695	**0.01**	0.72	0.44	**0.005**	0.46
rs5742667	0.42	0.63	0.15	0.08	0.17
rs7136446	0.78	0.97	0.7	0.81	0.78
rs7965399	**0.0001**	0.91	0.17	0.44	0.44
rs10735380	**0.0015**	**0.0009**	**0.0001**	**0.04**	0.44
rs10860860	**0.0002**	**0.02**	**0.03**	0.97	0.62

SNP- single nucleotide polymorphism; SGA- small for gestational age; LGA- large for gestational age; P values <0.05 are underlined.

Postnatal growth and BP at 3 years of age

The association of the *IGF1* genotype with growth parameters at 6 months, 12 months, 2 years and 3 years of age are provided in tables 4.3.5, 4.3.6, 4.3.7 and 4.3.8 respectively. The number of subjects available for these analyses reduced to 73% of the original birth cohort at 6 months, 66% at 1 year, 57% at 2 years and 44% at 3 years.

Some of the *IGF1* genotypes demonstrated significant association of *IGF1* genotype with anthropometric parameters. SNP rs7136446 was significantly associated with triceps and quadriceps skinfolds at 6 months, 1 year and 2 years of age.

IGF1 SNP rs10735380 was significantly associated with systolic BP (AA:91.7 ± 11.6, AG: 92.5 ± 9.5, GG: 87.0 ± 10.6; df=205; F=3.0 p = 0.05) while SNP rs7136446 (p=0.05) was significantly associated with diastolic BP (TT:57.9 ± 8.0, TC: 60.48 ± 11.0, TT: 56.80 ± 6.9, df=207; F=3.0; p = 0.05).

Table 4.3.5 Association of infant *IGF1* genotype with growth parameters at 6 months of age (n = 566). P value calculated by one-way analysis of variance (ANOVA).

SNP	Wt SDS	Lt SDS	BMI SDS	HC	MAC	TSF	SSSF	QSF
rs 6214	0.98	0.94	0.74	0.41	0.51	0.73	0.80	0.76
rs 35767	0.51	0.60	0.80	0.66	0.74	**0.04**	0.38	0.60
rs 2946834	0.44	0.66	0.49	0.25	0.71	0.77	0.63	0.97
rs 4764695	0.16	0.30	0.40	0.31	0.60	0.17	0.74	0.16
rs 5742667	0.68	0.57	0.31	0.45	0.56	0.97	0.60	0.92
rs 7136446	0.23	0.81	0.21	0.62	0.06	**0.03**	0.71	**0.01**
rs 7965399	0.93	0.39	0.61	0.29	0.51	0.23	0.57	0.80
rs 10735380	0.53	0.13	0.15	0.92	0.23	0.23	0.60	0.41
rs 10860860	0.80	0.32	0.88	0.88	0.72	0.06	0.65	0.25

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF-triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; P values <0.05 are underlined.

Table 4.3.6 Association of infant *IGF1* genotype with growth parameters at one year of age (*n* = 512). P value calculated by one-way analysis of variance (ANOVA).

SNP	Wt SDS	Lt SDS	BMI SDS	HC	MAC	TSF	SSSF	QSF
rs 6214	0.79	0.61	0.99	0.66	0.55	0.81	0.66	0.30
rs 35767	0.34	0.72	0.96	0.82	0.93	0.15	0.15	0.80
rs 2946834	0.71	0.21	0.87	0.21	0.29	0.22	0.66	0.50
rs 4764695	0.10	0.09	0.58	0.10	0.26	0.83	0.95	0.37
rs 5742667	0.46	0.46	0.37	0.87	0.54	0.28	0.16	0.76
rs 7136446	0.92	0.40	0.11	0.52	0.11	**0.04**	0.25	0.07
rs 7965399	0.82	0.41	0.28	0.85	0.14	0.81	0.08	0.64
rs 10735380	0.13	0.36	0.17	0.63	0.56	0.95	0.21	0.60
rs 10860860	0.74	0.95	0.66	0.70	0.76	0.20	0.51	0.26

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF-triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; P values <0.05 are underlined.

Table 4.3.7 Association of infant *IGF1* genotype with growth parameters at two years of age (*n* = 440). P value calculated by one-way analysis of variance (ANOVA).

SNP	Wt SDS	Lt SDS	BMI SDS	HC	MAC	TSF	SSSF	QSF
rs 6214	0.97	0.63	0.83	0.15	0.99	0.84	0.56	0.23
rs 35767	0.47	0.24	0.91	0.67	0.82	0.57	0.43	0.82
rs 2946834	0.41	0.53	0.52	**0.006**	0.77	0.58	0.81	0.94
rs 4764695	0.23	0.75	0.20	0.49	0.96	1.0	0.27	0.14
rs 5742667	0.95	0.92	0.79	0.22	0.39	0.73	0.16	0.74
rs 7136446	0.14	0.35	0.13	0.16	**0.03**	**0.016**	0.67	**0.03**
rs 7965399	0.68	0.83	0.74	0.39	0.94	0.06	0.31	0.23
rs 10735380	**0.006**	0.06	0.09	0.27	0.79	0.74	0.29	0.39
rs 10860860	0.56	0.67	0.57	0.72	0.09	0.49	0.68	0.54

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF-triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold P values <0.05 are underlined.

Table 4.3.8 Association of *IGF1* genotype with growth parameters (*n* = 342) and blood pressure (n=210) at three years of age. *P* value calculated by one-way analysis of variance (ANOVA).

SNP	Wt SDS	Lt SDS	BMI SDS	HC	MAC	TSF	SSSF	QSF	SBP	DBP
rs 6214	0.10	**0.004**	0.62	**0.05**	0.42	**0.01**	0.70	0.86	0.55	0.26
rs 35767	0.83	0.35	0.35	0.32	0.69	0.45	0.42	0.34	0.23	0.10
rs 2946834	0.12	0.14	0.44	0.79	0.63	0.92	0.94	0.23	0.70	0.95
rs 4764695	0.35	0.70	0.44	**0.05**	0.18	**0.01**	0.13	0.68	0.71	0.07
rs 5742667	0.24	0.60	0.31	0.20	0.26	0.20	0.34	0.69	0.66	0.80
rs 7136446	0.42	0.38	0.29	0.58	0.70	0.20	0.92	0.24	0.09	**0.05**
rs 7965399	0.86	0.59	0.45	0.87	**0.01**	0.27	0.41	0.67	0.86	0.89
rs 10735380	0.13	0.30	0.30	0.33	0.81	0.55	0.43	0.56	**0.05**	0.66
rs 10860860	0.21	0.54	0.15	0.23	0.67	0.46	0.94	0.14	0.56	0.50

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF-triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; SBP-systolic blood pressure; DBP-diastolic blood pressure; P values <0.05 are underlined.

4.3.4 Discussion

In a cohort of white European pregnant women and their offspring, this study reports findings of the association of *IGF1* genotypes with antenatal growth, birth size, postnatal growth parameters and body composition until 3 years of age, catch-up growth and blood pressure at 3 years of age. We did not find any significant positive association between *IGF1* genotypes and antenatal growth, nor consistent associations between the *IGF1* genotypes and postnatal growth parameters until 3 years of age. Some of the genotypes were represented in greater proportions in SGA (rs4764695, rs7965399, rs10735380 and rs10860860), AGA infants (rs10735380 and rs10860860) and in LGA infants (rs35767, rs10735380 and rs10860860). The *IGF1* SNPs rs2946834 and rs10735380 were significantly associated with catch-up growth.

Previous studies investigated the association of antenatal growth and postnatal growth with genetic variations of *IGF1* genotypes have mainly investigated polarised groups such as SGA infants while this study cohort represents a continuum across the birth size spectrum in the general population.

IGF1 genotypes, fetal growth and birth size

In the UCL-FGS cohort, detailed ultrasound assessment of fetal growth in each trimester is available. This provided an opportunity to ascertain the role of *IGF1* genotypes during different stages of fetal growth. In the UCL-FGS cohort, no clear and consistent association of *IGF1* genotypes were found on serial ultrasonographic measures of antenatal growth and size at birth. This suggests that the selected genetic variations in the *IGF1* gene do not influence antenatal growth or birth size.

The 192-bp CA repeat polymorphism located 841 bp upstream from exon 1 is by far the most extensively investigated polymorphism in the promoter region. The CA repeat polymorphism in the *IGF1* gene is located near the promoter region with the dinucleotide repeats ranging from 10 to 24 in number (i.e. 20-48 base pairs). The most common allelic variant in the white Caucasian population contains 19 CA repeats (Rietveld et al., 2003). RNA polymerase binds to the promoter region facilitating DNA transcription. Genetic variations of the promoter region have the potential to influence gene function and account for genetic variability in the population. High linkage disequilibrium exists from the promoter

area through into intron 3 (Zhao et al., 2007). In the UCL-FGS study we have investigated the *IGF1* genotype SNP rs35767 that is in linkage disequilibrium with the 192-bp allele (Ester et al., 2009b) and found no association of this genotype with antenatal growth and size at birth.

Similar to the UCL-FGS cohort, the Generation R Study prospectively collected data from fetal life to early childhood in a cohort consisting of 738 children. In this cohort no association was found between the *IGF1* 192-bp allele and the birth weight and length. Compared to carriers of homozygous and heterozygous 192-bp alleles, noncarriers of the 192-bp allele demonstrated small mid-pregnancy fetal size followed by an increased growth rate from mid-pregnancy to early infancy (Geelhoed et al., 2008). In our study, we did find association of *IGF1* genotypes rs4764695 with second trimester femur length and abdominal circumference, rs7136446 with first trimester crown rump length, rs10735380 with first trimester biparietal diameter and rs7965399 with third trimester abdominal circumference. However, none of the associations were consistent in longitudinal measurements and hence we conclude that the associations are likely to be chance findings.

Several studies have investigated the relationship between birth weight and the common 192-bp genetic polymorphism on the *IGF1* promoter (Arends et al., 2002; Johnston et al., 2003; Vaessen et al., 2002). Vaessen et al studied four hundred and sixty three participants from a sub-group of the Rotterdam Study, which recruited 1110 subjects. Absence of the 192-bp allele was associated with a 215 g reduction in birth weight compared with individuals homozygous for the allele (Vaessen et al., 2002). An association of the 192-bp allele genotype was also found with postnatal weight gain. Birth weight data in this study was collected retrospectively and was not adjusted for gestational age while in the UCL-FGS cohort, longitudinal data was collected and adjusted for age. While the UCL-FGS cohort consisted of uncomplicated pregnancies, subjects in the Rotterdam study were derived from a population with diabetes with some of the pregnancies complicated by maternal diabetes, which in itself influences birth weight.

In another Dutch study te Velde et al found a gender specific influence of the *IGF1* 192 bp allele in the Amsterdam Growth and Health Longitudinal study cohort (te Velde et al., 2005). Males (n=112) carriers of variants of the CA repeat

allele had a 0.2 kg lower birth weight than men with the common type allele.

Kytnarova et al investigated 196 AGA and 26 SGA infants born at term in a cohort derived from the Czech Republic. The average birth weight and length in AGA wild type (CA)19 homozygotes of the 192-bp promoter allele were lower in comparison with AGA carriers of various (CA)n polymorphisms (Kytnarova et al., 2009). The observed anthropometric differences however disappeared at the age of 18 months (Kytnarova et al., 2009). No association of the 192-bp allele polymorphism was found in the SGA infants at birth and at 12 months of age.

Results of Dutch (te Velde et al., 2005; Vaessen et al., 2002) and Czech (Kytnarova et al., 2009) studies were however, not replicated in other cohorts. In a UK population of 640 individuals no measures of fetal growth, including birth weight were associated with the *IGF1* 192-bp allele (Frayling et al., 2002). In another UK cohort of 627 men and 426 women, representing the Hertfordshire, cohort in which the birth weight associations with late onset diseases was first identified, no association of the *IGF1* CA repeat 192-bp allele was found with birth weight and catch-up growth. Similarly, in a Japanese cohort of 160 subjects, no association of the IGF1 CA repeat 192-bp allele was found with birth weight. Landmann et al investigated association of the 192-bp allele in a cohort of 768 children recruited in Germany (Landmann et al., 2006). Cohort characteristics were similar to the UCL-FGS cohort with children from white Caucasian background born to mothers with uncomplicated pregnancies. No association of the 192-bp allele was found with birth weight and length SDS (Landmann et al., 2006). The genotype was however significantly associated with catch-up growth with the *IGF1* 192-bp allele represented less frequently in children with accelerated weight gain. Similarly Johnston et al showed that the region ranging from 1.2 kb in the promoter until intron 2 was associated with catch-up growth of the SGA-born child (Johnston et al., 2003).

Other studies have not shown consistent significant effect of other IGF1 SNPs on growth (Ester and Hokken-Koelega, 2008).

In the UCL-FGS SNPs rs4764695, rs10860860, rs10735380 and rs7965399 were significantly overrepresented in the SGA population while SNPs rs10860860, rs10735380 and rs35767 were significantly overrepresented in the LGA population. In the UCL-FGS numbers of SGA and LGA infants are too small to derive any meaningful conclusion from these data.

IGF1 genotypes and head circumference

The *IGF1* gene has an important role in brain growth. Overexpression of *IGF1* in transgenic mice spares brain growth retardation caused by undernutrition in early postnatal life (Lee et al., 1999). IGF-I functions in an insulin-like manner to increase brain glucose utilisation during brain development (Cheng et al., 2000).

The association of head size with *IGF1* genotypes has been investigated. In a Japanese study of 160 infants, the CA repeat 192/192-bp homozygous in the promoter region were associated with a larger head circumference and increased serum IGF-I concentrations. In 635 white Caucasian SGA infants, Ester et al reported an association of SNP rs35767 with small head size and less brain sparing, particularly in those with the lowest birth weight (Ester et al., 2009b). In two European SGA cohorts strong linkage disequilibrium was found between the promoter region until intron 2 (Johnston et al., 2003). Within this haplotype block, *IGFI.PCRI* genotype was associated with a smaller head circumference at 1.3 years of age. Eusar et al in their cohort of 285 individuals born at a gestational age <32 weeks found homozygotes for the 192-bp allele had a slower cranial growth from birth until age 5 years, and a tendency towards less brain sparing and slower linear growth compared to the other two genotype groups (Euser et al., 2011).

In the UCL-FGS we have not demonstrated any association of SNP rs35767 (in linkage disequilibrium with IGFI.PCRI and the promoter CA repeat 192-bp alleles) with fetal head size (BPD), head size at birth or at 3 years of age. In our study, associations of head size were noted with rs2946834 at 2 years of age and SNPs rs6214 and rs4764695 at 3 years of age. Previous associations of *IGF1* genotypes and head circumference have been reported in polarised population of SGA infants (Ester et al., 2009b; Johnston et al., 2003) or in a general population with a small sample size (Kinoshita et al., 2007). The UCL-FGS has a larger sample size and represents the general population with continuum of birth weight. SNPs associated with head circumference in our study may reflect their association at different stages of growth and development or could be simply represent a chance finding as none showed a longitudinal association during antenatal or postnatal growth.

