Genetic and Environmental Factors in Pre- and Postnatal Growth Disorders

Studies in children born small for gestational age (SGA), with and without postnatal short stature

Genetische factoren en omgevingsfactoren in pre- en postnatale groeistoornissen

Studies in te klein geboren (SGA) kinderen, met en zonder een persisterende te kleine lengte

Wietske Arianne Ester

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Voor Lo en David

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Chapter 1

General Introduction

General introduction

This thesis describes genetic and environmental factors which are important in pre- and postnatal growth disorders and specifically focuses on children born small for gestational age (SGA) with or without postnatal catch-up growth. It also presents a subclassification of short SGA subjects according to length, weight and head circumference at birth. In addition, it reports investigations in three genes which have been found to play a central role in growth regulation: the insulin-like growth factor 1 (*IGF1*), the insulin-like growth factor 1 receptor (*IGF1R*) and the insulin (*INS*) gene and their associations with short SGA and SGA catch-up subjects. In addition, the thesis describes the results of a study into relatively large deletions and duplications (also called "copy number changes") in 18 growth-related genes, performed in short SGA subjects. The last chapter describes phenotypic data of a large group of subjects participating in the Network of European Studies in Genes in growth (NESTEGG). This group consisted of subjects born SGA, either with persistent short or normal stature, idiopathic short stature (ISS) and controls. Finally, the aims of the study and outline of this thesis are described.

1. Small for gestational age (SGA)

Definitions

According to the consensus reached by the International SGA advisory board [1,2], children being born small for gestational age (SGA) had a birth length and or birth weight below -2.0 standard deviation score (SDS).

SGA refers to the size of the infant at birth, and not to intrauterine growth. The term intrauterine growth retardation (IUGR) is used to describe reduced growth velocity in the fetus. This can be observed by ultrasound measurements as a deviation of the fetal growth chart, as registered by at least two intrauterine growth measurements. Although SGA is often related to IUGR, not all SGA infants have suffered from IUGR, and infants who are born after a short period of IUGR are not necessarily SGA. Figure 1 shows the fetal growth pattern of SGA and IUGR newborns.

SGA born children can be grouped by their birth length (L), birth weight (W), or both being ≤ -2.00 SDS, termed as SGA_L, SGA_W or SGA_{L+W} [3]. In this population-based postnatal growth study, 8.1% were classified SGA. Children born short and light for gestational age were shorter as adults than those born either short or light [3]. In addition, others showed that some children born both short and light for gestational age also had a smaller postnatal head circumference [4].

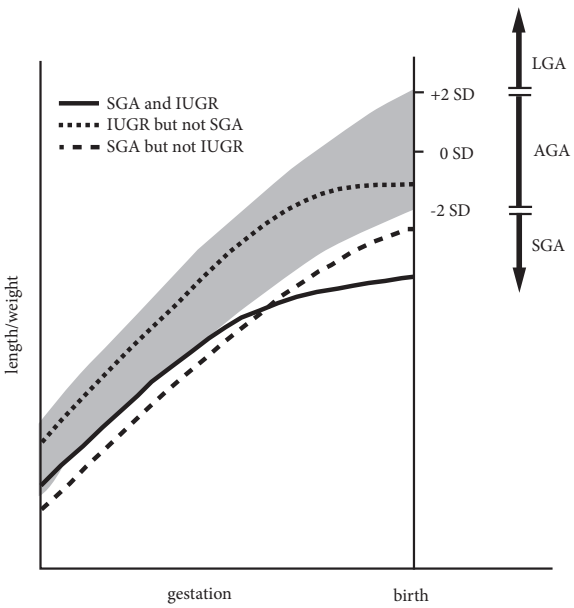

Figure 1. Fetal growth chart showing the difference between SGA and IUGR newborns.

Prevalence and etiology

When SGA is defined as a birth length and/or weight below -2 SDS for gestational age, 2.3% of all live-born infants are born SGA. In 2006, 10.46 live births per 1000 inhabitants of the European Union were reported (www.euro.who.int/HFADB) with a mid year population of 490 million. According to the definition, 118,000 European children were born SGA in 2006.

SGA can be caused by several factors, including fetal, maternal, placental and demographic factors [5-7]. Identification of the cause of SGA is important, as underlying mechanisms may affect prognosis and treatment. Table 1 shows several factors that were associated with SGA birth.

Fetal factors include chromosomal abnormalities, genetic defects, metabolic problems and congenital anomalies.

Maternal factors can be divided into medical conditions and environmental factors. Medical conditions include: chronic vascular diseases (secondary to hypertension, diabetes mellitus type 2, renal disease, collagen vascular disease), conditions associated maternal hypoxemia, infections (particularly toxoplasmosis, rubella, cytomegalovirus and herpes virus) and malnutrition. Environmental factors include cigarette smoking, alcohol abuse, use of illicit drugs (heroin, cocaine), and therapeutic drugs (e.g. anticonvulsants, anticoagulants).

Placental factors are associated with problems in placental perfusion resulting in reduced fetal oxygenation. These include structural abnormalities of the placenta, maternal or fetal thrombophilia, infarctions and suboptimal implantation site.

Demographic factors involve maternal age, parental race and height, obstetric history, and multiple gestation, particularly in case of shared fetal circulation.

Table 1. Factors associated with an increased incidence of infants who are born SGA. Adapted from Bernstein and Divon [5], Pollack and Divon [6], Wollmann [7] and Keller et al. [8].

Fetal factors	
Karyotypic abnormalities	Trisomy 21 (Down syndrome)
	Trisomy 18 (Edward syndrome)
	Monosomy X (Turner Syndrome)
	Trisomy 13 (gonadal dysgenesis)
Other chromosomal abnormalities	Autosomal deletions
	Ring chromosomes
Genetic diseases	Achondroplasia
	Bloom syndrome
Congenital anomalies	Potter syndrome
	Cardiac abnormalities
Maternal factors	
Medical conditions	Hypertension
	Renal disease
	Diabetes mellitus (advanced stages)
	Collagen vascular diseases (e.g. systemic lupus erythematosus)
	Maternal hypoxemia (cyanotic heart disease, chronic anemia, chronic pulmonary disease)
Infection	Toxoplasmosis
	Rubella
	Cytomegalovirus
	Herpes virus
	Malaria
	Trypanosomiasis
	Human immunodeficiency virus
Nutritional status	Low pre-pregnancy weight
	Low pregnancy weight with poor weight gain during pregnany
Substance use/abuse	Cigarette smoking
	Alcohol
	Illicit drugs

Maternal factors *(continued)*	
	Therapeutic drugs (e.g. warfarin, anticonvulsants, antineoplastic agents, folic acid antagonists)
Uterine/placental factors	
Gross structural placental factors	Single umbilical artery
	Velamentous umbilical cord insertion
	Bilobate placenta
	Placental hemangiomas
	Infarcts, focal lesions
Maternal and/or fetal thrombophilia	
Insufficient uteroplacental perfusion	Suboptimal implantation site
Placenta praevia	
Low-lying placenta	
Placental abruption	
Demographic factors	
Maternal age	Very young age
	Older age
Maternal height	
Maternal weight	
Maternal and paternal race	
Parity	Nulliparity
	Grand multiparity
Maternal history	Previous delivery of SGA infants
Multiple gestation	Particularly severe in syndromes associated with shared fetal circulation

Genetic factors

Although several factors have been identified in the etiology of SGA born children, the cause remains unidentified in up to 40% of the cases. SGA birth, as well as short stature, might be genetically determined. For that reason we investigated several genes, using a candidate-gene approach. In this approach specific genes are selected of which the protein is known to be involved in pre- and postnatal growth. Three genes were investigated which might be important in the development of the SGA neonate: the insulin-like growth factor 1 (*IGF1*), the insulin-like growth factor 1 receptor (*IGF1R*) and the insulin (*INS*) gene. All three genes are part of the growth hormone (GH)- IGF- IGF binding protein (IGFBP) axis (Figure 5).

IGF1 gene

The *IGF1*-gene (OMIM*147440, gene map locus: 12q22-q24.1, Figure 2) is thought to be an important candidate gene for children born SGA, being a major pre- and postnatal growth factor [9,10]. Prenatally, *IGF1* absence results in a 40% reduction in birth weight. During postnatal life, surviving *IGF1* knock-out mice become 30% of normal adult weight [9]. Several case-reports have described point-mutations and deletions in the *IGF1* [11-13]. Only a limited number of studies have investigated the relation of common genetic polymorphisms in the *IGF1* gene with SGA children [14,15].

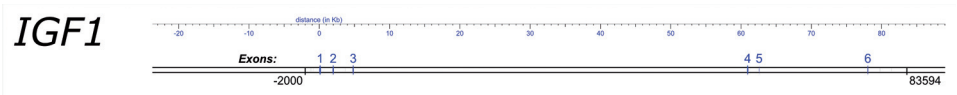

Figure 2. The *IGF1* gene structure.

IGF1R gene

The *IGF1R*-gene (OMIM*147370, gene map locus: 15q25-q26, Figure 3) is an important candidate gene for children born SGA, playing a major role in prenatal and postnatal growth, as *IGF1R* knockout mice did not survive postnatally [9,10]. Several case-reports have described point-mutations and deletions in the *IGF1R* gene in SGA children [16-29]. No studies on genetic polymorphisms in the *IGF1R*-gene in groups of SGA children have been published.

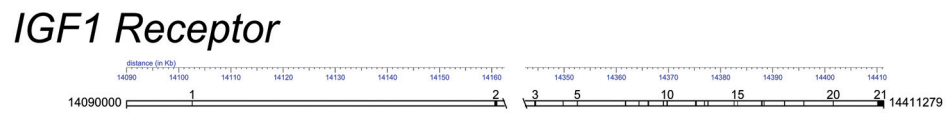

Figure 3. The *IGF1R* gene structure.

INS gene region

The insulin (*INS*) gene region (OMIM+176730, gene map locus 11p15.5, Figure 4) is considered to be a major candidate gene for the SGA phenotype as insulin is involved in fetal and early postnatal growth [30-33].

The most widely studied polymorphism in the *INS* gene is a variable number of tandem repeats (VNTR, OMIM *147510) which is located 600bp upstream of the *INS* transcription start site and comprises 14-25bp tandemly repeated sequence units. The insulin minisatellite has been associated with variations in birth size [31,34,35], obesity [32,36-39], diabetes mellitus

type 1 and type 2 [30], and polycystic ovary syndrome [33]. However, other studies could not demonstrate that the *INS* VNTR was associated with accelerated weight gain, reduced beta cell function or adiposity [40-43].

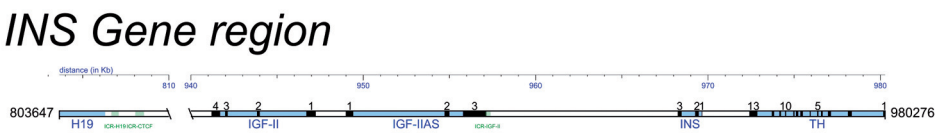

Figure 4. The *INS* gene region.

2. The GH-IGF-IGFBP axis, fetal and postnatal growth

Growth hormone (GH), insulin-like growth factors (IGFs) and IGF-binding proteins (IGFBPs)

The physiology of the GH-IGF-IGFBP axis is shown in Figure 5. GH is secreted by the pituitary gland under control of the hypothalamic hormones, GH-releasing hormone (GHRH) and somatostatin, as well as ghrelin, a hormone mainly produced in the stomach [44]. GHRH and ghrelin bind to their respective receptors in the pituitary and stimulate GH secretion. Somatostatin inhibits GH release. Most of the anabolic actions of GH are mediated by IGF-I, but GH has also many cellular effects that are independent of IGF-I [45].

The IGF system consists of 3 primary ligands: IGF-I, IGF-II, insulin, and three closely related membrane-bound receptors. IGF-I is an important member of the IGF system and shows structural and functional similarities with insulin and has important anabolic and metabolic effects. IGF-I is present in 2 forms: the circulating IGF-I which is primarily liver-derived and the extra-hepatic autocrine/paracrine form of IGF-I, which is produced by local tissues [46].

Most of the circulating IGF-I is bound to IGFBPs, of which six classes have been identified. IGFBP3 is the major carrier protein of IGF-I and binds 70-95% of IGF-I as a binary complex or a ternary complex together with acid-labile subunit (ALS) [47]. IGFBP3 and ALS are both regulated by GH. IGFBP-1 binds only a small fraction of IGF-I, but has direct inhibitory effects on IGF-I mediated processes *in vivo* [47]. The hepatic production of IGFBP-I is inversely regulated by the portal supply of insulin [48]. Less than 1% of IGF-I is unbound and circulates in its free form. Free IGF-I is believed to be the biological active form and several studies have indicated the importance of free versus total IGF-I in not only short-term dynamic metabolic changes, but also in long-term steady-state changes, like linear growth [49,50].

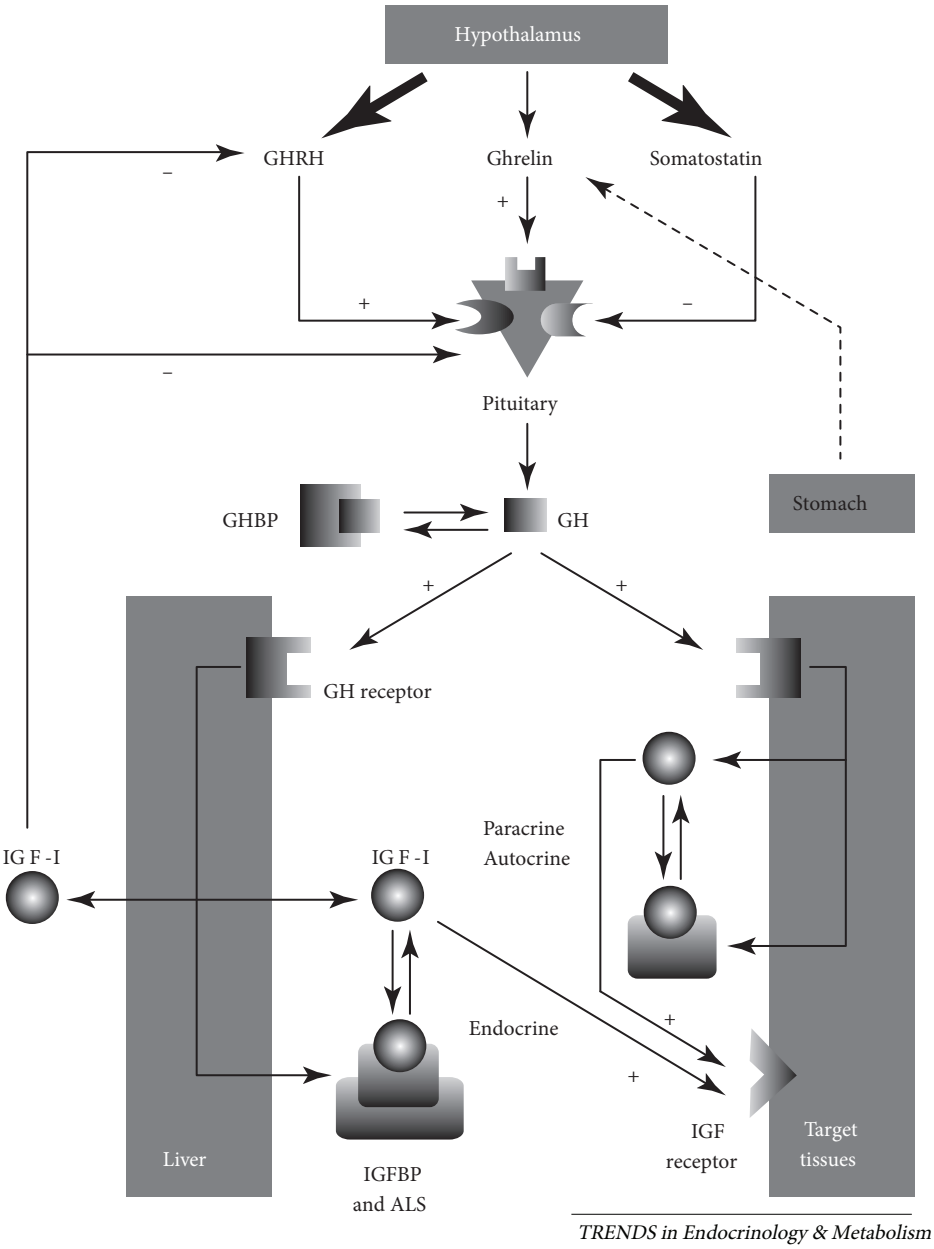

Figure 5. Physiology of the GH-IGF-IGFBP axis. Adapted from Holt [44].

Fetal growth

The exact role of GH in fetal growth remains unclear. Although high levels of GH in the fetal circulation have been reported [51,52], it appears that GH is not a major regulator of fetal growth, since the number of GH receptors in prenatal life is low [53]. On the other hand, in neonates with congenital GH deficiency, birth length was on average 1 SDS lower compared with healthy neonates, suggesting that GH does have a small effect on fetal growth [54]. In SGA fetuses, GH levels were found to be similar to appropriate-for-gestational age (AGA) fetuses during the second half of gestation [55]. In SGA neonates, however, most studies reported elevated GH concentrations in cord blood as compared with AGA neonates [55-58]. The latter suggests that SGA neonates have a state of GH resistance, which is characterized by increased GH levels [54].

Fetal IGF-I, IGF-II and insulin are the most important endocrine determinants of fetal growth and their concentrations are mainly regulated by genetic factors and fetal nutrition. IGF-I and IGF-II are already detectable in cord blood from the first trimester and show a 2-3 fold increase during the last trimester [59,60]. In newborn SGA infants, IGF-I and IGF-II levels were significantly reduced compared with AGA infants, suggesting an important role for low IGF-I levels in fetal growth retardation [55,59,60]. Gene deletion studies in mice demonstrated that IGF-I and IGF-II knock-out mice had a birth weight which was 60% of normal [10]. The growth-promoting effect of IGF-II is also exerted via the IGF-I receptor. Hence inactivation of the IGF-I receptor even resulted in a more severe reduction in birth weight to 45% of normal, due to loss of both IGF-I and IGF-II action. Interestingly, inactivation of hepatic IGF-I production, as in the liver IGF-I deficient (LID)-mice, had only a minimal effect on fetal growth, despite strongly reduced levels of circulating IGF-I [61]. Serum levels of free IGF-I were similar for LID-mice and wild-type controls, suggesting that normal growth in utero may result from circulating, free IGF-I levels. It might also be that the extra-hepatic autocrine/paracrine production of IGF-I was sufficient to maintain normal growth. In SGA fetuses, IGFBP-3 levels were lower than in AGA fetuses, wheras IGFBP-1 levels were elevated [59,62]. IGFBP-1 has been inversely related to insulin levels and it might be that low insulin levels in the growth-retarded fetus are responsible for the elevation of IGFBP-1 [44].

Initially, insulin was thought to be the major growth-promoting hormone in fetal life. More recently, it has been shown that insulin acts directly via stimulation of cellular nutrient (glucose) uptake and indirectly by stimulation of IGF-I production [63]. Glucose availability and the subsequent increase in fetal insulin are the major regulators of fetal IGF-I production. Fetal pancreatectomy in sheep resulted in low fetal IGF-I levels and caused severe intrauterine growth retardation [64]. In addition, intrafetal infusions with either glucose or insulin increased fetal IGF-I.

Postnatal growth

The pathophysiology of persistent short stature in some children born SGA is not fully understood. Disturbances in the GH-IGF-IGFBP-axis may contribute to poor postnatal catch-up growth as several studies have demonstrated that up to 60% of SGA children with persistent short stature show a reduced spontaneous GH secretion during a 24-hour GH-profile and/or low GH peaks during GH provocation tests [65-69]. Serum IGF-I and IGFBP-3 levels were also reduced in short SGA children when compared with healthy controls with normal stature [67, 70,71]. Some short SGA children have, however, normal or high GH-levels together with low levels of IGF-I and IGFBP-3, suggesting a reduced functioning of the GH-receptor [68]. High levels of GH and IGF-I have also been reported in some SGA individuals, which suggests that these children have a reduced function of the IGF-I receptor [72].

Abnormalities in the GH-IGF-IGFBP axis, as well as a lower birth weight of subjects, has been associated with higher risks of diabetes mellitus type 2 and cardiovascular disease in adulthood [73-75]. Therefore, in SGA subjects, disturbances in the GH-IGF-IGFBP axis may be related to the future development of these diseases.

3. Clinical aspects and hypotheses

Short stature

SGA is a common cause of short stature in childhood and adulthood, accounting for 20% of all cases [76]. Most children born SGA, show a spontaneous catch-up growth to a normal height above -2 SDS. However, approximately 10% of them do not attain a normal height and thus remain short throughout life. In a Swedish cohort of 111 infants born SGA, with a birth length below the -2 SDS, 13% still had a height below -2 SDS at the age of 2 years [3]. In another group of 724 infants born SGA, defined as a birth length below -1.88 SDS, Hokken-Koelega et al. found that 15% did not show catch-up growth to a normal height at the age of 2 years [77]. Catch-up growth is most pronounced during the first 6 months and was usually completed at the age of 2 years [77]. However, in premature SGA infants catch-up growth might take longer]77,78[.

Chaussain et al. reported that SGA children with a birth length below the -2 SDS who remained short during childhood reached a mean adult height of 161.9 (8.0) cm (~-2.1 SDS) for boys and 147.6 (7.0) cm (~-2.8 SDS) for girls [79,80]. These values were significantly lower than the target heights of these children ($p < 0.001$) [79]. If a normal height above -2.0 SDS has not been achieved by 2 years of age, there is a 7-fold increased risk for short stature for those

born with a low birth length and a 5-fold increased risk for those born with a low birth weight [76].Therefore, a child born SGA who is still short at 3 years of age, should be referred to a paediatrician with expertise in endocrinology [2].

Cardiovascular disease associated with SGA

In epidemiological studies, an inverse relation was found between birth weight and the risk of hypertension, cardiovascular disease and diabetes mellitus type 2 in adulthood [81-83]. Insulin resistance plays an important role in the pathogenesis of these diseases]84,85[. The exact mechanisms underlying these associations are, however, still unknown. Several hypotheses have been proposed:

Fetal origins hypothesis

Based on a number of epidemiological observations linking reduced birth weight and adult diseases, such as hypertension, hyperlipidemia, diabetes mellitus type 2, coronary artery disease and metabolic syndrome, Barker et al. suggested that fetal malnutrition results in permanent metabolic alterations and changes in organic structures in the fetus [81,82,84,85]. This re-programming would be in favor of short-term survival, but detrimental on the long-term as it would result in adult diseases (Figure 6).

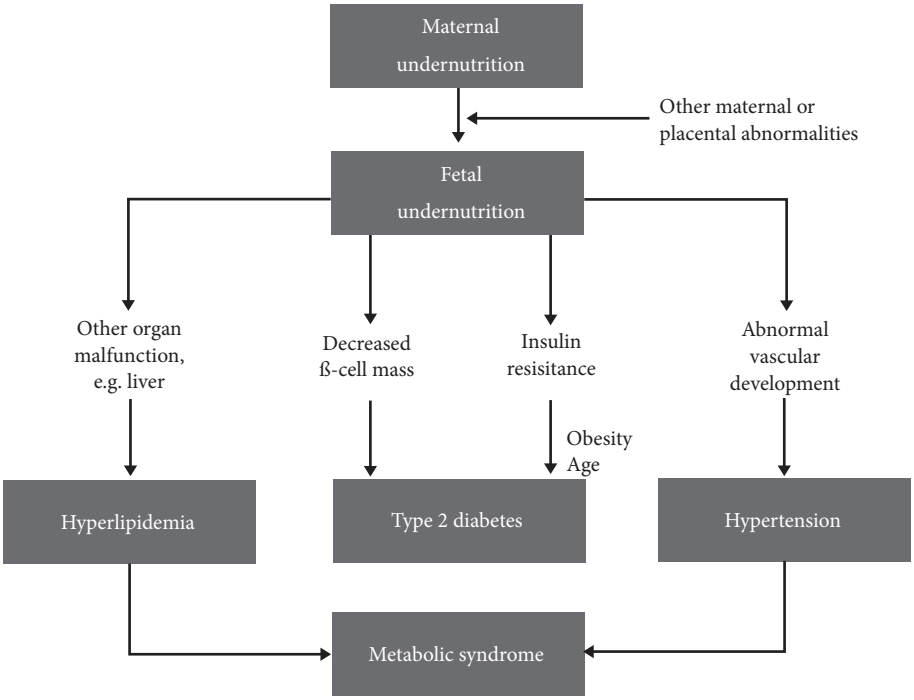

Figure 6. Representation of the fetal origins of the metabolic syndrome. Adapted from Barker et al. [82,86].

Fetal insulin hypothesis

The fetal insulin hypothesis was formulated by Hattersley et al. It postulated that the association between low birth weight and adult insulin resistance is principally genetically determined [87]. Genes involved in insulin resistance would result in low-insulin-mediated fetal growth as well as insulin resistance in childhood and adulthood (Figure 7).

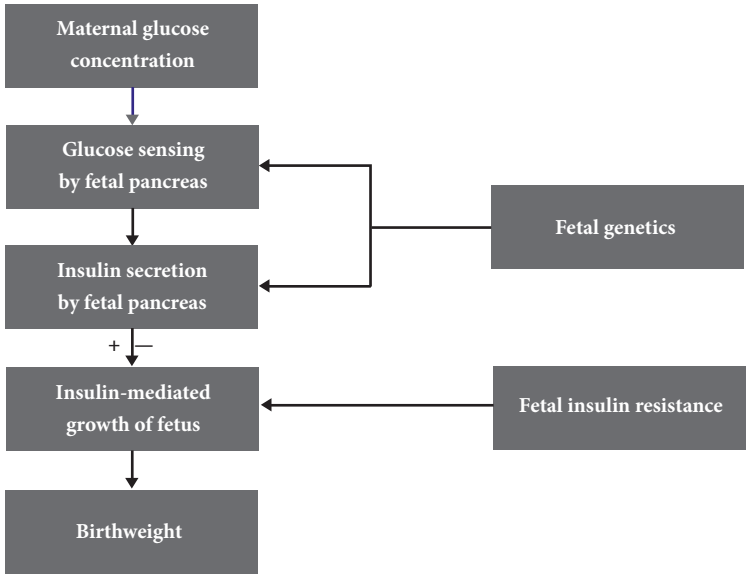

Figure 7. Simplified representation of fetal insulin hypothesis. Adapted from Hattersley et al. [87].

Growth acceleration hypothesis

Singhal and Lucas suggested that rapid postnatal growth rather than birth weight per se, could have adverse long-term effects and might result in adult diseases (Figure 8) [88].

Figure 8. Simplified representation of growth acceleration hypothesis. Adapted from Singhal and Lucas [88].

Fat accumulation hypothesis

Based on detailed measurements on body composition by dual energy x-ray absorptiometry (DXA), Leunissen et al. took the growth acceleration hypothesis by Singhal and Lucas a step further by specifying growth acceleration into fat accumulation (Figure 9) [89]. According to this hypothesis, small size at birth followed by growth in height and weight as such is not a problem as long as a subject continues to have a normal amount of fat for its age, gender and height.

Figure 9. Simplified representation of fat accumulation hypothesis. Adapted from Leunissen et al. [89].

4. Genetic studies in Dutch SGA children

Since 1991, we have performed prospective cohort studies to evaluate the effects of growth hormone (GH) treatment in short SGA children [4,71,90]. The children who participated in these studies were enrolled in the genetic study when the parents and/or their child agreed to participate and when children fullfilled the in- and exclusion criteria (Appendix A). Our genetic study has given us the unique opportunity to study genetic variations in a large group of short SGA children with detailed phenotypic data, before and during GH treatment.

Since 2003, the Programming Factors for growth and Metabolism (PROGRAM) study was started in which both short SGA, SGA catch-up, idiopathic short stature (ISS) and control young adults were enrolled [89,91]. The in- and exclusion criteria were similar to the NESTEGG study (Appendix B).

5. The Network of European Studies in Genes in growth (NESTEGG)

In 2001, the Network of European Studies in Genes in Growth (NESTEGG) was initiated by pediatric endocrinologists and molecular geneticists to study the etiology of pre- and postnatal growth failure in children and their parents (Appendix B) [92]. The primary aim of NESTEGG was to identify genetic influences on fetal and postnatal growth by studying subjects born small for gestational age (SGA) with and without postnatal catch-up growth and children with idiopathic short stature (ISS) (Figure 10). The secondary aim of NESTEGG was to study phenotypic characteristics of SGA and ISS subjects and their parents in order to identify parent-of-origin effects of genetic polymorphisms and to study growth response in growth hormone (GH) treated patients in relation to phenotype and genotype.

Children were collected in The Netherlands, France, Germany and the United Kingdom. SGA children were included when birth weight and/or birth length was less than ≤ –1.88 standard deviation score (SDS) according to the national growth charts (The Netherlands and Germany [93]; France [94]; United Kingdom [95]). Height at or after 3 years of age determined whether a child had caught up (> –1.88 SDS: SGA catch-up) or remained short (< –1.88 SDS:

short SGA) (The Netherlands [96]; France [97]; United Kingdom [95]; Germany [98]). Subjects in the ISS group had a birth weight and/or birth length above –1.88 but less than +2.00 SDS (The Netherlands [96]; France [97]; United Kingdom [95]; Germany (98]) and a actual height < -1.88 SDS (The Netherlands [96]; France [97]; United Kingdom [95]; Germany [98]).

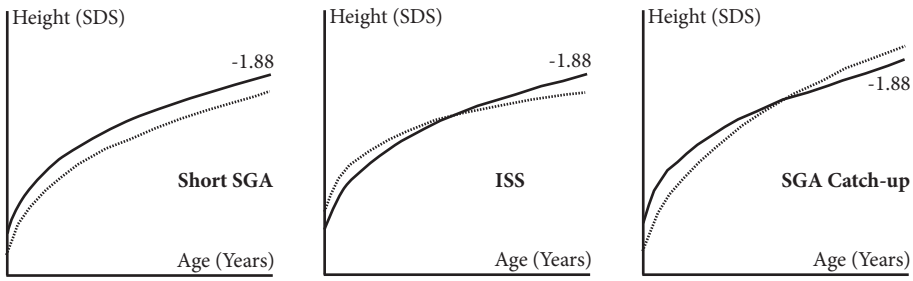

Figure 10. NESTEGG groups: Growth patterns of short SGA, SGA catch-up and ISS subjects.

6. Genetic variations, strategy and techniques in the Dutch study

In this study, several genetic variations were investigated among SGA subjects. The genetic variations which were investigated were single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTRs) and relatively large deletions and duplications.

Genetic variations

Genetic variations comprise a diversity of variations in the genome. They can be subdivided in genetic polymorphisms and mutations. A genetic polymorphism is a variant, not necessarily related to disease, which occurs in more than 1% of the normal population. A mutation is a change in genetic information, by definition occurring in less than 1% of the normal population and often related to disease.

Genetic polymorphisms can be subdivided in polymorphisms which comprise one base pair, also called single nucleotide polymorphisms or SNPs, or polymorphisms which consist of a tandemly repeating sequence in the genome at a particular locus, also called "variable number of tandem repeats" (VNTRs).

The most frequent mutations are single base-pair changes (so called point mutations). Point mutations can change the DNA sequence. Depending on the effect of the point mutation on the DNA code, the mutation is named: silent, missense or nonsense. Silent mutations code for

the same amino acid. Missense mutations code for another amino acid and nonsense mutations induce a stopcode which results in a truncated protein.. Also (relatively) large parts of the genome might be deleted or duplicated, these are also called copy number variants (CNVs).

Strategy

In the Dutch study genetic polymorphisms and copy number variations were studied.

Genetic polymorphisms

To investigate genetic polymorphisms in SGA children, we have used a candidate-gene approach. The IGF1, IGF1R and INS gene were studied by the following strategy:

Step 1:

Investigation of literature for the genetic polymorphisms in the gene and specifically for polymorphisms which are associated with SGA-related outcomes. Reports of genetic polymorphisms with functional studies showing the effect of the genetic polymorphism on RNA or protein level, were collected.

Step 2:

Investigation of genomic databases which contain single nucleotide polymorphisms throughout the genome. The presence of SNPs in coding regions, being either non-synonymous or synonymous, intronic regions or the promoter and 3'UTR region were noted.

Step 3:

Investigation of genetic databases which describe linkage disequilibrium (LD) in the gene and the tagging SNPs per LD-block per gene.

Step 4:

An integration of Step 1-3 was performed in which genetic polymorphisms reported in literature were selected when associated with SGA-related outcomes and especially when functional studies had been performed. These genetic polymorphisms were integrated with the SNPs collected from Step 2, in which the LD was taken into account and also particular gene regions like the promoter which is known for the binding of transcription factors regulating gene expression. Transcription factor binding site databases were investigated to identify transcription factors which change their binding depending on the allelic status of the genetic polymorphisms. In addition, a power calculation was performed in which the delta (difference

in population means) and sigma (the within group standard deviation) were estimated from the current literature. Depending on the group size, allele frequency or previous studies, cases with homozygous carriers of the minor allele were grouped together with the heterozygous carriers.

Step 5:
When the allelic status of the selected genetic polymorphisms was assessed in the population, the LD was determined. Subsequently haplotypes were constructed according to the observed LD patterns.

Step 6:
Association studies between the genetic polymorphisms, haplotypes and the SGA-related outcomes were performed.

Web resources which were used for this approach were:
- Celera genome browser: http://www.celera.com/
- HapMap genomic database: http://www.hapmap.org/
- Haploview v3.2: http://www.broad.mit.edu/mpg/haploview/
- NCBI SNP browser: http://www.ncbi.nlm.nih.gov/entrez/
- PHASE: http://www.stat.washington.edu/stephens/software.html
- Power and sample size calculation: http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize
- PubMed: http://www.ncbi.nlm.nih.gov/entrez
- TESS: Transcription Element Search System: http://www.cbil.upenn.edu/tess
- TFSEARCH: Searching Transcription Factor Binding Sites: http://www.cbrc.jp/research/db/TFSEARCH.html

Copy number variants
To investigate copy number variants in growth-related genes of SGA children, we used a broader candidate-gene approach by investigating 18 growth associated genes. This strategy consisted of:

Step 1:
Investigating a short SGA population for copy number variants in 18 growth associated genes by using a multiplex ligation-dependent probe amplification (MLPA) kit.

Step 2:
Examining all exonic area's of the gene in which a deletion or duplication was observed at Step 1, also by MLPA-kit.

Step 3:
Specify the extent of the deletion or duplication by testing multiple SNPs in the aberrant region, using the SNP array technique.