IGF1 genotypes and cord serum IGF-I concentration

Cord serum IGF-I concentrations was available in infants from the of UCL-FGS cohort. Therefore the present study has investigated the association of selected *IGF1* SNPs with cord serum IGF-I concentration. SNPs rs10735380 was significantly associated with the cord serum IGF-I concentration implying that it may influence serum IGF-I concentration. The SNP rs10735380 is associated with head circumference at birth, weight SDS at 2 years of age, catch up growth and the genotype has increased representation in the SGA, AGA and LGA population. These association could be mediated through influence of SNP rs10735380 in modulating IGF-I concentration.

Most association studies of *IGF1* gene and serum IGF-I concentration have focused on the 192-bp CA repeat genotype. Kinoshita et al found a positive association of 192-bp CA repeats with serum IGF-I concentrations in 160 neonates (Kinoshita et al., 2007). IGF-I concentrations in this study were not adjusted for age and sex of the infants. Frayling et al, in their study of young adult population (n=640), found carriers of the homozygous 192-bp allele had lower serum IGF-I concentrations, whereas height did not differ between the genotypic groups (Frayling et al., 2002). Results of the association of *IGF1* genotypes with serum IGF-I concentration have not been consistent with conflicting reports. Arends et al found no association of serum IGF-I concentrations with the 192-bp allele in 124 short SGA trios (Arends et al., 2002). Johnston et al could not find any association between the 192-bp allele and serum IGF-I concentration in a short SGA population during childhood and in a control population in late puberty (Johnston et al., 2003).

IGF1 genotypes, postnatal catch-up growth and body composition

IGF-I is an important growth factor for postnatal growth. Variations in the *IGF1* gene can influence early postnatal growth, the alterations in which can increase the risk for insulin resistance and related diseases (Euser et al., 2005). Landmann et al found an increased risk for rapid weight gain in early infancy in non-carriers of the *IGF1* 192-bp allele (Landmann et al., 2006). Geelhoed et al in the Generation R study also demonstrated increased growth rate from mid-pregnancy to early infancy in non-carriers of IGF1 192-bp allele (Geelhoed et al., 2008). In a recent Dutch study, Kharagjitsingh et al similarly showed an influence

of *IGF1* promoter genetic variation on early childhood growth in 271 children (146 type 1 diabetes patients and 125 siblings) with non-carriers of the IGF1*194 allele positively associated with accelerated first year growth in both patients and siblings, independent of disease (Kharagjitsingh et al., 2012). No association of this genotype was found with development of diabetes in children (Kharagjitsingh et al., 2012).

In the UCL-FGS cohort we have found a significant associations of *IGF1* SNP rs4764695 (p=0.005) and SNP rs10735380 (p=0.04) with catch-up growth. The *IGF1* SNP rs10735380 was also associated with cord serum IGF-I concentration with higher IGF-I concentrations in carriers of rare allele GG who were also represented in higher proportion in infants with catch-up growth implying that it may have a role in influencing early postnatal growth.

Unfavourable body composition is an independent risk factor for the development of the metabolic syndrome. In the UCL-FGS *IGF1* SNP rs7136446 was significantly associated with various parameters of body composition upto 2 years of age. Association between the *IGF1* gene polymorphism and body fatness was examined by Voorhoeve et al in two comparable young Dutch cohorts with a generational difference of around 20 years (older cohort n= 359; younger cohort n=258) (Voorhoeve et al., 2006). They found that body weight, BMI, fat mass, waist circumference and hip circumference were higher in female variant carriers of the *IGF1* polymorphism in the younger cohort but not in the older cohort (Voorhoeve et al., 2006). They hypothesise that these differences possibly reflect a gene–environmental interaction of this polymorphism and that an environment that promotes obesity leads to a more pronounced fat accumulation in carriers of this IGF-I polymorphism (Voorhoeve et al., 2006). Maas et al however, were unable to demonstrate the role of *IGF1* promoter genotype with childhood body composition in 627 children from the Generation R study (Maas et al., 2010). Similarly, in the UCL-FGS we were unable to demonstrate the influence of body composition in a cohort similar to the Generation R study.

IGF1 genotypes and early childhood blood pressure (BP)

In the UCL-FGS cohort BP measurements were available in children at 3 years of age (n=207). *IGF1* SNP rs10735380 (p=0.05) was significantly associated with systolic BP while SNP rs7136446 (p=0.05) was significantly associated with diastolic BP. The *IGF1* SNP rs10735380 was also significantly associated with cord serum IGF-I concentrations. Higher systolic BP (AA: 91.7 ± 11.6, AG: 92.5 ± 9.5, GG: 87.0 ± 10.6; df=205; F=3.0, *p*=0.05) was noted in genotypes associated with lower IGF-I concentrations (AA: 68.5 ± 2.4, AG: 68.4 ± 2.7, GG: 77.8 ± 2.9; df=716; F=5.4, *p*=0.005) implying that IGF-I concentration may have an inverse relationship with systolic blood pressure. This is in keeping with previous observations that IGF-I concentrations in the lower end of the normal range increase the risk of cardiovascular diseases (Ezzat et al., 2008). As the *IGF1* SNP rs10735380 was associated with catch-up growth (p=0.04) and with weight at 2 years of age (p=0.006), association with BP could be an effect of size.

The 192-bp allele on the IGF1 promoter gene is the most commonly investigated variation on early childhood BP. In the Generation R study (n=538) significantly lower systolic and diastolic BP was found in the non-carriers of 192-bp alleles (van, V et al., 2008). No association of the genotype was found with left heart dimensions at 2 years of age. Landmann et al in a German cohort of 768 infants however, did not find any association of BP with *IGF1* promoter variations.

Studies in adults have demonstrated an association of the *IGF1* promoter genotype with increased risk of mortality in patients with myocardial infarction (Yazdanpanah et al., 2006) and an increased risk of heart failure in subjects aged 55-75 years (Bleumink et al., 2004). The common variant -1411C>T in the IGF1 upstream promoter P1 has significantly reduced activity and is associated with a 27% lower risk of hypertension as well as lower SBP and DBP in normotensive individuals (n=745 hypertensive patients; n=769 normotensive control subjects) (Telgmann et al., 2009). Normotensive subjects carrying the protective T allele displayed a significant decrease in diastolic (P=0.036) and systolic (P=0.024) blood pressure. This may relate to decreased local IGF-I expression in blood vessels(Telgmann et al., 2009).

Genetic variation studies on BP are best performed in early childhood, as at later age environmental factors can influence BP. While in the UCL-FGS cohort and in the Generation R Study BP was measured within 3 years of age, Landmann et al

measured BP at 6 years of age. Influence of *IGF1* genotypes could be age related or the association obtained could be a chance finding especially as numbers in each cohort were small.

Summary discussion and conclusion

In this study, several *IGF1* genotypes have been found to be associated with parameters of fetal growth (rs4764695, rs7136446, rs10735380 and rs7965399), catch-up growth (rs4764695) and with postnatal anthropometry (rs5742667, rs2946834 and rs6214). We anticipate that if the *IGF1* gene polymorphisms were truly associated with growth parameters in the antenatal and postnatal period, associations with longitudinally measured growth patterns would be stronger than with only one or two growth measurements. With the decrease in the cohort size seen with age, the number of subjects in our follow up studies may be too small to show the longitudinal effects. For every genetic association study, there is always a possibility that association has arisen by chance, especially if they are novel (Ester et al., 2009a). It is likely therefore that some of these associations occur by chance, as none of the associations are consistently present in longitudinal measurements at later ages.

Although data are limited, several of the SNPs have shown multiple and longitudinal associations such as rs10735380 (IGF-I concentration, head circumference at birth, catch-up growth, weight at 2 years of age and systolic BP at 3 years of age) and rs7136446 (body composition at 6 months, 1 and 2 years of age). It could be hypothesised that these SNPs influence serum IGF-I concentration either directly or are in linkage disequilibrium with another regulatory element which may be directly influencing *IGF1* transcription. With age the cohort size of UCL-FGS decreases and hence numbers are small to make any meaningful conclusion from these associations. Chance findings can therefore not be excluded. Studies in larger cohorts are required to confirm association of these genotypes with anthropometric parameters and BP. Furthermore, we have only selected tagging *IGF1* SNPs with MAFs >5%. There is still a possibility that rare SNPs <5% MAF may influence growth. To investigate such an association, a very large cohort size would be required for sufficient statistical power to be achieved.

Absence of association of *IGF1* genotypes with pre- and postnatal growth in UCL-FGS cohort are similar to genome wide association studies (GWAS) which found no association of *IGF1* genotypes with birth weight (Freathy et al., 2010) or adult height (Lettre, 2011). GWAS have been performed in larger cohorts and hence have the statistical power to prove or disprove association. Unlike our study, GWAS, do not have follow-up data on patient cohorts because of practical difficulties of longitudinally collecting data on a very large population size. Most GWAS approaches are therefore less able to assess the role of genotypes at different phases of antenatal and postnatal growth.

GWAS relies on tagging SNPs to scan the whole genome. Because of practical difficulties of performing longitudinal studies on a large cohort, most of the GWAS are based on point measurements. There is also a possibility that in GWAS selected tagging SNPs may not represent the genetic variability of the whole of *IGF1* gene and hence may fail to identify actual associations. Longitudinal studies on larger cohort sizes with advanced genotyping techniques may help in further elucidating the association of *IGF1* genotypes with genetic variability on antenatal and postnatal growth and early childhood BP.

4.4 Genetic variations in the *IGF2* gene and fetal and postnatal growth

4.4.1 Introduction

Insulin-like growth factor II (*IGF2*; OMIM*147470, NM_000612, gene map locus: 11p15.5), also known as somatomedin A, is an important hormone for fetal growth. The *IGF2* gene spans over 30 kb and consists of 9 exons and five promoters (Monk et al., 2006). Exons 7, 8 and 234 nucleotides of exon 9, codes for the human IGF-II preprohormone (Fig. 4.4.1). Exons 1 and 6 are non-coding and form a 5'-untranslated region of the *IGF2* RNA. By initiating transcripts at five different promoter regions, the *IGF2* gene generates multiple mature transcripts with different 5' untranslated regions (UTRs) but identical coding regions and 3' UTRs (Fig. 4.4.1) (Nielsen, 1992). Initiation of transcripts from the five promoter regions is imprinted and occurs in a tissue- and development-specific way (Vu and Hoffman, 1994).

IGF-II is a 67 amino acid (7.5kDa) plasma protein. It is produced by post-translation cleavage of the 180 amino acid IGF-II preprohormone that contains a carboxy-terminal peptide of 89 amino acid and a signal peptide of 24 amino acids. It has structural and functional homology with insulin and IGF-I.

IGF-II has a critical role in fetal anabolism. Production of IGF-II in the fetus is induced by placental growth hormone and placental lactogen. It is widely distributed in human fetal tissues and is present in high concentration in the fetus, suggesting that it has an important role in fetal anabolism dependent on the availability of insulin and nutrients (Han et al., 1987). IGF-II binds to two structurally similar and high affinity IGF receptors, type 1 and type 2. Binding of IGF-II to type 1 receptor mediates many of its biological effects. The type 2 receptor also known as IGF/mannose-6-phosphate receptor is involved in internalisation and degradation of IGF-II (Humbel, 1990). Both type 1 and type 2 receptors are abundantly present in embryonic and fetal tissues where they influence IGF mediated fetal and placental growth.

The use of genetically modified mice has helped elucidate the importance of IGF-II and its receptors. The birth weight of *Igf2* null mice is reduced by 40%, with *Igf2* null mice exhibiting smaller placentas (Baker et al., 1993; DeChiara et al., 1990).

IGF-II mRNA is abundantly expressed in placenta at all gestational ages, throughout the chorionic villi, chorionic plate, basal plate, and fetal membranes (Han et al., 1996). Placental IGF-II mRNA correlates positively with placental weight (Liu et al., 1996). Deletion of the *Igf2* gene in the placenta reduces placental weight by 30–50% near term (Fowden and Forhead, 2009b).

The physiological role of IGF-II in postnatal period is poorly understood. Rather high levels of IGF-II persist in adult human serum, whereas, in rodents, IGF-II concentrations are very low (Rogler et al., 1994). Transgenic mice with elevated concentrations of IGF-II are smaller than controls, their lean body mass is reduced by 5-8%, whereas fat mass is reduced between 44 and 77% (Rogler et al., 1994).

Numerous studies have investigated an association between birth weight or adult body mass and variations in the *IGF2* gene, although replication has not been consistent (Adkins et al., 2010; Gaunt et al., 2001; Gomes et al., 2005; Gu et al., 2002; Kaku et al., 2007; Nagaya et al., 2009; Rodriguez et al., 2006; Zhang et al., 2006). This study has therefore investigated common genetic variations in the *IGF2* gene and their relationship to fetal growth, birth size, postnatal growth and blood pressure at 3 years of age.

4.4.2 Material and Methods

Subjects for this study were recruited from the University College London Hospital Fetal Growth Study (UCL-FGS). Suitable DNA samples for this study were available in 774 infants. Details of the cohort are provided in Chapter 3

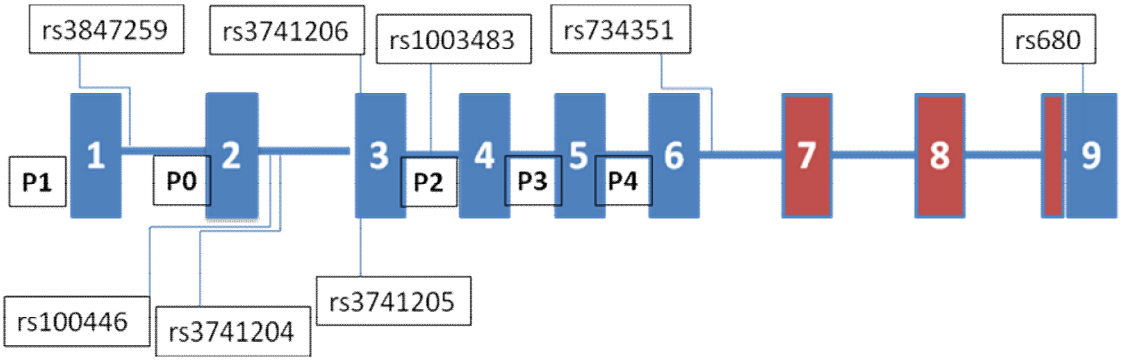


Figure 4.4.1- Schematic scale diagram of the human *IGF2* gene with positions of the SNPs genotyped. Although not to scale, the relative spacing of exons are represented. The coding exons 7, 8 and 234bp of 9 are shaded. Five promoter regions of the IGF2 gene are indicated P0-P4.

Choice of SNPs

The NCBI SNP database (dbSNP, http://www.ncbi.nlm.nih.gov/snp) lists 481 SNPs in human *IGF2* gene. Of these 44 are present in the coding region (24 nonsynonymous and 20 synonymous). For the SNPs to influence size and to provide sufficient power for sample size we have investigated only SNPs with minor allele frequencies (MAF) greater than 5%. SNPs were selected based on previously published associations with birth weight or body mass related phenotypes or for the purpose of tagging haplotypes (Table 4.4.1; Fig 4.4.1) (Gaunt et al., 2001; Gomes et al., 2005; Gu et al., 2002; Kaku et al., 2007). The choice of SNPs was therefore based on selected tag SNPs that were in linkage disequilibrium on the *IGF2* gene from the genotype data of the CEU panel (Americans of European ancestry) of the phase II HapMap Project.

Genotype methods

Genotyping for SNPs on the *IGF2* gene was performed by restriction fragment length polymorphism analysis, which has been described in Chapter 3.4.2. Genotyping of rs680 was also performed by KBioscience Competitive Allele Specific PCR genotyping System (KASP) analysis. Details of the method adopted have been described in Chapter 3.4.4. The genotypes of control samples and the rare allele AA of SNP rs680 were further confirmed by direct sequencing.

Statistics

All data were assessed for normal distribution by exploring the data for skewness and kurtosis. Infants were defined as small for gestational age (SGA) and large for gestational age (LGA) if birth weights were below the 10th centile or above the 90th centile, respectively, for gestational age. Postnatal catch-up or catch-down growth was defined as an increase or decrease in weight SDS of 0·6 over the first year of life (Dunger et al., 1998).

Chi-square tests were used to compare frequency distributions of the *IGF2* genotypes. One-way Analysis of Variance (ANOVA), with the Tukey's honest significant differences (HSD) *post hoc* test, was used to determine differences between the mean anthropometric measures in the *IGF2* genotypes. Multiple stepwise linear regression was used to determine factors influencing placental weight and birth weight SDS.

A sample size of 774 was estimated to have a power of 90% to identify a 35 g difference in placental weight and a 75 g difference in birth weight at the 1% level of significance between the SGA and LGA groups.

4.4.3 Results

Genotype frequency for the eight SNPs tested in the *IGF2* gene and χ^2 test for the Hardy-Weinberg equilibrium are provided in Table 4.4.1. All the tested SNPs except rs3741206 (p=0.001) were in Hardy-Weinberg equilibrium.

Table 4.4.1. Genotype frequencies and Hardy-Weinberg equilibrium test of *IGF2* genotypes. P value calculated by χ^2 test.

SNP	N	1/1 (%)	1/2(%)	2/2(%)	P value for H-W
rs1004446 1=T;2=C	778	23	45	32	0.57
rs3741204 1=A;2=G	778	51	34	15	0.70
rs3741205 1=T, 2=G	778	48	42	10	0.24
rs3741206 1=A;2=G	782	68	22	10	0.0015
rs1003483 1=G;2=T	779	15	50	35	0.1612
rs734351 1=C;2=T	775	18	52	30	0.36
rs3842759 1=A;2=T	776	59	35	6	0.97
rs680 1=G;2=A	743	52	40	8	0.99

Antenatal growth

Table 4.4.2 shows the association of the *IGF2* genotype with antenatal growth parameters measured by ultrasonography. *IGF2* SNP rs680 was significantly associated with third trimester abdominal circumference and biparietal diameter (Table 4.4.2 and 4.4.5).

Table 4.4.2. Association of infant *IGF2* genotype with antenatal growth (*n* = 774). P value calculated by ANOVA.

IGF1 SNP	First Trimester (12-14 wk GA)		Second Trimester (18-20 wk GA)			Third trimester (30-34 wk GA)		
SNPs	**CRL**	**BPD**	**FL**	**AC**	**BPD**	**FL**	**AC**	**BPD**
rs1004446	0.38	0.24	0.47	0.39	0.50	0.72	0.18	0.52
rs3741204	0.92	0.63	0.22	0.44	0.57	0.74	0.67	0.84
rs3741205	0.73	0.60	0.12	0.82	0.64	0.30	0.57	0.78
Rs3741206	0.62	0.64	0.60	0.53	0.98	0.79	0.93	0.75
rs1003483	0.61	0.65	0.22	0.32	0.80	0.51	0.47	0.81
rs734351	0.60	0.97	0.90	0.48	0.94	0.44	0.81	0.09
rs3842759	0.22	0.66	0.23	0.39	0.99	0.85	0.82	0.51
rs680	0.15	0.90	0.70	0.53	0.95	0.27	**0.008**	**0.03**

SNP- single nucleotide polymorphism; CRL- crown rump length; BPD-biparietal diameter; FL-femoral length; AC- abdominal circumference; P values <0.05 are underlined.

Anthropometric parameters at birth

Table 4.4.3 provides data of the association of the *IGF2* genotype with growth parameters at birth. ANOVA testing demonstrated significant association of SNP rs680 with birth weight SDS, birth length SDS, head circumference SDS, placental weight, cord IGFI and IGFBP3 concentrations (Table 4.4.3 and 4.4.5). Compared to the AA genotype, infants with the genotype GG had a higher placental weight of 58g; birth weight of 0.62 SDS (300g); birth length of 0.63 SDS (1.2cm) and head circumference of 0.12 SDS (0.25cm).

The *IGF2* SNP rs3741204 was also significantly associated with birth length SDS (AA: -0.01 ± 1.1, AG: -0.25 ± 1.2, GG: 0.25 ± 1.1; df=754; F=3.8 *p* = 0.023); rs3741206 with head circumference SDS (AA: 0.01 ± 1.1, AG: 0.05 ± 1.2, GG: -0.3 ± 1.1; df=754; F=3.67 *p* = 0.04) and cord insulin concentration (AA: 7.8 ± 7.3, AG: 6.7 ± 6.7, GG: 5.7 ± 6.3; df=696; F=3.18 *p* = 0.042) and rs1004446 with cord serum IGF-I concentration (TT: 71.5 ± 26.5, TC: 70.9 ± 26.4, CC: 65.0 ± 24.8; df=712; F=3.68 *p* = 0.025).

The proportion of genotype distribution in SGA, AGA and LGA infants and association of genotypes in catch-up and catch-down growth are presented in the Table 4.4.4. *IGF2* genotypes rs680 (χ^2=80.1, df=2, p=0.0001), rs3741205 (χ^2=9.02, df=2, p=0.011) and rs3842759 (χ^2=6.28, df=2, p=0.049) were significantly associated with SGA infants while rs680 (χ^2=6.28, df=2, p=0.043) and rs3741205 (χ^2=6.63, df=2, p=0.036) were significantly associated with LGA infants.

Tables 4.4.6 to 4.4.12 provide data on multiple stepwise linear regression models of the association of the *IGF2* rs680 genotype with anthropometric data at birth. Using this model, birth weight (Table 4.4.6) and placental weight (Table 4.4.7) were significantly influenced by SNP rs680 while birth length SDS (Table 4.4.8), head circumference SDS (Table 4.4.9), cord serum IGF-I (Table 4.4.10), IGFBP-3 (Table 4.4.11) and IGF-II (Table 4.4.12) concentrations were not.

Table 4.4.3. Association of infant *IGF2* genotype with growth parameters at birth (*n* = 774). P value calculated by ANOVA.

SNP	Bw SDS	Bl SDS	HC SDS	Pw	CIgf1	CIgf2	CGh	CIgfBp3	CIns
rs1004446	0.22	0.41	0.26	0.78	**0.03**	0.29	0.70	0.76	0.58
rs3741204	0.74	**0.02**	0.44	0.57	0.38	0.21	0.33	0.57	0.76
rs3741205	0.22	0.08	0.66	0.66	0.29	0.50	0.42	0.27	0.55
rs3741206	0.17	0.19	**0.04**	0.88	0.90	0.29	0.66	0.90	**0.04**
rs1003483	0.14	0.67	0.16	0.15	0.06	0.34	0.08	0.28	0.16
rs734351	0.44	0.97	0.74	0.11	0.55	0.50	0.16	0.78	0.42
rs3842759	0.91	0.85	1.00	0.94	0.80	0.17	0.07	0.25	1.00
rs680	**0.0001**	**0.001**	**0.006**	**0.02**	**0.013**	0.35	0.15	**0.02**	0.19

SNP- single nucleotide polymorphism; BW- birth weight; Bl- birth length; HC- head circumference; Pw- placental weight; CIgf1- cord insulin-like growth factor-1, CIgf2- cord insulin-like growth factor 2; CGh- cord growth hormone; CIgfBp3- cord insulin-like growth factor 3; CIns- cord insulin; P values <0.05 are underlined.

Table 4.4.4. Association of infant *IGF2* genotype with SGA, AGA and LGA at birth and with catch-up and catch-down growth in the first year of age. P value calculated by χ^2 test.

SNP	SGA	AGA	LGA	Catch-up	Catch-down
rs1004446	0.40	0.96	0.22	0.74	0.86
rs3741204	0.64	0.94	0.15	0.95	0.95
rs3741205	**0.011**	0.96	**0.036**	0.94	0.91
Rs3741206	0.25	1.0	0.40	0.90	0.25
rs1003483	0.08	0.97	0.45	0.98	0.81
rs734351	0.19	1.0	0.83	0.58	0.71
rs3842759	**0.049**	0.86	0.10	0.62	0.59
rs680	**0.0001**	0.93	**0.043**	0.9	0.5

SNP- single nucleotide polymorphism; SGA- small for gestational age; LGA- large for gestational age; P values <0.05 are underlined.

TABLE 4.4.5. Association of infant *IGF2 rs680* genotype with antenatal growth, placental weight and anthropometric measurements at birth (n=774).

	***IGF2* rs680 genotype**				
Anthropometric measurements	**GG (48%)**	**AG (45%)**	**AA (7%)**	***df***	***p* Value**
First Trimester (12-14 wks of gestational age)					
Crown rump length (cm)	5.5 (1.8)	5.2 (1.7)	5.6 (1.8)	576 (2)	0.15
Bi parietal diameter (cm)	2.3 (0.5)	2.4 (0.43)	2.4 (0.3)	286 (2)	0.90
Second Trimester (18-20 wks of gestational age)					
Femur length (cm)	3.24 (0.2)	3.24 (0.2)	3.22 (0.3)	717 (2)	0.97
Abdominal circumference (cm)	15.6 (1.1)	15.7 (1.1)	15.6 (1.1)	727 (2)	0.53
Bi parietal diameter (cm)	4.93 (0.30)	4.92 (0.35)	4.93 (0.34)	727 (2)	0.95
Third Trimester (30-34 wks of gestational age)					
Femur length (cm)	6.2 (0.3)	6.2 (0.2)	6.1 (0.3)	673 (2)	0.27
Abdominal circumference (cm)	28.7 (1.5)	28.4 (1.5)	28.2 (1.4)	673 (2)	**0.008**
Bi parietal diameter (cm)	8.44 (0.35)	8.38 (0.32)	8.37 (0.43)	588 (2)	**0.03**
Birth					
Placental weight (g)	676 (135)	668 (134)	618 (130)	990 (2)	**0.02**
Birth weight SDS	0.23 (1.0)	0.07 (1.0)	-0.39 (1.0)	1045 (2)	**0.001**
Birth length SDS	0.00 (1.1)	-0.18 (1.2)	-0.63 (1.0)	1018 (2)	**0.001**
Head circumference SDS	0.11 (1.0)	-0.13 (1.1)	-0.01 (1.0)	1019 (2)	**0.006**

Data shown as mean with SD in parentheses, P value calculated by One Way Analysis of Variance (ANOVA). P values <0.05 are underlined. df= degree of freedom within group with between group degree of freedom shown in parentheses.

TABLE 4.4.6. Multiple stepwise linear regression analysis of factors influencing birth weight SDS with *IGF2* genotype rs680 as one of the covariants.

Covariate	Coefficient	t-ratio	*p* value
Placental weight	0.57	18.3	0.0001
Gestational delivery	-0.3	-9.8	0.0001
Booking Weight	0.13	4.47	0.0001
Cigarette smoking	-0.12	-3.95	0.001
Parity	0.12	3.74	0.001
***IGF2* genotype rs680**	**-0.1**	**-3.02**	**0.003**
Maternal age	0.07	2.06	0.04

Dependent variable: Birth weight SDS, (R^2 = 0·44; P = 0·0001, df = 7)

TABLE 4.4.7. Multiple stepwise linear regression analysis of factors influencing placental weight with *IGF2* genotype rs680 as one of the covariants.

Covariate	Coefficient	t-ratio	*p* value
Gestational delivery	0.3	7.4	0.0001
Birth weight	0.28	6.9	0.0001
Parity	0.12	1.80	0.07
Booking Weight	0.11	2.91	0.004
***IGF2* genotype rs680**	**-0.08**	**-2.3**	**0.021**

Dependent variable: Placental weight, (R^2 = 0·10; P = 0·023, df = 5). Statistically insignificant variables excluded from the model are cigarette smoking and maternal age.

TABLE 4.4.8. Multiple stepwise linear regression analysis of factors influencing birth length SDS with *IGF2* genotype rs680 as one of the covariants.

Covariate	Coefficient	t-ratio	*p* value
Birth Weight SDS	0.39	10.58	0.0001

Dependent variable: birth length, (R^2 = 0·40; P = 0·98, df = 4). Statistically insignificant variables excluded from the model are placental weight, cigarette smoking, maternal age, parity, gestational age of delivery and IGF2 rs680 genotype.

TABLE 4.4.9. Multiple stepwise linear regression analysis of factors influencing head circumference SDS at birth with *IGF2* genotype rs680 as one of the covariants.

Covariate	Coefficient	t-ratio	*p* value
Birth Weight SDS	0.56	18.3	0.0001
Maternal age	0.10	3.20	0.001

Dependent variable: head circumference, (R^2 = 0·34; P = 0·98 df =4). Statistically insignificant variables excluded from the model are placental weight, cigarette smoking, maternal age and IGF2 rs680 genotype.

TABLE 4.4.10 Multiple stepwise linear regression analysis of factors influencing IGF-I concentration with *IGF2* genotype rs680 as one of the covariants.

Covariate	Coefficient	t-ratio	*p* value
Birth Weight SDS	0.421	11.65	0.0001
Parity	0.11	3.0	0.003
Gestational delivery	0.09	-2.42	0.02

Dependent variable: Cord serum IGF-I concentration, (R^2 = 0·23; P = 0·98 df =4). Statistically insignificant variables excluded from the model are placental weight, cigarette smoking, maternal age and IGF2 rs680 genotype.

Table 4.4.11. Multiple stepwise linear regression analysis of factors influencing IGFBP-3 concentration with *IGF2* genotype rs680 as one of the covariants.

Covariate	Coefficient	t-ratio	*p* value
Birth Weight SDS	0.39	10.58	0.0001
Gestational delivery	0.08	2.24	0.025

Dependent variable: Cord IGFBP-3 concentration, (R^2 = 0·15; P = 0·98, df = 4). Statistically insignificant variables excluded from the model are placental weight, cigarette smoking, maternal age, parity and IGF2 rs680 genotype.

Table 4.4.12. Multiple stepwise linear regression analysis of factors influencing IGF-II concentration with *IGF2* genotype rs680 as one of the covariants.

Covariate	Coefficient	t-ratio	*p* value
Birth Weight SDS	0.16	4.01	0.0001
Gestational delivery	0.08	2.26	0.024

Dependent variable: Cord serum IGF-II concentration, (R^2 = 0·15; P = 0·13, df = 4). Statistically insignificant variables excluded from the model are placental weight, cigarette smoking, maternal age, parity and IGF2 rs680 genotype.