Techniques
The main genetic techniques described in this thesis are the Taqman genotyping assay and the multiplex ligation dependent probe amplification (MLPA, Appendix C). The Taqman genotyping assay was used to detect the allelic status of single nucleotide polymorphisms (SNPs). By the MLPA, relatively-large deletions and duplications were detected. Also, variable number of tandem repeats were determined by using the Genescan technique. To specify the exact copy number variant of the MLPA findings, the Affymetrix 262K *NspI* array was used.

Aims of the study

Subclassification of short SGA children
As short SGA children form a heterogeneous population including several subgroups, we subclassified the short SGA population according to their length, weight and head circumference at birth. We wanted to investigate whether these subgroups would demonstrate different postnatal growth patterns and would have a differential response to GH treatment.

Genetic polymorphisms of the *IGF1*, *IGF1R* and *INS* gene
To assess genetic variations in the the *IGF1*, *IGF1R* and *INS* gene and their surrounding region in a large group of children born SGA, with either short or normal stature. Linkage disequilibrium was investigated and haplotypes were associated with birth size, as well as postnatal and GH-induced growth. In addition, we performed association studies between *INS* genetic variations and blood pressure.

Relatively large deletions and duplications in growth-related genes
To assess the presence of deletions or duplications in 18 growth-associated genes in a large group of short SGA children. In addition, a detailed genetic and phenotypic work-up was performed in two short SGA patients with a deletion in the *IGF1R* gene.

Gestational factors in short SGA, SGA catch-up and ISS subjects

To evaluate the frequency of hypertension, smoking and alcohol use during gestation in a large group of short SGA, SGA catch-up and idiopathic short stature (ISS) subjects (NESTEGG). In addition, we investigated associations between the gestational factors and spontaneous postnatal growth and growth during GH treatment.

Outline of the thesis

Chapter 1 gives an introduction in the topics described in this thesis.

Chapter 2 proposes a subclassification of small for gestational age children with persistent short stature, based on birth weight, birth length and head circumference at birth. In addition, the spontaneous and GH-induced postnatal growth pattern was determined in the various subgroups. **Chapter 3** reviews the current literature with regard to genetic polymorphisms in the *IGF1* and *IGF1R* gene.

Chapter 4 reports associations between birth size, spontaneous postnatal and GH-induced growth, in relation with *IGF1* gene polymorphisms and haplotypes.

Chapter 5 shows associations which were found in a much larger population consisting of both short SGA and SGA catch-up children.

Chapter 6 describes a genetic association study of *IGF1R* polymorphisms and haplotypes with spontaneous postnatal growth in short SGA and SGA catch-up children.

Chapter 7 presents associations between insulin gene polymorphisms and haplotypes with spontaneous postnatal growth in short SGA and SGA catch-up children.

Chapter 8 shows the results of a detailed study in which relatively large deletions and duplications of 18 growth-associated genes in short SGA children were investigated.

Chapter 9 describes the characteristics and spontaneous postnatal growth and growth during GH-treatment of short SGA, SGA catch-up and ISS subjects (NESTEGG) and the relations with gestational hypertension, smoking and alcohol use.

Chapter 10 discusses our data in relation to current literature and comments on the clinical implications and conclusions of our study results.

Chapter 11 summarizes our findings in English.

Chapter 12 presents a Dutch summary.

Appendix A. Genetic studies in Dutch SGA patients

Inclusion criteria

- Born small for gestational age (SGA), defined as a birth length ≤ –2.00 SDS [93]
- Persistent short stature, defined as a height ≤ -2.00 SDS at the age ≥ 3 years [96]
- Catch-up growth, defined as a height > -2.00 SDS at the age ≥ 3 years [96]
- Caucasian descent
- Uncomplicated postnatal period

Exclusion criteria

- severe chronic illness
- endocrine disorders
- chromosomal or genetic abnormalities
- positive endomysial or transglutaminase antibodies
- skeletal disorders
- psychosocial dwarfism
- growth failure caused by other syndromes (except Silver Russell syndrome)

Design

The Dutch genetic studies started in 2000. The initial study population in 2002 consisted of 201 short SGA children. The total populaion consisted of 635 SGA children either short stature or normal stature. The genetic studies were performed at the ErasmusMC, Sophia Children's Hospital, Rotterdam.

Appendix B. Network of European Studies in Genes in Growth (NESTEGG)

Aim

The primary aim of NESTEGG was to identify genetic influences on fetal and postnatal growth by studying subjects born small for gestational age (SGA) with and without postnatal catch-up growth and children with idiopathic short stature (ISS).

The second and third aims of NESTEGG included the study of phenotypic characteristics of SGA and ISS subjects and their parents, to identify parent-of-origin effects of genetic polymorphisms and to study growth hormone response in treated patients and relate this to phenotype and genotype. Growth hormone therapy was not a requirement for inclusion in this study.

Patients

Recruitment of all subjects was performed according to standard operating procedures in four paediatric endocrinology departments, namely, Sophia Children's Hospital Erasmus University Medical Centre Rotterdam; University Children's Hospital Toulouse; University Children's Hospital Tübingen and Bart's and the Royal London Hospitals London. Each centre gained ethical approval from their regional ethics review board. Subjects were free to refuse to participate in this study without altering their future medical management. The short subjects were recruited from the growth clinics, the SGA catch-up group was recruited from studies in young adult or from particular targeted recruitment, based on identification through birth cohorts.

Inclusion criteria

- SGA was defined as a birth weight and/or birth length ≤ –1.88 standard deviation score (SDS) according to the national growth charts (The Netherlands and Germany [93]; France [94]; United Kingdom [95]).
- Height at or after 3 years of age determined whether a child had caught up (> –1.88 SDS: SGA catch-up) or remained short (< –1.88 SDS: short SGA) (The Netherlands [96]; France [97]; United Kingdom [95]; Germany [98]).

- ISS children were selected by having a birth weight and/or birth length > −1.88 to +2.00 SDS and a prepubertal height after 3 years of age ≤ −1.88 SDS (The Netherlands [96]; France [97]; United Kingdom [95]; Germany [98]).
- Gestation ≥ 30 weeks with uncomplicated postnatal period (ventilation < 72 hours not including CPAP).
- Subjects were included only when they were of Caucasian origin and all grandparents and parents must have been born and living in Western Europe.

Exclusion criteria

- syndrome or dysmorphic features (except Russell Silver syndrome)
- chromosomal or genetic abnormality
- severe chronic illness or endocrine disease
- positive gliadin, endomysial or reticulin antibodies
- growth hormone deficiency
- severe disproportionate short stature
- psychosocial dwarfism
- any psychiatric, neurodegenerative, or chronic illness in parents, which may affect their ability to give fully informed consent or donate blood
- adoption of the child

Appendix C. Genetic techniques

The main genetic techniques described in this thesis are the Taqman genotyping assay and the multiplex ligation dependent probe amplification (MLPA).

Taqman: Taqman ® SNP genotyping assays, Applied Biosystems

Taqman SNP genotyping assay is based on the design of two Taqman probes, specific for the wild type and the mutant allele. Each of the two probes is labelled with a different fluorescent reporter (usually VIC or FAM), and each is designed with the genetic variation affecting the middle part of the probe sequence. Each of the reporters is "quenched" (silenced) by TAMRA (6-carboxyl-tetramethyl-rhodamine) attached by the left arm located at the 3'end of each probe. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence. During PCR, forward and reverse primers hybridize to a specific sequence of the target DNA. The Taqman probe hybridizes to a target sequence within the PCR product. The AmpliTaq Gold enzyme cleaves the Taqman probe with its 5'-3'nucelease activity. The reporter dye and quencher dye are separated upon cleavage. The 3'end of the Taqman probe is blocked to prevent extension of the probe during PCR. The separation of the reporter dyes from the quencher dye results in increased fluorescence for each of the VIC and FAM reporters. Both primer and probe must hybridize to their targets for amplification and cleavage to occur. The fluorescence signals are generated only if the target sequences for the probes are amplifying during PCR. The increase in fluorescence is measured, and is a direct consequence of target amplification during PCR (Figure 11).

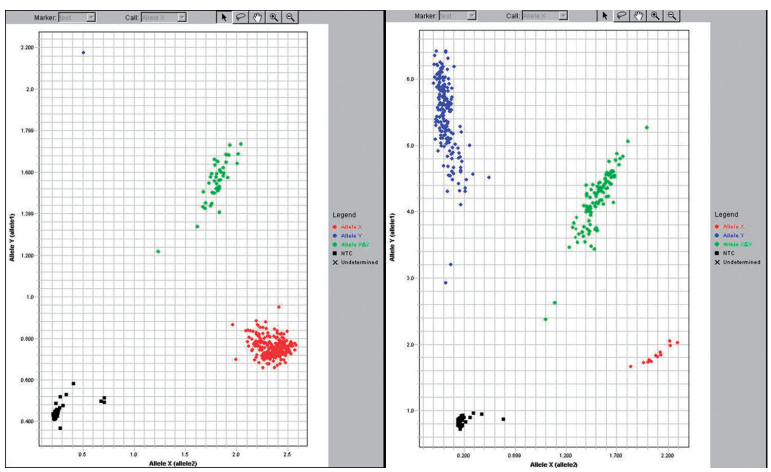

Figure 11. Example of a Taqman assay result on a genotyped population.

Multiplex ligation dependent probe amplification (MLPA)

MLPA can be used to detect the copy number of approximately 45 sequences in one test. Each probe consists of a two oligonucleotides, recognizing adjacent target sites on the DNA. When the two probes are hybridized to their target sequence, they can be ligated to each other (Figure 12). Only ligated probes will be amplified by universal primer pairs. The amplification product of each probe has a unique length, which makes it possible to separate and quantify the PCR products using electrophoresis (Figure 13).

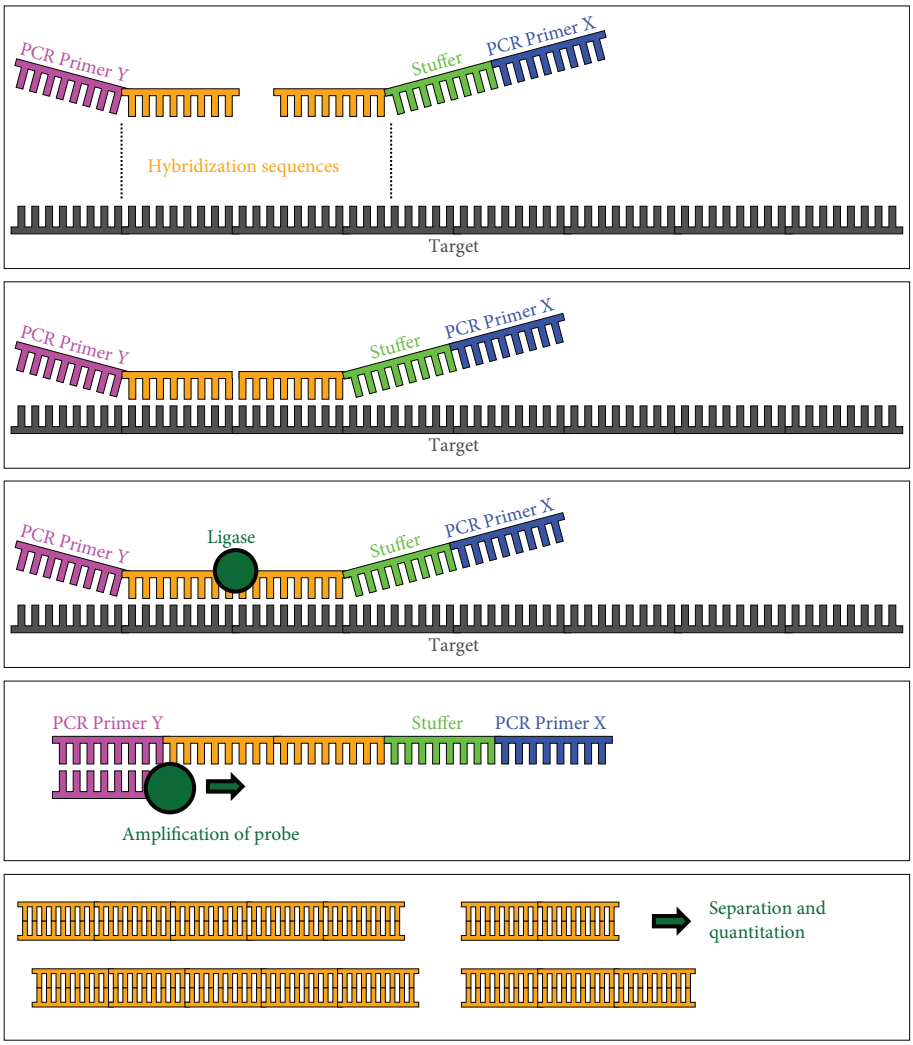

Figure 12. The principle of the MLPA technique.

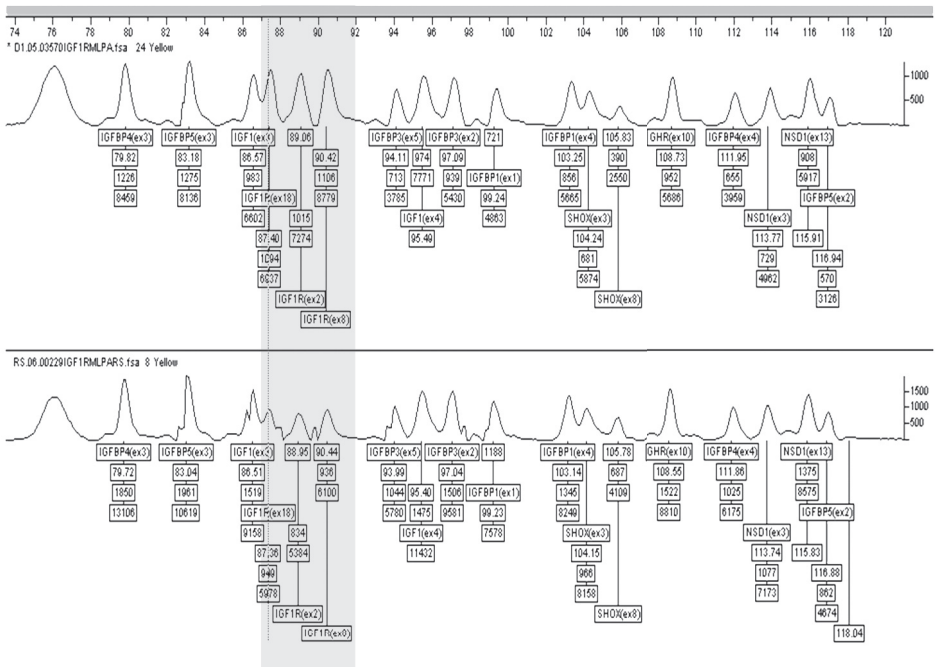

Figure 13. Example of an MLPA results in which the peak height indicates a copy number change of the probe target sequence. The two lanes represent DNA of a case and a control study participant whose peak heights can be compared.

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Chapter 2

Subclassification of small for gestational age children with persistent short stature: Growth patterns and response to GH treatment

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Abstract

Aim

We determined whether subclassification of short SGA children according to birth anthropometrics could delineate different patterns in gestation, delivery, postnatal growth, response to growth hormone (GH) treatment and parental height.

Methods

201 short SGA children were divided over 3 groups, SGA_L, SGA_{L+W} and SGA_{L+W+HC}, according to birth length (L), weight (W) and head circumference (HC) ≤ -2.00 standard deviation score (SDS).

Results

SGA_{L+W+HC} children were born after the shortest gestational age and more often by caesarean section than SGA_L children (36.3 vs. 38.1 weeks, 68.4 vs. 24.4%). SGA_{L+W} children had an intermediate pattern and experienced most gestational hypertension (P = 0.01). At birth, SGA_{L+W+HC} children were shorter than SGA_L or SGA_{L+W} (-4.12 vs. -2.67 and -3.72 SDS, P ≤ 0.001). During the first 3 years of life, SGA_{L+W+HC} children exhibited an increased growth in height (0.98 SDS) and HC (1.28 SDS) than SGA_L (height, -0.06 SDS; HC, -0.30 SDS) and SGA_{L+W} (height, 0.62 SDS; HC, -0.31 SDS). However, HC SDS remained smaller for SGA_{L+W+HC} than the other groups at age 3. The groups did not differ in growth response during GH treatment. SGA_L children tended to have shorter parents and target height than SGA_{L+W+HC} children.

Conclusions

Our study shows that subclassification of short SGA children might be a useful method for investigating SGA children as the subgroups revealed a different gestation, delivery and postnatal growth pattern. Response to GH treatment was not different between the groups.

Introduction

Small for gestational age (SGA) is the term used to describe a group of children born with a birth weight and/or birth length below the normal range of a reference population, corrected for gestational age at birth. Since reduced size at birth may result from any number of fetal, maternal, placental or demographic influences, children born SGA comprise a heterogeneous group with a broad spectrum of clinical characteristics. While many children born SGA achieve sufficient growth to normalize their stature by 2 years of age, approximately 10–15% maintain a height below -2 standard deviation scores (SDS) and continue to be short throughout adolescence and adulthood [1]. Furthermore, in a population-based postnatal growth study of 3650 infants, of whom 8.1% were classified SGA according to their birth weight and birth length SDS, children born both short and light for gestational age were found to be shorter as adults than those born either short or light [2].

Short SGA children appear to benefit from growth hormone (GH) treatment in terms of height increment. However, even after accounting for differences in parental height, age and duration of treatment, there appears to be a wide variation in the response to GH treatment that has been difficult to explain. The recent European approval of GH therapy to improve growth in short SGA children highlights the clinical relevance of ascertaining which short SGA children are most likely to benefit from treatment because potentially 23000 European children meet the criteria for GH therapy annually [1-3].

We have previously demonstrated that mean head circumference (HC) is significantly smaller ($P < 0.001$) in short SGA children with a reduced birth weight and birth length (≤ -2.00 SDS) than in short SGA children born with merely a short birth length (≤ –2.00 SDS) [4]. The objective of this study was to determine whether subclassification of short SGA children according to their birth anthropometrics could delineate differences in gestation, type of delivery, postnatal growth, response to GH treatment and parental height.

Methods

Patients

Data was evaluated of 201 SGA children with persistent short stature who were participating in prospective cohort trials evaluating the effect of growth hormone (GH) treatment [4-9]. SGA was defined as a birth length ≤ –2.00 SDS [10]. Persistent short stature was defined as a height ≤ -2.00 SDS at the age ≥ 3 years [11]. Children were divided into three groups based on anthropometrics at birth: group 1, comprised children with a reduced birth length (≤ –2.00 SDS) and normal birth weight and HC (SDS > -2.00) (SGA_L), group 2 comprised children with reduced birth length and weight (≤ –2.00 SDS) and normal HC (SDS > -2.00) (SGA_{L+W}), and group 3 included children with a reduced birth length, weight and HC (SDS ≤ –2.00) SGA_{L+W+HC} [10]. All children fulfilled the inclusion criteria of being Caucasian and having an uncomplicated postnatal period. Children with severe chronic illness or endocrine disorders, chromosomal or genetic abnormalities, positive endomysial or transglutaminase antibodies, skeletal disorders, psychosocial dwarfism, and growth failure caused by other syndromes (except Silver Russell syndrome), were excluded from the study.

The efficacy of 12 months GH treatment was evaluated in a subpopulation of 143 prepubertal children who were prepubertal at start and after one year of GH treatment. Prepuberty was defined as a bilateral testicular volume of 3 ml or less for boys and Tanner stage M1 for girls. Children receiving medication for induction or postponement of puberty were excluded from the analysis. The study protocol was approved by local Medical Ethics Committees and written informed consent was obtained from the parents/guardians of each child.

Clinical parameters

Data regarding gestational age and birth measurements were retrieved from medical records of 201 children. Maternal medical records of 120 children were traced for the type of delivery. Data regarding gestational hypertension, smoking (by mother, father or both) and use of alcohol were retrieved by questionnaires. Postnatal growth patterns from birth to 3 years of age were assessed using growth measurements from primary health care records, hospital records and baseline data from GH treatment studies of all 201 children. All measurements were plotted on a growth chart per child and checked for outliers to reduce within and between observer errors.

Ponderal index was calculated by ((birth weight in grams/ birth length in cm^3)*100) and expressed as SDS [12]. Body mass index was calculated by (weight in kg/ height in $meters^2$) and SDS calculated according to Dutch reference values [11]. A measure for birth head size compared to length was obtained by subtracting birth head circumference SDS from birth

length SDS. Height, weight, HC and sitting height were measured at start and after one year of GH treatment according to standardized methods [4,5]. Sitting height/ standing height ratio's for age were expressed as SDS using Dutch reference values [12].

Parental heights of 160 mothers and 157 fathers were assessed using a Harpenden stadiometer and expressed as SDS using Dutch reference values [13]. Target height (TH) SDS was calculated using Dutch reference values according to the formula: 1/2 * (Height father+ Height mother+ 13) + 4.5 for boys and 1/2 * (Height father+ Height mother– 13) + 4.5 for girls, where the addition of 4.5 cm represents the secular trend [11]. During GH treatment, biosynthetic GH was injected subcutaneously once daily at a dose of 1 to 2 mg/m^2 body surface area/day.

Statistical analysis

Anthropometric data were analyzed from birth until 3 years of age and before and after 12 months GH treatment. Analyses from birth to 3 years of age were performed on the total cohort of 201 children by univariate analysis of variance (ANOVA). Absolute birth size was adjusted for sex and gestational age to adjust for prematurity. Anthropometric SDS up to 3 years of age were also corrected for gestational age. The standard deviation (SD) of the adjusted means was approximated by SD = SE * (√n). The Chi-square test was used to analyze data on delivery and gestational factors.

A subgroup analysis on the effect of GH treatment on growth was performed in a group of 143 prepubertal children. Analysis of the growth response was performed by using ANOVA. Baseline measurements were corrected for age at start of GH treatment, as covariate in ANOVA. The growth response during 12 months of GH treatment was adjusted for GH dose and age at start of GH treatment, also as covariate in ANOVA. Standard deviations (SD) of the adjusted means were approximated by SD = SE * (√n). Parental and target heights were compared by using ANOVA. Parental heights were compared to 0 SDS by one-sample t-test.

If an overall test comparing the three groups was significant (P-value ≤ 0.05), post-hoc tests with Bonferroni correction were performed. Statistical tests were performed using SPSS 11.0 package (SPSS Inc., Chicago, IL, USA).

Results

Birth anthropometrics and delivery

As shown in Table 1, there were statistically significant differences in anthropometric birth data between the $SGA_{L,}$ SGA_{L+W} and SGA_{L+W+HC} children, with the latter being the most severely affected ones with regard to all anthropometric data (Table 1). Whereas SGA_{L+W+HC} children were most proportional as expected, SGA_{L+W} children were most disproportional with a low birth length SDS in combination with a spared head in the normal range. SGA_{L} children displayed an intermediate pattern. The type of delivery was different between groups. The majority (76%) of the SGA_{L} group was born after vaginal delivery in contrast to the SGA_{L+W} and SGA_{L+W+HC} children (46 and 32%, Table 1). Whereas the frequency of acute caesarean sections was not different between the groups, the frequency of elective caesarean sections was different. Within the SGA_{L+W+HC} group, 53% of children were born after an elective caesarean section in contrast to 41% of the SGA_{L+W} and 13% of the SGA_{L} group.

Gestational hypertension occurred most frequently in the SGA_{L+W} children, in contrast with SGA_{L} and SGA_{L+W+HC} children (37.9 vs. 13.6 and 28.3 %). Smoking during pregnancy was highly prevalent (49.7%) but did differ between the groups. Alcohol use during gestation was reported in 7.4% of the total population and tended to be the most frequent in the SGA_{L+W} children, although not statistically different between the groups.

Change in height, weight and HC during the first three years of life

During the first 3 years of postnatal life, the extent of spontaneous growth differed between SGA_{L} children and children in the SGA_{L+W} and SGA_{L+W+HC} groups (Figure 1; Table 2). Growth in height was greatest for SGA_{L+W+HC} children. At birth, children in the SGA_{L+W+HC} group were shorter than those in the other groups (Table 1), but by 6 months of age they had attained a height SDS that was similar to SGA_{L+W} children (-3.21 SDS) although they remained significantly shorter than SGA_{L} children (-2.62 SDS; $P = 0.001$, Table 2). In contrast, the height SDS of SGA_{L} children remained virtually unchanged during this period (Figure 1).

Whereas the SGA_{L} children had a substantial reduction in weight SDS from birth until the age of 3 years (Figure 2; Table 2), SGA_{L+W} and SGA_{L+W+HC} children maintained their weight SDS.

At birth, HC was by definition significantly ($P < 0.001$) smaller in SGA_{L+W+HC} children than in the SGA_{L} and SGA_{L+W} children (Table 1). During the first 6 months of postnatal life there was a substantial increase in HC SDS in SGA_{L+W+HC} children from -2.9 SDS at birth to -1.9 SDS at 6 months followed by a further, albeit slower increase to -1.6 SDS at 3 years of age (Figure 3; Table 2). Despite this

marked increase, HC was at 3 years of age significantly smaller ($P < 0.001$) in the SGA_{L+W+HC} children as compared to children in the other two groups. For children born SGA_L or SGA_{L+W}, HC decreased slightly during the first 6 months of life and remained below their HC SDS at birth until the age of 3 years (Table 2).

Table 1. Baseline characteristics of the total study population and the short SGA groups.

		Total	SGA_L	SGA_{L+W}	SGA_{L+W+HC}	P-value*
Sex	boys/girls	109/92	36/31	35/35	38/26	0.55
GA	weeks	36.8 (3.5)	38.1 (3.1)	36.0 (3.7)	36.3 (3.7)	0.0004[1]
Birth length	cm	41.6 (4.9)	42.8 (2.1)	41.3 (2.0)	40.7 (2.0)	<0.000001[1]
	SDS	-3.50 (1.30)	-2.67 (0.54)	-3.72 (1.30)	-4.12 (1.42)	<0.00001[1]
Birth weight	grams	1921 (716)	2347 (238)	1762 (234)	1649 (233)	<0.000001[3]
	SDS	-2.46 (1.11)	-1.25 (0.62)	-2.88 (0.64)	-3.27 (0.79)	<0.000001[3]
Birth HC	cm	31.5 (3.0)	32.4 (1.2)	32.4 (1.2)	29.7 (1.2)	<0.000001[2]
	SDS	-1.34 (1.45)	-0.64 (0.88)	-0.59 (1.11)	-2.89 (0.98)	<0.000001[2]
Birth HC-Birth Length	SDS	2.16 (1.71)	2.03 (1.09)	3.13 (1.89)	1.23 (1.48)	<0.000001[3]
Height mother	cm	162.3 (7.3)	160.6 (9.0)	163.3 (5.7)	163.0 (6.3)	0.09
	SDS	-0.97 (1.17)	-1.24 (1.45)	-0.80 (0.93)	-0.85 (1.01)	0.09
Height father	cm	176.5 (6.9)	176.2 (7.7)	175.4 (6.9)	178.4 (5.7)	0.07
	SDS	-0.82 (1.04)	-0.87 (1.15)	-0.99 (1.03)	-0.53 (0.85)	0.07
TH	SDS	-0.51 (0.86)	-0.66 (0.86)	-0.52 (0.87)	-0.29 (0.83)	0.12
Birth length-TH	SDS	-2.89 (1.59)	-1.95 (1.12)	-3.07 (1.38)	-3.81 (1.74)	<0.000001[3]
Height-TH	SDS	-2.61 (0.85)	-2.53 (0.85)	-2.63 (0.85)	-2.72 (0.83)	0.70
Type of delivery:						
Vaginal	% (n)	52.5 (63)	75.6 (34)	45.9 (17)	31.6 (12)	0.0002[3]
	GA (weeks)	38.9 (2.1)	39.1 (2.2)	38.6 (2.5)	38.6 (1.4)	0.66
Acute CS	% (n)	13.3 (16)	11.1 (5)	13.5 (5)	15.8 (6)	0.82
	GA (weeks)	37.1 (3.3)	35.8 (4.3)	37.4 (3.9)	37.8 (1.7)	0.61
Elective CS	% (n)	34.2 (41)	13.3 (6)	40.5 (15)	52.6 (20)	0.001[3]
	GA (weeks)	35.0 (2.8)	37.5 (2.1)	33.8 (2.3)	35.1 (2.9)	0.02 [4]

(continued)		Total	SGA_L	SGA_{L+W}	SGA_{L+W+HC}	P-value*
Gestational factors:						
Hypertension	% (n)	26.4 (43)	13.6 (8)	37.9 (22)	28.3 (13)	0.01[4]
Smoking	% (n)	49.7 (42)	50.0 (25)	46.0 (23)	53.3 (24)	0.77
Alcohol	% (n)	7.4 (12)	3.4 (2)	12.3 (7)	6.5 (3)	0.18

*Values are means (standard deviation) unless indicated otherwise. *= The overall P-value is presented by ANOVA for continuous and by chi-square test for categorical variables. Post-hoc testing showed; [1]= SGA_L differed in comparison to the SGA_{L+W} and SGA_{L+W+HC} groups; [2]= SGA_{L+W+HC} differed in comparison to the SGA_{L+W} and SGA_L groups; [3]= All groups differed significantly from each other, [4]= SGA_L differed in comparison to the SGA_{L+W} group. GA= gestational age, HC= head circumference, TH= target height, CS= caesarean section, SDS= standard deviation score.*

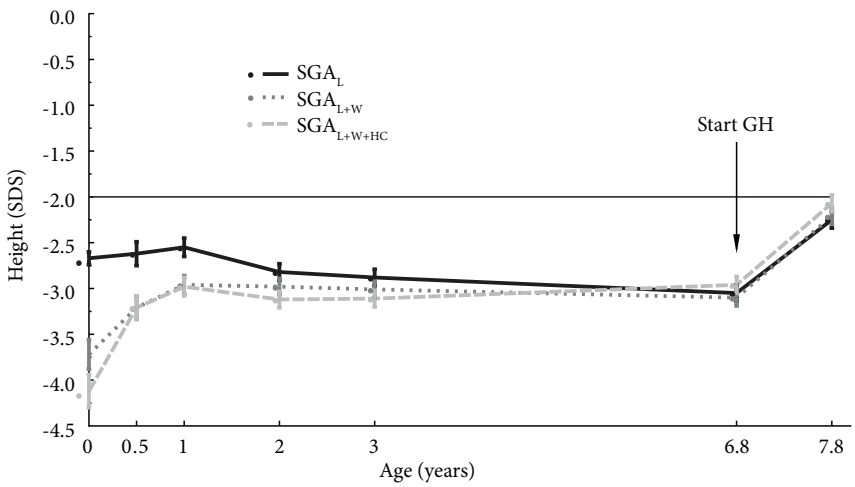

Figure 1. Height (SDS) during the first three years of life and during one year of GH treatment for the short SGA groups. Data shown are means with standard errors.

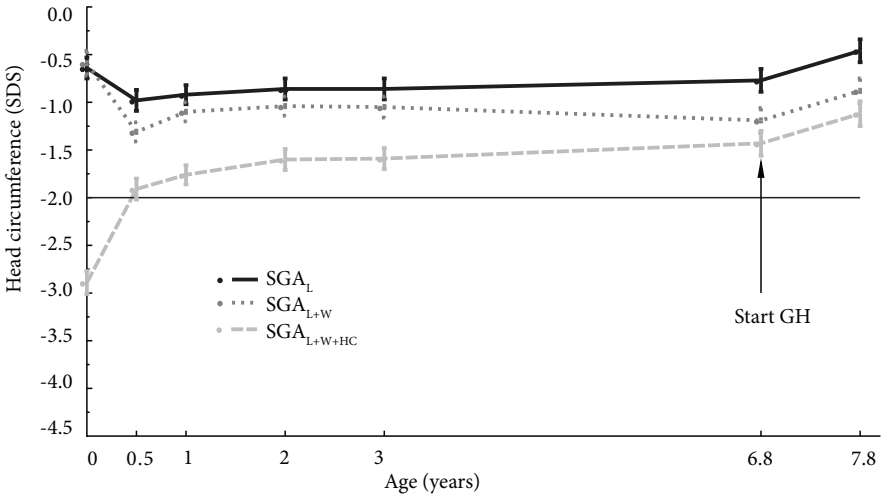

Figure 2. Weight (SDS) during the first three years of life and during one year of GH treatment for the short SGA groups. Data shown are means with standard errors.

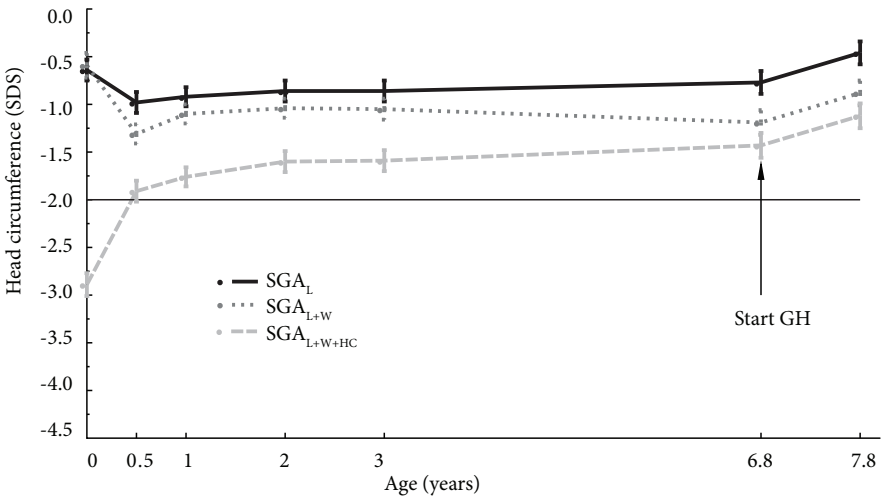

Figure 3. Head circumference (SDS) during the first three years of life and during one year of GH treatment for the short SGA groups. Data shown are means with standard errors.

Table 2. Spontaneous growth during the first 3 years of life in the total population and the three groups of short SGA children.