Postnatal growth and BP at 3 years of age

The associations of the *IGF2* genotype with growth parameters at 6 months, 12 months, 2 years and 3 years of age are provided in tables 4.4.13, 4.4.14, 4.4.15 and 4.4.16, respectively. Numbers of subjects recruited were reduced to 73% at 6 months, 66% at 1 year, 57% at 2 years and 44% at 3 years of the original birth cohort. The *IGF2* SNP rs680 was significantly associated with weight SDS, BMI

SDS and mid-arm circumference at 6 months of age and with weight SDS, BMI SDS, head circumference, triceps skin fold, sub-scapular skin fold and quadriceps skin fold at 3 years of age. However, no significant association of the SNP with growth parameters at 1 year and 2 years were demonstrated.

The *IGF2* SNPs rs3842759 was significantly associated with systolic BP and SNP rs3741204 with diastolic BP at 3 years of age.

Table 4.4.17 provides data of association of anthropometric measurements at 3 years of age with *IGF2* rs680 polymorphism. Compared to the AA genotype, children with the GG genotype at 3 years of age had statistically significant greater weight of 0.8 SDS; BMI of 0.91 SDS, head circumference of 2 cm; triceps skin fold thickness of 0.1cm and quadriceps skin fold thickness of 0.3 cm.

Some of the *IGF2* SNPs have shown cross sectional association with anthropometric parameters at different ages of early childhood growth (Tables 4.4.13-4.4.16). At 6 months of SNP rs3741205 was significantly associated with sub-scapular skin fold thickness (TT: 0.8 ± 0.18, TG: 0.85 ± 0.20, GG: 0.78 ± 0.17; df=548; F=4.05 p = 0.018) and rs3842759 with triceps skin fold thickness (AA: 1.24 ± 0.25, AT: 1.19 ± 0.24, TT: 1.25 ± 0.21; df= 533; F=3.8; p = 0.02). At one year of age rs1004446 was significantly associated with length SDS (TT: 0.51 ± 1.1, TC: 0.23 ± 1.0, CC: 0.43 ± 1.0; df=503; F=3.3 p = 0.036). At two years of age rs1004446 was significantly associated with head circumference SDS (TT: -0.43 ± 1.1, TC: -0.46 ± 1.1, CC: 0.73 ± 0.92; df=422; F=3.0 p = 0.049) and rs3741204 with length SDS (AA: -0.01 ± 1.1, AG: 0.31 ± 1.1, GG: 0.21 ± 1.2; df=427; F=3.3 p = 0.036) and triceps skin fold thickness (AA: 1.26 ± 0.24; AG: 1.26 ± 0.24, GG: 1.16 ± 0.24; df=401; F=3.8 p = 0.014). At three years of age *IGF2* SNPs rs3741204 was significantly associated with quadriceps skin fold (AA: 1.26 ± 0.24; AG: 1.26 ± 0.24, GG: 1.16 ± 0.24; df=275; F=3.08 p = 0.048) and SNP rs1004446 with triceps skin fold thickness (TT: 1.34 ± 0.25, TC: 1.29 ± 0.22, CC: 1.26 ± 0.18; df=279; F=3.66 p = 0.027).

Table 4.4.13. Association of infant *IGF2* genotype with growth parameters at six months of age (*n* = 566). P value calculated by ANOVA.

SNP	Wt SDS	Lt SDS	BMI SDS	HC	MAC	TSF	SSSF	QSF
rs1004446	0.42	0.10	0.16	0.41	0.44	0.92	0.45	0.94
rs3741204	0.38	0.13	0.99	0.11	0.62	0.54	0.15	0.80
rs3741205	0.81	0.50	0.68	0.58	0.23	0.32	**0.02**	0.44
Rs3741206	0.33	0.44	0.54	0.33	0.53	0.45	0.53	0.95
rs1003483	0.54	0.60	0.10	0.96	0.50	0.93	0.20	0.31
rs734351	0.63	0.59	0.97	0.65	0.16	0.38	0.23	0.68
rs3842759	0.31	0.55	0.42	0.38	0.13	**0.02**	0.11	0.20
rs680	**0.01**	0.43	**0.001**	0.54	**0.03**	0.25	0.31	0.23

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF-triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; P values <0.05 are underlined.

Table 4.4.14. Association of infant *IGF2* genotype with growth parameters at one year of age (*n* = 512). P value calculated by ANOVA.

SNP	Wt SDS	Lt SDS	BMI SDS	HC	MAC	TSF	SSSF	QSF
rs1004446	0.28	**0.04**	0.92	0.94	0.41	0.99	0.57	0.67
rs3741204	0.20	0.24	0.68	0.06	0.35	0.90	0.07	0.49
rs3741205	0.85	0.96	0.90	0.57	0.97	0.46	0.67	0.82
Rs3741206	0.18	0.26	0.40	0.59	0.09	0.40	0.63	0.43
rs1003483	0.66	0.79	0.44	0.92	0.63	0.54	0.65	0.62
rs734351	0.81	0.88	0.39	0.85	0.64	0.46	0.91	0.26
rs3842759	0.44	0.63	0.52	0.89	0.49	0.29	0.42	0.68
rs680	0.16	0.68	0.14	0.81	0.64	0.57	0.40	0.45

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF-triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; P values <0.05 are underlined.

Table 4.4.15. Association of infant *IGF2* genotype with growth parameters at two years of age (*n* = 440). P value calculated by ANOVA.

SNP	Wt SDS	Lt SDS	BMI SDS	HC	MAC	TSF	SSSF	QSF
rs1004446	0.41	0.20	0.97	**0.05**	0.17	0.42	0.77	0.63
rs3741204	0.30	**0.04**	0.48	0.09	0.11	**0.01**	0.12	0.54
rs3741205	0.84	0.79	0.90	0.61	0.87	0.45	0.41	0.19
Rs3741206	0.17	0.64	0.09	0.33	0.87	0.13	0.20	0.26
rs1003483	0.86	0.61	0.97	0.66	0.94	0.43	0.70	0.65
rs734351	0.76	0.74	0.35	0.35	0.75	0.13	0.59	0.10
rs3842759	0.39	0.09	0.97	0.66	0.19	0.72	0.33	0.38
rs680	0.25	0.46	0.27	0.52	0.33	0.89	0.38	0.76

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF-triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; P values <0.05 are underlined.

Table 4.4.16 Association of *IGF2* genotype with growth parameters (*n* = 342) and blood pressure (n=210) at three years of age. P value calculated by ANOVA.

SNP	Wt SDS	Lt SDS	BMI SDS	HC	MAC	TSF	SSSF	QSF	SBP	DBP
rs1004446	0.31	0.17	0.33	0.52	0.54	**0.03**	0.60	0.24	0.89	0.98
rs3741204	0.35	0.06	0.97	0.41	0.36	0.87	0.64	**0.05**	0.15	**0.03**
rs3741205	0.29	0.62	0.20	0.10	0.88	0.44	0.32	0.63	0.92	0.29
rs3741206	0.12	0.70	0.06	0.09	0.56	0.31	0.19	0.61	0.12	0.74
rs1003483	0.91	0.65	0.40	0.33	0.77	0.45	0.21	0.67	0.31	0.86
rs734351	0.42	0.47	0.56	0.88	0.76	0.60	0.17	0.42	0.97	0.98
rs3842759	0.10	0.19	0.36	0.70	0.47	**0.02**	0.28	0.38	**0.02**	0.24
rs680	**0.0001**	0.392	**0.0001**	**0.002**	0.88	**0.002**	0.06	**0.01**	0.15	0.35

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF-triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; SBP-systolic blood pressure; DBP-Diastolic blood pressure; P values <0.05 are underlined.

TABLE 4.4.17. Association of infant *IGF2* rs680 genotype with anthropometric measurements at three years of age (n=342) and blood pressure (n=210). P value calculated by ANOVA.

	IGF2 rs680 genotype				
Anthropometric parameters	**GG (48%)**	**AG (45%)**	**AA (7%)**	***df***	***p* Value**
Weight SDS	0.13 (1.0)	-0.09 (0.9)	-0.67 (0.9)	328 (2)	**0.0001**
Length SDS	0.28 (1.1)	0.16 (0.9)	0.05 (1.0)	576 (2)	0.392
BMI SDS	0.16 (1.0)	-0.03 (0.9)	-0.75 (0.9)	286 (2)	**0.0001**
Head circumference (cm)	50.3 (1.4)	50.3 (1.3)	48.3 (8.1)		**0.002**
MAC (cm)	27.8 (1.4)	31.7 (1.6)	15.5 (1.2)	717 (2)	0.88
TSF (cm)	1.3 (0.25)	1.3 (0.2)	1.2 (1.7)	727 (2)	**0.002**
SSSF (cm)	7.6 (0.5)	6.7 (0.2)	6.0 (0.1)	727 (2)	0.06
QSF (cm)	1.9 (0.7)	1.7 (0.3)	1.6 (0.2)		**0.01**
SBP (mmHg)	92 (11)	90 (9)	96 (13)	673 (2)	0.15
DBP (mmHg)	59 (9)	58 (8)	61 (13)	673 (2)	0.35

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF-triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; SBP-systolic blood pressure; DBP-Diastolic blood pressure; P values <0.05 are underlined.

4.4.4 Discussion

In a cohort of white European pregnant women and their offspring, this study reports findings of association of *IGF2* genotype with antenatal growth, birth size, postnatal growth parameters and body composition until 3 years of age, catch-up growth and BP at 3 years of age. We surveyed eight SNPs in the *IGF2* gene. We found significant positive associations between the *IGF2* genotype rs680 with antenatal growth, birth size and postnatal growth parameters at 3 years of age. Some of the *IGF2* genotypes were represented in greater proportions in SGA (rs680, rs3741204 and rs3842759) and in LGA infants (rs680, rs3741204 and rs3741205). These findings indicate that *IGF2* gene variants are associated with antenatal and postnatal growth.

Previous studies on association of antenatal growth and postnatal growth with genetic variations of *IGF2* genotypes have mainly investigated cross-sectional anthropometric parameters at birth or adult size. This study extends these observations to evaluate associations between *IGF2* genotypes and antenatal and postnatal growth parameters and early childhood blood pressure in infants born to mothers with uncomplicated pregnancies, with anthropometric data collected prospectively.

The 11p15.5 region of the human chromosome contains *IGF2-INS-TH (IDDM2)*, which encodes IGF-II, insulin and tyrosine hydroxylase, genes that are major fetal growth factors. Genetic variations in the *IDDM2* region have been found to be associated with metabolic syndrome, type 2 diabetes and coronary heart disease (Gaunt et al., 2001; Gomes et al., 2005; Gu et al., 2002; Rodriguez et al., 2006). Genome wide linkage scan has associated the 11p15.5 region with abdominal subcutaneous and visceral fat, especially in the *IGF2* region (Rice et al., 2002). Studies have also demonstrated the presence of differential mRNA expression or transcription of the *IGF2* gene corresponding to genetic variations (Jones et al., 2001; Zaina et al., 2003).

Of the eight *IGF2* SNPs investigated in this study, rs680 is significantly associated with antenatal and postnatal growth (Table 4.4.5 and 4.4.17). Genotype GG of SNP rs680 is associated with increased fetal size at the third trimester; increased placental weight and increased birth weight, length and head circumference. Genotype AA conferred a decrease in the anthropometric size parameters above with subjects leaner and lighter. Heterozygote subjects (AG),

showed intermediate mean values (Table 4.4.5). Genotype GG was also associated with higher IGF-I and IGFBP3 concentrations. On longitudinal assessment the influence of GG and AA genotypes persists at 3 years of age where it is significantly associated with weight, BMI, head circumference and body composition (Table 4.4.17). Children with the GG genotype are heavier and have higher BMI with increase in triceps and quadriceps skin fold thickness.

IGF-II plays a significant role in the development and growth of the placenta facilitating maternal-fetal nutrient transport. Placental weight is significantly associated with the *IGF2* SNP rs680 (ANOVA p=0.02). Given the role of IGF-II in influencing the size and morphology of the placenta and assisting in nutrient transfer this result would appear to have a physiological basis. Our data would suggest that the association of the *IGF2* SNP rs680 on placental weight is less than that upon the birth weight (ANOVA p=0.001 *vs.* p=0.02) but given the interaction of placental weight and birth size it is not easy to dissect this further. In the multiple linear regression models, birth weight (R^2 = 0·44; P = 0·0001, df = 7) as well as placental weight (R^2 = 0·10; P = 0·023, df = 5) is influenced by *IGF2* SNP rs680 implying that both parameters are influenced by the genotype but it would be wise not to infer too much due to the close interplay between placental size and birth weight.

A role for genetic variations in the *IGF2* gene impacting upon birth size and adult anthropometric parameters has been previously investigated. In 2650 healthy adult British White Caucasian males, O'Dell et al found an association of the *IGF2* SNP rs680 associated with mean body weight; GG homozygotes were 4kg heavier than AA homozygotes (O'Dell et al., 1997). GG homozygotes incurred a 1.67-fold risk of pathological BMI (>> 30 kg/m^2) compared with AA homozygotes (O'Dell et al., 1997). Serum IGF-II concentrations in 48 GG and 44 AA randomly selected homozygotes from the cohort showed a significant difference; 614.0 ± 124.0 ng/ml in GG and 683.3 ± 146.9 ng/ml in AA homozygotes (P= 0.01).

In the present study, we observed a significant difference in anthropometric parameters at the third trimester, at birth and at 3 years of age among the three rs680 genotypes; GG, GA and AA. Children with a GG genotype proportionately increase their weight from birth. However, similar increases were not seen in height, resulting in an increase in BMI and skin fold thickness at 3 years of age. This suggests that genotypes of rs680 starts influencing size from third trimester

and the effect persists during early childhood and, taking O'Dell et al's data into consideration, into late adulthood.

The association between the *IGF2* polymorphism rs680 and size at birth remains controversial. In a retrospective assessment of birth weight, through medical records in 693 Hertfordshire men and women, Sayer et al found highest birth weight in the GG genotype but the differences were not statistically significant (Sayer et al., 2002). In a Brazilian study of 294 healthy volunteers (95 men and 199 women; 18-30y), Gomes et al reported that the *IGF2* polymorphism rs680 was not significantly associated with BMI and/or birth weight. They however, observed a statistically significant correlation of 0.33 ($p < 0.023$) between birth weight and BMI in GG subjects whose birth weight was higher than 3.5 kg ($n = 47$) (Gomes et al., 2005). They hypothesized that the G allele of the IGF2 polymorphism rs680 is not a null factor and might be associated with predisposition to high BMI in young adults (Gomes et al., 2005).

Similar to our study, a Japanese study of 884 neonates found a significant difference ($p=0.04$) in birth weight SDSs among the three neonatal genotypes of SNP rs680 of *IGF2* gene (Kaku et al., 2007). AA homozygotes had a mean birth weight SDS of 0.18 lower than that of GG homozygotes ($p = 0.01$), and heterozygotes showed an intermediate mean value. There was also a significant difference in birth weight among the three neonatal c.901C > G genotypes of the *IGF2R* gene; CC, CG and GG, indicating that both *IGF2* and *IGF2R* gene variants may be associated with fetal growth (Kaku et al., 2007).