		Total	SGA_L	SGA_{L+W}	SGA_{L+W+HC}	P-value*
Height						
0.0 year	SDS	-3.50 (1.30)	-2.67 (0.54)	-3.72 (1.30)	-4.12 (1.42)	<0.00001[1]
0.5 year	SDS	-3.01 (1.08)	-2.62 (1.03)	-3.21 (1.01)	-3.21 (1.00)	0.001[1]
1.0 year	SDS	-2.83 (0.84)	-2.55 (0.83)	-2.96 (0.81)	-2.98 (0.81)	0.004[1]
2.0 years	SDS	-2.97 (0.73)	-2.82 (0.74)	-2.98 (0.73)	-3.12 (0.73)	0.07
3.0 years	SDS	-3.00 (0.70)	-2.88 (0.72)	-3.01 (0.71)	-3.11 (0.70)	0.20
Change 0-3	SDS	0.50 (1.37)	-0.06 (1.23)	0.62 (1.15)	0.98 (1.19)	<0.00001[1]
Weight						
0.0 year	SDS	-2.46 (1.11)	-1.25 (0.62)	-2.88 (0.64)	-3.27 (0.79)	<0.000001[3]
0.5 year	SDS	-2.74 (1.28)	-2.08 (1.16)	-2.95 (1.14)	-3.21 (1.13)	<0.00001[1]
1.0 year	SDS	-2.75 (1.14)	-2.14 (1.07)	-2.94 (1.05)	-3.19 (1.05)	<0.00001[1]
2.0 years	SDS	-2.78 (1.02)	-2.21 (0.96)	-2.94 (0.94)	-3.21 (0.94)	<0.00001[1]
3.0 years	SDS	-2.82 (0.96)	-2.34 (0.92)	-2.96 (0.91)	-3.19 (0.90)	0.00001[1]
Change 0-3	SDS	-0.36 (1.10)	-1.04 (0.98)	-0.10 (0.99)	0.07 (0.95)	<0.00001[1]
HC						
0.0 year	SDS	-1.34 (1.45)	-0.64 (0.88)	-0.59 (1.11)	-2.89 (0.98)	<0.000001[2]
0.5 year	SDS	-1.40 (0.94)	-0.98 (0.84)	-1.31 (0.83)	-1.91 (0.83)	<0.000001[3]
1.0 year	SDS	-1.26 (0.89)	-0.92 (0.81)	-1.10 (0.80)	-1.76 (0.80)	<0.00001[2]
2.0 years	SDS	-1.15 (0.87)	-0.86 (0.82)	-1.04 (0.81)	-1.60 (0.81)	<0.00001[2]
3.0 years	SDS	-1.14 (0.89)	-0.86 (0.84)	-1.05 (0.83)	-1.59 (0.83)	0.00002[2]
Change 0-3	SDS	0.17 (1.35)	-0.30 (1.17)	-0.31 (1.10)	1.28 (1.16)	<0.00001[2]
		Total	SGA_L	SGA_{L+W}	SGA_{L+W+HC}	**P-value***
PI and BMI						
0 year	PI (SDS)	1.57 (3.31)	3.86 (2.86)	0.66 (2.84)	0.17 (2.88)	<0.00001[1]
1	BMI (SDS)	-1.24 (1.23)	-0.59 (1.17)	-1.40 (1.17)	-1.73 (1.10)	<0.00001[1]
3	BMI (SDS)	-1.08 (1.03)	-0.55 (0.94)	-1.24 (0.94)	-1.44 (0.94)	<0.00001[1]

continued		Total	SGA_L	SGA_{L+W}	SGA_{L+W+HC}	P-value*
Change BMI 1-3	BMI (SDS)	0.17 (0.81)	0.05 (0.86)	0.16 (0.86)	0.29 (0.79)	0.26

*Values are means (standard deviation) which, at age 0.5 until 3 years were adjusted for gestational age. *= The overall P-value is presented of the SGA groups. Post-hoc testing showed; [1]= SGA_L differed in comparison to the SGA_{L+W} and SGA_{L+W+HC} groups; [2]= SGA_{L+W+HC} differed in comparison to the SGA_{L+W} and SGA_L groups; [3]= All groups differed significantly from each other; [4]= the SGA_L group differed from the SGA_{L+W+HC} group. HC= head circumference, PI= ponderal index, BMI= body mass index, SDS= standard deviation score.*

Ponderal- and body mass index (BMI) were investigated as measures for weight for height ratio at birth until the age of 3. SGA_L children had the highest ponderal index SDS at birth (3.86 SDS) in comparison with SGA_{L+W+HC} children (0.17 SDS). SGA_{L+W} children showed an intermediate pattern (0.66 SDS). Although SGA_L children had the highest ponderal index SDS at birth, they had a decrease in weight SDS especially during the first 6 months, resulting in a normal BMI SDS at age 3. From age 1 until age 3, SGA_{L+W} and SGA_{L+W+HC} children showed an increase in BMI (0.16 vs. 0.29 SDS), whereas SGA_L children remained at the same SDS.

Effect of GH treatment on height, weight and HC

Mean age (6.8 ± 2.3 years) at start of GH treatment was comparable between the SGA groups (P = 0.40, Table 3). From 3 years of age until start of GH treatment height SDS was maintained at a similar level within each group, and did not differ markedly between groups (Table 3). GH treatment for 12 months resulted in a similar increase in height SDS (mean increase = 0.86 SDS) in all groups (Figure 1). In all groups, height SDS after 12 months GH treatment was significantly (P < 0.001) higher than height SDS at start of treatment and was not different between groups (P = 0.35, Table 3).

Between 3 years of age and start of GH treatment, weight SDS remained at approximately the same level in all groups (Figure 2, Tables 2 and 3), with SGA_L children remaining at a weight SDS of approximately -2.5 SDS which was significantly higher than those in the other two groups during this period (Tables 2 and 3). GH treatment resulted in a significantly greater increment in weight SDS for the SGA_{L+W+HC} children than the SGA_L children (P = 0.02; Table 3).
At start of GH treatment, HC SDS was significantly greater in SGA_L children than in SGA_{L+W} or SGA_{L+W+HC} children (P = 0.002; Table 3, Figure 3). GH treatment resulted in a similar increase in HC in all groups (Table 3). Despite this increase, HC remained the smallest for SGA_{L+W+HC} and SGA_{L+W} children after 1 year of GH treatment at -1.12 SDS and -0.88 SDS in comparison with the children in the SGA_L group, being –0.46 SDS (P = 0.001).

At start of GH treatment, the sitting height to height ratio was not statistically significant between the groups, SGA_L children having a mean (SD) of 1.42 (1.63) SDS, SGA_{L+W} children 1.33 (1.22) SDS and SGA_{L+W+HC} children 1.51 (1.60) SDS (P = 0.86).

Table 3. The effect of growth hormone (GH) treatment on growth parameters in the total population and in the short SGA groups.

		Total	SGA_L	SGA_{L+W}	SGA_{L+W+HC}	P-value
Sex	boys/girls	75/68	24/29	23/22	28/17	0.24
Age at start	years	6.81 (2.29)	7.12 (2.42)	6.49 (2.28)	6.79 (2.14)	0.40
GH dose	mg/m^2/day	1.11 (0.29)	1.10 (0.28)	1.13 (0.31)	1.09 (0.29)	0.76
Height						
Start*	SDS	-3.04 (0.62)	-3.05 (0.62)	-3.10 (0.62)	-2.96 (0.62)	0.54
After 1 year**	SDS	-2.18 (0.65)	-2.25 (0.64)	-2.21 (0.64)	-2.07 (0.63)	0.35
Changes during 1 year**	SDS	0.86 (0.36)	0.81 (0.24)	0.89 (0.24)	0.89 (0.24)	0.13
Weight						
Start*	SDS	-3.00 (0.92)	-2.59 (0.85)	-3.32 (0.85)	-3.18 (0.85)	0.00007[1]
After 1 year**	SDS	-2.22 (0.87)	-1.92 (0.84)	-2.50 (0.84)	-2.29 (0.84)	0.003[1]
Changes during 1 year**	SDS	0.78 (0.43)	0.67 (0.37)	0.82 (0.37)	0.88 (0.37)	0.02[4]
HC						
Start*	SDS	-1.11 (0.88)	-0.77 (0.85)	-1.19 (0.85)	-1.43 (0.85)	0.002[1]
After 1 year**	SDS	-0.80 (0.92)	-0.46 (0.87)	-0.88 (0.87)	-1.12 (0.87)	0.001[1]
Changes during 1 year**	SDS	0.31 (0.39)	0.30 (0.36)	0.33 (0.37)	0.31 (0.36)	0.91

*Values are means (standard deviation). *= Means are adjusted for age at start of GH treatment; **= Means are adjusted for age at start of GH treatment and GH dose. The overall P-value is presented of the SGA groups. Post-hoc testing showed; [1]= SGA_L differed in comparison to the SGA_{L+W} and SGA_{L+W+HC} groups; [4]= the SGA_L group differed from the SGA_{L+W+HC} group. HC = head circumference, SDS= standard deviation score.*

Parental height

Parental heights (mother and father) were not significantly different between the SGA groups (Table 1). Parental height was lower than average (mother, -0.97 SDS; father, -0.82 SDS, in comparison to 0 SDS, both P < 0.001). Although mean height SDS for mothers of SGA_L children (-1.24 SDS) was lower than those of the other SGA groups, there was no statistical difference between the three groups (P = 0.09, Table 1). Target height (TH) SDS tended to be the lowest

for SGA_L children (-0.66 SDS) and the highest for SGA_{L+W+HC} children (-0.29 SDS), SGA_{L+W} children showing an intermediate pattern at -0.52 SDS. Height SDS corrected for TH SDS was significantly different at birth but not at start of GH treatment (Birth length-TH, Height-TH SDS, Table 1). All groups had a height SDS far below their TH SDS (Table 1).

Discussion

The aim of this study was to determine in short SGA children whether differences in birth anthropometrics, with respect to length, weight, and head circumference (HC), were associated with differences in gestation, type of delivery, postnatal growth, parental height, and response to GH treatment. Our results show that SGA_{L+W+HC} children experienced the most severe growth restriction during pregnancy. This is partly explained by their definition to have a length, weight and HC ≤ -2.00 SDS, but was often 1 until 2 SDS below -2.00 SDS. SGA_{L+W} children showed an intermediate pattern with regard to birth length and weight in comparison with SGA_L and SGA_{L+W+HC} children. Notably, SGA_{L+W+HC} had the greatest increase in height and HC during early postnatal life, although at the age of 3, HC SDS and weight SDS still were significantly lower then those born SGA_L. SGA subclassification could not predict a differential response to GH treatment as an increased growth was seen for all three groups. After 1 year of treatment however, HC and weight of SGA_{L+W+HC} and SGA_{L+W} children remained significantly below those of SGA_L children.

SGA_L children had no postnatal increase in growth, showed a marked decrease in weight and also a decline in HC SDS. In contrast, short SGA_{L+W} and SGA_{L+W+HC} children had a significant increase in growth during early postnatal life. These differences in postnatal growth patterns might result from different genetic and environmental parameters influencing SGA_L children as compared with the SGA_{L+W} and SGA_{L+W+HC} children. SGA_{L+W+HC} children suffered from severely impaired prenatal growth, but experienced a marked increase in growth after birth. In contrast, SGA_L children were mostly born after a vaginal delivery, were less small at birth and showed a decrease in growth in height and weight after birth. Also, parents of SGA_L children and especially their mothers, tended to be shorter than those of the other groups. These findings suggest that SGA_L children resemble a growth pattern as observed in children with idiopathic short stature (ISS) who are also mainly born after a vaginal delivery, have a decreased growth after birth and in many cases short parents. It might well be that subtle skeletal abnormalities play a role in some short SGA_L children instead of generalized fetal growth restriction, explaining the more frequent vaginal deliveries. For this group of SGA

children it might be interesting to perform genetic research into genes involved in pre- and postnatal bone development.

SGA_{L+W} children were characterized by their intermediate birth size, type of delivery, and postnatal growth pattern in comparison to the SGA_{L} and SGA_{L+W+HC} children. These children also had the highest frequency of gestational hypertension which might explain their short gestational age during elective cesarean section and their spared birth HC. This observation might suggest that SGA_{L+W} children, in contrast to SGA_{L} and SGA_{L+W+HC} children, might be a consequence of gestational hypertension. A previous report also showed that the combination of low birth weight and height is most frequent after gestational hypertension [14], which confirms our observation. We however could not observe that SGA_{L+W} children were more prone to have cardiovascular risk factors as a higher postnatal weight or BMI increase in comparison to the other groups. Further long-term follow-up of SGA_{L+W} children is needed, to identify a potential higher risk to cardiovascular risk factors.

SGA_{L+W+HC} children were the smallest at birth, with regard to length, weight and HC. We expected to find a comparable or somewhat smaller birth length SDS than in the other children, as birth length SDS ≤ -2.00 was the major inclusion criterion for all children in the study cohort. However, we found that SGA_{L+W+HC} children had a considerably shorter birth length than SGA_{L} and SGA_{L+W} children, despite of having the highest target height of the groups. Interestingly, SGA_{L+W+HC} children had the greatest increase in growth parameters of all groups, indicating that SGA_{L+W+HC} children might have had the most severe fetal growth restraint which disappeared after birth. Postnatal growth of the children with the most severe growth retardation might be explained by regression to the mean. However, if this growth would be due to regression to the mean then we would have expected growth of all growth parameters in the SGA_{L+W+HC} children. As there was no growth in weight, we suspect that regression had only a limited effect.

Of note was the finding that 68% of SGA_{L+W+HC} children were delivered by caesarean section in contrast to SGA_{L} children of whom 76% were born after vaginal delivery. It is likely that the SGA_{L+W+HC} children experienced growth retardation from early in pregnancy, also affecting head growth. It might well be that the percentage of deliveries by elective caesarean section was high due to this severe growth retardation which is often seen in combination with reduced placental blood flow.

Phenotypic variation is by definition determined by the interaction between genetic and environmental factors. Currently most genetic research in short SGA children is focused on the role of the insulin-like growth factor-I (IGF-I), IGF-I receptor (IGF-IR) and growth hormone receptor (GHR) genes in determining size at birth [15-21]. Of particular interest is

the finding that patients with mutations in the IGF-I or IGF-IR genes also had a reduced head circumference and various degrees of mental retardation [15,17-19]. SGA_{L+W+HC} children showed, however, a combination of severe prenatal growth restraint and remarkable postnatal growth in height and HC, which is not a characteristic of subjects with a deletion or mutation in the IGF-I or IGF-IR gene. For that reason, it is unlikely that SGA_{L+W+HC} children have major deletions in these genes but minor variants might play a role. Further research into gene-gene and gene-environment interactions are needed to elucidate the etiology of SGA_{L+W+HC}.

Evidence from several studies suggests that pre- and postnatal head circumference growth may influence cognitive function in SGA children [22-24]. For example, SGA children with reduced head circumference (≤ P10) at birth and at 9 months of age experienced widespread impairments in their verbal and nonverbal IQ ratings, phonological awareness skills, visual-motor integration skills, problem-solving abilities and literacy skills at age 7-9 [23]. Other studies have shown that SGA children with a reduced height and/ or insufficient growth in HC and/ or height had the highest risk for subnormal intellectual and psychological performance [22,24,25]. Hence identification of short SGA children with small HC SDS may be clinically relevant as it may help to identify children at particular risk for learning difficulties.

In this study, we have specifically evaluated SGA children with persistent short stature, in contrast to SGA catch-up children, having a height above -2.00 SDS at 3 years of age. As a first step in subphenotyping the heterogeneous SGA group, we have focused on short SGA children as they receive endocrine care for their growth retardation, often including growth hormone treatment.

In conclusion, our study suggests that SGA children with persistent short stature may be subclassified according to their birth length, weight and HC. Subclassification using these criteria may provide a useful framework to explore the mechanisms underlying differences in the extent of spontaneous postnatal growth that are observed in a population of short SGA children. Such a subclassification of short SGA children might also be useful for elucidating underlying genetic or environmental causes of SGA and future risk profiles with regard to adult diseases. Where SGA_{L} children appeared to experience the least fetal growth retardation and postnatal height increment as within ISS children, SGA_{L+W} children were most affected by gestational hypertension which might suggest effects on cardiovascular risk profiles in later life. SGA_{L+W+HC} children were born with the lowest HC but experienced a major increase in HC growth postnatally. In this group of children, further studies might be directed at specialized interventions such as remedial teaching on cognition during early infancy as well as evaluating the effect of GH treatment at an earlier age.

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Chapter 3

Polymorphisms in the *IGF1* and *IGF1R* gene and children born small for gestational age: Results of large population studies

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Abstract

Small for gestational age (SGA) is the term used to describe a group of children born with a birth weight and/or birth length below the normal range of a reference population, corrected for their gestational age. Although animal models have shown that the insulin-like growth factor 1 (*IGF1*) and insulin-like growth factor 1 receptor (*IGF1R*) are important candidate genes for reduced pre- and postnatal growth, only limited case-reports have been published, describing mutations. This might suggest that *IGF-I* and *IGF-IR* are such crucial growth factors that only common genetic polymorphisms are allowed to survive. Common *IGF1* and *IGF1R* gene polymorphisms, like single nucleotide polymorphisms (SNPs) and variable number of tandem repeats (VNTRs), have been investigated with conflicting results with respect to SGA related outcomes. The exact contribution of these polymorphisms to clinical practice remains to be elucidated.

Introduction

IGF1 gene, IGF1R gene and SGA

IGF1 (OMIM*147440, NM_000618, gene map locus: 12q22-q24.1) and the *IGF1R* (OMIM*147370, NM_000875, gene map locus: 15q25-q26) are thought to be important candidate genes for children born SGA, being major pre- and postnatal growth factors [1,2]. Prenatally, *IGF1* and *IGF1R* absence results in mice with a respectively 40% and 55% reduction in birth weight. During postnatal life, surviving *IGF1* knock-out mice become 30% of normal adult weight. In humans, growth hormone induced growth by IGF-I starts from 3 months of age. However as SGA might be the result of numerous maternal, foetal, and placental causes [3], it is unclear whether prenatal reduced *IGF1* and/or *IGF1R* expression result from etiological factors as maternal smoking or maternal alcohol by inducing malnutrition, or that prenatal reduced *IGF1* and *IGF1R* expression result from a genetic defect or variation in their respective genes [4]. Clinically, SGA children are defined as children born with a low birth weight and/or birth length for gestational age. Since reduced size at birth may result from foetal, maternal, placental or demographic influences, children born SGA comprise a heterogeneous phenotype with a broad spectrum of clinical characteristics. Around 2% of all life-born neonates are born SGA and 10% of them remain short. SGA children with a persistent short stature have been studied due to their participation in prospective GH-treatment studies [5-8]. Studies at start of GH treatment showed that SGA children had a reduced lean body mass, fat mass, skin fold measurement and body mass index [5,6,8] .They have a decreased caloric, fat and carbohydrate intake [7]. SGA children with catch-up growth until a height in the normal range are known for an increased risk for cardiovascular disease [9]. It seems likely that combinations of adverse genetic variations together with an adverse environmental factor result into intra-uterine growth retardation and SGA birth.

Heritability and genetic polymorphisms

Several twin studies have shown that serum IGF-I levels are highly heritable, especially at a young age. Heritability of serum IGF-I levels ranges from 77-93% for girls and boys in cord blood until 54-66% in free serum IGF-I levels of 6-18 year old school girls [10,11]. Twin studies at middle-aged subjects and in the elderly showed a heritability ranging between 38-63% [12, 13]. In this review we will focus on two common classes of genetic polymorphisms; single nucleotide polymorphisms (SNPs) and variable number of tandem repeats (VNTRs). SNPs are defined as a single nucleotide substitution by another nucleotide. Since coding DNA accounts for only about 1.5% of the human genome, most SNPs are found in non-coding DNA as introns

or intergenic sequences. There is no uniform spread of SNPs across the genome; there are large chromosomal regions with very few SNPs who are often adjacent to large regions containing many SNPs. Variable number of tandem repeats (VNTR) consist of tandemly repeated runs of simple sequences. An example of a VNTR is a microsatellite, consisting of a simple sequence which is one or several nucleotides long and the array length ranges from less than 10 to over 100 nucleotides.

Linkage disequilibrium

An important concept of research into genetic polymorphisms is linkage disequilibrium (LD) and haplotypes [14]. Linkage disequilibrium can be defined as the co-occurrence of closely linked alleles more frequently than expected by chance. A haplotype is a set of alleles of linked genes that tend to be inherited together. The total human genome consists of about 3 billion basepairs in which variation is determined by recombination. Recombination however doesn't occur evenly spaced throughout the genome but at certain hotspots. This means that areas without these hotspots are rather well conserved and tend to be inherited as one block together. Such a block is named a linkage disequilibrium block. Within these blocks, genetic polymorphisms have less variation than would be expected by chance. A combination of these limited combinations of genetic polymorphisms is called a haplotype. Haplotypes are interesting to investigate while due to the linkage disequilibrium-block it might not be necessary to investigate all the SNPs in the region but only the determining, or tagging, SNPs of the haplotype. An international project investigating linkage disequilibrium-blocks throughout the genome is the HapMap project (Web resources). Haplotypes and their tagging SNPs are of additional value above single genetic association studies due to their ability to identify combinations of SNPs with most associations.

Focus

This review will focus on genetic association studies in the *IGF1* and *IGF1R* gene with SGA-related outcomes and serum IGF-I levels. Studies were selected by searching Pubmed on the *IGF1* and *IGF1R* gene together with "SNP", "single nucleotide polymorphism", height and SGA. Excluded studies were veterinary studies, case-reports and reviews. Due to the limited number of publications, we could not apply stringent criteria with respect to the study-design. The questions we wanted to answer were: what is the contribution of the currently known genetic association studies in the *IGF1* and *IGF1R* gene into SGA-related outcomes, for clinical research and practice? What is the contribution of linkage disequilibrium and haplotype studies in the *IGF1* and *IGF1R* gene into SGA-related outcomes? We also wanted to identify the contribution of gene-gene interaction studies with the *IGF1* and *IGF1R* gene into these outcomes.

Polymorphisms in the insulin-like growth factor-1 gene

IGF-I is the product of a single-copy gene located on the long arm of chromosome 12 (12q22-q24.1, Figure 1). This gene is about 83kb in size and consists of 6 exons, of which exon 1, 2, 5 and 6 are alternatively spliced. Exon 1 and 2 code for the signal peptide, exon 3 and 4 code for the active protein and exon 5 and 6 code for the E domain (Figure 2). Alternative splicing depends on tissue type and hormonal environment [15]. Alternative splicing results in three isoforms, IGF-IEa, IGF-IEb, and IGF-IEc or mechano growth factor [16]. Whereas hormonal factors result in the IGF-IEa (systemic) and IGF-IEb isoforms, mechanical signals on muscles as stretch or damage result in mechano growth factor (MGF). The alternative splices result in different precursor peptides although they don't alter the structure of the mature peptide. It is unclear how these IGF-I isoforms would result in a tissue-specific IGF-I protein expression although it is hypothesized that mechano growth factor has a 52bp insert in the E-domain which alters the reading frame to the 3'end. This may result into binding to a different binding-protein of the splice variant, thus localizing its action [17].

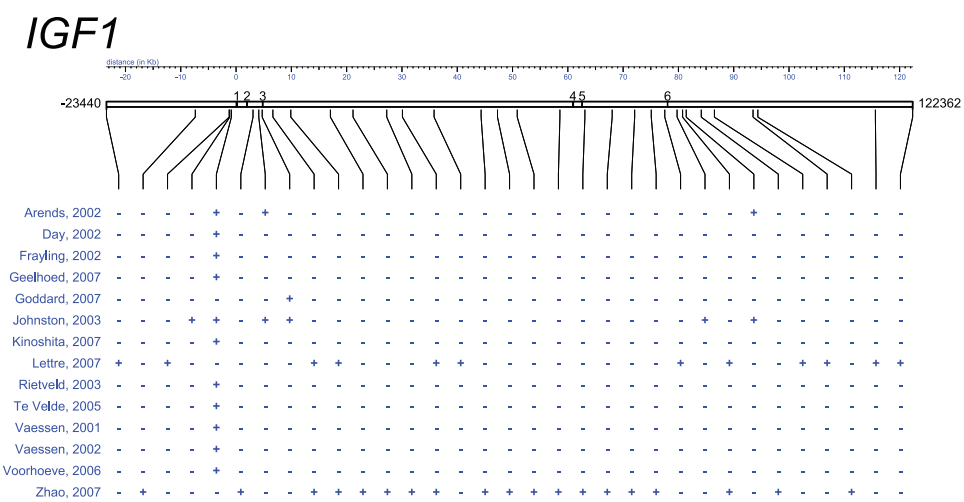

Figure 1. *IGF1* gene structure.

Since single nucleotide polymorphisms are estimated to occur every 100-1000 nucleotides (Web resources: NCBI, HapMap, UCSC), 85-850 SNPs are theoretically expected to be present in the 84648bp sized *IGF1* gene. SNP research is usually performed by investigating SNP databases and literature. Main SNP providing databases, as NCBI, HapMap and UCSC (Web resources),

provide respectively 449 SNPs, 140 SNPs, and 126 SNPs in the *IGF1* gene. Current literature provides numerous *IGF1* gene association studies based on systematic sequencing of patient-populations but also by studies focussing on a single SNP or VNTR.

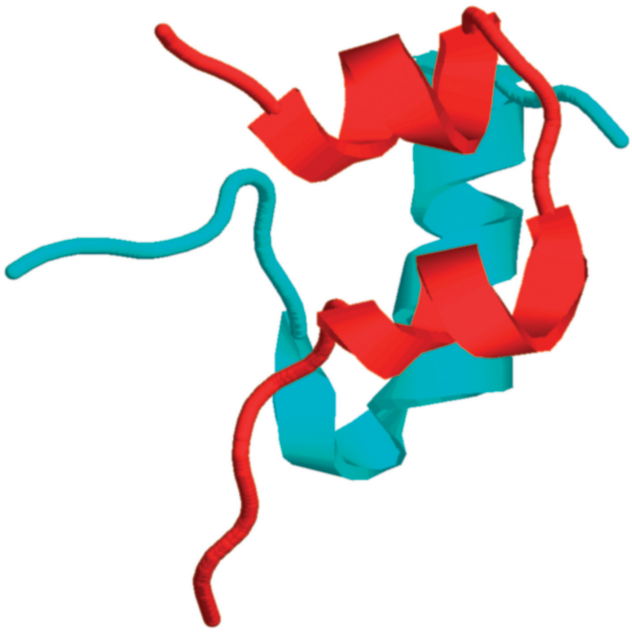

Figure 2. IGF-I protein structure with amino acid 1-38 (back: blue) and amino acid 39-66 (front: red), according to PDBID 1GZY (Web resources: Rasmol, RCSB PDB) [46,47].

There has been extensive research into *IGF1* genetic polymorphisms with respect to diverse outcomes such as physical growth, cardiovascular disease, and malignancies, but also to the location of the polymorphisms in the gene, linkage disequilibrium and haplotypes. Several studies have investigated the association with serum IGF-I level as an indication for the functional properties of the genetic polymorphism.

Although there has been quite some research with regard to *IGF1* genetic association studies, the studies reporting on the functionality of these polymorphisms are very limited. As most polymorphisms are, by definition, localized in non-coding areas of the gene, this is rather complex to perform in the *IGF1* gene. Additionally, there are hardly any non-synonymous SNPs reported in SNP databases and none reported in literature, to our knowledge. Circulating serum IGF-I levels are shown as a proxy for the effect of the SNP on protein level. In this chapter, we will focus on *IGF1* population studies who performed linkage disequilibrium and haplotype studies, investigating serum IGF-I levels with SGA-related outcomes.

Associations of the *IGF1* gene and SGA related outcomes

Promoter

Five polymorphisms have been investigated in the *IGF1* gene promoter region of which the 192bp CA-repeat located 841bp before start of exon 1 (Figure 1), is by far the most extensively studied *IGF1* polymorphism (Table 1). Upstream of the 192bp CA-repeat, four polymorphisms are located in a 22kb spanning area [18-20]. Linkage disequilibrium analyses by Zhao[18] and Johnston[20] showed that high linkage disequilibrium was present from the promoter area until intron 3. Whereas Zhao [18] did not find associations between haplotypes spanning the promoter until intron 3 area and age at menarche, Johnston [20] showed that the region ranging from 1.2kb in the promoter until intron 2 was important in determining catch-up growth of the SGA born child. Both studies showed that the linkage disequilibrium block was at least 19kb wide and that the highest linkage disequilibrium was downstream of the 192bp CA-repeat as the combination of the 192bp CA-repeat and intron 2 IGF1.PCR1 associated stronger with the short SGA phenotype than the combination of the T-1148C and 192bp CA-repeat. Lettre et al[19] also investigated a SNP in this promoter linkage disequilibrium block (rs35767), but did not find an association with adult height in 1132 tall (90th-95th percentile) and 1057 short (5th-10th percentile) subjects and replicated this finding in additional Polish and Framingham Heart Study cohorts.

Twelve studies have investigated associations between the 192bp CA-repeat and prenatal growth, birth size and postnatal anthropometrics. Whereas eight studies reported associations with growth related outcomes as catch-up growth, head size, chest circumference, height and weight, four studies could not confirm these associations (Table 1). The most discussed association concerned a higher birth weight in carriers of the 192bp-allele, which was shown by Vaessen [21] and te Velde [22] but not by Frayling [23], Day [24], Geelhoed [25] and Kinoshita [26]. This discrepancy was also seen for other outcomes like height. These conflicting results might be due to differences in the selected populations, data acquisition and data analysis. Despite these differences, however, it would have been plausible that a universal biological phenomenon like this association would be replicated in several populations. As this association could not be replicated in these studies, the universality of this association remains to be questioned. The same result is seen with outcomes such as head circumference. Kinoshita [26] observed a larger head circumference in 192/192 carriers. Arends [27], Frayling [23] and Geelhoed [25] did not observe this association. Many studies have investigated haplotypes, whereas Rietveld [28] and Vaessen [29] observed that carriers of the 192bp allele were taller than the remainder genotypes in an elderly population, Johnston et al. [20] showed that the

haplotype containing the 192bp CA-repeat associated with short SGA subjects. Voorhoeve [30] showed in 2 cohorts contradicting associations with height in males and Arends [27], Frayling [23], Day [24], Geelhoed [24] and te Velde [22] could not observe an association with height. Voorhoeve [30] also found that female carriers of the 192 and 194bp allele had a lower weight, BMI, fat mass and waist circumference in one of the cohorts. Arends et al. [27] investigated first transmission disequilibrium of all alleles of the 192bp CA-repeat and found that the rare 198bp-allele was less transmitted from the parents to the short SGA child. As only one child was carrier of the 198bp-allele, the association with serum IGF-I level could not be further investigated.

IGF1 gene

Four genetic polymorphisms have been investigated in intron 2 investigating associations with pre-eclampsia, age at menarche, head circumference and catch-up growth. Whereas no associations have been reported with pre-eclampsia and age at menarche, Arends [27] and Johnston [20] reported associations in SGA children. The 191bp-allele of IGF1.PCR1 associated with a smaller head circumference at 1.3 years of age which was at the same level as birth. Although non-carriers of the 191bp allele had the same head circumference at birth, head circumference was growing better in a selection of short SGA children with both a length and weight < -2.00 standard deviation score (SDS) at birth. Johnston[20] showed that IGF.PCR1 was part of the linkage disequilibrium -block ranging from at least, the promoter until intron 2. The IGF.PCR1 189bp-allele was associated with birth size, which was investigated by comparing SGA cases vs. controls, and also with persistent short stature in SGA children. The combination of the 189bp common allele of the IGF1.PCR1 and T-1148C SNP polymorphism associated with a short SGA phenotype. No associations were found between the A-23C polymorphism and the SGA phenotype.

Eleven polymorphisms have been studied in a 51kb area located in intron 3. Zhao [18] identified two linkage disequilibrium-blocks in the intron 3 region: one ranging from the promoter until intron 3, and a second ranging from intron 3 until intron 5. However, none of these polymorphisms and haplotypes showed associations with age at menarche. Lettre [19] investigated four polymorphisms in this area in relation to adult height. Three of these polymorphisms were identical with Zhao [18] and both did not observe associations except for rs10735380. Lettre [19] observed for rs10735380 and the combination of rs10735380 and rs7136446, an association with height in two of the three investigated cohorts.

Five polymorphisms have been investigated in intron 4 and 5 but Zhao [18] and Lettre [19] did not observe associations with age at menarche and adult height, respectively.

Table 1. Overview of *IGF1* genetic polymorphisms, their chromosomal position and SGA-related outcomes.

Location	Position	SNP / VNTR	IGF-I levels*	LD	Main results	Populations	Study
Intergenic	101421894	rs855228	ND	ND	NA with height	1132 tall and 1057 short European Americans, 506 tall and 512 short Polish, 1335 FHS related and 1533 FHS unrelated subjects	Lettre, 2007
Intergenic	101405826	rs35765	ND	+	NA with AAM	1048 females from 354 Caucasian nuclear families	Zhao, 2007
Promoter	101399699	rs35767	ND	ND	NA with height	See rs855228	Lettre, 2007
Promoter	101399602	T-1148C	NA	+	↑LD with 192bp and PCR1	114 short SGA and 60 SGA CU, 292 AGA	Johnston, 2003
Promoter	101399295	192bp**	NA	ND	NA	124 trio's of short SGA children	Arends, 2002
Promoter	101399295	192bp**	NA	+	↑LD with T-1148C and PCR1	See T-1148C	Johnston, 2003
Promoter	101399295	192bp**	192bp↓	ND	NA with birth size, height	640 subjects age 25 yrs, 348 diabetic and 365 control subjects	Frayling, 2002
Promoter	101399295	192bp**	ND	ND	NA with birthsize, postnatal size	627 men and 426 women and a subset of 225 men and 133 women	Day, 2002
Promoter	101399295	192bp**	ND	ND	192bp ↑foetal size, ↓growth rate	738 children	Geelhoed, 2007
Promoter	101399295	192bp**	192bp↑	ND	192bp ↑HC, CC	160 neonates	Kinoshita, 2007
Promoter	101399295	192bp**	192bp↓	ND	192bp ↓IGF-I with age	346 elderly subjects	Rietveld, 2003
Promoter	101399295	192bp**	192bp↑	ND	192+194bp ↑height	9278 elderly of which 359 had serum IGF-I level	Rietveld, 2003
Promoter	101399295	192bp**	192bp↑	ND	192bp ↑height, ↓MI and DM2	3 populations: 900 elderly, 220 DM2 and 596 controls and 477 with MI and 808 controls	Vaessen, 2001
Promoter	101399295	192bp**	ND	ND	192bp ↑birth weight	463 adults	Vaessen, 2002

Location	Position	SNP / VNTR	IGF-I levels*	LD	Main results	Populations	Study
Promoter	101399295	192bp**	ND	ND	192/194bp ↑birth weight in males, ↓LDL in all	264 subjects aged 36 yrs	Te Velde, 2005
Promoter	101399295	192bp**	ND	ND	192bp: ↓weight, BMI, fat mass, WC in females in 1 cohort	359 subjects 13-36 yrs, 258 subjects 8-14 yrs	Voorhoeve, 2006
Intron 2	101395389	rs2162679	ND	ND	NA with AAM	See rs35765	Zhao, 2007
Intron 2	101394352	IGF1.PCR1	191bp↓	ND	191bp ↓HC	See 192bp**	Arends, 2002
Intron 2	101394352	IGF1.PCR1	189bp↓	+	↑LD T-1148C and 192bp, birth size and CU growth	114 short SGA and 60 SGA CU, 292 AGA controls, 108 SGA CU and 12 short SGA, 144 AGA	Johnston, 2003
Intron 2	101393730	rs5742620	ND	ND	AC: ↑risk for PE	394 women with PE and 602 controls and their offspring	Goddard, 2007
Intron 2	101393730	A-23C	NA	ND	NA	108 SGA CU and 12 short SGA subjects and 144 AGA controls	Johnston, 2003
Intron 3	101391797	rs12821878	ND	ND	NA with height	See rs855228	Lettre, 2007
Intron 3	101391797	rs12821878	ND	+	NA with AAM	See rs35765	Zhao, 2007
Intron 3	101388555	rs1019731	ND	ND	NA with height	See rs855228	Lettre, 2007
Intron 3	101388555	rs1019731	ND	+	NA with AAM	See rs35765	Zhao, 2007
Intron 3	101381393	rs5742629	ND	ND	NA with AAM	See rs35765	Zhao, 2007
Intron 3	101377302	rs4764697	ND	ND	NA with AAM	See rs35765	Zhao, 2007
Intron 3	101371206	rs2033178	ND	+	NA with AAM	See rs35765	Zhao, 2007
Intron 3	101368366	rs10735380	ND	ND	with rs10860860: height in 3 out of 4 panels	See rs855228	Lettre, 2007
Intron 3	101368366	rs10735380	ND	+	NA with AAM	See rs35765	Zhao, 2007

Location	Position	SNP / VNTR	IGF-I levels*	LD	Main results	Populations	Study
Intron 3	101362645	rs7136446	ND	ND	with rs10735380: height in 3 out of 4 panels	See rs855228	Lettre, 2007
Intron 3	101354138	rs2288378	ND	+	NA with AAM	See rs35765	Zhao, 2007
Intron 3	101351175	rs2373721	ND	+	NA with AAM	See rs35765	Zhao, 2007
Intron 3	101347608	rs5742667	ND	+	NA with AAM	See rs35765	Zhao, 2007
Intron 3	101339888	rs5742671	ND	+	NA with AAM	See rs35765	Zhao, 2007
Intron 4	101335369	rs11111267	ND	+	NA with AAM	See rs35765	Zhao, 2007
Intron 4	101330435	rs4764883	ND	+	NA with AAM	See rs35765	Zhao, 2007
Intron 5	101326369	rs978458	ND	+	NA with AAM	See rs35765	Zhao, 2007
Intron 5	101323366	rs5742694	ND	+	NA with AAM	See rs35765	Zhao, 2007
Intron 5	101320921	rs1549593	ND	ND	NA	See rs855228	Lettre, 2007
3'UTR	101318603	T+1771C	NA	↓LD with D12S318	NA	See T-1148C	Johnston, 2003
3'UTR	101317699	rs6214	ND	ND	NA with height	See rs855228	Lettre, 2007
3'UTR	101317699	rs6214	ND	+	A: later AAM	See rs35765	Zhao, 2007
3'UTR	101316953	D12S318	NA	ND	NA	See 192bp**	Arends, 2002
3'UTR	101316953	D12S318	NA	↓LD with T+1771C	birth size and short SGA	See T-1148C	Johnston, 2003
3'UTR	101314322	rs6219	ND	+	G and rs6214: AAM	See rs35765	Zhao, 2007
3'UTR	101311944	rs2946834	ND	ND	NA with height	See rs855228	Lettre, 2007

Location	Position	SNP / VNTR	IGF-I levels*	LD	Main results	Populations	Study
3'UTR	101304963	rs10860860	ND	ND	with rs10735380: height in 3out of 4 panels	See rs855228	Lettre, 2007
3'UTR	101304092	rs2373720	ND	ND	NA with AAM	See rs35765	Zhao, 2007
3'UTR	101282832	rs4764876	ND	ND	NA with height	See rs855228	Lettre, 2007
3'UTR	101276092	rs1996656	ND	ND	NA with height	See rs855228	Lettre, 2007

*SNP= single nucleotide polymorphism, VNTR= variable number of tandem repeats, LD= linkage disequilibrium, ND= not determined, NA= not associated, FHS= Framingham heart study, AAM= age at menarche, HC= head circumference, CC= chest circumference, OGTT= oral glucose tolerance test, WC= waist circumference, PE= pre-eclampsia, SGA CU= SGA catch-up. *= Serum IGF-I levels in minor allele. **= 737/738=192bp VNTR= -841(CA)$_n$.*

3'UTR

Nine polymorphisms have been investigated in the 3'UTR area spanning 41kb. Two studies identified linkage disequilibrium. Zhao [18] reported a linkage disequilibrium block ranging between rs6214 and rs6219, being 3.3kb in size. Both rs6214 alone and the combination of its A-allele and the rs6219 G-allele associated with a 0.27 year later start of menarche. Johnston [20] investigated haplotypes of the D12S318 polymorphism and the upstream T+1771C 3'UTR polymorphism which did not show associations with birth size or postnatal growth in SGA subjects. However, when the D12S318 polymorphism was investigated separately, the most common allele associated with birth size and short stature in SGA subjects. Arends [27] also investigated the D12S318 polymorphism in a short SGA cohort by trio-design, but could not confirm these associations. Lettre [19] reported the previously described rs10735380 polymorphism in combination with rs7136446 and found an association with height in two of the three investigated cohorts. The other four polymorphisms from Lettre [19] and Zhao [18] were not associated with adult height and age at menarche, respectively.

Associations of the *IGF1* gene and serum IGF-I levels

Promoter

Two polymorphisms have been associated with serum IGF-I levels: the T-1148C SNP and the 192bp CA-repeat. Johnston [20] investigated the T-1148C SNP and serum IGF-I levels in a short SGA population during childhood, and in a control population in late puberty. No associations were observed between the T-1148C SNP and serum IGF-I levels, adjusted for age, sex and pubertal status.

Seven studies investigated the 192bp CA-repeat and serum IGF-I levels. Arends could not demonstrate an association between the 192bp CA-repeat and serum IGF-I levels in a population of 124 short SGA trios [27]. Johnston [20] investigated the 192bp CA-repeat and serum IGF-I levels in the same population as the T-1148C SNP, and found no associations. Frayling [23] investigated the 192bp CA-repeat with serum IGF-I levels in a young adult population (n = 640) and found that carriers of the 192/192 had a trend towards a lower serum IGF-I level whereas height was not different between the genotypic groups. Serum IGF-I levels were adjusted for sex and age. Kinoshita [26] investigated the 192bp CA-repeat and the serum IGF-I levels in 160 neonates, five days after birth. Carriers of the 192/192 genotype had a higher serum IGF-I level than the two other most frequent genotypes (192/196 and 196/196). Carriers of the 192bp allele had the longest duration of gestation, were the tallest and heaviest compared

with the 196bp allele carriers, although this was not statistically significant. Serum IGF-I levels were not adjusted for age and sex, but all blood samples were taken five days after birth with a similar sex distribution in the population. Rietveld [31] investigated the 192bp CA-repeat and serum IGF-I levels in 346 subjects of an elderly population. The population consisted of a randomly selected population-based sample of 196 subjects and 150 subjects who were selected on their *IGF1* genotype, namely 192/192bp, 192/non192bp and non192/non192bp carriers, each group consisting of 50 subjects. Carriers of the 192/192bp genotype showed a decline of their serum IGF-I level with increasing age, which was not seen in 192/non192bp and non192/non192bp carriers. Serum IGF-I levels were adjusted for age, sex and BMI. Rietveld [28] also investigated the previously described population with in addition 50 subjects who were selected on their genotype: homozygous carriers of less than 19 CA-repeats (< 192bp alleles), or carriers of more than 21 CA-repeats (> 194bp alleles). Homozygous carriers of the 192bp and 194bp allele had higher serum IGF-I levels than homozygous carriers of shorter or longer alleles. Serum IGF-I levels were adjusted for age, sex and BMI. Vaessen[29] investigated the same genotypic groups in the population Rietveld also described[31], in combination with their serum IGF-I levels (n = 150).

***IGF1* gene**

Both Arends [27] and Johnston [20] investigated the IGF1.PCR1 intron 2 polymorphism in combination with serum IGF-I levels. Arends [27] investigated first the transmission disequilibrium of all alleles of the IGF1.PCR1 polymorphism. The second most common allele, the 191bp allele, was transmitted more frequent from the parents to the short SGA child than the other alleles. Secondly, short SGA children with and without the 191bp allele were compared for their serum IGF-I level. Short SGA children with the 191bp allele had a lower serum IGF-I level than non191bp carriers (-1.1 vs. -0.5 SDS, respectively) but had no different height SDS. Serum IGF-I levels were adjusted for age and sex.

Johnston [20] also investigated the IGF1.PCR1 polymorphism in combination with serum IGF-I levels in an association study of two SGA populations and a control population. Carriers of the, most frequent, 189/189bp genotype, were associated with a lower IGF-I SDS than non-carriers. Their control population did not show this association between the 189bp allele and serum IGF-I level.

3'UTR

In the 3'UTR, two polymorphisms were investigated in combination with serum IGF-I levels. Johnston [20] investigated the T+1771C SNP and the D12S318 polymorphism in a short, SGA catch-up and control population without observing an association with serum IGF-I levels.

Polymorphisms in the insulin-like growth factor-i receptor gene

The IGF1R gene contains 21 exons, spans about 310kb and is mapped on chromosome 15q25-q26 (Figure 3) [32,33]. The gene for the IGF1R is homologous to the insulin receptor gene with respect to their exon and intron organisation and is for more than 50% identical. Both encode precursor proteins that undergo post-translational modification to result in receptors which are composed of two alpha and two beta subunits, connected by disulfide bonds to form an α2β2 heterotetrameric complex of generally similar design (Figure 4) [34]. The α-subunits are extracellular (exon 1-10), containing ligand-binding domains and the β-subunits are intracellular containing tyrosine kinase domains (exon 11-21). The region of the highest amino acid similarity in the IGF- and insulin-receptors (80-95%) consists of five exons (exon 16-20), encoding parts of the tyrosine kinase domain in the beta subunit. The 5'-flanking and untranslated region is GC-rich and contains numerous potential SP1 and AP2 binding sites [35]. The IGF1R consists of a 1367- amino acid receptor precursor, including a 30-residue signal peptide, which is removed during translocation of the nascent polypeptide chain. Cleavage of the precursor generates the alpha and beta subunits as in the case of the insulin receptor.

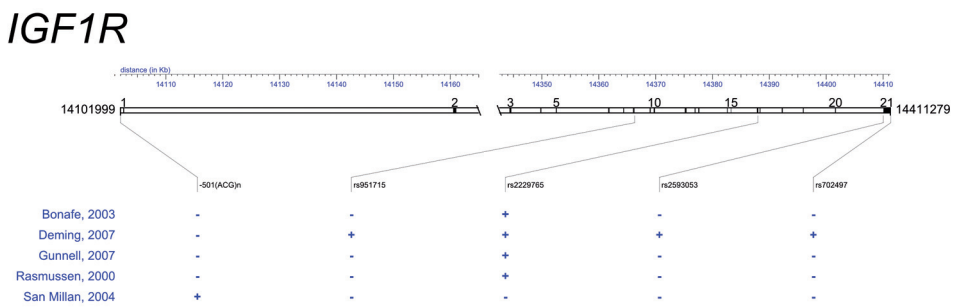

Figure 3. *IGF1R* gene structure.

Whereas the *IGF1* gene has been extensively investigated for genetic polymorphisms, these studies are rare for the IGF1R gene. We only identified one association study with SGA-related outcomes. Another four studies [36-39] described *IGF1R* polymorphisms with outcomes in polycystic ovary syndrome (PCOS), breast cancer, longevity and schizophrenia but not with SGA-related outcomes (Table 2). The absence of extensive genetic association studies, however, does not mean that the *IGF1R* gene is not polymorphic. The NCBI database provides at least 1274 SNPs in the *IGF1R* gene of which 24 synonymous, 5 non-synonymous and 1 frame-shift polymorphism (Web resources: NCBI). Many of these SNPs have a low allele frequency (often < 5%) and have been rarely investigated in gene association studies.

Table 2. Overview of investigated *IGF1R* genetic polymorphisms.

Location	Position	SNP / VNTR	LD	Main results	Populations	Study
Promoter	97295748	ACG VNTR	ND	NA	72 PCOS patients and 42 controls	San Millan, 2004
Intron 8	97274076	rs951715	-	G: ↑risk of death	1455 women with BRC	Deming, 2007
Exon 16	97295748	G/A	ND	A: ↓free IGF-I	496 subjects	Bonafe, 2003
Exon 16	97295748	G/A	ND	NA	1) 395 DM2 patients and 238 matched controls, 2) 349 young, healthy subjects	Rasmussen, 2000
Exon 16	97295748	rs2229765	-	NA	See rs951715	Deming, 2007
Exon 16	97295748	rs2229765	ND	NA	648 schizophrenia cases, 712 controls, 297 schizophrenia trios	Gunnell, 2007
Intron 20	97317779	rs2593053	-	NA	See rs951715	Deming, 2007
3'near gene	97319104	rs702497	-	NA	See rs951715	Deming, 2007

SNP= single nucleotide polymorphism, VNTR= variable number of tandem repeats, LD= linkage disequilibrium, ND= not determined, NA= not associated, PCOS= polycystic ovarian syndrome, BRC= breast cancer, DM2= diabetes mellitus type 2.

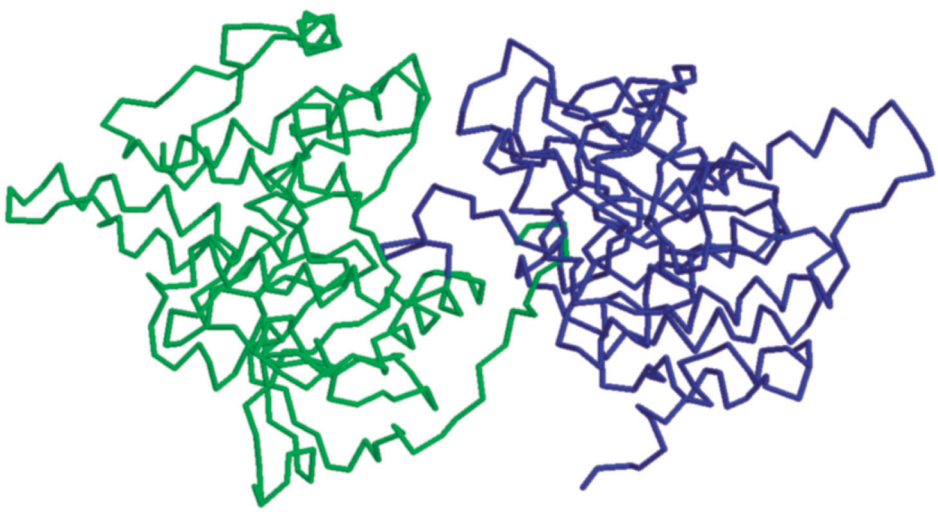

Figure 4. IGF-IR protein structure with amino acid chain A (left: green) and amino acid chain B (right: blue), according to PDBID 1P4O (Web resources: Rasmol, RCSB PDB) [47,48].

Associations of the *IGF1R* gene and SGA-related outcomes

IGF1R gene

Only one polymorphism has been investigated in the *IGF1R* gene in relation with SGA-related outcomes. Rasmussen et al. [40] sequenced all twenty-one *IGF1R* exons to identify polymorphisms in 82 probands of Danish families with diabetes mellitus type 2. Ten polymorphisms were found of which six were silent exonic and four were intronic variants. Only the exon 16 polymorphism (E1013E) was present in > 5% of the population. Further investigation, also in a population of 349 young, healthy subjects did not reveal associations with birth size and oral glucose tolerance test results in both populations.

IGF1 and *IGF1R* gene interaction studies and SGA-related outcomes

IGF1 gene

Lettre et al. [19] investigated gene-gene interaction with height in eight genes: *GHR, GHRH, GHRHR, IGF1, IGFALS, IGFBP3, JAK2, STAT5B*. All pairwise combinations between all 150 SNPs and 82 multimarker predictors were tested. Only one interaction remained after applying Bonferroni correction: an interaction between the *GHR* and *IGFALS* genes (rs12233949* rs11540961) in a European-American height panel. However, this finding could not be replicated in a Poland height panel. No other studies were found with gene-gene interactions involving SGA-related outcomes.

IGF1R gene

Rasmussen [40] did not describe gene-gene interaction studies. No other studies were found with gene-gene interaction studies involving SGA-related outcomes.

Concluding remarks

The objective of this review was to determine the contribution of the currently known genetic association studies in the *IGF1* and *IGF1R* gene with regard to SGA-related outcomes both for clinical research and practice. We also determined the contribution of linkage disequilibrium and haplotype studies in the *IGF1* and *IGF1R* gene in relation to SGA-related outcomes. Additionally, the contribution of gene-gene interaction studies with the *IGF1* and *IGF1R* genes and SGA-related outcomes was studied.

This review showed that many *IGF1* polymorphisms have been studied but that the 192bp CA-repeat in the promoter was most likely to associate with SGA-related outcomes. The region ranging from the promoter until intron 2 is part of a linkage disequilibrium block which is important in SGA-related outcomes as all association studies in SGA populations report associations with polymorphisms in this area [20,27]. Conflicting results arise especially in general populations and with associations between birth size measures and polymorphisms in this gene region [23-25,29]. There was only one study evaluating associations between *IGF1R* polymorphisms and SGA-related outcomes, which could not demonstrate any association [40]. Limited gene-gene interaction studies have been performed with SGA-related outcomes and no associations were found [19]. None of these genetic association studies are currently suitable as a diagnostic measure because no therapeutic outcome has been identified yet.

Due to the diverse outcomes, it was not possible to perform a meta-analysis. We applied guidelines for a systematic review of these studies. However, the diverse outcomes demand criteria to determine if a genetic polymorphism is associated to an outcome or not. The conflicting results of the 192bp CA-repeat promoter polymorphism showed that replication of results in a comparable but not the same cohort was not possible, which makes it hard to conclude on the contribution of the polymorphism to SGA-related outcomes. A system in which genetic polymorphisms are scored based on their potential effect on the protein level might be needed.

Another recurring problem is the use of different methods for correction of results for the number of performed tests. Lettre et al. [19] showed a large number of *IGF1* SNPs and corrected all P-values by Bonferroni [41,42]. Most studies discussed in this review did, however, not follow this approach, which makes them hard to compare.

Another complicating factor in comparing study results of SGA populations, is the varying SGA definition. Although an SGA-consensus meeting [3] stated that SGA should be defined as a birth length and/or weight below the -2.00 standard deviation score, this is not widely applied. Also, many studies in adult populations do not correct for gestational age and only report unadjusted birth weights and/or lengths. By not adjusting for gestational age, it is almost impossible to draw conclusions about SGA children as many associations will be the result of premature birth.

A complicating factor of genetic association studies in the SGA population is the low prevalence of SGA. By definition, SGA children comprise only 2% of the population and only 15% of them remains short. Thus it is almost impossible to ascertain a SGA population which is large enough to detect minor associations of their phenotype. A methodological concern in many association studies in SGA children is the occurrence of population stratification

in case-control designs. Population stratification is the situation in which cases and controls have different allele frequencies which is attributable to diversity in background population, unrelated of outcome status [43]. A method to control for population stratification is sample matching or estimation of the genetic differences by anonymous genetic markers. Anonymous genetic markers are at least 30 randomly selected SNPs throughout the genome which would reflect the background diversity between the case and control population so that they can be addressed statistically.

Another factor which is important in genetic research in SGA children, is epistasis or genetic interaction. SGA is the result of a complex of factors, so it is likely that genetic interactions determine parts of the SGA phenotype. However, only one study has investigated gene-gene interaction with SGA-related outcomes. This study could not demonstrate significant gene-gene interactions in relation to height [19]. Association studies into diverse adult diseases demonstrated potential gene-gene interactions between *IGF1* 192bp CA-repeat and the Insulin VNTR in a diabetic population [44] and an interaction between the *IGF1* 192bp CA-repeat, the oestrogen receptor α and oestrogen receptor β [45]. It remains to be investigated, whether these gene-gene interactions explain parts of the SGA phenotype.

Practice points

It is essential for both genetic and non-genetic research in SGA children that health care workers systematically measure and record height, weight and head circumference of infants at birth.

Testing the biological function of polymorphisms is needed, especially of the IGF1 192bp CA repeat, to show the effect of the polymorphism on protein level.

Many studies have reported associations between *IGF1* gene polymorphisms and SGA-related outcomes as birth size and postnatal growth. Results are, however, conflicting. It is unclear whether this is the result of small sample sizes, population stratification or the different definitions of SGA.

Few studies have reported *IGF1R* genetic polymorphisms and SGA-related outcomes.

Research agenda

- Association studies using etiological factors of the SGA phenotypes, should be performed.
- Collaboration of international research consortia is required in order to increase the SGA sample size.
- Functional studies are needed on the *IGF1* and *IGF1R* genetic polymorphisms.

– Studies into *IGF1* gene isoforms and their tissue-specific actions into the SGA phenotype are needed.
– Genome wide associations are to be performed into SGA populations to identify non-candidate, but important genes in the SGA phenotype.

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Web resources

URLs of the presented data are:

– HapMap genomic database: http://www.hapmap.org/
– NCBI SNP browser: http://www.ncbi.nlm.nih.gov/entrez/
– Open rasmol: http://www.openrasmol.org/
– PubMed: http://www.ncbi.nlm.nih.gov/entrez
– RCSB: http://www.rcsb.org/pdb/
– UCSC: http://genome.ucsc.edu/

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Chapter 4

Birth size, postnatal growth and growth during GH treatment in SGA children: Associations with IGF1 gene polymorphisms and haplotypes?

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Abstract

Background: Short SGA children experience pre- and postnatal growth restriction, which might be influenced by polymorphisms in the *IGF1* gene. The well-known -841$(CA)_n$/192bp polymorphism has been associated with birth size and cardiovascular disease.

Aims: To determine whether birth size, postnatal growth and growth during GH treatment, were associated with *IGF1* gene polymorphisms and haplotypes.

Methods: 201 short SGA children were investigated for four *IGF1* gene polymorphisms in the promoter (-G1245A, -841$(CA)_n$), intron 2 (+3703$(CT)_n$) and 3UTR (+A1830G). Spontaneous growth and growth during GH treatment were studied.

Results: The -1245 A-allele was identified as a marker-allele for the well-known -841$(CA)_n$/non192bp-allele, both part of Haplotype 2. The -1245 A-allele was not associated with head circumference at birth, but was associated with a postnatal 0.3 SDS smaller head circumference at age 1-3. The -1245 A-allele was also associated with a 1 week shorter gestational age which explained the association with a smaller absolute birth size. No associations were found with gestational age-adjusted birth size, height and weight SDS during postnatal life and with growth during GH treatment.

Conclusions: The –G1245A SNP appeared to be a marker for the well-known -841$(CA)_n$/192bp polymorphism. Haplotype 2, of which the -1245 A-allele was the marker, was associated with a smaller head circumference SDS during spontaneous postnatal growth, but not during GH treatment.

Introduction

Small for gestational age (SGA) is the term used to describe a child born with a low birth weight and/or birth length for gestational age. Since reduced size at birth may result from fetal, maternal, placental or demographic influences, children born SGA comprise a heterogeneous phenotype with a broad spectrum of clinical characteristics. Around 2% of all life-born neonates is born SGA and 10% of them remain short [1,2].

Phenotypic variation is determined by the interaction between genetic and environmental factors. Genes involved in pre- and postnatal growth are major candidate genes to explain pre- and postnatal growth restraint. Currently, most genetic research in short SGA children is focused on the role of the insulin-like growth factor 1 (*IGF1,* OMIM*147440, NM_000618, gene map locus: 12q22-q24.1), IGF-I receptor (*IGF1R,* OMIM*147370, NM_000875, gene map locus: 15q25-q26) and growth hormone receptor (*GHR*, OMIM*600946, NM_000163, gene map locus: 5p13-p12) genes [3-6]. The *IGF1* gene is a major candidate as animal studies have shown that *IGF1* knock-out mice had a 40% birth weight reduction and retarded postnatal growth in length and weight [7,8]. Additionally, case-reports have described patients with mutations in the *IGF1* and *IGF1R* gene with a specific phenotype, consisting of short SGA, sensorineural deafness, a small head circumference and a low performal IQ [5,9-13]. Although only very few short SGA children have all the abnormalities described in the case-reports, they have as a group a smaller head circumference and lower IQ than average [14].

The most common investigated genetic polymorphism in the *IGF1* gene is a CA-repeat located in the promoter. From sequence analysis, it is known that 19 CA-repeats resemble the most frequently investigated "192bp allele". The non-192bp allele is associated with a low birth weight, low adult height, low IGF-I serum level and a high risk for diabetes mellitus and myocardial infarction although conflicting results are reported [15-19]. At present, it is not known if the 192bp allele also associates with gestational age, birth head circumference and postnatal growth in short SGA children. Also it is unknown whether the 192bp allele associates with the response to GH treatment and whether nearby genetic variants might function as a genetic marker for the 192bp allele.

In the present study we evaluated whether anthropometric measurements at birth, during postnatal growth and GH treatment, were associated with *IGF1* gene polymorphisms and haplotypes in a population of 201 short SGA children.

Methods

Patients

The study population consisted of 201 SGA children with persistent short stature who participated in prospective cohort trials evaluating the effect of growth hormone (GH) treatment [14,20,21]. SGA was defined as a birth length ≤ –2.00 SDS [22]. Persistent short stature was defined as a height ≤ –2.00 SDS at ≥ 3 years of age [23]. For the present study, all children fulfilled the inclusion criteria of Caucasian ethnicity and an uncomplicated postnatal period. Children with severe chronic illness or endocrine disorders, chromosomal or genetic abnormalities, positive endomysial or transglutaminase antibodies, skeletal abnormalities, psychosocial dwarfism, and growth failure caused by syndromes, were excluded from the study. Data regarding gestational age at birth and length, weight and head circumference measurements from birth until 3 years of age were retrieved from primary health care records.

The efficacy of one year of GH treatment was evaluated in a subpopulation of 143 prepubertal children who were prepubertal at start and after one year of GH treatment. Prepuberty was defined as a bilateral testicular volume of 3 ml or less for boys and Tanner stage M1 for girls. Children receiving medication for induction or postponement of puberty were excluded from the analysis. The study protocol was approved by the Medical Ethics Committee and written informed consent was obtained from the parents/guardians of each child. Parents could indicate on the informed consent form if they wanted to be informed about the genetic results of the population.

Controls

A Dutch blood donor population of 207 individuals was used to determine allele frequencies in non-SGA subjects.

Selection of *IGF1* gene polymorphisms

Four *IGF1* gene polymorphisms were studied: the -G1245A (rs35767) single nucleotide polymorphism (SNP), the -841$(CA)_n$ variable number of tandem repeat (VNTR), the +3703$(CT)_n$ VNTR, and the +A1830G SNP (rs6220, Figure 1A). The two SNPs, -G1245A [24,25] and the +A1830G were identified by searching NCBI and Celera SNP databases (Web resources) and literature. The two VNTRs, -841$(CA)_n$ and +3703$(CT)_n$ were selected because of their previously reported associations with birth characteristics [3,4,16,19].

Genotyping

Genomic DNA was extracted from samples of peripheral venous blood according to salting out procedure [26]. Genotypes were determined using the Taqman allelic discrimination assay. The Assay-by-Design service (www.appliedbio-systems.com) was used to set up a Taqman allelic discrimination assay for the -G1245A and +A1830G SNPs. The -G1245A forward primer consisted of GGATTTCAAGCAGAACTGTGTTTTCA, and reverse primer of GGTGGAAATAACCTGGACCTTGAAT. The -G1245A forward probe consisted of VIC-TTTTTTCCGCATGACTCT and reverse probe of FAM-TTTTTTTTCCACATGACTCT. The +A1830G forward primer consisted of GAAAAGAAGGAATCATTGTGTTTTTCAAAATGAA and reverse primer of GCACTCACTGACTCTTCTATGCA. The +A1830G forward probe consisted of VIC-ATATCTAGTAAAACCTTGTTTAAT and reverse probe of FAM-CTAGTAAAACCTCGTTTAAT. The PCR reaction mixture included 5ng of genomic DNA, 0.125 μl TaqMan assay (40*, ABI), 2.5 μl Master mix (ABI) and 2.375 μl water. PCR was performed in 384 wells PCR plates in an ABI 9700 PCR system (Applied Biosystems Inc., Foster City, CA, USA) and consisted of initial denaturation for 10 minutes at 95° C, and 40 cycles with denaturation of 15 seconds at 92° C and annealing and extension for 60 seconds at 60° C. Results were analyzed by the ABI Taqman 7900HT using the sequence detection system 2.22 software (Applied Biosystems Inc., Foster City, CA, USA). The -841$(CA)_n$ and +3703$(CT)_n$ VNTR were genotyped as previously described [3].

Linkage Disequilibrium and Transcription Factor Binding Sites

The pairwise linkage disequilibrium (LD) coefficients (D') and correlation coefficients (R^2) were calculated with the use of Haploview software (Web resources). Gene polymorphisms which were in high LD (> 0.90) were used for haplotype construction. We inferred haplotypes of the -G1245A and the -841$(CA)_n$/192bp genotypes using PHASE 2.1 [27,28]. Identification of previously known LD blocks in the IGF1 gene took place by searching the HapMap genomic database and PubMed (Web resources). Transcription factor binding sites (TFBS) around the -G1245A SNP were searched in the "Transcription Factor search" program [29] and the "Transcription Element Search System" (Web Resources).

Statistical analysis

The power calculation was performed by the "Power and sample size calculation" tool of the Department of Biostatistics at Vanderbild University (Web resources). Cases consist of the homozygous carriers of the minor allele grouped together with the heterozygous carriers. The control group consists of the homozygous carriers of the major allele. As the genotype group

with the lowest allele frequency was above the minimal sample size of 52, we considered the power adequate.

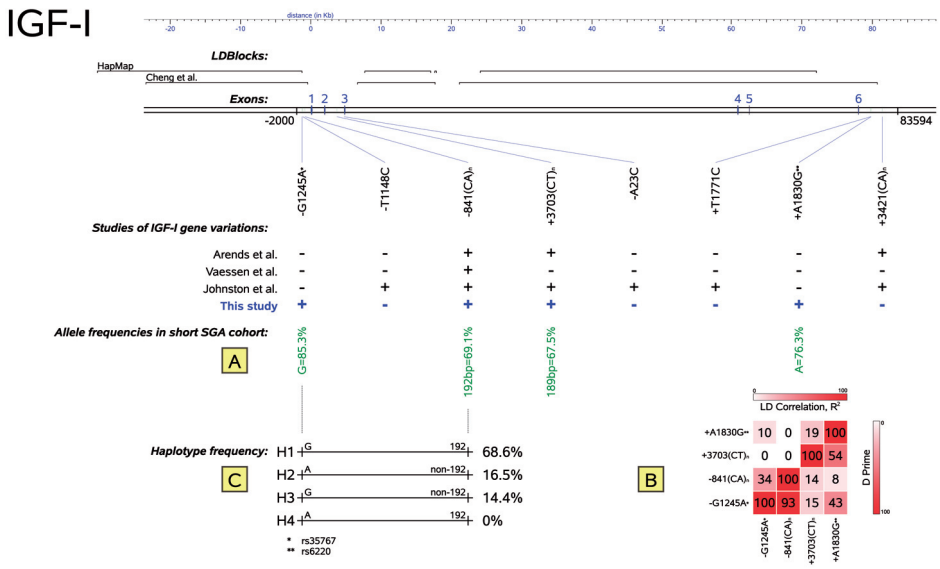

Figure 1. *IGF1 gene structure.* **A**: *IGF1* gene structure with the gene polymorphisms studied in this short SGA population and previous studies. The allele frequencies are presented of the studied gene polymorphisms. LD blocks were retrieved from the HapMap database and Cheng (Web resources) [25]. **B**: LD (D' and R^2) is shown for the -G1245A SNP, the -841(CA)$_n$/192bp VNTR and the +3703(CT)$_n$/189bp VNTR. **C**: Haplotypes were constructed of the -G1245A SNP and the -841(CA)$_n$/192bp VNTR. Their frequency is presented for the short SGA population.

Hardy-Weinberg equilibrium (HWE) was calculated by computing the chi-square test for deviations in HWE. Association studies with birth size, postnatal growth and growth during GH treatment were performed by analyzing the -G1245A SNP, the -841(CA)$_n$ and +3703(CT)$_n$ VNTRs and the +A1830G SNP separately. The gene polymorphisms were grouped according to a dominant model into homozygous carriers of the most frequent G-, 192bp-, 189bp- and A-allele, respectively, versus the remaining alleles. Secondly, haplotypes of the -G1245A SNP and the -841(CA)$_n$ VNTR were analyzed (Figure 1C). Haplotype 1 was grouped into 0 & 1 vs. 2 allele copies due to the combination of the most frequent alleles. Haplotype 2 and 3 were grouped into 0 vs. 1 & 2 allele copies. The genotypic groups were compared for all anthropometric parameters by univariate analysis of variance (ANOVA). Anthropometric measurements at 1,

2 and 3 years of age were corrected for premature birth by adding gestational age as a covariate in ANOVA. A subgroup analysis on the effect of GH treatment on growth was performed in 143 prepubertal children. Analysis of the growth response was performed by using ANOVA. Baseline measurements were corrected for age at start of GH treatment, as covariate in ANOVA. The growth response during one year of GH treatment was adjusted for GH dose and age at start of GH treatment, also as covariate in ANOVA. $P< 0.05$ was considered significant. Statistical tests were performed using SPSS 11.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinical characteristics

The study population consisted of 201 children (109 boys) and was characterized by their short birth length, low birth weight and below the mean head circumference, being respectively -3.50, -2.46 and -1.34 SDS. From birth until 3 years of age, height and head circumference increased by 0.5 and 0.2 SDS, respectively, whereas weight decreased by 0.4 SDS (Table 1).

There were 75 boys and 68 girls who were prepubertal at start and after 1 year of GH treatment. Their mean age was 6.8 years (SD = 2.3) and their GH dose was 1.11 (0.29) $mg/m^2/day$.