In a recent study Adkins et al investigated eight SNPs in the *IGF2* gene for associations with birth weight variation in 342 mother-newborn pairs (birth weight 2.1 – 4.7 kg at term) and 527 parent-newborn trios (birth weight 2.1 – 5.1 kg). Subjects consisted of African-American (n=830), white Caucasian (472) and other ethnicity (27) (Adkins et al., 2010). No association of parental or neonatal SNP rs680 was found with birth weight. Newborn SNP rs3741205 was significantly associated with birth weight (Adkins et al., 2010). In the present study, rs3741205 was not associated with birth weight. However, it was significantly overrepresented in SGA and LGA infants.

Results of association of *IGF2* genotypes with birth weight have not been consistent with conflicting reports. One reason for the inconsistent results could be differences in the ethnicity of the populations studied. While the present study

and the Japanese study corrected the anthropometric data of subjects for gestational age and gender by converting them into standard deviation score, other studies have used uncorrected data that can influence the result of associations of anthropometric data with genotypes.

Only one study has reported association of *IGF2* genotype rs680 with anthropometric parameters in late childhood. Dedoussis et al screened 795 peri-adolescent children (424 girls) aged 10–11 years old from the Gene and Diet Attica Investigation (GENDAI) paediatric cohort for the *IGF2* rs680 polymorphism (Dedoussis et al., 2010). Children homozygous for the common allele (GG) were taller (148.9±7 cm) compared with those with the A allele (148.1±7.9 cm), after adjusting for age, sex, Tanner Staging for puberty and dairy intake (Dedoussis et al., 2010). In multiple regression analysis, the *IGF2* rs680 polymorphism was independently associated with height *IGF2* rs680 (GG vs GA+AA; p=0.026) (Dedoussis et al., 2010).

Cord serum IGF-I, IGF-II and IGFBP-3 concentrations are available in infants of UCL-FGS cohort. We have investigated the association of selected *IGF2* SNPs with cord serum IGF-I, IGF-II and IGFBP-3 concentrations (Table 4.4.3). SNPs rs680 is significantly associated with the cord serum IGF-I and IGFBP-3 concentrations either implying that it directly influences their concentrations or it is in linkage disequilibrium with a tagging SNP on the *IGF1* and/or *IGFBP3* gene which influences their concentrations. This association could have resulted in the SNPs influence on antenatal growth, birth anthropometric parameters and early childhood growth implying that the association is a true association and not a chance finding. However, since cord plasma IGF-I, IGFBP-3, and GH concentrations relate to birth size (Geary et al., 2003), it is not easy to dissect this further. Our data would suggest that the statistical significance of the effect of SNP rs680 on cord IGF-1 (ANOVA, p=0.01) and IGFBP-3 (ANOVA, p=0.02) concentrations is less than that upon birth size (birth weight ANOVA p=0.0001) raising the possibility that the association is indirectly related to birth size rather than a direct association with the *IGF2* SNP rs680. In a multiple stepwise linear regression model, birth weight is influenced by *IGF2* SNP rs680 (R^2 = 0·347; P = 0·002, df = 6), while IGF-I (R^2 = 0·23; P = 0·98 df =4) and IGFBP-3 (R^2 = 0·15; P = 0·98, df = 4) concentrations are not, further suggesting that the *IGF2* genotype effect on IGF-I and IGFBP-3 is mediated through birth weight.

Functional studies of the IGF2 rs680 polymorphism have been attempted and have shown that the G allele of the polymorphism was associated with increased *IGF2* mRNA levels in leukocytes (Vafiadis et al., 1998). However, O'Dell et al found an opposite association as the mean serum IGF-II concentration in AA individuals was significantly higher than in GG individuals (O'Dell et al., 1997). Additionally, the association between the *IGF2* rs680 genotype and serum IGF-II concentrations was not observed by Roth et al (Roth et al., 2002). Genotypic variations could produce phenotypic variations without producing major alterations in gene function. Minor alterations in gene function would be relatively difficult to determine in *in vitro* studies.

IGF-II regulates glucose homeostasis, lipid metabolism and cardiovascular function. *IGF2* gene variants hence have the potential to influence BP. In the UCL-FGS cohort BP measurements are available in children at 3 years of age (n=207). The *IGF2* SNP rs3741204 was significantly associated with diastolic BP while IGF2 SNP rs3842759 was significantly associated with systolic BP. Faienza et al investigated IGF2 polymorphisms rs3842759 and rs680 in 227 obese children to evaluate the potential association between *IGF2* variants with either obesity or high BP (Faienza et al., 2010). They observed a significant association between the rs3842759 *IGF2* gene variant and systolic BP in obese children. Homozygote subjects for the T allele showed a higher risk of developing hypertension than those carrying the A allele. The three groups of genotype differed only for systolic BP (AA: 112.5 ± 11.6, AT: 114 ± 10.6, TT: 119 ± 15.3; p = 0.007). Although the same trend was observed, there were no statistically significant differences in diastolic BP (AA: 64.4 ± 9.3, AT: 65.4 ± 9.2, 67.2 ± 10.7; p = 0.3) (Faienza et al., 2010). This observation was also confirmed by comparing the 24-h blood pressure values between the three groups of genotypes (Faienza et al., 2010). The evaluation performed in the second group of obese subjects confirmed and corroborated this observation (Faienza et al., 2010). This result were not confirmed in a cohort of lean children (Faienza et al., 2010).

The present study also found a significant association of rs3842759 with early childhood systolic BP (AA: 89.6 ± 10.1, AT: 93.3 ± 11.5, TT: 96.3 ± 12; df=201; F=3.88 p = 0.02). Diastolic BP showed a similar trend but the results were not statistically significant (AA: 89.6 ± 10.1, AT: 93.3 ± 11.5, TT: 96.3 ± 12; df=201;

F=1.44 p = 0.24). Our cohort represents a continuum across the size spectrum in the general population and does not represent polarised size class groups, as was the case in the cohort of Faienza et al. Similar to Faienza et al we did not find any association of rs680 with childhood BP. Replication of the results raises a strong possibility of an association of *IGF2* SNP rs3842759 with early childhood BP, and further studies would be of interest in this regard.

Some of the SNPs have shown cross sectional association with anthropometric parameters. *IGF2* SNP rs3741204 was associated with birth length SDS; rs3741206 with birth head circumference SDS and cord insulin concentration and rs1004446 with cord serum IGF-I concentration At 6 months, SNP rs3741204 was associated with subscapular skin fold thickness and rs3842759 with triceps skin fold thickness, and at one year of age rs1004446 with length SDS. At two years of age, rs1004446 was associated with head circumference SDS and rs3741204 with length SDS and triceps skin fold thickness. At three years of age *IGF2* SNPs rs3741204 was associated with quadriceps skin fold thickness and SNP rs1004446 with triceps skin fold thickness. Unlike SNP rs680, none of these effects have demonstrated longitudinal association with antenatal or postnatal growth. For every genetic association study, there is always a possibility that association has arisen by chance, especially if they are novel (Ester et al., 2009a). It is likely that these random associations are by chance, as none of the associations are consistently present in longitudinal measurements.

Large genome wide association studies (GWAS) have not found any influence of the *IGF2* locus on height, body mass index and BP on adult variables studied (Lettre, 2011). This could simply mean that *IGF2* variation does not significantly influence growth or the SNP that tags *IGF2* in the GWAS does not recapitulate all the genomic variability at the locus, including other copy number variations, or rare variants (Bougneres, 2010).

Given the role of IGF-II in growth and metabolism, genetic variations at the *IGF2* regions are expected to explain the observed differences in intrauterine development, early childhood growth and BP. In the present study, we have demonstrated that genetic variations in the *IGF2* gene, the rs680 polymorphism contribute toward influencing fetal growth and early childhood growth and rs3842759 contributes towards early childhood blood pressure. However, it is highly probable that more complex mechanisms including epigenetic regulation

such as parental imprinting also contributes towards fetal development. Further studies of this model locus in larger longitudinal cohort will provide more detailed information on the genetic aspects of fetal and early childhood growth.

4.5 Genetic variations from Genome Wide Association Studies (influencing body mass index and blood pressure) and fetal and postnatal growth

4.4.2 Introduction

Genome wide association studies (GWAS) investigate specific genetic markers, usually SNPs, across the entire genome of a large population in order to determine genetic variations associated with polygenic traits and specific diseases.

Multiple genomic loci influence polygenic traits such as size and BP. The contribution of each genetic locus is small in influencing polygenic traits. Current knowledge of the patho-physiological basis of a polygenic trait is limited and hence the candidate gene approach is challenging to apply in determining all genetic variations that contribute towards polygenic traits. GWAS is a powerful tool as it is hypotheses free. Advances in high throughput, cost effective genetic technologies have assisted in scanning the whole genome in determining genetic variations that could contribute towards polygenic traits or specific common diseases, which are polygenic in their aetiology such as essential hypertension.

In the past few years, GWAS have identified many genetic loci that have been implicated for BMI and blood pressure BP in adults. The fat mass- and obesity-associated gene (*FTO*) gene was the first genetic locus that was related to BMI, which was reliably replicated in multiple studies including meta-analyses of multiple populations (Frayling et al., 2007). A recent meta-analyses of GWAS data of 249,796 adults of white European descent in the Genetic Investigation of Anthropometric Traits (GIANT) consortium has identified 18 new loci, thus setting the genetic loci likely associated with obesity to 42 (Speliotes et al., 2010). More elaborate meta-analyses of GWAS data continue to emerge. Each of the identified loci however explain only a relatively small proportion of individuals variability of BMI and in total contributes to only 0.17kg/m^2 of BMI variability (Speliotes et al., 2010). Meta-analyses of cohort studies have identified 13 new genetic loci for BP (Levy et al., 2009; Newton-Cheh et al., 2009). Association

effect of the variants however is very small and explains only a small proportion of the phenotypic variability between individuals.

The genesis of childhood polygenic traits is different from adults, as environmental impact on a polygenic trait increases with age. The dynamics of gene-gene and gene-environmental interaction also changes with age. However, extrapolating GWAS data from adults to explain genetic variations for childhood polygenic traits will give potentially important new factors that influence childhood growth and BP. Most of the studies on childhood BMI/obesity have investigated role of adult associated GWAS genes influencing BMI and BP (Cauchi et al., 2009; den et al., 2010; Elks et al., 2012; Hardy et al., 2010; Zhao et al., 2011; Zhao et al., 2009). One GWAS study on children has been performed in a small cohort size limiting the chances of detecting novel genetic variations, which can influence childhood BMI/obesity (Bradfield et al., 2012).

In studies performed so far, the timing of associations between common genetic variants for BMI or BP across the life course is still unclear. Compared to single measurements of anthropometric data at a specific age, repeated measures of growth parameters at varied ages provides more useful variation in the development of anthropometric traits determined by genetic components. This study has examined whether BMI and BP susceptibility loci identified by GWAS in adults are associated with fetal growth, early childhood anthropometric traits and BP in children from the University College London Fetal Growth Study (UCL-FGS) cohort.

4.5.2 Material and Methods

Subjects for this study were recruited from the University College London Hospital Fetal Growth Study. Suitable DNA samples for this study were available in 774 infants. Details of the cohort are provided in chapter 3.1.

Choice of SNPs

The selection of SNPs were based on studies reporting meta-analysis of GWAS data in cohorts that demonstrated genetic loci influencing BMI and BP, specifically the Genetic Investigation of ANthropometric Traits (GIANT) consortium which carried out a genome wide association meta-analysis of a total of 32,387 individuals of European ancestry from 15 cohorts of 1,094 to 5,433

individuals. Eight genetic loci which influence BMI were selected for analysis here (Willer et al., 2009). Similarly, the Cohorts for Heart and Aging Research in Genome Epidemiology (CHARGE) Consortium conducted meta-analysis of GWAS data in 29,136 participants of European descent who had undergone standardized BP measurements in six population-based cohort studies (Levy et al., 2009). Eight SNPs were selected from this study to identify their association with antenatal and postnatal growth and BP in UCL-FGS cohort.

Genotype methods

Genotyping for SNPs was performed by KBioscience Competitive Allele Specific PCR genotyping System (KASP) analysis. The details of the method adopted have been described in Chapter 3.4.4.

Statistics

All data were assessed for normal distribution by exploring the data for skewness and kurtosis. Infants were defined as small for gestational age (SGA) and large for gestational age (LGA) if birth weights were below 10th centile or above 90th centile, respectively, for gestational age. Postnatal catch-up or catch-down growth was defined as an increase or decrease in weight SDS of 0·6 over the first year of life (Dunger et al., 1998).

Chi-square tests were used to compare frequency distributions of the *IGF2* genotypes. One-way Analysis of Variance (ANOVA), with the Tukey's honest significant differences (HSD) *post hoc* test, was used to determine differences between the mean anthropometric measures in the *IGF2* genotypes.

A sample size of 774 was estimated to have a power of 90% to identify a 35 g difference in placental weight and a 75 g difference in birth weight at the 1% level of significance between the SGA and LGA groups.

4.5.3 Results

SNPs Influencing BMI on GWAS (BMI-GWAS)

Genotype frequency for the eight SNPs derived from BMI-GWAS data and χ^2 tests of Hardy-Weinberg equilibrium are provided in Table 4.5.1. All the tested SNPs were in the Hardy-Weinberg equilibrium.

Table 4.5.1. Genotype frequencies of BMI-GWAS SNPs and Hardy-Weinberg's equilibrium test. P value calculated by χ^2 test.

SNP	Gene	N	1/1 (%)	1/2(%)	2/2(%)	P value for H-W
rs1083873 1=A;2=G	***MTCH2***	733	44	52	4	0.299
rs10938397 1=A;2=G	***GNPDA2***	772	34	47	19	0.59
rs1108475 1=G;2=A	***KCTD15***	779	46	42	12	0.41
rs1778231 1=G;2=A	***MC4R***	778	58	36	6	0.053
rs2815752 1=T;2=C	***NEGR1***	782	39	47	14	0.811
rs6548238 1=A;2=G	***TMEM18***	776	70	26	4	0.624
rs7498665 1=A;2=G	***SH2B1***	764	41	46	13	0.42
rs9939609 1=T;2=A	***FTO***	783	32	51	17	0.365

Antenatal growth

Table 4.5.2 provides data of association of BMI-GWAS SNPs with antenatal growth parameters measured by ultrasonography. Second trimester femoral length was significantly associated with the rs1778231 variant of *MC4R* (GG: 3.22 ± 0.23, GA: 3.27 ± 0.23, AA: 3.3 ± 0.23; df=726; F=3.894 p = 0.021). At the third trimester the AA genotype of rs1778231 in *MC4R* still demonstrated greater femoral length but the results were not statistically significant (GG: 6.15 ± 0.26, GA: 6.20 ± 0.25, AA: 6.22 ± 0.35; df=682; F=2.85 p = 0.059). Third trimester biparietal diameter was significantly associated with the rs2815752 polymorphism in *NEGR1* (TT: 8.37 ± 0.33, TC: 8.43 ± 0.35, CC: 8.48 ± 0.35; df=726; F=3.69 p = 0.026)

Table 4.5.2. Association of infant BMI-GWAS SNPs with antenatal growth (*n* = 774). P value calculated by ANOVA.