Allelic frequencies of *IGF1* polymorphisms

Figure 1A presents the allele frequencies of the -1245G, -841$(CA)_n$/192bp, +3703$(CT)_n$/189bp and +1830A alleles in the short SGA population, being respectively 85.3, 69.1, 67.5 and 76.3%. All gene polymorphisms were found to be in Hardy-Weinberg equilibrium (HWE: $P = 0.45$, $P = 0.85$, $P = 0.71$ and $P = 0.68$, respectively). The allele frequency of the -G1245A SNP was not significantly different between the total short SGA population and the control population (14.7% vs. 17.4%, respectively).

Linkage disequilibrium in the *IGF1* gene

There was high LD between the -G1245A SNP and the well-known -841$(CA)_n$/192bp VNTR ($D' = 0.93$, $R^2 = 0.34$, Figure 1B) both located in the promoter region. Haplotypes were constructed. Haplotype 1, 2 and 3 had a respective frequency of 68.6, 16.5 and 14.4 %, Haplotype 4 (H4) was not present in the population (Figure 1C). LD between the -G1245A SNP and +3703$(CT)_n$ VNTR and between the -841$(CA)_n$ VNTR and the +3703$(CT)_n$ VNTR was low ($D'< 0.6$), so no haplotypes were generated (Figure 1B).

Table 1. Clinical characteristics of the total short SGA population at birth and during postnatal growth.

		Short SGA population*			
		Height	**Weight**	**Head circumference**	**IGF1**
Birth	cm/ g /cm	41.6 (4.9)	1921 (716)	31.5 (3.0)	-
Birth	SDS	-3.50 (1.30)	-2.46 (1.11)	-1.34 (1.45)	-
Age 1 (yrs) #	SDS	-2.83 (0.84)	-2.75 (1.14)	-1.26 (0.89)	-
Age 2 (yrs) #	SDS	-2.97 (0.73)	-2.78 (1.02)	-1.15 (0.87)	-
Age 3 (yrs) #	SDS	-3.00 (0.70)	-2.82 (0.96)	-1.14 (0.89)	-
At start GH*	SDS	-3.04 (0.62)	-3.00 (0.92)	-1.11 (0.88)	-0.95 (1.21)
After 1 yr GH**	SDS	-2.18 (0.65)	-2.22 (0.87)	-0.80 (0.92)	0.90 (1.29)

*Values are means (standard deviation). SDS= standard deviation score. The mean gestational age was 36.8 (3.5) weeks. Birth size SDS were determined by using a gestational age specific growth chart [22]. # = Corrected for gestational age, being a covariate in ANOVA, to adjust for premature birth. *= Means are adjusted for age at start of GH treatment and presented with standard error (SE); **= Means are adjusted for age at start of GH treatment and GH dose, and presented with SE.*

To investigate the consistency of high LD within the promoter region of the *IGF1* gene, we searched the HapMap genomic database (Web resources) and literature. Cheng [25] and the HapMap database showed a consistent pattern of high LD in the *IGF1* promoter region, confirming what was observed in our study (Figure 1A).

The study of Cheng and the HapMap database showed that the -G1245A SNP (rs35767) appeared to be a tagging SNP for 2 haplotypes described in a Caucasian population [25]. Our study describes 3 instead of 2 haplotype blocks which might well be due to the -841$(CA)_n$ VNTR which was not present in the study of Cheng and the HapMap database because in these studies only SNPs and not VNTRs were genotyped.

The -G1245A SNP

Carriers of the -1245 A-allele had a shorter birth length, smaller birth head circumference and tended to have a lower birth weight (Table 2). When birth measurements were adjusted for the shorter gestational age ([GG] = 37.1 vs. [GA+AA] = 36.0 weeks, P = 0.04), no differences between SD scores were found. We therefore only describe birth data which are adjusted for gestational age (SDS).

The -G1245A SNP did not associate with a different height SDS and weight SDS during the first 3 years of life (Table 2), but carriers of the -1245 A-allele had a 0.3 SDS smaller head circumference from 1 until 3 years of age (P = 0.02, 0.01 and 0.06, Figure 2). The -G1245A SNP was not associated with the gain in height, weight and head circumference and IGF-I levels during GH treatment.

The -841$(CA)_n$/192bp VNTR

The non192bp-allele was associated with a one week shorter gestational age (36.3 vs. 37.3 weeks in 192bp-allele carriers, P = 0.05). No associations were found with gestational age adjusted birth measurements and measurements during postnatal life (data not shown).

Promoter haplotypes

Three haplotypes could be constructed of the -G1245A SNP and the -841$(CA)_n$/192bp VNTR (Figure 1C). Haplotype 2, consisting of the -1245 A- and the -841$(CA)_n$/non192bp-allele, was the only haplotype in our population which included the -1245 A-allele. Haplotype 2 showed comparable associations with gestational age and head circumference as those of the -1245 A-allele separately. Haplotype 1 and 3 showed no associations (Table 3).

Table 2. Birth size and postnatal growth associations with the -G1245A SNP in the total short SGA population.

		Height		Weight		Head circumference	
		[GG]	**[GA+AA]**	**[GG]**	**[GA+AA]**	**[GG]**	**[GA+AA]**
Birth	Cm/ g /cm	42.1 (4.5)	40.4 (5.8) [2]	1975 (630)	1781 (811) [1]	31.8 (2.7)	30.7 (3.4) [2]
Birth	SDS	-3.41 (1.21)	-3.72 (1.50) [1]	-2.42 (1.05)	-2.57 (1.24)	-1.28 (1.29)	-1.49 (1.80)
Age 1*	SDS	-2.80 (0.07)	-2.88 (0.11)	-2.71 (0.10)	-2.87 (0.15)	-1.16 (0.81)	-1.52 (0.86) [2]
Age 2*	SDS	-2.95 (0.06)	-3.03 (0.10)	-2.77 (0.09)	-2.84 (0.14)	-1.04 (0.90)	-1.42 (0.85) [3]
Age 3*	SDS	-2.98 (0.06)	-3.04 (0.10)	-2.79 (0.08)	-2.94 (0.13)	-1.06 (0.90)	-1.36 (0.84) [1]

*Values are given as means (SD) unless otherwise specified. Birth size SDS were determined by using a gestational age specific growth chart [22]. *= Corrected for gestational age, being a covariate in ANOVA, to adjust for premature birth, presented with standard error. SDS= standard deviation score. P-values compare the [GG] vs. [GA+AA] genotype, [1]= P-value ≤ 0.10, [2]= P-value ≤ 0.05, [3]= P-value ≤ 0.01.*

Table 3. Gestational age and head circumference associations with the *IGF1* promoter haplotypes in the total short SGA population.

		H1 = [-1245G] + [-841/192bp]		**H2 = [-1245A] + [-841/n192bp]**		**H3 = [-1245G] + [-841/n192bp]**	
		H1	**nH1**	**H2**	**nH2**	**H3**	**nH3**
GA	Weeks	36.8 (3.6)	36.5 (3.5)	35.8 (4.1)	37.2 (3.3) [1]	36.7 (3.4)	36.9 (3.6)
Age 1*	HC SDS	-1.27 (0.07)	-1.28 (0.21)	-1.52 (0.12)	-1.17 (0.08) [2]	-1.21 (0.12)	-1.29 (0.08)
Age 2*	HC SDS	-1.16 (0.07)	-1.17 (0.22)	-1.42 (0.12)	-1.06 (0.08) [3]	-1.08 (0.12)	-1.20 (0.08)
Age 3*	HC SDS	-1.15 (0.07)	-1.15 (0.22)	-1.36 (0.13)	-1.07 (0.08) [1]	-1.08 (0.13)	-1.18 (0.08)

*Values are given as means (SD) unless otherwise specified. *= Corrected for gestational age, being a covariate in ANOVA, to adjust for premature birth, presented with standard error. H1, H2 and H3 represent the homozygous haplotype carriers vs. the heterozygous carriers of the haplotype together with non-carriers of the haplotype (nH1, nH2, nH3). GA=gestational age, SDS= standard deviation score, HC= head circumference. P-values compare the [GG] vs. [GA+AA] genotype, [1]= P-value ≤ 0.10, [2]= P-value ≤ 0.05, [3]= P-value ≤ 0.01.*

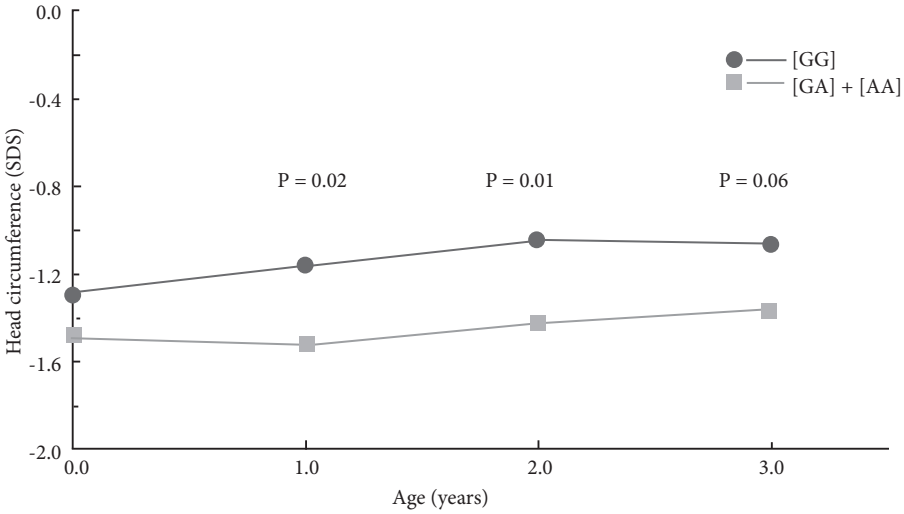

Figure 2. The -G1245A SNP in short SGA children: Difference in postnatal head circumference SDS. Means are presented with P-values above, SDS= standard deviation score**.**

Marker alleles within haplotypes

Haplotype 2 consisted of the -1245A- and -841$(CA)_n$/non192bp-alleles, who both were associated with a shorter gestational age. The -1245 A-allele only, showed an association with a smaller head size during the first 3 years of life. Therefore, the -1245 A-allele appeared to be the marker allele within Haplotype 2 (Figure 1C).

Transcription factor binding sites in -G1245A

Transcription factor binding site (TFBS) databases were searched for changes in transcription factor binding to the -1245 SNP sequence when either the G- or the A-allele was present. Blasting the -G1245A SNP and 20bp surrounding sequence on each site in TFBS-databases, showed that the A-allele induced a potential binding site for "upstream stimulatory factor" (USF: NCACGTGN, 88.6% homology). In case of a G instead of an A-allele, such a binding site was not found.

The +3703$(CT)_n$/189bp VNTR

The +3703$(CT)_n$ VNTR was not part of the promoter LD block, so association studies were performed with this VNTR separately. Carriers of the 189bp-allele had a shorter gestational age (P=0.04), but no associations were found with gestational age adjusted birth measurements and measurements during postnatal life.

The +A1830G SNP

Due to the large intermarker distance and low LD, association studies were performed with this SNP separately. There were no associations with gestational age adjusted birth measurements and measurements during postnatal life (data not shown).

Discussion

The aim of our study was to determine in short SGA children whether birth size, postnatal growth and growth during GH treatment were associated with *IGF1* gene polymorphisms and haplotypes. The -1245 A-allele was identified as a marker-allele for the well-known -841$(CA)_n$/non192bp-allele. The -1245 A-allele associated with a 0.3 SDS smaller head circumference during spontaneous postnatal growth, and a smaller absolute birth size but this was due to a one week shorter gestational age. Carriers of the intron 2 gene polymorphism had a shorter gestational age, whereas the 3UTR gene polymorphism did not show any association. No associations were found between the *IGF1* gene polymorphisms and length, weight and head circumference standard deviation scores (SDS) at birth, and with height and weight SDS during postnatal life. *IGF1* polymorphisms were not associated with growth during GH treatment. The frequency of the different genetic polymorphisms was similar in SGA cases and controls.

The -G1245A SNP in the *IGF1* gene promoter was investigated for the first time in relation to birth size, postnatal growth and growth during GH treatment. The -G1245A SNP is an informative SNP as it is in high linkage disequilibrium with the well-known -841$(CA)_n$/192bp VNTR. The latter being the most extensively investigated gene polymorphism in the *IGF1* gene region with numerous associations in childhood and adult life [3,4,15,25]. The -G1245A SNP and -841$(CA)_n$/192bp VNTR were investigated together. Haplotype analysis showed that the -G1245A SNP was the tagging SNP for the -841$(CA)_n$/192bp VNTR. This finding might enhance further research into this gene region as the -G1245A SNP is an easy to determine gene variant by Taqman assay compared to the -841$(CA)_n$/192bp VNTR which is mainly determined by Genescan.

The association between the -841$(CA)_n$ non192bp-allele and a shorter gestational age might explain the discrepant findings of Vaessen and Frayling [16,19]. Vaessen, reported an association between the non192bp-allele and a lower birth weight in grams, which could not be confirmed by Frayling. Vaessen did however, neither select, nor adjust for gestational age in contrast to Frayling who only selected subjects between 36 and 44 weeks of gestation. It might well be that the associations found by Vaessen would have disappeared after correction for

gestational age. The association with a shorter gestational age was in line with previous studies showing an association between intrauterine growth retardation, a shorter gestational age and low IGF-I levels [30,31]. The shorter gestational age might well be an effect of prenatal low IGF-I levels resulting in a severely growth-retarded SGA fetus, which is a recognized indication to induce labor by caesarean section or chemical induction of contractions , especially when the head circumference is small.

By searching transcription factor binding site databases, we identified a transcription factor known for growth inhibiting properties, upstream stimulatory factor (USF), which binds to the -1245 A-allele. In case of the common G-allele, USF is unable to bind the surrounding *IGF1* promoter sequence. The fact that USF has growth inhibiting properties [32], suggests that the -1245 A-allele might result in reduced IGF-I protein expression in the short SGA child. Previous characterization of the region surrounding the -G1245A SNP showed that the region ranging from -1630 until -926 base pairs before the start of exon 1 is important for the activation of the *IGF1* gene transcription [33]. Important regulatory elements might coincide with the promoter linkage disequilibrium block, containing the -G1245A SNP and the -841$(CA)_n$/192bp VNTR, located at -1245 and at -841 base pairs before the start of exon 1.

The *IGF1* gene contains 2 promoter regions which regulate tissue-specific expression through alternative RNA processing [34,35]. These 2 promoter regions are located in front of exon 1 (P1) and 2 (P2), respectively, and is the same region in which high linkage disequilibrium was found (this study, [4]). Polymorphisms in these regions might result in different *IGF1* mRNA levels showing a cell-type specific expression pattern. The specific association of the -1245 A-allele with a smaller head circumference SDS, might be such a tissue-specific effect. Cheng showed that brains of *IGF1* knock-out mice are highly dependent on locally produced IGF-I in promoting growth of the developing brain [36]. A reduced *IGF1* gene expression has been associated with a reduced brain size in mice whereas *IGF1* overexpression gives rise to increased brain growth, although the IGF-I level in brain tissue was among the lowest one measured in all body tissues [37,38]. These findings show that brain growth is extremely sensitive to local IGF-I levels. Although men and mice invariably differ, it might well be that part of the *IGF1* promoter induces brain-specific local IGF-I production.

IGF1 gene polymorphisms were not associated with growth and IGF-I levels during GH treatment. This might be due to a time- and tissue-specific effect of the -G1245A gene polymorphism which is important for head growth during early postnatal life. Animal studies have demonstrated that *IGF1* induces growth mostly by tissue-specific actions, as liver *IGF1*-deficient mice with a 75% decrease in circulating IGF-I levels displayed a comparable postnatal growth pattern as control mice [39]. The -1245A-allele has also been associated with low IGF-I levels in a breast cancer population, indicating that this SNP has effects on protein level [40].

For every genetic association study there is always a possibility that associations have arisen by chance, especially if they are novel. As the head circumference associations have also been demonstrated by Arends [3] in a selected, smaller population, we consider the observed head circumference associations as real. The association between gestational age and the -G1245A SNP is new, but many studies [16,19,41-44] have investigated the association between the -841/192bp VNTR and birth size. That gestational age might be an explanatory factor in these, sometimes, conflicting associations, is biologically plausible. However, replicative studies are needed to draw clinical conclusions from these associations.

Another explanation of novel associations is the occurrence of population stratification i.e. the situation in which cases and controls have different allele frequencies which is attributable to diversity in background population, unrelated of outcome status. Population stratification seems less probable in our population, as family based studies using a transmission disequilibrium test (TDT), also showed an association between this *IGF1* gene region and head size [3,45,46].

In conclusion, the -1245 A-allele in the *IGF1* promoter was the only polymorphism which associated with a smaller head circumference SDS during spontaneous postnatal growth and a shorter gestational age. No other associations were found with birth size and growth, neither spontaneously, nor during GH treatment. The -G1245A SNP appeared to be a marker for the well-known -841$(CA)_n$/192bp polymorphism.

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This research was an investigator-initiated/responsible project.

Web resources

- URLs for the presented data are:
- Celera genome browser: http://www.celera.com/
- HapMap genomic database: http://www.hapmap.org/
- Haploview v3.2: http://www.broad.mit.edu/mpg/haploview/
- NCBI SNP browser: http://www.ncbi.nlm.nih.gov/entrez/
- PHASE: http://www.stat.washington.edu/stephens/software.html
- Power and sample size calculation: http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize
- PubMed: http://www.ncbi.nlm.nih.gov/entrez
- TESS: Transcription Element Search System: http://www.cbil.upenn.edu/tess
- TFSEARCH: Searching Transcription Factor Binding Sites: http://www.cbrc.jp/research/db/TFSEARCH.html

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Chapter 5

The –G1245A *IGF1* polymorphism is related with small head size and less brain sparing in SGA born children

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Abstract

Context: SGA subjects experience pre- and postnatal growth restriction, which might be influenced by polymorphisms in the *IGF1* gene. The well-known -841$(CA)_n$/192bp polymorphism has been associated with birth size, cardiovascular disease and IGF-I levels, and is in linkage disequilibrium with the -G1245A SNP (rs35767).

Objective: To associate the –G1245A SNP with head circumference (HC) and brain sparing (a greater head compared to height standard deviation score (SDS)) in short SGA and SGA catch-up subjects.

Design: Gene association study.

Patients: 635 SGA subjects of which 439 remained short and 196 had a postnatal height > -2.00 SDS.

Measurements: The –G1245A SNP *IGF1* gene polymorphism and head size.

Results: All SGA subjects had a postnatal head size below the population mean (-1.01 SDS, $P < 0.001$). Whereas SGA catch-up subjects had a head size which was in proportion with their height, short SGA subjects displayed extensive brain sparing (HC – height: SGA CU: 0.01 vs. short SGA: 1.75 SDS, $P < 0.001$). The most severely SGA born subjects had a 0.4 SDS smaller postnatal head size and 0.6 SDS less brain sparing when carrying the -1245 A-allele in contrast to G-allele carriers ($P = 0.03$). The association between the –G1245A SNP and head size remained significant after correction for birth weight and postnatal height SDS ($P < 0.001$). Birth weight, birth length and postnatal height SDS were not related with the –G1245A SNP.

Conclusions: The -1245 A-allele of the *IGF1* promoter SNP is associated with a small head size and less brain sparing in SGA born subjects and particularly those with the lowest birth weight.

Introduction

Around 2% of all life-born neonates are born small for gestational age (SGA) and ~10% of them remain short [1,2]. Since reduced size at birth may result from fetal, maternal, placental or demographic influences, SGA subjects comprise a heterogeneous group. SGA subjects without catch-up growth are known to have a smaller head circumference and a lower IQ [3] compared to SGA subjects catching up in height [4,5]. Head size preservation, or "brain sparing" is defined as centralisation of the fetal circulation in the brain 6 and is essential for the growth retarded fetus to prevent brain damage [7].

The *IGF1* gene (*IGF1,* OMIM*147440, NM_000618, gene map locus: 12q22-q24.1) is a major candidate gene for explaining parts of the SGA phenotype as animal studies have shown that *IGF1* knock-out mice had a 40% birth weight reduction and retarded postnatal growth in length and weight [8,9]. Case-reports have described patients with *IGF1* gene mutations who were born SGA, had a persistent short stature and sensorineural deafness, a small head circumference (HC) and a low performance IQ [10-12].

The most common investigated polymorphism in the *IGF1* gene is the 192bp CA-repeat in the promoter. The non-192bp allele is associated with a low birth weight, low adult height, low IGF-I serum level and a higher risk for diabetes mellitus and myocardial infarction although conflicting results are reported [13-18]. The 192bp allele polymorphism and the –G1245A SNP (rs35767) are located in the same linkage disequilibrium (LD) block and studies have shown that the –G1245A SNP can be used as a marker for the 192-bp polymorphism (Hapmap, Web resources) [19,20]. A subgroup of the short SGA population which is described in the current study, has already previously shown that a haplotype, of which the -1245 A-allele was the marker, was associated with a smaller head circumference during spontaneous postnatal growth, but not during GH treatment in short SGA children [20]. Thus, the *IGF1* gene is involved in determining head size, and SGA subjects have a smaller head circumference and lower IQ depending on the extent of postnatal catch-up growth. It is unknown whether the *IGF1* 192bp gene polymorphism is related to head size in short SGA versus SGA catch-up subjects. In addition, it is unknown whether SGA severity modifies the relation between brain sparing and this genetic polymorphism.

Therefore, we investigated whether the –G1245A SNP was associated with head circumference and brain sparing in a large cohort of 439 short SGA and 196 SGA catch-up subjects.

Methods

Patients

All 635 subjects were born small for gestational age (SGA: birth length and/or weight ≤ -2 standard deviation score or SDS for their gestational age [21]) and fulfilled the inclusion criteria of Caucasian ethnicity and an uncomplicated postnatal period. Subjects with severe chronic illness or endocrine disorders, chromosomal or genetic abnormalities, positive endomysial or transglutaminase antibodies, skeletal abnormalities and psychosocial dwarfism were excluded from the study. The study protocol was approved of by the Medical Ethics Committee and written informed consent was obtained from the parents/guardians and subjects above 12 years.

SGA subjects were assigned to two subgroups according to their ability to attain a postnatal height in the normal range, consisting of 439 short [20] and 196 catch-up subjects. The SGA subjects who remained short (height ≤ -2.00 SDS: short SGA [22]) were investigated at start of GH treatment and were drawn from prospective cohort trials evaluating the effect of GH treatment [23,24]. SGA subjects who had catch-up growth to a normal height (height > -2.00 SDS: SGA catch-up [22]) were randomly selected from hospitals in the Netherlands, where they had been registered because of being small at birth.

Clinical and biochemical measurements

Birth data of the SGA subjects were retrieved from records of hospitals, community health services and general practitioners. Birth weight SDS was used as a proxy for SGA severity as birth length and birth head circumference measurements are not always performed in case of a severely growth-retarded newborn. Anthropometrical measurements of the short SGA subjects were performed at the start of the GH trials [24]. Anthropometrical measurements of the SGA catch-up group were obtained at the out-patient clinic. Delta height was calculated by subtracting birth length (SDS) from height (SDS). Body mass index was calculated (weight in kg/ height in meters^2) and adjusted for age and sex, expressed as SDS [25]. Brain sparing was calculated by subtracting height (SDS) from head circumference (SDS). Both height and head circumference measurements were present in a subset of 597 SGA subjects. Serum IGF-I levels were measured in the SGA subjects as previously described [26] and values were transformed in SDS by adjusting for sex and age [27].

Genotyping

Genomic DNA was extracted from samples of peripheral venous blood according to salting out procedure [28]. Genotypes were determined using the Taqman allelic discrimination assay.

The Assay-by-Design service (www.appliedbio-systems.com) was used to set up a Taqman allelic discrimination assay for the -G1245A SNP (rs35767). Primer sequences were: Forward: GGATTTCAAGCAGAACTGTGTTTTCA, reverse: GGTGGAAATAACCTGGACCTTGAAT. Probe sequences were for –G1245A, Forward: VIC-TTTTTTCCGCATGACTCT, reverse: FAM-TTTTTTTTCCACATGACTCT. The PCR reaction mixture included 5ng of genomic DNA, 0.125 µl TaqMan assay (40*, ABI), 2.5 µl Master mix (ABI) and 2.375 µl water. PCR was performed in 384 wells PCR plates in an ABI 9700 PCR system (Applied Biosystems Inc., Foster City, CA, USA) and consisted of initial denaturation for 10 minutes at 95° C, and 40 cycles with denaturation of 15 seconds at 92° C and annealing and extension for 60 seconds at 60° C. Results were analyzed by the ABI Taqman 7900HT using the sequence detection system 2.22 software (Applied Biosystems Inc., Foster City, CA, USA).

Statistical analysis

The total SGA population was compared to the average population, defined at 0.00 SDS, using T-test. Continuous data were compared between the short SGA and the SGA catch-up population by univariate analysis of variance (ANOVA). The Chi-square test was used to analyze categorical variables. Multiple regression analysis was performed by using head circumference SDS as dependent variable and gestational age, birth weight SDS, age and height SDS as independent variables. As height SDS and BMI SDS were highly interrelated, only height SDS was investigated in the regression analysis. Correlations were determined by Spearman correlation coefficient.

The –G1245A SNP was determined in the total SGA group of 635 subjects. The Hardy-Weinberg equilibrium (HWE) was calculated by computing the chi-square test for deviations in HWE. Allele frequencies were calculated and tested by chi-square. The polymorphism was grouped according to a dominant model into the [GG] genotype versus the [GA]+[AA] genotype. The genotypic groups were compared for all anthropometric parameters by univariate analysis of variance (ANOVA). P-value ≤ 0.05 was considered significant. Statistical tests were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinical data

The total SGA group had a mean birth length of -3.00 SDS and a birth weight of -2.34 SDS (Table 1). Postnatally, they had a significantly lower BMI and smaller head circumference than

the average population (P < 0.001), but compared to their height SDS, they had a large head (HC – height, 1.18 SDS). Their IGF-I level was significantly lower than the average population (-0.93 SDS), but relatively high compared to their height of -2.24 SDS.

Table 1. Clinical data of the total, short SGA and SGA catch-up group.

	Total SGA group	**SGA subgroups**		
Child:		**Short SGA**	**SGA catch-up**	**P-value**
Birth:	635	439	196	
Sex (boys/girls)	302/333	223/216	79/117	0.01
GA (weeks)	36.7 (3.6)	36.6 (3.8)	37.1 (3.0)	0.10
Length (SDS)	-3.00 (1.48) [a]	-3.14 (1.49) [a]	-2.70 (1.41) [a]	0.001
Weight (SDS)	-2.34 (1.01) [a]	-2.28 (1.09) [a]	-2.47 (0.81) [a]	0.03
At measurement:				
Age (years)	12.7 (7.2)	8.9 (5.1)	21.1 (2.6)	<0.001
Height (SDS)	-2.24 (1.27) [a]	-2.95 (0.63) [a]	-0.64 (0.84) [a]	<0.001
Height (SDS) – birth length (SDS)	0.81 (1.81) [a]	0.20 (1.60) [c]	2.04 (1.57)	<0.001
BMI (SDS)	-0.73 (1.34) [a]	-1.13 (1.16) [a]	0.15 (1.31)	<0.001
HC (SDS)	-1.01 (1.00) [a]	-1.19 (0.95) [a]	-0.64 (0.99) [a]	<0.001
HC (SDS) – height (SDS)	1.18 (1.38) [a]	1.75 (1.06) [a]	0.01 (1.22) [a]	<0.001
IGF-I level (SDS)	-0.93 (1.32) [a]	-0.99 (1.33) [a]	-0.45 (1.10) [b]	0.005

Values are means (standard deviation) unless indicated otherwise. When compared with 0.00 SDS: [a] P-value <0.001, [b] P-value <0.01, [c] P-value <0.05. The overall P-value is presented between the short SGA and the SGA catch-up group by ANOVA for continuous and by chi-square test for categorical variables. GA= gestational age, SDS= standard deviation score, HC= head circumference.

Short SGA subjects were born with a lower birth length SDS and tended to have a shorter gestational age than SGA catch-up subjects (Table 1). Short SGA subjects were younger at examination and by definition, shorter and had less catch-up growth in height from birth onwards compared to SGA catch-up subjects. Short SGA subjects also had a lower BMI, smaller head size and lower IGF-I levels than SGA catch-up subjects. Whereas there was no brain sparing within SGA catch-up subjects (0.01 SDS), short SGA subjects had a 1.75 SDS larger head than height SDS. Although SGA catch-up subjects had caught up to a normal height and head circumference, these measurements and their IGF-I level remained below the population mean of 0 SDS (P-value: < 0.001, < 0.001 and 0.005, respectively).

Factors associated with head circumference SDS

The –1245 A-allele was associated with a 0.3 SDS smaller head circumference in the total SGA population, although explaining a minor proportion of the variation in head size ($P < 0.006$, 1.3%, Table 2). Birth weight was positively associated with postnatal head circumference, each SDS in birth weight resulting in a 0.2 SDS increase in postnatal head circumference. The –G1245A SNP remained a significant explanatory factor, even after inserting birth weight and postnatal height into the model ($P < 0.001$, R^2 13.1%).

Table 2. Factors associated with head circumference SDS in the total SGA group.

Explanatory factors:	**Model 1**		**Model 2**		**Model 3**	
	B	**P-value**	**B**	**P-value**	**B**	**P-value**
-G1245A SNP*	-0.26	0.006	-0.23	0.01	-0.20	0.03
Birth weight (SDS)	-	-	0.17	<0.001	0.20	<0.001
Height (SDS)	-	-	-	-	0.24	<0.001
R^2	1.3%		3.9%		13.1%	
P-value	0.006		<0.001		<0.001	

**coded as: 0= [GG] and 1= [GA+AA].*

–G1245A polymorphism

The total SGA population consisted of 71.7 % [GG], 24.6% [GA] and 2.2% [AA] carriers. The short SGA and SGA catch-up group had comparable genotype frequencies ($P = 0.40$). Genotypes were in Hardy-Weinberg equilibrium ($P = 0.88$).

The association between head size and the –G1245A SNP was examined in each birth weight quartile in the total SGA population. Subjects who were most severe SGA at birth, being in the lowest birth weight quartile, had a significantly 0.4 SDS smaller head size when carrying the -1245 A-allele (Table 3, Figure 1). SGA subjects with the -1245 A-allele also had 0.6 SDS less brain sparing than [GG] carriers (Figure 1). Short SGA and SGA catch-up groups showed the same difference, although it did not reach statistical significance. No associations were found with birth length, birth weight and height SDS.

Table 3. The total, short SGA and SGA catch-up group associations between the –G1245A SNP and head size according to birth weight quartiles.

Total SGA group								
Birth weight quartiles:	1		2		3		4	
Birth weight range [SDS]:	[< -3.0]		[-3.0; -2.3]		[-2.3; -1.8]		[> -1.8]	
	[GG]	[GA+AA]	[GG]	[GA+AA]	[GG]	[GA+AA]	[GG]	[GA+AA]
Head circumference SDS	**-1.11 (1.16)**	**-1.54 (1.02)[a]**	-0.96 (0.99)	-1.16 (1.04)	-0.92 (0.96)	-1.11 (0.79)	-0.78 (0.88)	-0.89 (0.98)
HC - Height SDS	**1.27 (1.43)**	**0.69 (1.43)[a]**	0.75 (1.55)	0.77 (1.05)	0.97 (1.25)	1.13 (1.01)	1.91 (1.19)	1.89 (1.02)
Short SGA group								
Birth weight quartiles:	1		2		3		4	
Birth weight range [SDS]:	[< -3.0]		[-3.0; -2.3]		[-2.3; -1.8]		[> -1.8]	
	[GG]	[GA+AA]	[GG]	[GA+AA]	[GG]	[GA+AA]	[GG]	[GA+AA]
Head circumference SDS	**-1.29 (1.09)**	**-1.70 (1.09)[b]**	-1.20 (0.83)	-1.57 (0.88)	-1.21 (0.96)	-1.26 (0.81)	-0.87 (0.84)	-1.00 (0.82)
HC - Height SDS	1.61 (1.20)	1.32 (1.14)[c]	1.77 (1.06)	1.38 (0.81)	1.65 (0.93)	1.47 (0.81)	2.12 (1.04)	1.99 (0.94)
SGA catch-up group								
Birth weight quartiles:	1		2		3		4	
Birth weight range [SDS]:	[< -3.0]		[-3.0; -2.3]		[-2.3; -1.8]		[> -1.8]	
	[GG]	[GA+AA]	[GG]	[GA+AA]	[GG]	[GA+AA]	[GG]	[GA+AA]
Head circumference SDS	-0.53 (1.21)	-1.19 (0.75)[d]	-0.73 (1.08)	-0.65 (1.03)	-0.56 (0.83)	-0.72 (0.59)	-0.18 (0.89)	0.09 (1.71)
HC - Height SDS	0.21 (1.59)	-0.71 (0.92)[e]	-0.21 (1.32)	0.03 (0.83)	0.09 (1.04)	0.23 (0.95)	0.44 (1.15)	1.04 (1.45)

Values are means (standard deviation). P-values between the [GG] and [GA+AA] genotype are: [a] = 0.03, [b]= 0.08, [c]= 0.26, [d]=0.08, [e]= 0.06.

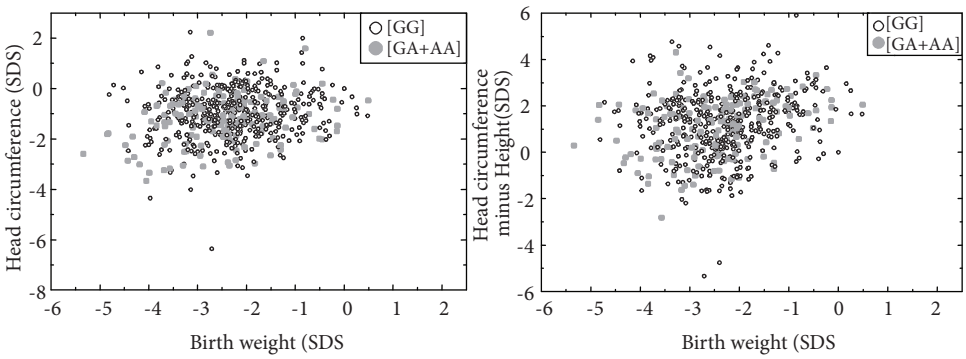

Figure 1. Scatterplot of the correlation in the total SGA population between: birth weight SDS and head circumference SDS (left), and birth weight SDS and brain sparing (HC-height SDS, right).