		First Trimester (12-14 wk GA)		Second Trimester (18-20 wk GA)			Third trimester (30-34 wk GA)		
SNPs	**Gene**	**CRL**	**BPD**	**FL**	**AC**	**BPD**	**FL**	**AC**	**BPD**
rs1083873	***MTCH2***	0.28	1.0	0.71	0.94	0.92	0.31	0.30	0.87
rs10938397	***GNPDA2***	0.52	0.77	0.53	0.91	0.63	0.53	0.29	0.69
rs1108475	***KCTD15***	0.77	0.97	0.33	0.33	0.74	0.49	0.74	0.60
rs1778231	***MC4R***	0.17	0.31	***0.02***	0.11	0.70	0.06	0.47	0.91
rs2815752	***NEGR1***	0.55	0.84	0.31	0.43	0.13	0.52	0.65	***0.03***
rs6548238	***TMEM18***	0.62	0.77	0.71	0.83	0.49	0.54	0.57	0.74
rs7498665	***SH2B1***	0.97	0.53	0.15	0.88	0.12	0.11	0.25	0.70
rs9939609	***FTO***	0.81	0.90	0.55	0.88	0.86	0.89	0.58	0.46

SNP- single nucleotide polymorphism; CRL- crown rump length; BPD-biparietal diameter; FL-femoral length; AC- abdominal circumference; P values <0.05 are underlined.

Anthropometric parameters at birth

Table 4.5.3 provides data of association of BMI-GWAS SNPs with growth parameters at birth. ANOVA tests did not demonstrate any significant association of genotypes with parameters of birth weight SDS, birth length SDS and placental weight. None of the genotypes were associated with cord hormone concentrations.

The proportion of genotypic distribution in SGA, AGA and LGA infants and association of genotypes in catch-up and catch-down growth are presented in Table 4.5.4. Some of the genotypes of BMI-GWAS SNPs rs1108475 in *KCTD15* (χ^2=10.37, df=2, p=0.0056), rs6548238 in *TMEM18* (χ^2=9.53, df=2, p=0.009) and rs9939609 in *FTO* (χ^2=9.55, df=2, p=0.009) were represent in significant proportion within SGA infants. Genotypes of SNP rs1083873 in *MTCH2* were significantly associated with catch-up (χ^2=9.38, df=2, p=0.009) and catch-down growth (χ^2=6.55, df=2, p=0.04).

Table 4.5.3. Association of infant BMI-GWAS SNPs with growth parameters at birth (*n* = 774). P value calculated by ANOVA.

SNPs	Gene	Bw	Bl	Hc	Pw	Igf1	Igf2	Gh	IgfBp 3	Ins
rs1083873	***MTCH2***	0.66	0.99	0.50	0.18	0.12	0.49	0.70	0.23	0.74
rs10938397	***GNPDA2***	0.79	0.21	0.55	0.48	0.74	0.48	0.23	0.70	0.68
rs1108475	***KCTD15***	0.53	0.23	0.10	0.66	0.80	0.79	0.04	0.66	0.14
rs1778231	***MC4R***	0.15	0.47	0.66	0.48	0.50	0.26	0.63	0.88	0.36
rs2815752	***NEGR1***	0.97	0.44	0.38	0.34	0.54	0.45	0.62	1.0	0.56
rs6548238	***TMEM18***	0.63	0.43	0.12	0.31	0.58	0.31	0.96	0.46	0.95
rs7498665	***SH2B1***	0.38	0.09	0.07	0.87	0.41	0.77	0.07	0.07	0.95
rs9939609	***FTO***	0.95	0.52	0.53	1.0	0.10	0.61	0.65	0.73	0.93

SNP- single nucleotide polymorphism; BW- birth weight; Bl- birth length; Hc- head circumference; Pw- placental weight; CIgf1- cord insulin-like growth factor-1, CIgf2- cord insulin-like growth factor 2; CGh- cord growth hormone; CIgfBp3- cord insulin-like growth factor 3; CIns- cord insulin; P values <0.05 are underlined.

Table 4.5.4. Association of infant BMI-GWAS SNPs with SGA, AGA and LGA at birth and with catch-up and catch-down growth in the first year of age. P value calculated by χ^2 test.

SNPs	Gene	SGA	AGA	LGA	Catch-up	Catch-down
rs1083873	***MTCH2***	0.87	0.52	0.28	**0.009**	**0.04**
rs10938397	***GNPDA2***	0.62	0.96	0.59	0.86	0.96
rs1108475	***KCTD15***	**0.006**	0.98	0.46	0.91	0.81
rs1778231	***MC4R***	0.04	0.98	0.97	1.0	0.59
rs2815752	***NEGR1***	0.08	0.95	0.98	0.74	0.98
rs6548238	***TMEM18***	**0.009**	0.56	0.27	0.06	0.27
rs7498665	***SH2B1***	0.20	0.83	0.18	0.84	0.59
rs9939609	***FTO***	**0.009**	0.97	0.50	0.90	0.70

SNP- single nucleotide polymorphism; SGA- small for gestational age; LGA-large for gestational age; P values <0.05 are underlined..

Postnatal growth and BP at 3 years of age

The associations of BMI-GWAS SNPs with growth parameters at 6 months, 12 months, 2 years and 3 years of age are provided in tables 4.5.5, 4.5.6, 4.5.7 and 4.5.8 respectively. Numbers of subjects recruited were reduced to 73% of the original birth cohort at 6 of months, 66% at 1 year, 57% at 2 years and 44% at 3 years.

At 6 months of age rs6548238 *TMEM18* was significantly associated with weight SDS (AA: 0.20 ± 1.1, AG: 0.00 ± 0.99, GG: -0.47 ± 1.2; df=540; F=3.98 *p* = 0.019).

At one year of age rs7498665 *SH2B1* was significantly associated with weight (AA: 0.19 ± 1.1, AG: -0.01 ± 1.0, GG: 0.35 ± 0.96; df=480; F=3.68 *p* = 0.026) and length (AA: 0.43 ± 1.1, AG: 0.27 ± 1.1, GG: 0.71 ± 1.0; df=477; F=4.67 *p* = 0.01) SDS.

At 2 years of age rs6548238 *TMEM18* was significantly associated with quadriceps skin-fold thickness (AA: 1.67 ± 0.37, AG: 1.77 ± 0.38, GG: 1.59 ± 0.41; df=383; F=3.53 p = 0.03) which remained significant at 3 years of age (AA: 1.71 ± 0.34, AG: 1.95 ± 0.86, GG: 1.73 ± 0.44; df=268; F=5.6 p = 0.004) along with sub-scapular skin-fold thickness (AA: 0.67 ± 0.21, AG: 0.81 ± 0.64, GG: 0.68 ± 0.19; df=271; F=3.38 p = 0.036). At 2 years of age rs7498665 *SH2B1* was significantly associated with triceps skin-fold thickness (AA: 1.24 ± 0.23, AG: 1.23 ± 0.23, GG: 1.32 ± 0.26; df=384; F=2.98 p = 0.05) which remained significant at 3 years of age (AA: 1.31 ± 0.20, AG: 1.29 ± 0.24, GG: 1.39 ± 0.22; df=270; F=3.6 p = 0.029).

At 3 years of age rs1083873 *MTCH2* was significantly associated with weight (AA: 0.11 ± 1.0, AG: -0.4 ± 1.0, GG: -0.34 ± 0.89 df=330; F=3.1 p = 0.05), length (AA: 0.40 ± 1.0, AG: 0.13 ± 1.0, GG: 0.03 ± 0.3; df=328; F=3.37 p = 0.038) and head circumference (AA: 50.4 ± 1.4, AG: 50.3 ± 1.3, GG: 48.6 ± 7.4; df=316; F=6.38 p = 0.002). SNP rs6548238 *TMEM18* was significantly associated with length SDS (AA: 0.30 ± 1.1, AG: 0.14 ± 0.90, GG: -0.68 ± 1.0; df=326; F=4.53 p = 0.011).

At 3 years of age SNP rs9939609 on the *FTO* gene was significantly associated with systolic (TT: 90.4 ± 11.0, TA: 90.6 ± 9.9, AA: 95.8 ± 12.0; df=201; F=3.1 p = 0.046) and diastolic BP (TT: 58.6 ± 8.7, TC: 56.8 ± 7.6, AA: 61.6 ± 11.2; df=201; F=3.2 p = 0.04).

Table 4.5.5. Association of infant BMI-GWAS SNPs with growth parameters at 6 months of age (n = 566). P value calculated by ANOVA.

SNP	Gene	Wt	Lt	BMI	HC	MAC	TSF	SSSF	QSF
rs1083873	***MTCH2***	0.46	0.77	0.55	0.51	0.71	0.28	0.26	0.71
rs10938397	***GNPDA2***	0.12	0.86	0.09	0.40	0.13	0.88	0.94	0.23
rs1108475	***KCTD15***	0.20	0.04	0.66	0.27	0.14	0.85	0.07	0.03
rs1778231	***MC4R***	0.81	0.75	0.66	0.48	0.48	0.83	0.24	0.36
rs2815752	***NEGR1***	0.75	0.22	0.46	0.38	0.87	0.86	0.31	0.37
rs6548238	***TMEM18***	**0.02**	0.08	0.21	0.07	0.24	0.86	0.77	0.90
rs7498665	***SH2B1***	0.05	0.24	0.15	0.33	0.35	0.92	0.94	0.88
rs9939609	***FTO***	0.20	0.33	0.09	0.16	0.10	0.20	0.38	0.06

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF- triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; P values <0.05 are underlined.

Table 4.5.6. Association of infant BMI-GWAS SNPs with growth parameters at one year of age (*n* = 512). P value calculated by ANOVA.

SNP	Gene	Wt	Lt	BMI	HC	MAC	TSF	SSSF	QSF
rs1083873	***MTCH2***	0.30	0.17	0.82	0.10	0.84	0.13	0.67	0.38
rs10938397	***GNPDA2***	0.99	0.50	0.69	0.93	0.94	0.18	0.81	0.57
rs1108475	***KCTD15***	0.80	0.06	0.50	0.63	0.98	0.73	0.24	0.64
rs1778231	***MC4R***	0.63	0.16	0.98	0.64	0.59	0.76	0.15	0.47
rs2815752	***NEGR1***	0.52	0.31	0.86	0.12	0.51	0.41	1.0	0.72
rs6548238	***TMEM18***	0.22	0.34	0.35	0.63	0.57	0.61	0.62	0.39
rs7498665	***SH2B1***	**0.03**	**0.01**	0.24	0.35	0.30	0.08	0.23	0.70
rs9939609	***FTO***	0.22	0.46	0.48	0.13	1.0	0.87	0.71	0.64

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF- triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; P values <0.05 are underlined.

Table 4.5.7. Association of infant BMI-GWAS SNPs with growth parameters at two years of age (*n* = 440). P value calculated by ANOVA.

SNP	Gene	Wt	Lt	BMI	HC	MAC	TSF	SSSF	QSF
rs1083873	***MTCH2***	0.38	0.49	0.19	0.47	0.69	0.16	0.84	0.07
rs10938397	***GNPDA2***	0.82	0.75	0.87	0.34	0.72	0.07	0.67	0.83
rs1108475	***KCTD15***	0.50	0.55	0.14	0.50	0.87	0.52	0.18	0.69
rs1778231	***MC4R***	0.30	0.30	0.56	0.67	1.0	0.56	0.86	0.23
rs2815752	***NEGR1***	0.18	0.21	0.67	0.16	0.31	0.33	0.60	0.20
rs6548238	***TMEM18***	0.13	0.13	0.49	0.08	0.50	0.40	0.63	**0.03**
rs7498665	***SH2B1***	0.22	0.14	0.75	0.77	0.23	**0.05**	0.35	0.28
rs9939609	***FTO***	0.77	0.70	0.67	0.48	0.32	0.98	0.96	0.68

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF- triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; P values <0.05 are underlined.

Table 4.5.8. Association of BMI-GWAS SNPs with growth parameters (*n* = 342) and blood pressure (n=210) at three years of age. P value calculated by ANOVA.

SNP	Gene	Wt	Lt	BMI	HC	MAC	TSF	SSSF	QSF	SBP	DBP
rs1083873	***MTCH2***	***0.05***	**0.04**	0.16	**0.002**	0.88	0.14	0.26	0.44	0.82	0.18
rs10938397	***GNPDA2***	0.52	0.82	0.43	0.28	0.28	0.41	0.89	0.27	0.58	0.28
rs1108475	***KCTD15***	0.89	0.67	0.54	0.33	0.89	0.19	0.68	0.52	0.70	0.06
rs1778231	***MC4R***	0.31	0.29	0.64	0.56	0.49	0.58	0.59	0.69	0.79	0.23
rs2815752	***NEGR1***	0.12	0.22	0.20	0.92	0.28	0.04	0.56	0.40	0.86	0.41
rs6548238	***TMEM18***	0.26	**0.01**	0.86	0.40	0.80	0.08	**0.04**	**0.004**	0.67	0.17
rs7498665	***SH2B1***	0.14	0.28	0.34	0.64	0.56	**0.03**	0.21	0.06	0.88	0.52
rs9939609	***FTO***	0.97	0.96	0.90	0.40	0.40	0.62	0.94	0.49	**0.05**	**0.04**

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF- triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; SBP-systolic blood pressure; DBP-Diastolic blood pressure; P values <0.05 are underlined.

SNPs Influencing BP on GWAS (BP-GWAS)

The genotype frequency for the eight SNPs derived from BP-GWAS data and the χ^2 test for the Hardy-Weinberg equilibrium are provided in Table 4.5.9. All the tested SNPs were in the Hardy-Weinberg equilibrium.

Table 4.5.9. Genotype frequencies of BP-GWAS SNPs and Hardy-Weinberg equilibrium test. P value calculated by χ^2 test.

SNP	Gene	N	1/1 (%)	1/2(%)	2/2(%)	P value for H-W
rs381815 1=C;2=T	***PLEKHA7***	760	52	38	10	0.71
rs1004467 1=A;2=G	***CYP17A1***	761	82	17	1	0.85
rs11014166 1=A;2=T	***CACNB2***	763	41	46	13	0.73
rs2384550 1=G;2=A	***TBX3***	767	41	45	14	0.33
rs2681472 1=A;2=G	***ATP2B1***	787	67	30	3	0.74
rs6495122 1=C;2=A	***CSK***	762	32	48	20	0.12
rs9815354 1=G;2=A	***ULK4***	770	68	29	3	0.12
rs3184504 1=C;2=T	***SH2B3***	771	29	48	23	0.68

Antenatal growth

Table 4.5.10 provides data of the association of BP-GWAS genotypes with antenatal growth parameters measured by ultrasonography. SNP rs1004467 on *CYP17A1* was significantly associated with 3rd trimester femoral length (AA: 6.16 ± 0.26, AG: 6.23 ± 0.27, GG: 6.1 ± 0.21; df=689; F=3.9 p = 0.021) and abdominal circumference (AA: 28.45 ± 1.5, AG: 28.8 ± 1.1, GG: 28.0 ± 1.4; df=689; F=3.3 p = 0.037). SNP rs11014166 on *CACNB2* was significantly associated with 3rd trimester biparietal diameter (AA: 8.43 ± 3.7, AT: 8.41 ± 3.1, TT: 83.0 ± 3.5; df=588; F=2.9 p = 0.05).

Table 4.5.10. Association of infant BP-GWAS SNPs with antenatal growth (*n* = 774). P value calculated by ANOVA.

		First Trimester (12-14 wk GA)		Second Trimester (18-20 wk GA)			Third trimester (30-34 wk GA)		
SNPs	**Gene**	**CRL**	**BPD**	**FL**	**AC**	**BPD**	**FL**	**AC**	**BPD**
rs381815	***PLEKHA7***	0.85	0.74	0.92	0.57	0.77	1.0	0.33	0.46
rs1004467	***CYP17A1***	0.91	0.57	0.81	0.84	0.35	**0.02**	**0.04**	0.11
rs11014166	***CACNB2***	0.12	0.41	0.12	0.66	0.53	0.66	0.16	**0.05**
rs2384550	***TBX3***	0.60	0.50	0.94	0.41	0.46	0.74	0.95	0.16
rs2681472	***ATP2B1***	0.60	0.45	0.33	0.36	0.75	0.70	0.28	0.11
rs6495122	***CSK***	0.12	0.41	0.66	0.63	0.58	0.75	0.34	0.20
rs9815354	***ULK4***	0.34	0.46	0.18	0.75	0.07	0.08	0.26	0.53
rs3184504	***SH2B3***	0.92	0.74	0.89	0.21	0.11	0.64	0.13	0.33

SNP- single nucleotide polymorphism; CRL- crown rump length; BPD-biparietal diameter; FL-femoral length; AC- abdominal circumference; statistically P values <0.05 are underlined.

Anthropometric parameters at birth

Table 4.5.11 provides data of association of BP-GWAS genotype with growth parameters at birth. SNP 11014166 on *CACNB2*, which was significant with 3rd trimester biparietal diameter, showed significant association with head circumference SDS at birth (AA: 0.02 ± 1.08, AT: 0.03 ± 1.07, TT: -0.27 ± 1.2; df=720; F=3.1 *p* = 0.046). None of the other SNPs from BP-GWAS data were significantly associated with anthropometric parameters at birth.

The proportion of genotypic distribution in SGA, AGA and LGA infants and association of genotypes in catch-up and catch-down growth are presented in Table 4.5.12. Some of the genotypes of SNPs rs11014166 in *CACNB2* (χ^2=14.59, df=2, p=0.0007)*,* rs2384550 in *TBX3* (χ^2=8.45, df=2, p=0.015) and rs3184504 in *SH2B3* (χ^2=10.8, df=2, p=0.0045) were present in significant proportion in the SGA infants. Some of the genotypes of SNPs rs2681472 *ATP2B1* (χ^2=7.66, df=2, p=0.02) and rs9815354 *ULK4* (χ^2=18.85, df=2, p=0.0001) were present in significant proportion in LGA infants. Genotypes of SNP rs3184504 in *SH2B3* was significantly associated with catch-up growth (χ^2=11.07, df=2, p=0.004).

Table 4.5.11. Association of infant BP-GWAS SNPs with growth parameters at birth (*n* = 774). P value calculated by ANOVA.

SNP		Bw	Bl	HC	Pw	Igf1	Igf2	Gh	IgfBp3	Ins
rs381815	***PLEKHA7***	0.84	0.76	0.42	0.92	0.68	0.50	0.94	0.90	0.61
rs1004467	***CYP17A1***	0.26	0.12	0.78	0.86	0.21	0.77	0.57	0.48	0.46
rs11014166	***CACNB2***	0.12	0.26	**0.05**	0.60	0.84	0.94	0.97	0.74	0.56
rs2384550	***TBX3***	0.66	0.30	0.30	0.70	0.14	0.14	0.26	0.28	0.99
rs2681472	***ATP2B1***	0.74	0.54	0.11	0.68	0.24	1.0	0.60	0.43	0.12
rs6495122	***CSK***	0.80	0.99	0.35	0.61	0.10	0.58	0.27	0.77	0.33
rs9815354	***ULK4***	0.23	0.07	0.62	0.37	0.85	0.70	0.40	0.75	0.12
rs3184504	***SH2B3***	0.67	0.15	0.17	0.16	0.91	0.79	0.43	0.74	0.43

SNP- single nucleotide polymorphism; BW- birth weight; Bl- birth length; HC- head circumference; Pw- placental weight; Igf1- cord insulin-like growth factor-1, Igf2- cord insulin-like growth factor 2; Gh- cord growth hormone; IgfBp3- cord insulin-like growth factor 3; Ins- cord insulin; P values <0.05 are underlined.

Table 4.5.12. Association of infant BP-GWAS SNPs with SGA, AGA and LGA at birth and with catch-up and catch-down growth in the first year of age. P value calculated by χ^2 test.

SNP	Gene	SGA	AGA	LGA	Catch-up	Catch-down
rs381815	***PLEKHA7***	0.44	0.20	0.18	0.45	0.98
rs1004467	***CYP17A1***	0.10	1.00	0.60	0.97	0.87
rs11014166	***CACNB2***	**0.0007**	0.95	0.59	0.26	0.77
rs2384550	***TBX3***	**0.01**	1.00	0.83	0.42	1.00
rs2681472	***ATP2B1***	0.42	1.00	**0.02**	1.00	0.74
rs6495122	***CSK***	0.18	0.97	0.12	0.64	0.97
rs9815354	***ULK4***	0.30	0.98	**0.0001**	0.60	0.91
rs3184504	***SH2B3***	**0.005**	0.96	0.10	**0.004**	0.97

SNP- single nucleotide polymorphism; SGA- small for gestational age; LGA-large for gestational age; P values <0.05 are underlined.

Postnatal growth and BP at 3 years of age

Association of BP-GWAS SNPs with growth parameters at 6 months, 12 months, 2 years and 3 years of age are provided in tables 4.5.13, 4.5.14, 4.3.15 and 4.3.16 respectively. The number of subjects recruited were reduced to 73% at 6 months, 66% at 1 year, 57% at 2 years and 44% at 3 years of age.

At 6 months SNP rs381815 *PLEKHA7* was significantly associated with length SDS (CC: 0.27 ± 1.0, CT: 0.18 ± 0.89, TT: 0.50 ± 1.1; df=526; F=3.9 p = 0.021). SNP rs3184504 *SH2B3* was significantly associated with head circumference SDS (CC: -0.20 ± 1.0, CT: -0.18 ± 0.90, TT: -0.46 ± 1.0; df=536; F=3.66 p = 0.027)

At 2 years of age, rs3184504 *SH2B3* was significantly associated with weight SDS (CC: 0.62 ± 1.0, CT: 0.41 ± 1.1, TT: 0.07 ± 1.1; df=421; F=7.16 p = 0.001), BMI SDS (CC: 0.25 ± 1.0, CT: 0.09 ± 1.12, TT: -0.27 ± 0.85; df=416; F=6.88 p = 0.001) and head circumference SDS (CC: -0.42 ± 1.0, CT: -0.45 ± 1.1, TT: -0.77

± 1.0; df=411; F=3.74 *p* = 0.025). SNP rs2681472 *ATP2B1* was significantly associated with sub-scapular (AA: 0.73 ± 0.19, AG: 0.72 ± 1.6, GG: 0.59 ± 0.09; df=397; F=4.1 *p* = 0.017) and quadriceps (AA: 1.7 ± 0.34, AG: 1.7 ± 0.4, GG: 1.4 ± 0.28; df=394; F=3.9 *p* = 0.02) skin-fold thickness**.** SNP rs381815 *PLEKHA7* was significantly associated with triceps skin fold thickness (CC: 1.28 ± 0.24, CT: 1.17 ± 0.22, TT: 1.23 ± 0.24; df=383; F=3.64 *p* = 0.027)*.*

At 3 years of age, rs3184504 *SH2B3* was significantly associated with weight SDS (CC: 0.19 ± 1.1, CT: 0.04 ± 0.93, TT: -0.77 ± 0.92; df=333; F=7.55 *p* = 0.001) and BMI SDS (CC: 0.27 ± 1.1, CT: 0.03 ± 0.97, TT: -0.27 ± 0.71; df=331; F=8.6 *p* = 0.0001). SNP rs381815 *PLEKHA7* was significantly associated with triceps skin fold thickness (CC: 1.34 ± 0.30, CT: 1.24 ± 0.19, TT: 1.3 ± 0.18; df=269; F=3.7 *p* = 0.026) and length SDS (CC: 0.18 ± 0.88, CT: -0.16 ± 1.4, TT: 0.32 ± 0.98; df=318; F=3.3 *p* = 0.038). SNP rs2681472 *ATP2B1* was significantly associated with head circumference (AA: 50.2 ± 1.4, AG: 50.4 ± 1.4, GG: 44.8 ± 1.4; df=322; F=18.4 *p* = 0.0001). None of these polymorphisms were associated with systolic or diastolic BP at 3 years of age (Table 4.5.16)

Table 4.5.13. Association of infant BP-GWAS SNPs genotype with growth parameters at 6 months of age (*n* = 566). P value calculated by ANOVA.

SNP	Gene	Wt	Lt	BMI	HC	MAC	TSF	SSSF	QSF
rs381815	***PLEKHA7***	0.22	**0.02**	0.33	0.23	0.25	0.23	0.44	0.44
rs1004467	***CYP17A1***	0.73	0.76	0.46	0.78	0.78	0.25	0.31	0.83
rs11014166	***CACNB2***	0.46	0.79	0.57	0.65	0.13	0.36	0.13	0.21
rs2384550	***TBX3***	0.63	0.62	0.27	0.29	0.73	0.33	0.36	0.55
rs2681472	***ATP2B1***	0.56	0.91	0.47	0.95	0.25	0.70	0.24	0.61
rs6495122	***CSK***	0.50	0.66	0.63	0.65	0.54	0.17	0.45	0.06
rs9815354	***ULK4***	0.62	0.54	0.90	0.90	0.77	0.52	0.57	0.74
rs3184504	***SH2B3***	0.36	0.22	0.36	**0.03**	0.22	0.33	0.53	0.13

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF- triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; P values <0.05 are underlined.

Table 4.5.14. Association of infant BP-GWAS SNPs genotype with growth parameters at one year of age (*n* = 512). P value calculated by ANOVA.

SNP	Gene	Wt	Lt	BMI	HC	MAC	TSF	SSSF	QSF
rs381815	***PLEKHA7***	0.08	0.20	0.24	0.43	0.30	0.30	0.33	0.45
rs1004467	***CYP17A1***	0.54	0.30	0.99	0.86	0.59	0.58	0.46	0.72
rs11014166	***CACNB2***	0.83	0.64	0.88	0.56	0.82	0.63	0.56	0.38
rs2384550	***TBX3***	0.88	0.64	0.81	0.29	0.99	0.74	0.10	0.24
rs2681472	***ATP2B1***	0.23	0.35	0.47	0.76	0.41	0.91	0.57	0.40
rs6495122	***CSK***	0.38	0.48	0.53	0.90	0.45	0.08	0.09	0.49
rs9815354	***ULK4***	0.64	0.89	0.58	0.52	0.38	0.53	0.84	0.74
rs3184504	***SH2B3***	0.11	0.22	0.33	0.27	0.37	0.80	0.58	0.38

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF- triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; P values <0.05 are underlined.

Table 4.5.15. Association of infant BP-GWAS SNPs with growth parameters at two years of age (*n* = 440). P value calculated by ANOVA.

SNP	Gene	Wt	Lt	BMI	HC	MAC	TSF	SSSF	QSF
rs381815	***PLEKHA7***	0.10	0.15	0.30	0.60	0.62	**0.03**	0.60	0.08
rs1004467	***CYP17A1***	0.08	0.27	0.19	0.62	0.24	0.78	0.94	0.89
rs11014166	***CACNB2***	0.60	0.89	0.38	0.87	0.75	0.60	0.15	0.98
rs2384550	***TBX3***	0.62	0.69	0.20	0.15	0.30	0.51	0.78	0.94
rs2681472	***ATP2B1***	0.23	0.12	0.84	0.74	0.24	0.06	**0.02**	**0.02**
rs6495122	***CSK***	0.89	0.98	0.68	0.22	0.42	0.82	0.93	0.60
rs9815354	***ULK4***	0.69	0.91	0.82	0.09	0.18	0.20	0.89	0.32
rs3184504	***SH2B3***	**0.001**	0.20	**0.001**	**0.03**	0.10	0.53	0.72	0.01

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF- triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; P values <0.05 are underlined.

Table 4.5.16. Association of BP-GWAS SNPs with growth parameters (*n* = 342) and blood pressure (n=210) at three years of age. P value calculated by ANOVA.

SNP	Gene	Wt	Lt	BMI	HC	MAC	TSF	SSSF	QSF	SBP	DBP
rs381815	***PLEKHA7***	0.14	**0.04**	0.88	0.43	0.46	**0.03**	0.25	0.86	0.78	0.98
rs1004467	***CYP17A1***	0.27	0.28	0.40	0.54	0.76	0.18	0.85	0.33	0.71	0.55
rs11014166	***CACNB2***	0.30	0.72	0.47	0.80	0.88	0.91	0.71	0.95	0.42	0.87
rs2384550	***TBX3***	0.40	0.60	0.22	0.89	0.34	0.32	0.15	0.16	0.13	0.41
rs2681472	***ATP2B1***	0.18	0.21	0.38	**0.0001**	0.59	0.07	0.41	0.70	0.66	0.28
rs6495122	***CSK***	0.22	0.65	0.13	0.44	0.33	0.39	0.67	0.52	0.06	0.51
rs9815354	***ULK4***	0.77	0.52	0.96	0.68	0.64	0.14	0.58	0.30	0.49	0.95
rs3184504	***SH2B3***	**0.001**	0.18	**0.0001**	0.30	0.34	0.16	0.45	0.20	0.21	0.75

Wt- weight; Lt-length; BMI- body mass index; HC- head circumference; MAC- mid-arm circumference; TSF- triceps skin fold; SSSF- subscapular skin fold; QSF- quadriceps skin fold; SBP- systolic blood pressure; DBP- Diastolic blood pressure; P values <0.05 are underlined.

4.5.4 Discussion

SNPs influencing BMI on GWAS (BMI-GWAS)

In a cohort of white European pregnant women and their offspring, this study reports findings of association of BMI-GWAS genotype with antenatal growth, postnatal size and BP at 3 years of age. We surveyed eight SNPs in the eight genes which were significantly associated with BMI in GIANT consortium GWAS study (Willer et al., 2009).

BMI-GWAS SNPs did not demonstrate any consistent association with antenatal growth or with birth size suggesting that they are not associated with birth size. Similar to the present study, meta-analyses data on 24,683 individuals of white European descent found little evidence that loci known to be associated with BMI in adult life affect weight at birth (Kilpelainen et al., 2011). The SNPs rs1108475 on *KCTD15,* rs6548238 on *TMEM18* and rs9939609 on *FTO* however were significantly overrepresented in polarised groups of SGA infants implying that they may have a role in influencing anthropometric parameters in this group. The small sample size of SGA infants did not allow assessment of these SNPs in detail in this group.

The *FTO* gene is the first robustly replicated common obesity gene. One of its variants rs9939609 within intron 1 has been associated with adult and childhood BMI in GWAS and meta-analyses of multiple GWAS studies (Frayling et al., 2007; Speliotes et al., 2010). Association of *FTO* (rs9939609) was first identified in a population of type 2 diabetes (Frayling et al., 2007). When the results were adjusted for increase in size, the apparent association with T2D was abolished, suggesting that *FTO* SNP rs9939609 predisposes to diabetes through an effect on BMI (Frayling et al., 2007).