Discussion

The aim of our study was to investigate whether the –G1245A SNP was associated with head circumference and brain sparing in a large cohort of 439 short SGA and 196 SGA catch-up subjects. The -1245 A-allele of the *IGF1* promoter SNP was associated with a small head size and less brain sparing in SGA born subjects and particularly those with the lowest birth weight. Birth weight, birth length and postnatal height SDS were not associated with the –G1245A SNP.

This study demonstrated that the relation between the –G1245A SNP, head size and brain sparing was present in SGA born subjects and especially in those with the smallest size at birth. The head sparing adaptation of these severely SGA born neonates was reduced when carrying the –G1245 A-allele. As the –1245 A-allele is known to be in close linkage with the non192bp-allele in the *IGF1* gene promoter, our study has shown another feature of carriers of this allele. Our study also showed that the association between the –G1245A SNP, head size and brain sparing, was present in the total SGA population which is suggestive for a general feature of SGA born children.

Several studies have shown the important role of the *IGF1* gene in brain growth [29,30]. Lee et al [29] demonstrated that transgenic mice overexpressing *IGF1* could ameliorate brain growth retardation caused by undernutrition, which resulted in a comparable brain growth as the well-fed control mice. They also demonstrated that *IGF1* overexpression resulted in postnatal brain growth, which was localized in cerebral cortex, hippocampus and diencephalon. Also Simmons et al [30] suggested a tissue-specific effect of *IGF1*, showing that glucose transport in

the brain was preserved and unaffected by treatment with IGF-I or insulin. Cheng showed that brains of *IGF1* knock-out mice are highly dependent on locally produced IGF-I in promoting growth of the developing brain [31]. A reduced *IGF1* gene expression has also been associated with a reduced brain size in mice whereas *IGF1* overexpression gave rise to increased brain growth, although the IGF-I level in brain tissue was among the lowest one measured in all body tissues [32,33]. This might also be applicable to short SGA subjects having the -1245 A-allele which might result in less local IGF-I expression in the brain and a smaller head size. All together these observations indicate that short SGA subjects might experience a differential *IGF1* gene expression which influenced their head size.

Several studies have demonstrated that short stature and a small head size are important predictors for subnormal intellectual performance [4,34,35]. Our study showed that a small birth size in combination with the -1245 A-allele, was associated with the smallest head circumference in SGA subjects, and especially in short SGA subjects (A-allele carriers: -1.70 SDS). Unfortunately, we do not have data on intellectual performance in these subjects, but it would be interesting to study the influence of the A-allele on intellectual performance in future studies.

This study did not show associations between the –G1245A SNP and birth size, postnatal height and IGF-I level in the short SGA and SGA catch-up group. Although animal studies and case-reports of subjects with *IGF1* gene mutations have shown that the *IGF1* gene is important in pre- and postnatal growth, the short SGA subjects only had an association with head size. The absence of associations with pre- and postnatal growth in these SGA populations confirm previous genome wide and large association studies in which *IGF1* was not one of the genes that could explain variations in height [36-41].

Additionally, animal models in which hepatic IGF-I levels were eliminated, have shown that mice kept growing with 75% reduced serum circulating IGF-I levels [42]. These observations underline that the processes determining height are multifactorial in nature and that the *IGF1* gene is not a main contributor in determining adult height.

Due to the ascertainment, short SGA subjects were investigated at 9 years of age and SGA catch-up subjects were investigated in early adulthood at 21 years of age. Several studies have demonstrated that SGA subjects do not show any catch-up growth in height after the age of five years [1,43]. We therefore consider the difference in age unlikely to explain the observed associations.

We observed that the most severely SGA born subjects had a 0.4 SDS smaller head size and 0.6 SDS less brain sparing when carrying the -1245 A-allele in contrast to G-allele carriers, which might be of clinical relevance. Our study has shown for the first time that the –G1245A SNP is related to postnatal head size in SGA subjects regardless of their postnatal growth.

In conclusion, we found in a large group of SGA subjects that the -1245 A-allele of the *IGF1* promoter SNP was associated with a small head size and less brain sparing in SGA born subjects and particularly those with the lowest birth weight.

Web resources

URLs for the presented data are:
HapMap genomic database: http://www.hapmap.org/
NCBI SNP browser: http://www.ncbi.nlm.nih.gov/entrez/

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Chapter 6

IGF1R promoter variation is related to postnatal weight of SGA subjects with persistent short stature

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Abstract

Context: The insulin-like growth factor 1 receptor (*IGF1R)* gene is an important factor in pre- and postnatal growth. The *IGF1R* gene promoter has multiple transcription factor binding sites affecting *IGF1R* protein expression.

Objective: To determine whether haplotypes of single nucleotide polymorphisms (SNPs) in the IGF1R gene promoter, rs4965425 and rs1319868, associate with postnatal growth in a large cohort of SGA subjects.

Patients: Cohort of 635 SGA subjects (439 short SGA, 196 catch-up SGA).

Results: The rs4965425 C-allele was associated with a 0.3 standard deviation score (SDS) lower weight, BMI and catch-down in weight in short SGA subjects in contrast to the T-allele. There were no associations with the rs1319868 SNP. Both SNPs were in complete linkage disequilibrium (D' = 1.00, R^2 = 0.14). The minor allel frequency of the rs1319868 SNP was significantly higher in the total SGA population compared to controls. Haplotype analysis showed that Haplotype 1, consisting of the rs4965425 C-allele and the rs1319868 G-allele, was associated with a 0.4 SDS lower weight, BMI and catch-down in weight in short SGA subjects. This was most pronounced in short SGA subjects who had a birth length and -weight <-2.00 SDS as they had a 0.8 SDS lower postnatal weight and BMI, and the largest postnatal catch-down in weight compared to non-carriers (P = 0.002, P = 0.002 and P = 0.02, respectively). The rs4965425 C-allele drove these associations. Growth of SGA catch-up subjects was not associated with any SNP.

Conclusions: Short SGA subjects with the rs4965425 C-allele have a lower weight and BMI SDS due to a postnatal catch-down in weight compared to non-carriers. This was most pronounced in short SGA subjects born with both a birth length and -weight <-2.00 SDS. Our findings suggest that genetic variation in the *IGF1R* gene promoter contributes to postnatal weight development of short SGA subjects.

Introduction

Around 2% of all life-born neonates are born SGA and 10% of them remain short [1,2]. SGA subjects with a persistent short stature have a reduced lean body mass, fat mass, skin fold measurement and body mass index [3-5]. They also have a decreased caloric, fat and carbohydrate intake [6]. SGA subjects with an accelerated postnatal growth are known for an increased risk for cardiovascular disease [7-9]. In addition to classical risk factors, modifier genes may be of importance to postnatal growth of SGA subjects.

The insulin-like growth factor 1 receptor gene (*IGF1R*: OMIM*147370, NM_000875, gene map locus: 15q25-q26) is an important candidate gene for subjects born SGA, being a major pre- and postnatal growth factor [10,11]. *IGF1R* knockout mice have a 55% reduction in birth weight and do not survive postnatally. The *IGF1R* promoter region is rich in GC nucleotides and contains numerous potential SP1 binding sites affecting *IGF1R* gene expression [12-14].

Several case-reports have described subjects born SGA with persistent short stature, who had mutations in the *IGF1R* gene [15-18]. Although these case-reports are limited, they illustrate that the *IGF1R* gene is involved in pre- and postnatal growth.

IGF1R gene polymorphisms have rarely been investigated in SGA subjects. Several studies have investigated *IGF1R* polymorphisms but only one polymorphism has been investigated in relation with SGA-related outcomes. Namely, Rasmussen et al [19] observed no associations between the synonymous E1013E polymorphism (Rs2229765) and birth size or oral glucose tolerance.

It is unknown whether *IGF1R* gene polymorphisms in the promoter are associated with postnatal growth of SGA subjects. It might well be that promoter polymorphisms play a role in the degree of postnatal catch-up growth in weight and height of SGA subjects as the *IGF1R* gene promoter contains several transcription regulating area's which influence protein expression. In this study we investigated whether the rs4965425 and rs1319868 polymorphisms in the *IGF1R* gene promoter were associated with postnatal growth in a large population of 635 SGA subjects.

Methods

Study participants

We included 635 SGA subjects, and according to their ability to attain a postnatal height in the normal range, we divided the cohort into 439 short and 196 catch-up subjects. SGA was defined as a birth length and/or weight ≤ -2 SDS for gestational age [20]. The SGA subjects who remained short (height ≤ -2.00 SDS: short SGA, [21]) were drawn from a prospective cohort at start of GH treatment [4,22,23]. SGA subjects who had catch-up growth to a normal height (height ≥ age 3: > -2.00 SDS: SGA catch-up [21]) were randomly selected from hospitals in the Netherlands, where they had been registered because of being small at birth.

The total SGA group was subdivided in SGA_L and SGA_{L+W} subjects. SGA_L was defined as a birth length ≤ -2.00 SDS and a birth weight >-2.00 SDS, whereas SGA_{L+W} was defined as a birth length and a birth weight ≤ -2.00 SDS [24]. Within the total SGA group, 553 subjects had birth length and -weight available, 104 were SGA_L and 203 SGA_{L+W}.

All subjects were of Caucasian ethnicity and had an uncomplicated postnatal period. Subjects with severe chronic illness or endocrine disorders, chromosomal or genetic abnormalities, positive endomysial or transglutaminase antibodies, skeletal abnormalities and psychosocial dwarfism were excluded from the study. The study protocol was approved of by the Medical Ethics Committee and written informed consent was obtained from the parents/ guardians and subjects of age 12 years and older. A healthy control population of 100 subjects was acquired from a local bloodbank.

Anthropometrical measurements

Birth data and postnatal measurements of the SGA subjects were retrieved from records of hospitals, community health services, general practitioners and from baseline data of the GH trials. Height and weight were available from all subjects. All anthropometric measurements in the subjects were performed twice and the mean value was used for analysis. Body mass index was calculated (weight in kg/ height in meters^2) and adjusted for age and sex, expressed as standard deviation score (SDS) [21].

Selection of SNPs and Linkage Disequilibrium

Two SNPs within the *IGF1R* gene promoter region were identified by using the SNP selection tool, tagger in the HapMap database (Web resources). The rs4965425 [C/T] and rs1319868 [G/T] SNPs were identified as tagging SNPs for the linkage disequilibrium block in the promoter of the *IGF1R* gene.

Linkage Disequilibrium

The pairwise linkage disequilibrium (LD) coefficients (D' and R^2) were calculated with the use of Haploview software (Web resources). Gene polymorphisms which were in high LD (D' > 0.90) were used for haplotype construction. We inferred haplotypes of the rs4965425 and rs1319868 SNPs using PHASE 2.1 [25,26].

Genotyping

Genomic DNA was extracted from samples of peripheral venous blood according to salting out procedure [27]. Genotypes were determined using the Taqman allelic discrimination assay. The Assay-by-Design service (www.appliedbio-systems.com) was used to set up a Taqman allelic discrimination assay for the rs4965425 and rs1319868 SNPs.

Primer sequences for the rs4965425 and rs1319868 SNPs were: forward: GTGAATGTTTCCAATCTCGCTTTTCT and GAAGCCTCCAGTTTCCATTTTGG, reverse: CACCAAGTCCAGGACACAGT and GGCCTGGGCCAAGAGT. Probe sequences for the rs4965425 and rs1319868 SNPs were, Forward: CTTATGTTA**C**GTGATTCC and CTCCAGG**G**TGCCTAT, reverse: CTTATGTTATGTGATTCC and TCTCCAGGTTGCCTAT. The PCR reaction mixture included 5ng of genomic DNA, 0.125 µl TaqMan assay (40*, ABI), 2.5 µl Master mix (ABI) and 2.375 µl water. PCR was performed in 384 wells PCR plates in an ABI 9700 PCR system (Applied Biosystems Inc., Foster City, CA, USA) and consisted of initial denaturation for 10 minutes at 95° C, and 40 cycles with denaturation of 15 seconds at 92° C and annealing and extension for 60 seconds at 60° C. Results were analyzed by the ABI Taqman 7900HT using the sequence detection system 2.22 software (Applied Biosystems Inc., Foster City, CA, USA).

Statistical analysis

Continuous variables were compared between the short SGA and SGA catch-up subjects using univariate analysis of variance (ANOVA), corrected for age and gender by SDS. The Chi-square test was used to analyze categorical variables.

The Hardy-Weinberg equilibrium (HWE) was calculated by computing the chi-square test for deviations in HWE. Differences in allele frequencies were tested by a chi-square test. Subjects were grouped according to their genotypes. Haplotype 3 was grouped as carriers vs. non-carriers of haplotype 3, due to low frequency. The genotypic and haplotype groups were compared for all anthropometric parameters by univariate analysis of variance (ANOVA). P-values ≤ 0.05 were considered significant. All statistical tests were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinical data

SGA subjects had a mean birth length of -3.00 SDS and birth weight of -2.34 SDS (Total SGA group, Table 1). Both length and weight at birth and at examination were significantly below the population mean of 0 SDS (all P-values < 0.001, Table 1). Short SGA subjects were born with a shorter birth length SDS and were younger at examination. They were also shorter, lighter and had less catch-up growth in height and weight compared to SGA catch-up subjects.

Table 1. Clinical data of the total SGA group and the short SGA and SGA catch-up subgroups.

	Total SGA group	SGA subgroups		
		Short SGA	SGA catch-up	P-value*
Sex (boys/girls)	302/333	223/216	79/117	0.01
Birth:				
Gestational age (wks)	36.7 (3.1)	36.6 (3.8)	37.1 (3.0)	0.10
Length (SDS)	-3.00 (1.48) [a]	-3.14 (1.49)	-2.70 (1.41)	<0.001
Weight (SDS)	-2.34 (1.01) [a]	-2.28 (1.09)	-2.47 (0.81)	0.03
At examination:				
Age (yrs)	12.7 (7.2)	8.9 (5.1)	21.1 (2.6)	<0.001
Height (SDS)	-2.24 (1.27) [a]	-2.95 (0.63)	-0.64 (0.84)	<0.001
Change in height (SDS)	0.81 (1.81) [a]	0.20 (1.60)	2.04 (1.57)	<0.001
Weight (SDS)	-2.01 (1.67) [a]	-2.79 (1.16)	-0.25 (1.24)	<0.001
Change in weight (SDS)	0.33 (1.88) [a]	-0.51 (0.37)	2.22 (1.46)	<0.001
BMI (SDS)	-1.29 (1.01) [a]	-1.13 (1.16)	0.15 (1.31)	<0.001

*Values are means (standard deviation) unless indicated otherwise. *= The overall P-value is presented by ANOVA for continuous variables and by chi-square test for categorical variables. The total SGA group SDS is compared to 0 SDS by T-test: [a] P-value <0.001. SDS= standard deviation score.*

Linkage disequilibrium and haplotypes

There was high LD between the rs4965425 and rs1319868 SNPs (D' = 1.00, R^2 = 0.14, Figure 1), both located in the promoter region. We inferred haplotypes of the rs4965425 and rs1319868 SNPs using PHASE 2.1 (Figure 1, Table 3) [25,26]. Haplotypes 1-3 occurred in > 10% of the population.

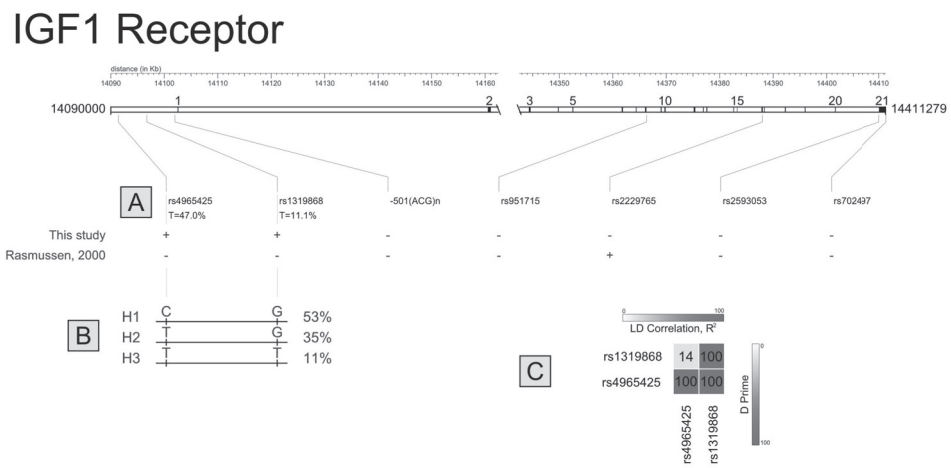

Figure 1. *IGF1R* gene structure. A. The *IGF1R* gene structure is presented with the currently investigated rs4965425 (C/T) and rs1319868 (G/T) *IGF1R* SNPs and their allele frequencies in the currently investigated SGA population. Also previously performed association studies are shown with the location of the investigated polymorphisms. **B**. Haplotypes of the rs4965425 (C/T) and rs1319868 (G/T) *IGF1R* SNPs are presented and their frequencies in the currently investigated SGA population of 635 subjects is shown. **C**. Linkage disequilibrium (D' and R^2) between the rs4965425 (C/T) and rs1319868 (G/T) *IGF1R* SNPs is shown.

IGF1R SNPs: allele frequencies

The SGA subjects had a higher rs4965425 and rs1319868 minor allele frequency than healthy controls, the latter being significantly different (rs4965425: 47.0% vs. 39.9%, P = 0.08 and rs1319868: 11.1% vs. 6.1%, P = 0.02). The minor allele frequency of the rs4965425 and rs1319868 SNPs were not significantly different between the short SGA and SGA catch-up population (rs4965425: 48.6% and 43.4%, P = 0.09; rs1319868: 11.8% and 9.7%, P = 0.27). The total SGA population was in Hardy-Weinberg equilibrium for the rs4965425 and rs1319868 SNPs (P = 0.71 and P = 0.14, respectively).

IGF1R SNPs: associations with postnatal growth

The rs4965425 C-allele was not associated with postnatal growth in the total SGA and SGA catch-up population. However, in short SGA subjects, this SNP was associated with a 0.3 SDS lower postnatal weight, 0.3 SDS lower BMI and 0.4 SDS catch-down in weight from birth onwards compared to the T-allele (Table 2). There was no association between the rs4965425 SNP and birth length, birth weight, postnatal height and the change in height in short SGA

subjects. In addition, we exploratively analyzed in a small subpopulation of short SGA subjects of 18 years of age and older (n = 42), whether their rs4965425 genotype was related to their systolic blood pressure SDS. Although not statistically significant, [CC] carriers tended to have a lower systolic blood pressure SDS than the other genotypes (mean (SD): 0.73 (1.00) vs. 1.27 (1.09), respectively, P = 0.14).

The rs1319868 SNP was not associated with postnatal growth in the total SGA population, and with the short SGA and SGA catch-up subjects (data not shown).

IGF1R haplotypes: associations with postnatal growth

Short SGA subjects with Haplotype 1 (-C-G-, Figure 1B) had a 0.4 SDS lower postnatal weight, 0.4 SDS lower BMI and 0.4 SDS catch-down in weight (Table 3) compared to non-carriers of Haplotype 1. Haplotype 2 carriers (-T-G-) also displayed comparable associations with weight, BMI and change in weight, however in an opposite direction. The change in weight was borderline significant (P = 0.08). SGA catch-up subjects did not show any association between postnatal growth and the investigated haplotypes (data not shown). Haplotype 3 was not associated with the postnatal growth of both the short SGA and SGA catch-up group.

As Haplotype 1 contained the rs4965425 SNP C-allele, which was the only allele that was not present in Haplotype 2 and 3, it might be concluded that the C-allele of the rs4965425 SNP was driving the association with postnatal weight, BMI and catch-up growth in weight of short SGA subjects.

IGF1R haplotypes: associations in short SGA_L and SGA_{L+W} subpopulations

To further delineate the association between Haplotype 1 and the change in weight gain in the short SGA group, we exploratively investigated the association in the short SGA_L and short SGA_{L+W} subgroups. Whereas, short SGA_L subjects did not show any association with Haplotype 1, short SGA_{L+W} subjects with Haplotype 1 had a 0.8 SDS lower postnatal weight, 0.8 SDS lower BMI and 0.7 SDS catch-down in weight from birth onwards (Table 4). When the SGA catch-up group was stratified according to SGA_L and SGA_{L+W} subgroups, no associations were found.

Table 2. The rs4965425 *IGF1R* SNP in the short SGA and SGA catch-up group.

	SGA subgroups							
	Short SGA				SGA catch-up			
	[CC]	[CT]	[TT]	P	[CC]	[CT]	[TT]	P
Birth:	109	221	97		62	91	37	
Length (SDS)	-3.31 (1.55)	-3.09 (1.61)	-3.15 (1.20)	NS	-2.81 (1.69)	-2.63 (1.41)	-2.69 (0.92)	NS
Weight (SDS)	-2.29 (1.10)	-2.27 (1.11)	-2.35 (1.07)	NS	-2.47 (0.66)	-2.47 (0.91)	-2.52 (0.77)	NS
At examination:								
Height (SDS)	-2.93 (0.65)	-2.99 (0.63)	-2.88 (0.60)	NS	-0.64 (0.91)	-0.68 (0.80)	-0.48 (0.84)	NS
Change in height (SDS)	0.35 (1.59)	0.11 (1.73)	0.26 (1.36)	NS	2.17 (1.88)	1.94 (1.56)	2.16 (0.99)	NS
Weight (SDS)	-2.85 (1.22)	-2.90 (1.07)	-2.55 (1.27)	**0.05**	-0.28 (1.36)	-0.22 (1.22)	-0.19 (1.13)	NS
Change in weight (SDS)	**-0.56 (1.34)**	**-0.63 (1.30)**	**-0.21 (1.54)**	**0.04**	2.20 (1.40)	2.25 (1.58)	2.33 (1.21)	NS
BMI (SDS):	**-1.19 (1.19)**	**-1.22 (1.11)**	**-0.89 (1.24)**	**0.05**	0.12 (1.43)	0.21 (1.26)	0.12 (1.20)	NS

Values are means (standard deviation). The overall P-value is presented, which compares the difference between the [CC], [CT] and [TT] genotypic groups by ANOVA. SDS= standard deviation score.

Table 3. Haplotypes of the rs4965425 and rs1319868 *IGF1R* SNPs in the short SGA population.

	Short SGA population							
	H1 (-C-G-: 53%)				**H2 (-T-G-: 35%)**			
	[H1/H1]	**[H1/nH1]**	**[nH1/nH1]**	**P**	**[H2/H2]**	**[H2/nH2]**	**[nH2/nH2]**	**P**
Number	104	215	95		49	209	156	
Birth:								
Length (SDS)	-3.37 (1.56)	-3.12 (1.62)	-3.15 (1.22)	NS	-3.24 (1.26)	-3.07 (1.53)	-3.34 (1.59)	NS
Weight (SDS)	-2.34 (1.10)	-2.28 (1.12)	-2.35 (1.08)	NS	-2.26 (1.02)	-2.31 (1.13)	-2.32 (1.09)	NS
At examination:								
Height (SDS)	-2.95 (0.65)	-2.99 (0.64)	-2.88 (0.60)	NS	-2.80 (0.63)	-2.98 (0.62)	-2.97 (0.65)	NS
Change in height (SDS)	0.39 (1.62)	0.14 (1.75)	0.27 (1.36)	NS	0.40 (1.41)	0.10 (1.63)	0.37 (1.69)	NS
Weight (SDS)	-2.87 (1.24)	-2.89 (1.08)	-2.52 (1.25)	0.03	-2.34 (1.24)	-2.85 (1.14)	-2.89 (1.16)	0.01
Change in weight (SDS)	-0.53 (1.36)	-0.62 (1.31)	-0.17 (1.52)	0.03	-0.07 (1.38)	-0.53 (1.40)	-0.57 (1.34)	0.08
BMI (SDS):	-1.20 (1.22)	-1.22 (1.12)	-0.85 (1.22)	0.03	-0.72 (1.34)	-1.17 (1.13)	-1.21 (1.15)	0.03

Values are means (standard deviation). The overall P-value is presented which compares the difference between the haplotype groups by ANOVA. SDS= standard deviation score.

Table 4. *IGF1R* haplotypes in the short SGA_L and SGA_{L+W} subgroups.

	Haplotype 1 (-C-G-)							
	Short SGA_L				Short SGA_{L+W}			
	[H1/H1]	[H1/nH1]	[nH1/nH1]	P	[H1/H1]	[H1/nH1]	[nH1/nH1]	P
Number	24	49	22		53	93	49	
Birth:								
Length (SDS)	-2.71 (0.62)	-2.75 (0.58)	-2.66 (0.47)	NS	-4.11 (1.50)	-4.00 (1.57)	-3.63 (1.25)	NS
Weight (SDS)	-1.14 (0.69)	-1.29 (0.55)	-1.28 (0.60)	NS	-3.05 (0.73)	-3.06 (0.71)	-2.94 (0.67)	NS
At examination:								
Height (SDS)	-3.02 (0.56)	-3.03 (0.63)	-2.91 (0.49)	NS	-2.99 (0.77)	-3.00 (0.71)	-2.87 (0.66)	NS
Change in height (SDS)	-0.31 (0.82)	-0.28 (0.91)	-0.24 (0.57)	NS	1.12 (1.60)	0.99 (1.75)	0.76 (1.46)	NS
Weight (SDS)	-2.29 (1.09)	-2.40 (1.21)	-2.16 (0.68)	NS	**-3.28 (1.28)**	**-3.15 (1.12)**	**-2.49 (1.37)**	0.002
Change in weight (SDS)	-1.15 (1.35)	-1.11 (1.17)	-0.88 (0.99)	NS	**-0.23 (1.16)**	**-0.09 (1.26)**	**0.46 (1.52)**	0.02
BMI (SDS):	-0.42 (1.02)	-0.61 (1.12)	-0.34 (0.67)	NS	-1.64 (1.15)	-1.52 (1.16)	-0.83 (1.26)	0.002

Values are means (standard deviation). The overall P-value is presented which compares the difference between the haplotype groups by ANOVA. SDS= standard deviation score.

Discussion

Our study shows that short SGA subjects with the rs4965425 C-allele have a lower weight and BMI due to postnatal catch-down in weight compared to non-carriers. This was most pronounced in those who were born with both a birth weight and birth length <-2.00 SDS. Our findings suggest that genetic variations in the *IGF1R* gene promoter play a role in postnatal catch-down in weight development of short SGA subjects. The minor allel frequency of the rs1319868 SNP was significantly higher in the total SGA population compared to controls. The minor allele frequency of the rs4965425 SNP showed the same tendency, although not significantly different.

This study has shown for the first time that the IGF1R rs4965425 promoter SNP might have a role in modifying postnatal weight, thereby influencing the risk for cardiovascular disease. It might well be that short SGA subjects carrying the rs4965425 C-allele and having both a low birth weight and –length, experience a long-term advantage by having a catch-down in weight. Although the majority of short SGA subjects experience varying degrees of postnatal catch-up growth, this study shows that a genetic variation in the *IGF1R* promoter contributes to postnatal catch-down in weight by which short SGA subjects remain lean. Leanness of short SGA subjects is often interpreted as a result of minimal appetite. This study has shown that genetic factors, like the investigated *IGF1R* promoter polymorphism, play a role in weight development of short SGA children. By our knowledge, it is unknown whether *IGF1R* promoter polymorphisms and appetite are related, although relationships have been reported between the *IGF1R* and food intake in animal models [28,29].

The association between the rs4965425 C-allele and postnatal catch-down in weight of short SGA subjects was most pronounced in the most severe SGA subjects, having a birth length and weight ≤ -2.00 SDS instead of the subjects having only a birth length ≤ -2.00 SDS. It might well be that rs4965425 polymorphism has also contributed to the severity of the SGA condition due to reduced prenatal *IGF1R* expression which reduces prenatal growth. This was also found in *IGF1R* knockout mice [10,11].

The *IGF1R* promoter contains a large linkage disequilibrium block ranging from rs4965425 until 2 kb before start of exon 1 (Web resources: Hapmap). It might well be that the tagging promoter SNPs which we investigated in this study, are markers for variations in *IGF1R* expression due to changes in the transcription factor binding sites in this region. The *IGF1R* promoter has been extensively studied with regard to the regulation of *IGF1R* gene expression [30]. Several studies have shown that transcription factors in the *IGF1R* promoter induce an increased *IGF1R* expression which would result in increased *IGF1R* protein levels [31,32-37].

The significantly higher minor allele frequency of the rs1319868 SNP in the SGA population might indicate a relation between the allelic status of the SNP and the SGA phenotype. However, further studies are needed to clarify an effect of the SNP on protein expression.

We were not able to perform functional studies of the rs4965425 SNP on *IGF1R* expression levels. However, preliminary searches in a transcription factor database (Web Resources: TESS) revealed an induction of a glucocorticoid receptor (GR) binding place in case of an rs4965425 T-allele. In case of the rs4965425 C-allele, the binding site for GR was not present the *IGF1R* promoter sequence. Glucocorticoid receptors are well-known transcription regulators [38,39] which dose-dependently influence target genes. Thus, it might well be that that the GR acts as a growth enhancer in case of an rs4965425 T-allele. Our findings merit further investigations, including functional studies.

A limitation of this study is the absence of a control group born appropriate for gestational age (AGA) with a detailed growth parameters. However, we do not expect that these *IGF1R* polymorphisms associate with postnatal growth in the normal population, due to the specific relation of the *IGF1R* gene, as shown by knock-out mice, with a reduced prenatal growth [10]. To determine the universality of this finding, it is necessary to replicate this study in an independent short SGA cohort with detailed birth and postnatal anthropometric measurements available.

In conclusion, short SGA subjects with the rs4965425 C-allele have a lower weight and BMI due to postnatal catch-down in weight compared to non-carriers. This was most pronounced in those who were born with both a birth weight and birth length <-2.00 SDS. Our findings suggest that genetic variations in the *IGF1R* gene promoter contribute to weight development of short SGA subjects. Studies are warranted to identify the risk for cardiovascular disease in short SGA rs4965425 T-allele carriers.

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Web Resources

URLs for the presented data are:

HapMap genomic database: http://www.hapmap.org/

Haploview v3.2: http://www.broad.mit.edu/mpg/haploview/

NCBI SNP browser: http://www.ncbi.nlm.nih.gov/entrez/

PHASE: http://www.stat.washington.edu/stephens/software.html

PubMed: http://www.ncbi.nlm.nih.gov/entrez

TESS: http://www.cbil.upenn.edu/cgi-bin/tess/tess

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Chapter 7

Blood pressure of SGA subjects is related to Insulin gene haplotypes and most pronounced after postnatal weight gain

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Abstract

Context: The *INS* gene region is an important region with regard to fetal growth, coronary heart disease and hypertension. It is unknown whether polymorphisms in the *INS* gene are related to blood pressure in subjects born SGA.

Objective: To determine whether haplotypes of three polymorphisms in the insulin gene region, rs7924316, rs3842748 and the INS VNTR, associate with blood pressure in a large cohort of SGA subjects.

Patients: 635 SGA subjects (439 short SGA and 196 SGA catch-up subjects).

Results: We analyzed SNPs and haplotypes in short SGA and SGA catch-up subjects. The tagging SNP was investigated in the total SGA population after correction for potential confounders and according to weight gain stratification. All polymorphisms were in high linkage disequilibrium (D' = 96-100, R^2 = 32-68). SGA catch-up subjects had a significantly higher rs7924316 T-allele (P = 0.007) and rs689 T-allele (P = 0.03) frequency than short SGA subjects. The association between the rs7924316 and the INS *VNTR* and systolic blood pressure was most pronounced in SGA catch-up subjects (P = 0.003 and P = 0.007). These associations were not seen in short SGA subjects. SGA catch-up subjects with Haplotype 1, consisting of the rs7924316 G-allele, rs3842748 G-allele and the class I *INS* VNTR, had a 0.6 SDS higher systolic and 0.5 SDS higher diastolic blood pressure. The rs7924316 G-allele drove these associations. Rs7924316 and the INS *VNTR* were associated with systolic blood pressure in the total SGA population (P = 0.05 and P = 0.007) and remained significant after correction for change in weight and age. The association of blood pressure with rs7924316 and the INS *VNTR* was most pronounced in subjects with postnatal weight gain (> 0.67 SDS: P = 0.006 and P = 0.01).

Conclusions: Subjects born SGA have a higher blood pressure when carrying the *INS* rs7924316 G-allele and class I *INS* VNTR. Our findings suggest that genetic variation in the *INS* gene may identify a higher risk for development of hypertension especially after postnatal weight gain.

Introduction

Around 2% of all life-born neonates are born small for gestational age (SGA) and 90% of them will attain a height in the normal range in early postnatal life (SGA catch-up) [1,2]. SGA catch-up subjects have an increased prevalence of cardiovascular disease compared to SGA subjects who do not show an accelerated postnatal growth [3-5].

The insulin (*INS*) gene (Figure 1) is considered to be a major candidate gene for the SGA phenotype as insulin is involved in fetal and early postnatal growth [6-9].

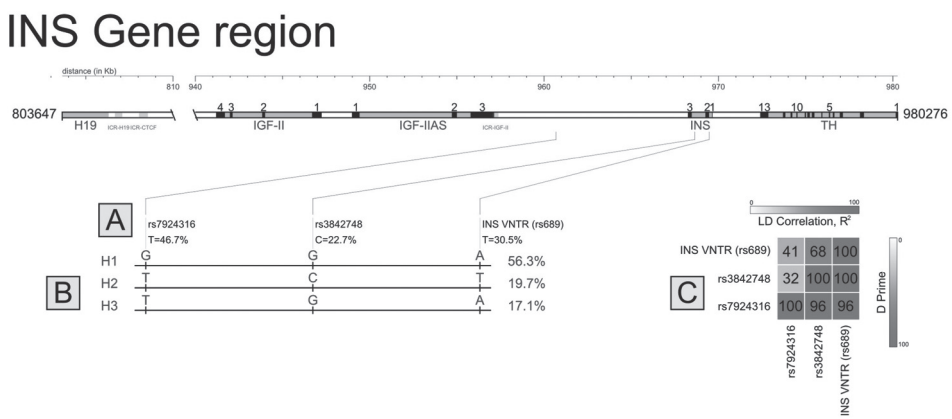

Figure 1. Genetic variation in the insulin gene region (11p15.5). A. *INS* gene structure with SNPs: rs7924316, rs3842748 and rs689, which were studied in the total SGA group. The allele frequencies are presented below the SNP rs number. B. Haplotypes were constructed of the rs7924316, rs3842748 and rs689 SNPs and their frequency is presented in the total SGA group. C. Linkage disequilibrium (D') is shown for SNPs rs7924316, rs3842748 and rs689.