Fto null mice demonstrate postnatal growth retardation with a significant reduction in adipose tissue and lean body mass (Fischer et al., 2009). These mice have increased energy expenditure and systemic sympathetic activation, despite decreased spontaneous locomotor activity and relative hyperphagia implying that *Fto* in mice is functionally involved in energy homeostasis by the control of energy expenditure (Fischer et al., 2009).

In humans, *FTO* is ubiquitously expressed and hence its primary role in BMI variance is not clear. Data from multiple human studies suggest involvement of the *FTO* SNP rs9939609 in food intake with the risk allele ‘A’ associated with

greater intake of food, impaired satiety responsiveness and more frequent loss of eating control than did those homozygous for the non-risk allele 'T' (Cecil et al., 2008; Speakman et al., 2008; Tanofsky-Kraff et al., 2009).

In the UCL-FGS cohort data presented here, the *FTO* genetic variant is not associated with antenatal growth and birth size or with BMI at 3 years of age. Previous studies have also failed to associate *FTO* variants with birth size (den et al., 2010; Dina et al., 2007; Jess et al., 2008). Hebebrand has hypothesised that intrauterine environment may restrict the influence of the *FTO* genetic variant on intrauterine growth while genetic make-up becomes prominent postnatally (Hebebrand, 2010). The strength of the association of *FTO* with BMI is stronger after 4 years of age and hence we may have failed to demonstrate association in our cohort (den et al., 2010; Dina et al., 2007; Hardy et al., 2010).

However, in the UCL-FGS data the *FTO* gene variant rs9939609 was significantly associated with systolic and diastolic BP at 3 years of age. AA genotype children had a systolic BP 5.4 mmHg higher than those with a TT genotype (TT: 90.4 ± 11.0, TA: 90.6 ± 9.9, AA: 95.8 ± 12.0; df=201; F=3.1 p = 0.046). AA genotype children also had a diastolic BP 3 mmHg higher than those with a TT genotype (TT: 58.6 ± 8.7, TC: 56.8 ± 7.6, AA: 61.6 ± 11.2; df=201; F=3.2 p = 0.04). Our findings are similar to those of Pausova et al. In 485 adolescents recruited from a French Canadian founder population the *FTO* genotype rs9939609 showed higher systolic BP (Pausova et al., 2009) independent of its association of body weight. AA genotype adolescents had systolic BP 4.2 mmHg and diastolic BP 1.2 mmHg higher than those with a TT genotype. The authors hypothesised an independent association of *FTO* with BP secondary to powerfully modulated sympathetic vasomotor tone by the paraventricular and dorsomedial nuclei of the hypothalamus, which show particularly high *FTO* expression (Pausova et al., 2009).

In the UCL-FGS cohort, none of the other BMI-GWAS SNPs demonstrated significant association with BMI on serial measurements until 3 years of age. This may imply that these SNPs are not associated with early childhood growth and they exert their influence on BMI after 3 years of age. Sample size of this study may have failed to detect mild effects of these SNPs on antenatal and postnatal growth, which were noted in adult studies performed in larger study population.

In terms of growth patterns, the SNP rs1083873 on *MTCH2* was significantly associated with catch-up growth and with weight SDS, height SDS and head circumference at 3 years of age. Children with a AA genotype were heavier by 0.45 SDS, taller by 0.37 SDS and had larger head circumference by 1.8 cm. The AA genotype was not associated with BMI as children of this genotype were proportionately of larger size. Association of the *MTCH2* SNP rs1083873 with catch-up growth and size at 3 years of age suggests that this is not a chance finding. This is however, a novel finding as previous studies of *MTCH2* on childhood growth has revealed no association with BMI or size (Zhao et al., 2009). The exact functions of the *MTCH2* gene (encoding mitochondrial carrier 2) and mechanisms behind a potential association with adult BMI are currently not known (Manco and Dallapiccola, 2012).

SNPs on *SH2B1* and *TMEM18* have been found to be associated with adult and childhood BMI (Almen et al., 2010; Willer et al., 2009; Zhao et al., 2011; Zhao et al., 2009). Out of 25 SNPs investigated by Zhao et al in a cohort of 6078 children of European descent, the strongest effect for obesity was found near *TMEM18* SNP (Zhao et al., 2009). A similar association of TMEM18 SNP rs6548238 was found in 2042 children (9.7 ± 0.4 years) and adolescents (15.5 ± 0.5 years) from European descent with strong association with BMI and sum of skin fold thickness (den et al., 2010; Zhao et al., 2009). In the UCL-FGS cohort, we did not find associations of SNPs on *SH2B1* and *TMEM18* with BMI but they were significantly associated with skin-fold thickness at 2 and 3 years of age. *TMEM18* is widely expressed in the majority of neurons in all brain regions and might be powerfully involved in the modulation of energy homeostasis (Almen et al., 2010).

SNPs Influencing BP on GWAS (BP-GWAS)

In general, birth size and early childhood growth have a positive association with blood pressure (Tu et al., 2010). Data from diverse populations show that the evidence for BP tracking from childhood into adulthood is strong (Chen and Wang, 2008). One may therefore argue that genetic variations influencing BP in adults will influence childhood size and BP. In a cohort of white European pregnant women and their offspring, this study reports findings of association of BP-GWAS genotype with antenatal growth, postnatal size and blood pressure at 3 years of age. We surveyed eight SNPs in the eight genes which were

significantly associated with BP in the CHARGE consortium GWAS study (Levy et al., 2009).

SNP rs1004467 on *CYP17A1* was significantly associated with 3rd trimester femoral length and abdominal circumference. The influence however was not strong enough to affect birth size. In a small sample size of 134 pregnant Japanese women, Yamada H et al demonstrated an association of the polymorphisms of CYP17 that encodes for the cytochrome p450c 17alpha enzyme with fetal growth restriction (Yamada et al., 2004). None of the other SNPs from BP-GWAS were significantly associated with birth size. Some of the SNPs on *CACNB2, TBX3 and SH2B3* were significantly associated with polarised group of SGA infants.

SNP rs3184504 in *SH2B3* was significantly associated with catch-up growth and with weight SDS and BMI SDS at 2 and 3 years of age. At 3 years, subjects with CC genotype were heavier by 0.96 SDS and had higher BMI by 0.54 SDS compared to TT genotype. Association of the *SH2B3* rs3184504 genotype with catch-up growth and consistent longitudinal association with size at 2 and 3 years of age suggest that it is not a chance finding but a true association. *SH2B3* codes for lymphocyte adaptor protein (LDP) which mediates the interaction between extracellular T-cell receptors and intracellular signalling pathways.

SNP rs3184504 in *SH2B3* is associated with multiple traits. In earlier GWAS studies, this SNP was found to be associated with BP and increased odds of autoimmune diseases including type 1 diabetes (Todd et al., 2007), celiac disease (Hunt et al., 2008), myocardial infarction and higher eosinophil and other blood counts (Gudbjartsson et al., 2009). The role for SNP rs3184504 in *SH2B3* on BP is not clear. It is hypothesised that loss of LDP function alters the endothelial cell atherothrombosis and smooth muscle tone thereby influencing BP (Arora and Newton-Cheh, 2010). Given the recent evidence of altered immune system contributing towards pathogenesis of obesity, genetic variations in *SH2B3* may have a role in contribution towards genetic variance in BMI (Batra and Siegmund, 2012; de Heredia et al., 2012).

In the UCL-FGS cohort, none of the other BP-GWAS SNPs demonstrated significant association with BP measurements at 3 years of age. This may imply that these SNPs are not associated with early childhood BP and they exert their influence on BP after 3 years of age. Sample size of this study may have failed to

detect mild effects of these SNPs on early childhood BP, which were noted in adult studies performed in a larger study population.

Summary discussion and conclusion

In the UCL-FGS cohort we have found associations of genetic variants in the *FTO* gene with BP at 3 years of age; *MTCH2* with catch-up growth and size at 3 years of age; *SH2B1* and *TMEM18* with skin-fold thickness and *SH2B3* with weight SDS and BMI SDS at 2 and 3 years of age. None of the BMI-GWAS were associated with weight or BMI at 3 years of age and none of the BP-GWAS were associated with BP at 3 years of age. These may imply that genetic variations in BMI-GWAS and BP-GWAS are not associated with BMI and BP respectively in early childhood and may exert their influence after 3 years of age. It is also a possibility that our sample size may have failed to identify mild effects of these SNPs which have been previously identified in larger adult cohorts. The strength of our study is the prospective collection of longitudinal data until 3 years of age. Previous replications of BMI-GWAS and BP-GWAS in childhood cohorts have either collected cross-sectional data or retrospectively collected data through recall or medical notes. Independent GWAS studies on the childhood population with prospective collection of longitudinal data on a larger sample size will lead to identification of new genetic variants as well as elucidate a role of adult BMI-GWAS and BP-GWAS findings on childhood size and BP.

Chapter 5

Conclusion

Most polygenic traits are influenced by genetic variations and environmental factors. It is difficult to dissect the relative contributions of genetic and environmental factors to polygenic traits. In most polygenic traits, genetic variations contributes towards 40-60 % variation. Polygenic phenotypes such as height, weight, head circumference and BP are controlled by multiple genomic loci. Selection may act on such traits by altering allele frequencies at a single associated polymorphism or by changing allele frequencies at many associated polymorphisms. The contribution of each genetic locus in influencing phenotypic variations is small and it is anticipated that small contributions of each locus produces net variation of observed phenotype in each individual.

Intrauterine and postnatal growth influences future risks for adult health and conditions such as metabolic syndrome. These conditions have major personal, social and economical consequences. Understanding the origins of such conditions is essential for developing new screening tests and interventions, with the aim of ultimately improving personal and population health. The development of the “fetal origins of adult disease” hypotheses was an important breakthrough in addressing some of the potential early life events associated with late disease, and the recent explosion in genomic knowledge and technology is now allowing some of these potential effects to be addressed at a genetic level.

In the present study, we have identified genetic variations in the *IGF1*, *IGF2* and *GHR* genes influencing antenatal growth, birth size, early childhood growth and BP at 3 years of age. We have also indentified genetic variations in GWAS derived gene loci of adult BMI and BP associated with early childhood growth and BP at 3 years of age.

Our main conclusions are:

1. The *GHR* exon 3 deletion genotype was significantly associated with birth weight and placental weight.
2. Several *IGF1* genotypes have been found to be associated with parameters of fetal growth (rs4764695, rs7136446, rs10735380 and rs7965399), catch-up growth (rs4764695), with postnatal anthropometry (rs5742667, rs2946834 and rs6214) and BP (rs10735380) at 3 years of age. However, none demonstrated significant consistent longitudinal association with parameters investigated.

3. *IGF2* SNPs were significantly associated with intrauterine growth (rs680), birth weight (rs680), placental weight (rs680) and BP (rs3842759) at 3 years of age.
4. Several SNPs in genes found to be associated with adult BMI and BP from GWAS were significantly associated with early childhood size (*MTCH2, SH2B3*), body composition (*SH2B1, TMEM18*) and BP (*FTO*).

However, these genetic variations and others identified in GWAS explain only a small fraction of genetic variance for the polygenic traits. While GWAS are relatively simple to perform using high-throughput genotyping technologies, interpretation of results are neither straightforward nor replicable in multiple ethnic populations. GWAS have been utilised to identify genetic variations in single nucleotides around a gene, but the effects of potentially important rarer allelic variants will be missed. Furthermore, current advances in technology allow identification of copy number variants (McCarroll, 2008), which may also contribute significantly towards genetic variance of polygenic traits.

Besides genetic variations, DNA expression and epigenetic factors also contribute towards variability of polygenetic traits. Recent advances in automated high-throughput array-based technologies have made large-scale methylation studies possible (Kidambi et al., 2012). Use of such studies is required to identify epigenetic variants that might contribute towards the missing heritability of the polygenic traits and those that mediate differential gene expression from environmental influences (Wang and Snieder, 2010).

Further longitudinal studies on large sample sizes using advanced technologies will assist in identifying genetic variations that contribute towards heritability of polygenic traits.

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Appendix 1: List of laboratory equipment used

Benchtop centrifuge: Sorvall Legend RT (swinging buckets rotor 7500 6445), Sorvall, Germany.
Benchtop microcentrifuge: Heraeus Pico 17, Thermo Fisher Scientific, UK.
ChemiDoc system: Bio-Rad Laboratories Ltd., UK.
Class II biological safety cabinet: HeraSafe HS12, Thermo Fisher Scientific.
Cold room (4°C): Stancold, UK.
Drying cabinet: Unitemp, LTE Scientific, UK.
Electronic multichannel pipettes (0.5-10 µl; 5-100 µl): Research pro, Eppendorf AG, Germany.
Electronic pipette filler: Easypet, Eppendorf.
Electrophoresis power supply: PowerPac 300, Bio-Rad.
Electrophoresis system: Sub-Cell GT, Bio-Rad.
Freezer (-20°C): ISU57, Lec, UK.
Freezer (-80°C): Ultra Low model U57085, New Brunswick Scientific, UK.
High performance stand alone centrifuge: Sorval RC 5C Plus (rotor SA-600), Sorvall.
Ice flake machine: KF75, Porkka, UK.
Incubator: Economy incubator size 2, Sanyo Gallenkamp, UK.
Laboratory glasswasher: Mielabor G7783, Miele, Germany.
Luminescence/fluorescence plate reader: FLUOstar OPTIMA, BMG Labtech Ltd., UK.
Mastercycler PCR machine (Eppendof Scientific, Stevenage, UK)
Microwave oven: Proline, UK
Precision micropipettes (0.5-10 µl; 2-20 µl; 20-200 µl; 100-1000 µl): Discovery
Refrigerated benchtop microcentrifuge: Accuspin Micro R, Thermo Fisher Scientific.
Scale: BL 150S, Sartorius, UK.
Vortex: Vortex-Genie 2, Scientific Industries Inc., USA.
Waterbath: Grant W14, Grant Instruments (Cambridge) Ltd., UK.

Appendix 2: Publication and presentations from this thesis

Publication in peer reviewed journal:

- **Padidela** R, Bryan SM, Abu-Amero S, Hudson-Davies RE, Achermann JC, Moore GE, Hindmarsh PC. The growth hormone receptor gene deleted for exon three (GHRd3) polymorphism is associated with birth and placental weight. Clin Endocrinol (Oxf). 2012 Feb;76(2):236-40. doi: 10.1111/j.1365-2265.2011.04207.x.

Presentations at national and international conferences:

- **Padidela** R, Bryan SM, Hudson-Davies RE, Achermann JC, Hindmarsh PC. A common polymorphism in the Insulin-like growth factor 2 gene is associated with late antenatal growth, birth size and size at 3 years of age. 92nd Meeting and Expo of the Endocrine Society (ENDO 2010), San Diego, CA, USA.
- **Padidela** R, Bryan SM, Abu-Amero S, Hudson-Davies RE, Achermann JC, Moore GE, Hindmarsh PC. The growth hormone receptor exon three deleted polymorphism is associated with birth and placental weight. 38th Meeting of British Society of Paediatric Endocrinology and Diabetes, 2010, Manchester, UK.