The most widely studied polymorphism in the *INS* gene, is a variable number of tandem repeats (VNTR, OMIM *147510) which is located 600bp upstream of the *INS* transcription start site and comprises 14-25bp tandemly repeated sequence units. In Caucasian populations there are two main classes; short so-called "class I alleles" (26-63 repeat units) and long, so-called "class III" alleles (141-209 repeat units) [10,11].

The *INS* gene region has been recognized as an important region in relation to disease in later life. The insulin minisatellite (INS VNTR, rs689) class III, has been associated with variations in birth size [7,12,13], obesity [8,14-17], type I and type II diabetes mellitus [6] and

polycystic ovary syndrome [9]. However, other studies could not demonstrate that the *INS* VNTR was associated with accelerated weight gain, reduced beta cell function or adiposity [18-21].

The objective of this study was to determine whether genetic variation across the insulin gene region was associated with blood pressure in a large group of short SGA and SGA catch-up subjects. To determine the universality of the genetic associations we analyzed SNPs and haplotypes in short SGA and SGA catch-up subjects. The tagging SNP was investigated in the total SGA population after correction for potential confounders and according to weight gain stratification as reported in previous studies [13,22]. In this study, three polymorphisms in the insulin gene region were studied, rs7924316, rs3842748 and the insulin VNTR (rs689).

Methods

Subjects

We included 635 SGA subjects and divided these in two groups of 439 short SGA and 196 SGA catch-up subjects according to their ability to attain a postnatal height in the normal range. SGA was defined as a birth length and/or weight ≤ -2 standard deviation score (SDS) for gestational age [23]. The SGA subjects who remained short after age 3 (height ≤ -2.00 SDS: short SGA, [24]) participated in prospective Dutch cohort trials evaluating the effect of growth hormone (GH) treatment [25-27]. SGA subjects who had catch-up growth to a normal height (height > -2.00 SDS: SGA catch-up [24]) were randomly selected from hospitals in the Netherlands, were they had been registered because of being born SGA.

All subjects were of Caucasian ethnicity and had an uncomplicated postnatal period. Subjects with severe chronic illness or endocrine disorders, chromosomal or genetic abnormalities, positive endomysial or transglutaminase antibodies, skeletal abnormalities and psychosocial dwarfism were excluded from the study. The study protocol was approved by the Medical Ethics Committee and written informed consent was obtained from the parents/guardians and subjects aged 12 years and older. A healthy control population of 100 subjects was acquired from a local bloodbank.

Clinical and biochemical measurements

Birth data of the SGA subjects were retrieved from records of hospitals, community health services and general practitioners. Postnatal measurements of the short SGA subjects were retrieved from the baseline visit of the GH trials. Postnatal height, weight and blood pressure of

the SGA catch-up group were obtained at the outpatient clinic. All anthropometric measurements were performed twice and the mean value was used for analysis. Blood pressure was measured three times at the same visit by an automatic blood pressure monitor (Omron Healthcare, Inc.) and the mean was used for analysis. Body mass index was calculated (weight in kg/ height in meters^2) and expressed as standard deviation score (SDS), adjusting for age and sex, [24]. Blood pressure of the SGA subjects was expressed as SDS by adjusting for sex and height [28].

Selection of SNPs and Linkage Disequilibrium

Three SNPs across the *INS* gene region capturing most of the common genetic variation, were identified by using the SNP selection tool "tagger" in the HapMap database (Web resources) [29]. The rs7924316 (G to T), rs3842748 (G to C) and rs689 (A to T) SNPs were identified as tagging SNPs for the linkage disequilibrium block surrounding the *INS* gene. The rs689 SNP was previously identified as a marker for the *INS* VNTR polymorphism, as the A-allele tagged the class I VNTR alleles and the T-allele tagged the class III VNTR alleles [19].

The pairwise linkage disequilibrium (LD) coefficients (D' and R^2) were calculated with the use of Haploview software (Web resources). Gene polymorphisms which were in high LD (D' > 0.90) were used for haplotype construction. We inferred haplotypes of the rs7924316, rs3842748 and rs689 SNPs using PHASE 2.1 [30,31].

Genotyping

Genomic DNA was extracted from samples of peripheral venous blood according to salting out procedure [32]. Genotypes were determined using the Taqman allelic discrimination assay. The Assay-by-Design service (www.appliedbio-systems.com) was used to set up a Taqman allelic discrimination assay for the rs7924316, rs3842748 and rs689 SNPs, the latter tagging the *INS* VNTR. The rs689 A-allele tagging the class I VNTR allele and the rs689 T-allele tagging the class III VNTR allele [19].

Primer sequences were for the rs7924316 SNP: forward GCACCGCCAGTAAATCCATATTG and reverse CCTCTTCTCTAATTCCCAAGGTTT.

Primer sequences were for the rs3842748 SNP: forward GGAAGGAGGTGGGACATGTG and reverse GGCTGGACCCAGGTTAGAG. Probe sequences for the rs7924316 SNP were: forward CATGACCG**G**AGCTAC and reverse CCATGACCG**T**AGCTAC. Probe sequences for the rs3842748 SNP were: forward CCCACA**G**TGGGTGTG and reverse CCCACA**C**TGGGTGTG.

The probe sequence of the rs689 SNP was: CATGGCAGAAGGACAGTGATCTGGG [A/T] GACAGGCAGGGCTGAGGCAGGCTGA. The PCR reaction mixture included 5ng of genomic DNA, 0.125 μl TaqMan assay (40*, ABI), 2.5 μl Master mix (ABI) and 2.375 μl water.

PCR was performed in 384 wells PCR plates in an ABI 9700 PCR system (Applied Biosystems Inc., Foster City, CA, USA) and consisted of initial denaturation for 10 minutes at 95° C, and 40 cycles with denaturation of 15 seconds at 92° C and annealing and extension for 60 seconds at 60° C. Results were analyzed by the ABI Taqman 7900HT using the sequence detection system 2.22 software (Applied Biosystems Inc., Foster City, CA, USA).

Statistical analysis

Continuous variables were compared between the short SGA and SGA catch-up subjects by univariate analysis of variance (ANOVA). The Chi-square test was used to analyze categorical variables.

The rs7924316, rs3842748 and rs689 SNPs were determined in the total SGA group of 635 SGA subjects. The Hardy-Weinberg equilibrium (HWE) was calculated by computing the chi-square test for deviations in HWE. Allele frequencies were calculated and tested by chi-square. All genotypes for individual SNPs and genotypes for haplotypes were grouped according to a co-dominant model. The genotypic and haplotype groups were compared for all anthropometric parameters by univariate analysis of variance (ANOVA). Post-hoc pairwise tests employed a Bonferroni correction for multiple comparisons. Change in weight SDS was stratified in three groups: (1) a decrease in weight SDS by > 0.67 SDS indicated "catch-down" in weight, (2) a clinically unchanged weight SDS ranging from -0.67 SDS until 0.67 SDS, (3) a gain in weight of > 0.67 SDS indicating clinically significant "catch-up" in weight [7].

Multiple linear regression analysis was used to investigate the contribution of the genotype on blood pressure (SDS) and to investigate potential confounders. P-values ≤ 0.05 were considered significant. All statistical tests were performed using SPSS 11.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinical data

SGA subjects had a mean birth length of -3.00 SDS and birth weight of -2.34 SDS (Total SGA group, Table 1). They had a significantly higher systolic and diastolic blood pressure than the average population ($P < 0.001$).

SGA catch-up subjects were born with a higher birth length SDS (Table 1). SGA catch-up subjects were older at examination and they were, by definition, taller and had more catch-up growth in height from birth onwards (height SDS – birth length SDS). Whereas systolic

blood pressure tended to be higher in short SGA subjects, SGA catch-up subjects had a higher diastolic blood pressure SDS.

Table 1. Clinical data of the total SGA group and the short SGA and SGA catch-up subgroups.

	Total SGA group	SGA subgroups		
		Short SGA	SGA catch-up	P-value[b]
	635	439	196	
At birth:				
Sex (boys/girls)	302/333	223/216	79/117	0.01
Gestational age (weeks)	36.7 (3.1)	36.6 (3.8)	37.1 (3.0)	0.10
Length (SDS)	-3.00 (1.48)[a]	-3.14 (1.49)	-2.70 (1.41)	<0.001
Weight (SDS)	-2.34 (1.01)[a]	-2.28 (1.09)	-2.47 (0.81)	0.03
At follow-up:				
Age (years)	12.7 (7.2)	8.9 (5.1)	21.1 (2.6)	<0.001
Height (SDS)	-2.24 (1.27)[a]	-2.95 (0.63)	-0.64 (0.84)	<0.001
Height (SDS) – birth length (SDS)	0.81 (1.81)	0.20 (1.60)	2.04 (1.57)	<0.001
BMI (SDS)	-1.29 (1.01)[a]	-1.13 (1.16)	0.15 (1.31)	<0.001
Weight (SDS) – birth weight (SDS)	0.33 (1.88)	-0.51 (0.37)	2.22 (1.46)	<0.001
Systolic BP (SDS)	0.92 (1.05)[a]	0.98 (1.08)	0.80 (0.97)	0.06
Diastolic BP (SDS)	0.66 (0.98)[a]	0.44 (0.99)	1.15 (0.77)	<0.001

Values are means (standard deviation) unless indicated otherwise. [a] P-value < 0.001when the SDS of the total SGA group was compared with 0 SDS. [b] The overall P-value for the difference between short SGA and SGA catch-up subjects is presented by ANOVA for continuous and by chi-square test for categorical variables. SDS= standard deviation score, BP= blood pressure.

Linkage disequilibrium and haplotypes

There was high LD between the rs7924316, rs3842748 and rs689 SNPs (D = 96-100, R^2 = 32-68, Figure 1), both located in the promoter region. We inferred haplotypes of the rs7924316, rs3842748 and rs689 SNPs (Figure 1, Table 3). Haplotypes 1-3 occurred in >10% of the population.

Allele frequencies

The total SGA group was in Hardy-Weinberg equilibrium for the rs7924316, rs3842748 and rs689 SNPs (P = 0.74, P = 0.34, P = 0.65). The total SGA group had an rs7924316 T-allele,

rs3842748 C-allele and rs689 T-allele frequency of 46.7%, 22.7% and 30.5%, respectively (Figure 1). Controls showed an rs7924316 T-allele, rs3842748 C-allele and rs689 T-allele frequency of 31.3 %, 32.4 % and 32.2%, respectively. Short SGA subjects had an rs7924316 T-allele, rs3842748 C-allele and rs689 T-allele frequency of 45.0%, 21.5% and 28.4%, respectively. SGA catch-up subjects had an rs7924316 T-allele, rs3842748 C-allele and rs689 T-allele frequency of 50.5%, 25.7% and 34.6%, respectively.

The allelefrequencies of the rs7924316, rs3842748 and rs689 SNPs, were not statistical different between the total SGA population and the controls. When the allelefrequencies of the three SNPs were compared between short SGA vs. SGA catch-up subjects, the SGA catch-up subjects had a significantly higher rs7924316 T-allele (P = 0.007) and rs689 T-allele (P = 0.03) frequency than short SGA subjects.

Short SGA vs. SGA catch-up: **polymorphisms and blood pressure**

When short SGA and SGA catch-up subjects were compared, SGA catch-up subjects with the rs7924316 [GG] genotype had a 0.6 SDS higher systolic and 0.3 SDS higher diastolic blood pressure than the [TG] genotype (P = 0.003, P = 0.04, Table 2). However, no associations were seen in short SGA subjects. Similarly, SGA catch-up subjects with the rs689 [AA] genotype had a 0.5 SDS higher systolic and 0.3 SDS higher diastolic blood pressure (P = 0.007, P = 0.01), whilst no relation was found in short SGA subjects. Short SGA and SGA catch-up subjects did not show associations between these SNPs and weight or BMI SDS at follow-up.

Short SGA vs. SGA catch-up: **haplotypes and blood pressure**

We inferred haplotypes of the rs7924316, rs3842748 and rs689 SNPs using PHASE 2.1 (Table 3) [30,31]. Seven haplotypes were identified of which three haplotypes occurred in >10% of the population (Figure 1). SGA catch-up subjects who were homozygous Haplotype 1 carriers had a 0.6 SDS higher systolic and a 0.5 SDS higher diastolic blood pressure (P = 0.003 and P = 0.008, respectively) compared to heterozygous carriers of Haplotype 1. No associations were found between Haplotype 2 and 3 and blood pressure in SGA catch-up subjects. Short SGA subjects did not show any associations. Thus, Haplotype 1 was significantly associated with blood pressure SDS in SGA catch-up subjects, in contrast to Haplotype 2 and 3. As the G-allele of the rs7924316 SNP was the only allele that was not present in Haplotype 2 and 3, it can be concluded that the G-allele of the rs7924316 SNP was driving the association with blood pressure in SGA catch-up subjects.

Table 2. Blood pressure and *INS* polymorphisms in short SGA and SGA catch-up subjects.

	Short SGA				SGA catch-up			
rs7924316	**[GG]**	**[TG]**	**[TT]**	**P-value**	**[GG]**	**[TG]**	**[TT]**	**P-value**
	129	214	86		49	90	51	
Systolic BP (SDS)	1.02 (1.10)	0.95 (1.09)	1.00 (1.08)	0.86	**1.16 (1.05)** [2]	**0.58 (0.93)** [2]	0.84 (0.91)	0.003
Diastolic BP (SDS)	0.36 (1.01)	0.52 (1.00)	0.37 (0.96)	0.30	**1.35 (0.90)** [1]	**1.01 (0.69)** [1]	1.20 (0.69)	0.04
	Short SGA				**SGA catch-up**			
rs3842748	**[GG]**	**[CG]**	**[CC]**		**[GG]**	**[CG]**	**[CC]**	
	257	144	18		104	58	17	
Systolic BP (SDS)	1.03 (1.11)	0.92 (1.00)	1.37 (1.38)	0.23	0.96 (0.99)	0.61 (0.96)	0.77 (0.83)	0.09
Diastolic BP (SDS)	0.39 (1.02)	0.48 (0.95)	0.68 (1.04)	0.40	1.23 (0.84)	1.03 (0.66)	1.30 (0.77)	0.21
	Short SGA				**SGA catch-up**			
INS VNTR (rs689)	**[AA]**	**[AT]**	**[TT]**		**[AA]**	**[AT]**	**[TT]**	
	215	180	32		82	87	23	
Systolic BP (SDS)	1.05 (1.09)	0.90 (1.07)	1.01 (1.19)	0.37	**1.04 (1.00)** [2]	**0.58 (0.93)** [2]	0.90 (0.89)	0.007
Diastolic BP (SDS)	0.41 (1.02)	0.47 (0.96)	0.36 (1.05)	0.76	**1.32 (0.85)** [1]	**0.99 (0.65)** [1]	1.32 (0.76)	0.01

Values are given as means (SD). BP= blood pressure, SDS= standard deviation score. The overall P-values are presented and were calculated by comparing the three genotype groups. P-value between the marked subgroups was: [1] <0.05, [2] <0.01. [3] rs689: A-allele tags class I VNTR alleles and the T-allele tags class III VNTR alleles

Table 3. Blood pressure and *INS* gene haplotypes in short SGA and SGA catch-up subjects.

	Short SGA				SGA catch-up			
Haplotype 1	**[H1/H1]**	**[H1/nH1]**	**[nH1/nH1]**	**P-value**	**[H1/H1]**	**[H1/nH1]**	**[nH1/nH1]**	**P-value**
Systolic BP (SDS)	1.08 (1.10)	0.96 (1.08)	1.02 (1.08)	0.66	**1.27 (1.07)** [1]	**0.64 (0.93)** [1]	0.81 (0.90)	**0.003**
Diastolic BP (SDS)	0.37 (1.01)	0.51 (1.00)	0.34 (0.98)	0.32	**1.47 (0.92)** [1]	**1.02 (0.69)** [1]	1.20 (0.71)	**0.008**
	Short SGA				**SGA catch-up**			
Haplotype 2	**[H2/H2]**	**[H2/nH2]**	**[nH2/nH2]**		**[H2/H2]**	**[H2/nH2]**	**[nH2/nH2]**	
Systolic BP (SDS)	1.30 (1.45)	0.93 (1.01)	1.04 (1.10)	0.37	0.77 (0.83)	0.60 (0.99)	0.95 (0.98)	0.10
Diastolic BP (SDS)	0.72 (1.07)	0.48 (0.96)	0.39 (1.02)	0.35	1.30 (0.77)	0.99 (0.63)	1.24 (0.83)	0.13
	Short SGA				**SGA catch-up**			
Haplotype 3	**[H3/H3]**	**[H3/nH3]**	**[nH3/nH3]**		**[H3/H3]**	**[H3/nH3]**	**[nH3/nH3]**	
Systolic BP (SDS)	1.54 (1.33)	0.98 (0.98)	1.00 (1.12)	0.33	0.87 (0.81)	0.82 (0.94)	0.83 (1.01)	0.99
Diastolic BP (SDS)	0.49 (0.81)	0.39 (1.04)	0.45 (0.99)	0.82	1.44 (0.69)	1.11 (0.74)	1.19 (0.79)	0.61

Values are given as means (SD). H= haplotype, BP= blood pressure, SDS= standard deviation score. P-value between the marked subgroups was: [1] *<0.01.*

Total SGA population: polymorphisms and potential confounders

Both rs7924316 G-allele and rs689 A-allele carriers had a higher systolic blood pressure SDS in the total SGA population ([GG] vs. [TG+TT], 1.06 (1.08) vs. 0.87 (1.05), P=0.05; [AA] vs. [AT+TT], 1.05 (1.06) vs. 0.82 (1.04), P= 0.007, representing mean (SD)). The rs3842748 SNP was not associated with systolic blood pressure in the total SGA population. Also diastolic blood pressure was not associated with any of the three SNPs in the total SGA population. The rs7924316 SNP remained significantly associated with systolic blood pressure SDS after adjustment for change in weight SDS and age (Table 4).

In addition, we investigated whether the association between the rs7924316 SNP and the SGA catch-up group was influenced by height gain or weight gain since birth. Weight gain was significantly related to the rs7924316 SNP but the gain in height was not associated with the rs7924316 SNP (P = 0.01 vs. P = 0.60, respectively).

Table 4. Systolic blood pressure and the rs7924316 *INS* SNP in the total SGA population.

Systolic BP SDS Total SGA population	**Model 1**		**Model 2**		**Model 3**	
	B	**P-value**	**B**	**P-value**	**B**	**P-value**
rs7924316 [a]	-0.19	0.05	-0.20	0.04	-0.19	0.04
Change in weight (SDS) [b]	-	-	0.06	0.01	0.08	0.008
Age at measurement (years)	-	-	-	-	-0.009	0.26
Overall P-value	0.05		0.006		0.01	
R^2 corrected	0.5%		1.3%		1.4%	

BP= blood pressure, SDS= standard deviation score. [a] Genotype coding is: 0= [GG], 1= [TG+TT]. [b] Change is calculated by measurement SDS at follow-up minus birth SDS.

Total SGA population: weight gain stratification

In a next step, the total SGA population was stratified by their catch-up in weight from birth onwards (Table 5). SGA subjects with the highest gain in weight (> 0.67 SDS), had a 0.5 SDS and 0.4 SDS higher systolic blood pressure when the [GG] genotype of the rs7924316 SNP and the [AA] genotype of the rs689 SNP, were compared to their heterozygous genotypes (P = 0.006 and P = 0.008).

Table 5. Blood pressure and the rs7924316 *INS* SNP and the *INS* VNTR in the total SGA population, stratified by change in weight.

Delta wt group:		1			2			3	
Delta wt range [SDS]:		[< -0.67]			[-0.67; 0.67]			[> 0.67]	
rs7924316:	**[GG]**	**[TG]**	**[TT]**	**[GG]**	**[TG]**	**[TT]**	**[GG]**	**[TG]**	**[TT]**
	69	97	41	47	94	29	61	113	67
Systolic BP	0.87 (1.14)	0.70 (0.99)	0.91 (0.96)	0.94 (1.10)	0.99 (1.08)	0.83 (1.20)	**1.35 (0.96)** [1]	**0.82 (1.09)** [1]	0.99 (0.98)
***INS* VNTR (rs689)** [2]**:**	**[AA]**	**[AT]**	**[TT]**	**[AA]**	**[AT]**	**[TT]**	**[AA]**	**[AT]**	**[TT]**
	109	90	10	80	72	14	107	105	31
Systolic BP	0.86 (1.09)	0.78 (1.00)	0.74 (0.69)	1.08 (1.04)	0.79 (1.12)	1.08 (1.35)	**1.22 (1.03)** [1]	**0.80 (1.01)** [1]	0.99 (1.06)

Values are given as means (SD). BP= blood pressure, SDS= standard deviation score. P-value between the marked subgroups was: [1] <0.01. [2] Rs689: A-allele tags class I VNTR alleles and the T-allele tags class III VNTR alleles

Discussion

Our study has shown that SGA subjects have a higher blood pressure when carrying the class I *INS* VNTR and the rs7924316 G-allele. Although rapid postnatal growth is a well-known factor associated with a higher blood pressure in later life [3,5], our study shows for the first time that there is a polymorphism in the *INS* gene region which independently influences blood pressure in SGA subjects.

In our study we firstly analyzed the rs7924316, rs3842748 and the INS *VNTR* in a group of short SGA and SGA catch-up subjects. SGA catch-up subjects had a significantly higher rs7924316 T-allele (P = 0.007) and rs689 T-allele (P = 0.03) frequency than short SGA subjects. We showed that SGA catch-up subjects carrying the rs7924316 G-allele and the class I *INS* VNTR allele had a significantly higher systolic and diastolic blood pressure. No associations were found in short SGA subjects. The rs3842748 SNP was not associated with blood pressure in any group.

Secondly, we performed haplotype analysis of the three SNPs to identify the driving genetic variant of the association between SGA catch-up subjects and an increased systolic and diastolic blood pressure. Our data showed that the rs7924316 G-allele was driving these associations indicating that the rs7924316 SNP in the *INS* gene region is either functional itself or strongly linked with other functional polymorphisms in the LD-block.

Because the difference between short SGA and SGA catch-up is an arbitrary cut-off of -2.00 SDS in height, we also investigated the rs7924316 SNP and the INS *VNTR* in the total SGA population. In this analysis, we corrected the association between the rs7924316 G-allele and systolic blood pressure for possible confounders, as age at examination and postnatal weight gain, within the total SGA population. Regression analysis showed that the rs7924316 remained significantly associated with systolic blood pressure of SGA subjects after correction for these confounders, indicating that the rs7924316 SNP is an independent genetic factor enhancing blood pressure in SGA subjects.

In addition, we analyzed whether the extent of weight gain was influencing the association between systolic blood pressure and the rs7924316 G-allele in SGA catch-up subjects by weight gain stratification. We used cut-offs of 0.67 SDS which were based on "centile bands" on standard growth charts, indicating significant "catch-up" or "catch-down" growth from birth onwards [7]. This analysis showed that marked postnatal weight gain was an enhancing factor for the association between systolic blood pressure and the rs7924316 G-allele in SGA catch-up subjects. As both, growth in height and weight accumulation concern different physiological mechanisms in SGA subjects, we added both stratifications to the analysis.

Our results are in line with previous studies showing that children with a postnatal catch-up in weight (> 0.67 SDS), also had a higher BMI at age 7 when having the *INS* class I-allele vs. the class III/III genotype [13]. In addition, it was previously shown that the *INS* VNTR was associated with head and birth size implying a perinatal survival genotype [7]. However, also contrasting results have been reported on associations between the *INS* VNTR and early postnatal growth and the metabolic syndrome [18,19,22,33,34].

Our haplotype study showed that class I of the *INS* VNTR and especially the rs7924316 G-allele was the determining SNP in the haplotype association with blood pressure in SGA catch-up subjects. Other studies have also demonstrated that haplotypes capturing the *INS* gene region were associated with insulin production, and would predispose to an elevated blood pressure, fat mass or triglyceride level [16].

Our study demonstrated that the heterozygote class I/III *INS* and especially rs7924316 [TG] carriers had the lowest systolic and diastolic blood pressure in SGA catch-up subjects. These results indicate that the combination of a T- and G-allele in case of the rs7924316 SNP, gives rise to the most protective effect on blood pressure. The phenomenon that the heterozygous state is associated with a certain phenotype which is different from the two homozygous states, has been previously reported [35-37]. Heterozygous advantage or disadvantage of SNPs in regulatory regions, as the *INS* gene region [38], may be the result of allele-dependent regulation of gene expression. The central process in allele-dependent regulation of gene expression is *trans*-regulation, which means that an enhancer from one allele acts in *trans* to activate transcription from the regulatory region of the second allele [39,40]. Transcription factors can bind two DNA molecules simultaneously, functioning as a protein bridge and mediating communication between two homologues [41]. *Trans* regulation might explain advantages or disadvantages of heterozygosity as found in several studies [42,43] and in the rs7924316 and the rs689 SNPs in our study.

Functional studies have shown that shorter class I alleles of the *INS* gene are associated with higher *INS* gene expression in the pancreas and higher *IGF2* gene expression in the placenta [38,44-48]. Our results might be in line with these observations as an increased insulin production will be particularly associated with accelerated postnatal growth and particularly weight gain, giving a predisposition to cardiovascular disease. However, these functional studies did not investigate the influence of the rs7924316 G-allele in SGA catch-up subjects. Thus, our data merit further studies to investigate how the rs7924316 G-allele influences the *INS* VNTR and thereby *INS* and *IGF2* gene expression in SGA subjects.

In conclusion, our study has shown that the rs7924316 SNP is a contributing factor in blood pressure regulation of subjects born SGA, particularly in those with postnatal weight

gain. This study shows for the first time that there is a polymorphism in the *INS* gene region which independently influences blood pressure in SGA subjects.

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Web resources:

URLs for the presented data are:
HapMap genomic database: http://www.hapmap.org/
Haploview v3.2: http://www.broad.mit.edu/mpg/haploview/
NCBI SNP browser: http://www.ncbi.nlm.nih.gov/entrez/
PHASE: http://www.stat.washington.edu/stephens/software.html
PubMed: http://www.ncbi.nlm.nih.gov/entrez

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Chapter 8

Two novel short SGA cases with *IGF1R* haploinsufficiency illustrate the heterogeneity of its phenotype

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Abstract

Context: Small for gestational age (SGA) born children comprise a heterogeneous group in which only few genetic causes have been identified.

Objective: To determine copy number variations in 18 growth-related genes in 100 SGA children with persistent short stature.

Methods: Copy number variations in 18 growth-related genes (*SHOX, GH1, GHR, IGF1, IGF1R, IGF2, IGFBP1-6, NSD1, GRB10, STAT5B, ALS, SOCS2,* and *SOCS3*) were determined by an "in house" multiplex ligation-dependent probe amplification (MLPA) kit. The deletions were further characterized by SNP array analysis.

Results: We found two short SGA children with a *de novo IGF1R* deletion. No deletions or insertions were found in the remaining genes. Patient A had a heterozygous deletion of the complete *IGF1R* gene (15q26.3, exon 1-21) including distally flanking sequences. Surprisingly, she also had a low IGF-I level (-2.78 SDS), probably because of a co-existing growth hormone (GH) deficiency. In the DNA of patient B, heterozygosity for a deletion comprising exon 3-21 of the *IGF1R* gene was detected which extended further into the telomeric region. Both children increased their height during GH treatment, with a GH dose of 1 mg/m^2/day (Patient A: after 4 yrs GH: delta height +1.65 SD; Patient B: after 4 yrs GH: delta height +1.69 SD) and continue to receive GH until their adult height will be attained.

Conclusions: We demonstrate that *IGF1R* haploinsufficiency was present in 2 out of 100 short SGA children. GH therapy gave moderate catch-up growth in our patients. A review of the literature shows that small birth size, short stature, small head size, relatively high IGF-I levels, developmental delay and micrognathia are the main predictors for an *IGF1R* deletion.

Introduction

Children born with a low birth weight and/or birth length corrected for gestational age (small for gestational age, SGA) comprise a heterogeneous group with a broad spectrum of clinical characteristics. Reduced size at birth may result from fetal, maternal, placental and/or genetic factors. While many children born SGA achieve sufficient growth to normalize their stature by 2 years of age, approximately 15% maintain a height below -2 standard deviation scores (SDS) and continue to be short throughout adolescence and adulthood [1]. Short SGA children have a reduced lean body mass, fat mass, skinfolds and body mass index [2-4], as well as a lower caloric, fat and carbohydrate intake [5]. SGA children with a persistent short stature and/or a small head size have a higher risk of subnormal intellectual and psychological performance [6-9].

Genetic causes have only been found in a small proportion of short SGA children, including point-mutations and deletions in the *IGF1* [10-12] and *IGF1R* genes [13-26]. The availability of the complete sequence of the human genome and the introduction of high throughput DNA scanning techniques provides us with novel tools to investigate the genetic basis of short stature. In this study we used MLPA to rapidly investigate whether copy number variations in growth-related genes (*SHOX, GH1, GHR, IGF1, IGF1R, IGF2, IGFBP1-6, NSD1, GRB10, STAT5B, ALS, SOCS2, SOCS3*) were present in a group of 100 children born SGA with persistent short stature. The extent of the two deletions that were found was determined with SNP array analysis.

Methods

Study population

The first hundred short SGA children participating in four prospective cohort trials evaluating the effect of GH treatment [3,27] were included in the study. Small for gestational age was defined as a birth length and/or weight ≤ -2 standard deviation score (SDS) for their gestational age [28] and only children were included who remained short in postnatal life (at age 3: height ≤ -2.00 SDS: short SGA [29]). All children were Caucasian and had an uncomplicated postnatal period. Severe chronic illness or endocrine disorders, chromosomal or genetic abnormalities, positive endomysial or transglutaminase antibodies, skeletal abnormalities and psychosocial dwarfism were reasons to exclude children from the study. The study protocol was approved by the Medical Ethics Committee of the ErasmusMC (Rotterdam) and written informed consent was obtained.

Clinical and biochemical measurements

Birth and growth data before start of GH treatment were retrieved from records of hospitals, community health services and general practitioners. Children were systematically measured at the start and during the GH trials [3,27]. Height and head circumference were expressed as SDS [29]. Body mass index was calculated (weight in kg/ height in meters^2) and adjusted for age and sex, expressed as SDS [30]. Body proportion was assessed by the sitting height/height ratio and expressed in SDS [31]. GH production was assessed by arginine and clonidine GH provocation tests and GH was measured by AutoDelphia [Perkin Elmer) and standardized according to WHO 80/505 guidelines. Serum IGF-I and IGFBP-3 levels were measured in the SGA subjects as described previously [32,33] and values were transformed to SDS by adjusting for sex and age [33]. Bone age was determined according to Greulich and Pyle [34]. Dysmorphological examination was performed by an experienced clinical geneticist (LCPG).

Genetic analysis

Genomic DNA was extracted from peripheral blood samples [35]. DNA from control samples was isolated from leucocytes using Puregene™ nucleic acid purification chemistries for the Autopure LS Instrument (Gentra Systems, Minneapolis, USA).

The "in house" probe kit was designed according to the criteria described in White et al [36]. The kit contained 34 probes (supplementary data) in 18 different growth-related genes (*SHOX, GH1, GHR, IGF1, IGF1R, IGF2, IGFBP1-6, NSD1, GRB10, STAT5B, ALS, SOCS2, SOCS3*). Reactions were performed as described by Walenkamp et al [25]. MLPA of all 21 *IGF1R* exons was performed using the MRC Holland P217 MLPA kit according to the manufacturer's instructions (MRC Holland, Amsterdam, The Netherlands).

All MLPA kits that were used were validated with DNA from patients that have been diagnosed with a deletion in that particular gene with other molecular techniques. As a positive control a patient that was previously published [25] was used and a number of normal individuals, including their parents, and a blank (no DNA) were used as negative controls.

The Affymetrix GeneChip Human Mapping 262K *NspI* array was used according to the instruction provided in the Affymetrix GeneChip Human Mapping 500K Manual (http://www.affymetrix.com). SNP copy number was assessed using CNAG (Copy Number Analyser for GeneChip®) Version 2.0 [37].

IGF1 gene sequencing of all four exons and flanking intron-exon boundaries was performed according to standard procedures (primers and conditions available upon request).

Results

All 100 short SGA children were investigated with the "in house" MLPA growth-kit and two patients were identified with a deletion of the *IGF1R* gene. No copy number variants (CNV's) in the other growth related genes were detected.

Patient A

Clinical description

Patient A was a girl who was born spontaneously after 40 weeks of gestation as the third child of non-consanguineous parents. As maternal age was 36 years, her mother chose for amniocentesis showing a 46 XX karyotype. The pregnancy was complicated by vaginal bleeding and limited fetal movements. Her birth weight was 2890 g (-1.28 SDS), and birth length 47 cm (-2.21 SDS). The height of her father was 184.7 cm (0.40 SDS) and of her mother 176.6 cm (1.34 SDS) resulting in a (secular-trend corrected) target height of 178.8 cm (1.25 SDS). She had bilateral hip dysplasia and clubfeet for which she had hip casting from 4 to 12 months of age. Her bilateral hearing loss improved by tympanostomy tubes, which were implanted at 3 years of age. Psychomotor development was delayed. At 2.3 years of age, her height was 78.9 cm (-3.46 SDS), weight 10.3 kg (-2.13 SDS weight for height) and head circumference 47.2 cm (-0.82 SDS). Arginine and clonidine stimulation tests were performed at age 2 with a maximal GH response of 19.1 mU/L and 14.1 mU/L, respectively. She had a delayed dentition, starting at age 2. At age 3 her bone age was 2 years. Her IGF-I level was 46 ng/ml (-1.61 SDS) and IGFBP-3 level 1.17 mg/L (-1.63 SDS). Cardiovascular, respiratory and abdominal examinations were all normal. On magnetic resonance imaging of the hypothalamic and pituitary region no abnormalities were seen.

From age 4 onwards, GH treatment was initiated at a dose of 1 mg/m^2/day (Figure 1). At start of GH treatment, her height was 90.6 cm (-3.42 SDS), BMI 14.3 kg/m^2 (-1.02 SDS), sitting height/height ratio 0.60 (0.00 SDS) and head circumference 48.6 cm (-0.95 SDS). Her serum total IGF-I level was 34 ng/ml (-2.78 SDS) , IGFBP-3 level 1.35 mg/l (-1.33 SDS) and her bone age was 1 year behind. After 1 year of GH treatment, height had increased by 1.02 SD and head circumference by 0.58 SD. Her serum total IGF-I level was 197 ng/ml (1.51 SDS) and IGFBP-3 level was 2.62 mg/l (0.92 SDS). After 4 years of GH treatment, she had an increase in height of +1.65 SD and an increase in IGF-I level of +5.75 SD. Currently, at age 8.3, her height is 123.3 cm (-1.68 SDS). Cardiac ultrasound showed an undulating shape of the left ventricular wall which could not be further specified.

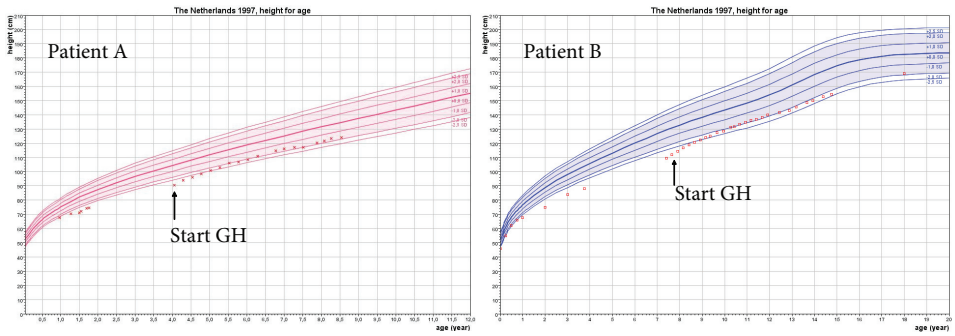

Figure 1. Growth charts of the two children with a *IGF1R* deletion.

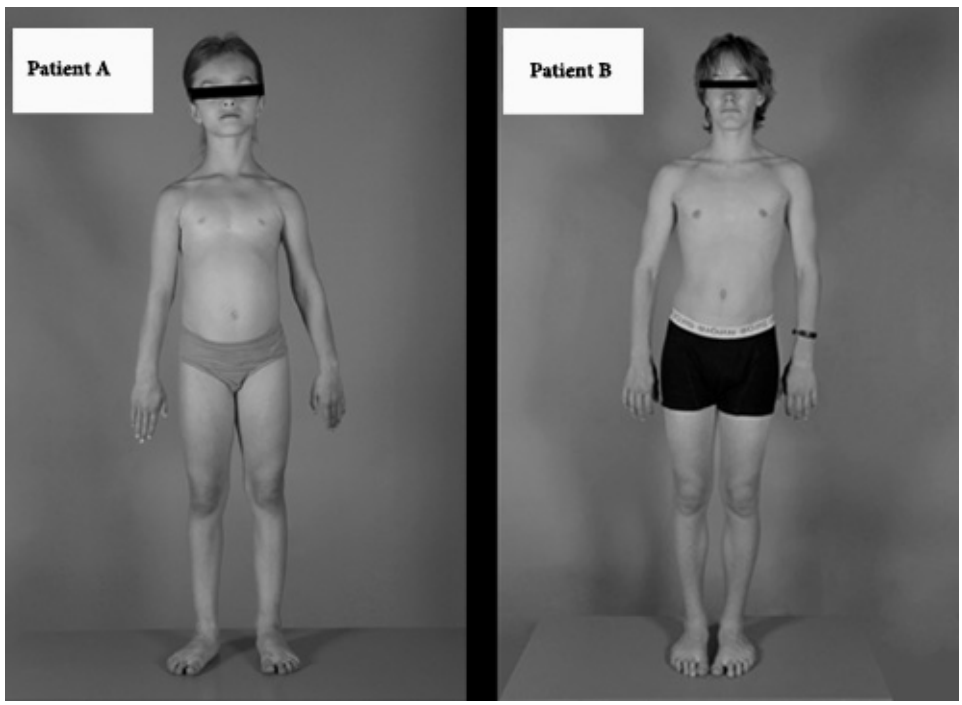

Figure 2. Physical characteristics of Patient A, at age 9, and of Patient B at 18 years of age.

Dysmorphological examination showed hypertelorism, hypocanthal folds, medial flaring of the eyebrows, broad nasal bridge and thick hair (Figure 2). She had a triangular face, large mouth, short and pigmented upper lip, low placed, posterior rotated ears. Her abdomen was protruded. She had bilateral clinodactyly, short fingers and proximally placed, broad thumbs. She had a bilateral sandal gap and broad forefeet with pes planus. There was hyperlaxity of the joints, especially of the elbows and knees (Beighton score 7/9). Dimples were present at the right flank

and in the lumbar region. These dysmorphic features were absent in her parents. She goes to a regular primary school. Testing of her verbal and performance intelligence quotient showed an average intellectual level, with a score of 93 and 121 points, respectively. These scores indicate a disharmonic intellectual profile although being in the normal range.

Her two brothers were born after 40 weeks gestation, had a normal birth size and grew normally (data not shown).

Genetic analysis

Patient A had a deletion of all three probes in the *IGF1R* gene on the MLPA growth kit. Confirmation of this result with the MRC Holland P217 MLPA kit showed a heterozygous deletion of all probes in the *IGF1R* gene (exon 1-21) including the two telomeric control probes which were located at 2.0 and 2.8 Mb downstream of the *IGF1R* gene. Her parents and both brothers did not carry this deletion. SNP array-analysis showed a terminal deletion from location rs12912857, the first detected SNP probe located at 95.883.282 bp until rs7169385, the last detected SNP probe located at 100.192.115 bp (Ensembl release 49), comprising a 4.5 Mb region on chromosome 15 (Figure 3). No other pathogenic CNV's were observed with SNP array analysis. Additional sequence analysis of the coding region of the *IGF1* gene, to exclude a mutation as a cause of the low IGF-I level revealed no mutation.

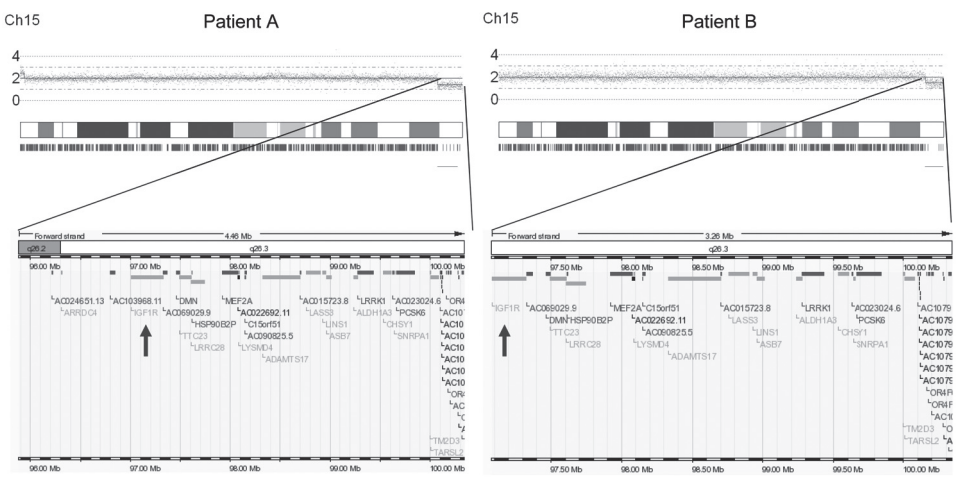

Figure 3. Array analysis of chromosome 15 of Patient A showed a heterozygous deletion of 15q26.2 to 15qter, from rs12912857 to rs7169385 comprising the whole *IGF1R* gene. Patient B showed a heterozygous deletion of 15q26.3 to 15qter from rs11857366 to rs7169385. This deletion starts within intron 2 of the *IGF1R* gene.

Patient B

Clinical description

Patient B was a boy born spontaneously after 40 weeks of gestation as the first child of non-consanguineous parents. A decrease in fetal growth was observed during the last trimester by ultrasound. His birth weight was 2600 g (-1.91 SDS) and birth length 47 cm (-2.21 SDS). The height of his father and mother was 187.6 cm (0.84 SDS) and 165.2 cm (-0.50 SDS), respectively. His secular-trend corrected target height was 187.4 cm (0.49 SDS). Bone age was 2 years delayed at the chronological age of 4 years. At age 2 a rotation deformity of both tibial bones was observed and tympanostomy tubes were implanted because of recurrent ear infections. At 3.0 years of age, his height was 84.0 cm (-3.84 SDS), and his weight was 11.2 kg (-2.65 SDS). A clonidine stimulation test was performed at age 5 with a maximal GH response of 69.7 mU/L. Cardiovascular, respiratory and abdominal examinations were all normal.

From age 7 onwards, he started GH treatment in a dose of 1 mg/m^2/day (Figure 1). At start of GH treatment, his height was 109.4 cm (-3.57 SDS), BMI 16.5 kg/m^2 (0.49 SDS), sitting height/ height ratio of 0.55 (-1.86 SDS) and head circumference 51.3 cm (-0.53 SDS). IGF-I was 208 ng/ml (1.25 SDS) and IGFBP-3 level 2.99 mg/l (1.24 SDS). After 1 year of GH treatment, his height had increased by 0.83 SD, and head circumference by 0.29 SD. His serum total IGF-I level was 356 ng/ml (2.28 SDS) and IGFBP-3 level was 1.99 mg/l (-0.40 SDS). After 4 years of GH treatment, he had an increase in height of +1.69 SD and an increase in IGF-I of +0.39 SD. Currently, at age 17, his height is 168.9 cm (-1.89 SDS).

From age 11 onwards, he used methylfenidate (10/5/5 mg) because of attention-deficit hyperactivity disorder. After primary school, he started a secondary school for children with hearing and speech difficulties. Currently, he receives training for becoming a baker. Cardiac evaluation showed no abnormalities.

Dysmorphological examination showed hypertelorism, upward slant, thin upper lip, bilateral extra nipple, proximal implanted thumbs and broad feet (Figure 2). These dysmorphic features were absent in his parents and brother. His brother has a height within the normal range.

Genetic analysis

MLPA analysis showed a deletion of two out of three *IGF1R* probes (exon 8 and 18). The MRC-Holland P217 MLPA kit showed a heterozygous deletion of exon 3-21 of the *IGF1R* gene comprising also both telomeric control probes located at 2.0 and 2.8Mb downstream of the *IGF1R* gene. His parents and brother did not carry the deletion. SNP array analysis showed a terminal deletion of 3.1 Mb on chromosome 15 ranging from rs11857366, first detected SNP probe at 97081324 bp until rs7169385, last detected SNP probe at 100192115 bp, containing 282 SNP probes (Figure 3). No other pathogenic CNV's were observed in the SNP array analysis.

Discussion

This study shows that *IGF1R* haploinsufficiency was detected in 2 out of 100 short SGA children and that no other copy number variants were found in 18 other growth-associated genes.

As dominant *de novo* deletions are a well-recognized sign of pathogenicity, it is very likely that the observed deletions explain the short stature of both patients. This is supported by previous clinical studies reporting subjects with *IGF1R* point-mutations and deletions [13-15, 17-24, 26], including functional analysis of a patient with a missense mutation and one with a complete deletion of the *IGF1R* gene [16,25]. In addition, according to the database of genomic variants (http://projects.tcag.ca/variation/) no deletion of the *IGF1R* gene region has been reported yet in the general population.

Both children responded to GH treatment, increasing their height by 1.02 SD and 0.83 SD respectively, after 1 year of GH treatment, and a further increase to -1.68 and -1.89 SD in the following years. This growth response was comparable to the mean 1 year growth response in short SGA children (approximately 0.8 SD in prepubertal short SGA children on the same GH dosage) [38]. This may be a combination of the direct effect of GH on the epiphyseal chondrocytes which is independent of the biological actions of serum IGF [39], and elevated serum IGF-I levels which may partially overcome the diminished sensitivity.

When previous case reports of children with an *IGF1R* deletion or mutation were reviewed, only six other children had received GH treatment (Table 1). The children who received a GH dose of 1mg/m^2/day, increased their height with ~1.0 SD per year of GH treatment. Their serum IGF-I level increased with ~1.0 SD except for Patient A (discussed in the next paragraph). The other children received a higher GH dose but showed a similar variable response: a similar response in two of them, and no apparent response in two other patients [13,16]. Thus, six out of eight children showed a beneficial effect of GH treatment.

An unexpected finding in Patient A was the low serum IGF-I level, in contrast to a high or normal serum IGF-I level usually observed in patients with an *IGF1R* mutation or deletion. Since no abnormalities in the *IGF1* gene were found, we hypothesize that this may be explained by a partial GH deficiency. The marginal response of GH in the provocation test and the observation that IGF-I and IGFBP-3 strongly rose by +4.3 SD and +2.2 SD after 1 year of GH treatment supports our hypothesis. Low IGF-I serum levels (1.3 SDS, Dr. H.J. van der Kamp, personal communication) were also observed in the child with an *IGF1R* missense mutation [26]. However after realimentation by a gastrostoma her IGF-I levels increased up to +2.9 SDS.

Table 1. Literature overview of short stature and/or SGA patients with a heterozygous *IGF1R* mutation or terminal chromosome 15q deletion who received GH treatment.

	Patient A	***Patient B***	**Abuzzahab**	**Abuzzahab**	**Inagaki**	**Walenkamp**	**Siebler**	**Walenkamp**
			(13)	(13, 42)	(16)	(26, 43)	(23)	(25)
Molecular characterisation:	**del(15) (q26.2)**	**del(15) (q26.3)**	**IGF1R R108Q/ K115N**	**IGF1R R59X**	**IGF 1R R481Q**	**IGF1R E1050K**	**del(15) (q26.1)**	**del(15) (q26.2)**
Birth:								
GA (weeks)	40	40	38	At term	41	39	39	39
Length (SDS)	-2.21	-2.74	NA	-5.89	-5.63	-2.94	NA	-1.95
Weight (SDS)	-1.28	-1.91	-4.33	-3.22	-3.06	-6.13	-3.05	-2.93
At start GH:								
Age (years)	4.0	7.0	4.5	6.4	13.6	1.6	3.5	5.3
Height (SDS)	-3.42	-3.57	~ -4.0[1]	-2.51	-5.0	-2.3	~ -5.0[1]	-3.5
IGF-I (SDS)	-2.78	1.25	"normal" [2]	+1.20	↑↑[3]	2.1	NA[2]	2.5
GH dose (mg/m^2/day)	1.0	1.0	1.6	0.9	2.1	1.4	1.6[5]	1.0
After 1 yr GH:								
Height (SDS)	-2.40	-2.74	~ -4.0[1]	-1.96	-5.0[4]	-1.5[4]	~ -4.0[1]	-2.0[6]
IGF-I (SDS)	1.51	2.28	↑↑[2]	+1.77	NA	3.6	NA	3.5[6]
Change ht (SD)	1.02	0.83	0	0.55	0	0.8	~ 1.0	1.5
Change IGF-I (SD)	*3.53*	↑↑	↑↑[2]	*0.57*	*NA*	*1.5*	*NA*	*1.0*

NA= not available. All birth weight and birth lengths are presented as standard deviation scores according to the Usher growth charts. [1]= estimated from the growth chart, [2]= No SDS is provided, [3]= IGF-I SDS not available, but level was above the normal range and unchanged after GH treatment, [4]= measured after 6 months of GH treatment, [5]= 3 different GH doses were used of which this dose was maintained for the longest period (age 8-10 years), [6]= measurements are provided when height and IGF-I level stabilized.

Table 2. Overview of phenotypic characteristics in short SGA patients with a heterozygous *IGF1R* mutation or terminal chromosome 15q deletion.

Genotype		Phenotype										
Mutation or deletion	Study	Birth weight ≤ -2.0 SDS	Birth length ≤ -2.0 SDS	Birth HC ≤ -2.0 SDS and/ or microcephaly	Postnatal height ≤ -2.0 SDS	IGF-1 level > +1.0 SDS	Developmental delay and/ or MR	Micrognathia/ triangular facies	Proximal placed dig I	Cardiac disorders	Total	Extra diagnoses :
15q26.2→qter	Patient A	-	+	-	+	-	+	+	+	+	6	Club feet, hip dysplasia, aberrant left ventricular wall
15q26.3→qter	Patient B	-	+	-	+	+	+	-	+	-	5	
IGF1R exon 2	R108Q/ K115N (13)	+	NA	-	+	+	+	-	-	-	4	
IGF1R exon 2	R59X (13)	+	+	+	+	+	+	-	-	-	6	
IGF1R exon 7	R481Q (16)	+	+	NA	+	+	-	+	-	-	5	
IGF1R exon 11	R709Q (17)	-	-	NA	+	+	+	-	-	-	3	
IGF1R exon 11	R709Q (17)	+	NA	NA	+	-	NA	NA	NA	-	2	
IGF1R exon 16	E1050K (26)	+	-	+	+	+	-	-	-	-	4	
IGF1R exon 16	E1050K (26)	+	+	+	+	+	-	+	-	-	6	Oligohydramnios
15q26.1→qter	(14)	+	+	+	NA	NA	NA	-	-	+	4	Right sided aorta, VSD, tethered spinal cord. Death at 5 months.
15q26.1→qter	(15)	-	NA	NA	NA	NA	NA	NA	+	+	2	Diaphragmatic hernia. Bicuspid aortic valve, aortic coarctation.

Genotype		Phenotype										
Mutation or deletion	Study	Birth weight ≤ -2.0 SDS	Birth length ≤ -2.0 SDS	Birth HC ≤ -2.0 SDS and/or microcephaly	Postnatal height ≤ -2.0 SDS	IGF-I level > +1.0 SDS	Developmental delay and/or MR	Micrognathia/ triangular facies	Proximal placed dig I	Cardiac disorders	Total	Extra diagnoses :
15q26.1→qter	(23)	+	+	NA	†	†	†	+	-	+	4	Lung hypoplasia, diaphragmatic hernia
15q26.1→qter	(23)	+	NA	NA	+	NA	+	+	+	-	5	Cubitus valgus
15q26.1→qter	(21)	+	+	+	+	NA	+	+	-	-	6	Oligohydramnios, hypoplastic lungs
15q26.1	(24)	+	+	+	+	NA	+	+	-	+	7	Gastroesophagal reflux
15q26.2→qter	(19)	+	+	+	+	+	+	+	+	-	8	Seizures, Gastroesophagal reflux
15q26.2→qter	(20)	+	+	+	+	-	+	+	-	+	7	Oculocutaneous albinism, subluxation radial heads
15q26.2→qter	(25)	+	-	+	+	+	-	-	-	-	4	Severe myopia
15q26.2	(22)	+	NA	+	+	NA	+	+	-	-	5	Club feet, genu recurvatum
15q26.2	(18)	+	NA	-	+	NA	-	+	-	+	4	
Total (number)		16	11	10	17	9	11	11	5	7		
Total (%)		80	79	71	100	75	69	61	26	35		

*†Died directly post partum, HC= head circumference, MR= mental retardation, NA= not available. IGF1R is located at 15q25-26 (OMIM *147370). All birth weight and lengths were calculated as standard deviation scores (28).*

The children we identified had a relatively mild phenotype compared to the other patients with a terminal *IGF1R* deletion who had lung hypoplasia, atrial and/or ventricular septum defects, hypoplastic left atrial or ventricular heart, dextrocardia and diaphragmatic hernia (Table 2) [19-25]. We believe that these clinical signs are primarily linked to other genes in the area, as in children with an *IGF1R* mutation a much smaller number of additional characteristics has been observed. Poot et al indicated *IGF1R* flanking genes which might be responsible for several characteristics of the variation in the phenotypes of children with an *IGF1R* deletion [20].

Both patients described here had hearing problems, showed mild developmental delay and had proximal implanted thumbs. Hearing problems and developmental delay have been described in previous case-reports of mutations in the *IGF1* and *IGF1R* gene and might also be due to their tissue-specific expression in the auditory and central nervous system [40,41].

To our knowledge, there are no diagnostic criteria for children with an *IGF1R* mutation or deletion. In Table 3 we have summarized clinical features of children with an *IGF1R* mutation or deletion according to their organ system. We identified major criteria which are predominantly related to growth restriction and minor criteria which are based on dysmorphic features and signs of joint hypermobility. Based on the information summarized in Table 2, we propose the combinations of major and minor criteria which indicate a high likelihood of an *IGF1R* mutation or deletion. Interestingly, joint hypermobility is related to the fibrillin (*FBN1*) gene which is located upstream of the *IGF1R* gene on 15q21. *FBN1* gene mutations are responsible for Marfan syndrome which however, instead of short stature, is associated with tall stature. Both phenotypes however display joint hypermobility. Further research is needed to identify whether the *IGF1R* and *FBN1* gene together might explain variations in height and hypermobility in short SGA patients.

The remaining 98 short SGA children had no CNV's in the 18 growth-associated genes we selected, but we did not exclude mutations in these genes. Future studies are needed to investigate whether deletions or mutations in other genes, or combinations of several gene defects, are associated with the short SGA phenotype.

In summary, this study has shown that *IGF1R* haploinsufficiency was present in 2 out of 100 short SGA children. This study illustrates that the combination of a small birth size, short stature, small head size, relatively high IGF-I level, developmental delay and micrognathia is suggestive for children with an *IGF1R* deletion. Since GH therapy leads to a moderate catch-up growth of ~1.0 SD in the first year, we recommend that the SGA children with a persistent short stature are tested for an *IGF1R* deletion, particularly if some of the major or minor criteria are present. The multiplex ligation-dependent probe amplification (MLPA) has shown to be a valuable tool in rapidly identifying these relatively large deletions in short SGA children.

Table 3. Suggested clinical indicators for a heterozygous *IGF1R* mutation or terminal chromosome 15q deletion based on published cases.

	Major	**Minor**
1. Skeletal	Height ≤ -2.00 SDS	Triangular face/ micrognathia
	Birth length ≤ -2.00 SDS	Proximal placed thumb
	Birth weight ≤ -2.00 SDS	
	Head circumference ≤ -2.00 SDS	
	IGF-I level SDS > 1.00 SDS	
2. Nervous system	Developmental delay	Tethered spinal cord
	Mental retardation	Seizures
3. Cardiac	Right sided aorta, Bicuspid aortic valve, aortic coarctation	
	VSD	
	Aberrant left ventricular wall	
4. Gastro-intestinal		Diaphragmatic hernia
		Gastroesophagal reflux
5. Connective tissue		Club feet
		Hip dysplasia
		Cubitus valgus
		Genu recurvatum
		Subluxation radial heads
		Severe myopia
6. Skin		Oculocutaneous albinism

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Supplementary data

Table 1. Probe sequences of the "in house" MLPA kit.

Gene name	exon	Upstream (U)/ Down stream (D)*	Sequence	Total fragment length (bp)	label
IGF1R	2	U	GATGTGTGAGAAGACCACCATCAACA	92	HEX
		D	ATGAGTACAACTACCGCTGCTGGACCACAA		
	8	U	CTACATGGGCTGAAGCCCTGGACTCAG	94	HEX
		D	TACGCCGTTTACGTCAAGGCTGTGACCCTCA		
	18	U	CAGTCCTAGCACCTCCAAGCCTGAGCA	90	HEX
		D	AGATGATTCAGATGGCCGGAGAGATTG		
IGF1	3	U	GAGTGCTGCTTCCGGAGCTGTGATCT	88	HEX
		D	AAGGAGGCTGGAGATGTATTGCGCAC		
	4	U	GTACATTTGAAGAACGCAAGTAGAGGGAGT	98	HEX
		D	GCAGGAAACAAGAACTACAGGATGTAGGAAGA		
GH1	4	U	CAACAGCCTGGTGTACGGCGCCTCTGACAGCAACGTCTATGA	116	FAM
		D	CCTCCTAAAGGACCTAGAGGAAGGCATCCAAA		
GHR	4	U	GCCACTGGACAGATGAGGTTCATCATGGTACAAAGAACC	112	FAM
		D	TAGGACCCATACAGCTGTTCTATACCAGAAG		
	10	U	GGTACTTTGGTGGCCACATAAGCCATTATTCACTAGTATGA	110	HEX
		D	CTAGTTGTGTCTGGCAGTTTATATTTAACTCTC		
SHOX	3	U	GGATTTATGAATGCAAAGAGAAGCGCGAGGACG	106	HEX
		D	TGAAGTCGGAGGACGAGGACGGGCAGACCAAGCTGAA		
	8	U	CCCTGAGTTTCTCTGGTGACGCCCTCATTCTCCTAA	108	HEX
		D	CGTTCAATAATCTCAATGTTGAGTTGCAGCAACAGA		
IGFBP1	1	U	CAAACTTATTTTGAACACTCAGCTCCTAGCGTGCG	102	HEX
		D	GCGCTGCCAATCATTAACCTCCTGGTGCAAG		
	4	U	GTGAGACATCCATGGATGGAGAGGCGGGACTCTGCT	104	HEX
		D	GGTGCGTCTACCCTTGGAATGGGAAGAGGATC		

Gene name	exon	Upstream (U)/ Down stream (D)*	Sequence	Total fragment length (bp)	label
IGFBP2	2	U	CACTCAGAAGGAGGCCTGGTGGAGAACCACGT	100	FAM
		D	GGACAGCACCATGAACATGTTGGGCG		
	4	U	CAAGATGTCTCTGAACGGGCAGCGTGGGGAGTGCT	108	FAM
		D	GGTGTGTGAACCCCAACACCGGGAAGCTGAT		
IGFBP3	2	U	GCTAAAGACAGCCAGCGCTACAAAGTTGACTACG	100	HEX
		D	AGTCTCAGAGCACAGATACCCAGAACTTCT		
	5	U	CTGGAGCTCACAGCCTTCTGTGGTGTCATT	96	HEX
		D	TCTGAAACAAGGGCGTGGATCCCTCAACCA		
IGFBP4	3	U	GACCTCTACATCATCCCCATCCC	84	HEX
		D	CAACTGCGACCGCAACGGCAACTTC		
	4	U	CAGAGTCAGAGGAGAAGAGACATGTACCTTGACCATCGTCC	114	HEX
		D	TTCCTCTCAAGCTAGCCAGAGGGTGGGAGCCTAAGGA		
IGFBP5	2	U	GATCTTCCGGCCCAAACACACCCGCATCTCCGAGCTGAAGGCT-GAAG	120	HEX
		D	CAGTGAAGAAGGACCGCAGAAAGAAGCTGACCCAGTC		
	3	U	GTGCTGTGTACCTGCCCAATTGT	86	HEX
		D	GACCGCAAAGGATTCTACAAGAGAAAG		
IGFBP6	2	U	CCACAGGATGTGAACCGCAGAGA	92	FAM
		D	CCAACAGAGGAATCCAGGCACCTCTAC		
	3	U	CAAACACTCTACGTGCCCAATTGTG	90	FAM
		D	ACCATCGAGGCTTCTACCGGAAG		
ALS	2	U	GAACCTCTCTGGGAACTGTCTCCGG	88	FAM
		D	AACCTTCCGGAGCAGGTGTTC		
Stat5b	2	U	GATACAAGCTCAGCAGCTCCAAGGAGAAGCCCT	102	FAM
		D	TCATCAGATGCAAGCGTTATATGGCCA		
	14	U	GAGAATTTACCAGGACGGAATTACACTTTCTG	104	FAM
		D	GCAATGGTTTGACGGTGTGATGGAAGTGTT		

Gene name	exon	Upstream (U)/ Down stream (D)*	Sequence	Total fragment length (bp)	label
Socs3	2	U	CTTCAGCATCTCTGTCGGAAGACCGTCAACG	98	FAM
		D	GCCACCTGGACTCCTATGAGAAAGT		
GRB10	3	U	GATGTGGACCTGGAAGCCCTGGTGAACGAT	96	FAM
		D	ATGAATGCATCCCTGGAGAGCCTG		
	10	U	CAGAGGACGAGCAAACCAGGACGTGCT	94	FAM
		D	GGATGACAGCGTTCAGACTCCTCAA		
NSD1	3	U	GAGATCTCATCTGGGCAAAATTCAAGAGACGCCCATGGTGGCCCT	116	HEX
		D	GCAGGATTTGTTCTGATCCGTTGATTAACACACAT		
	13	U	CACTGTTATGCAGAACAAGGGCTTCCGGTGCTCCCTCCACATCT-GTA	118	HEX
		D	TAACCTGTCATGCTGCTAATCCAGCCAATGTTTCT		
	20	U	CTCGGTTCATGAATCATTGCTGCCAGCCCAACTGTGAAACACA-GAAG	120	FAM
		D	TGGTCTGTGAATGGAGATACCCGTGTAGGCC		
IGF2	2	U	GAATCCCAATGGGGAAGTCGAT	86	FAM
(11p15)		D	GCTGGTGCTTCTCACCTTCTTG		
	4	U	CATCCTGCAGCCTCCTCCTGACCACGGACGTTTCCATCAGGTT	114	FAM
		D	CCATCCCGAAAATCTCTCGGTTCCACGTC		
SOCS2	1	U	GCCGCGGCCTCAACTAAAAGTGGCCATTGACCTTTCAA	110	FAM
		D	GCTTTCGAGCAGTGATGCAATAGAATAGAA		

**The Upstream hybridising sequence (U) was extended with a labelled primer 5'-GGGTTCCCTAAGGGTTGGA-3'; the downstream hybridizing sequence was extended with an unlabelled primer 5'-GTGCCAGCAAGATCCAATCTAGA-3'.*

Chapter 9

Postnatal spontaneous and GH-induced growth of short SGA, SGA catch-up and ISS subjects: relation with gestational hypertension, smoking and alcohol. The Network of Studies into Genes in Growth (NESTEGG).

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Abstract

Background: The network of studies into genes in growth (NESTEGG) is a growth genomics project in which subjects born small for gestational age (SGA), subjects with idiopathic short stature (ISS) and controls are studied.

Objectives: To determine postnatal and GH-induced growth and their relation with maternal hypertension, smoking and alcohol during gestation.

Methods: 464 short SGA, 336 SGA catch-up, 464 ISS and 724 control subjects were investigated. GH treatment data were available of 262 short SGA and 109 ISS prepubertal subjects. Growth data were collected from medical records and gestational data were retrieved from questionnaires.

Results: Birth length and postnatal height were significantly different between short SGA, SGA catch-up, ISS and control subjects (short SGA: -2.88 vs. -2.82 SDS, SGA catch-up: -2.50 vs. -0.49 SDS, ISS: -0.59 vs. -2.49 SDS and controls: -0.01 vs. 0.05 SDS, respectively). Short SGA subjects increased 0.2 SDS more in height than ISS subjects during 1 year of GH treatment ($P < 0.001$). Mothers of SGA catch-up subjects had more gestational hypertension than mothers of short SGA, ISS and control subjects (27.9% vs. 16.4%, 6.0% and 12.3%, $P<0.001$). Mothers of short SGA subjects smoked more during gestation than mothers of ISS and control subjects (35.1% vs. 20.3% and 23.5%, $P<0.001$). During spontaneous postnatal growth, short SGA subjects increased more in height when their mothers had gestational hypertension compared to short SGA subjects without (0.64 SDS vs. -0.06 SDS, $P<0.001$).

Conclusions: Prenatal growth restriction of SGA subjects was related to gestational hypertension and smoking. Spontaneous postnatal catch-up growth of short SGA subjects was most pronounced when their mothers had gestational hypertension. Gestational factors were not related to the ISS phenotype.

Introduction

In 2001 the network of studies into genes in growth (NESTEGG) was initiated by paediatric endocrinologists and molecular geneticists to investigate etiological factors of children with pre- and/or postnatal growth failure [1]. This cross-sectional study was performed in The Netherlands, France, Germany and the United Kingdom. Clinical data on growth and cardiovascular outcomes were collected in subjects born small for gestational age (SGA) with and without catch-up growth, subjects with idiopathic short stature (ISS) and controls. In addition, clinical data during GH treatment were attained from a subgroup of short SGA and ISS subjects.

The aim of the Network of Studies into Genes in Growth (NESTEGG), is to describe the phenotype of short SGA, SGA catch-up and ISS subjects and to identify genes that contribute to foetal or postnatal growth. These genetic factors may allow for a more specific diagnosis and may be the base of more targeted treatment possibilities. In addition, novel genetic factors affecting growth might give rise to new treatment modalities.

The aim of the current study is to describe pre- and postnatal growth in SGA and ISS children compared with controls and to identify whether gestational factors are related to postnatal growth and growth during GH treatment. Previous studies have shown that gestational hypertension, maternal smoking and gestational alcohol influence pre- and postnatal growth [2-4). However, it is unknown to what extent these gestational factors influence spontaneous postnatal and GH-induced growth in short SGA, SGA catch-up, ISS and control subjects.

This is a unique insight into the relationship of gestational hypertension, gestational smoking and alcohol use with spontaneous postnatal and GH-induced growth in short SGA, SGA catch-up, ISS and control subjects in a large international cohort.

Methods

Study population

The NESTEGG study comprises short SGA, SGA catch-up, ISS subjects, their parents and controls. All subjects were recruited by paediatric endocrinologists of the Sophia Children's Hospital, Erasmus medical Centre, Rotterdam; University Children's Hospital, Toulouse, University Children's Hospital, Tübingen and Bart's and the Royal London Hospitals, London. Short SGA and ISS subjects were recruited from the growth clinics, where GH treatment was in some cases prescribed. SGA catch-up and ISS subjects who did not use GH treatment were additionally recruited from young adult studies or on identification through birth cohorts. Controls were acquired from blood banks, hospital staff and student populations.

SGA was defined as a birth weight and/or birth length ≤ -1.88 standard deviation score (SDS) according to the national growth charts (The Netherlands and Germany [5]; France [6]; United Kingdom [7]). Height at or after 3 years of age determined whether a child had caught up (> -1.88 SDS: SGA catch-up) or remained short (< -1.88 SDS: short SGA) (The Netherlands [8]; France [9]; United Kingdom [7]; Germany [10]). ISS was defined as a birth weight and/or birth length > -1.88 to +2.00 SDS and a prepubertal height after 3 years of age ≤ –1.88 SDS (The Netherlands [8]; France [9]; United Kingdom [7]; Germany [10]). Inclusion criteria were: gestation ≥ 30 weeks with uncomplicated postnatal period (ventilation < 72 hours not including CPAP). Subjects were included only when they were of Caucasian origin and all grandparents and parents must have been born and living in Western Europe. Exclusion criteria were: a known syndrome or dysmorphic features (except Russell Silver syndrome), known chromosomal or genetic abnormality, known severe chronic illness or endocrine disease, or positive gliadin, endomysial or reticulin antibodies, growth hormone deficiency, severe disproportionate short stature, psychosocial dwarfism, any psychiatric, neurodegenerative, or chronic illness in parents, which may affect their ability to give fully informed consent or donate blood and if the child was adopted. All centres obtained approval of their Medical Ethics Committee. Children and/or their parents signed informed consent before participation in the study.

Clinical parameters

Birth measurements and gestational age were obtained from birth records. Birth weight as qualifying parameter was available for all subjects. Anthropometric data during postnatal life were cross-sectionally collected at the out-patient clinic or retrospectively when GH therapy was started earlier. Anthropometric measurements were performed by standardized methods. Pubertal status was determined by the physician according to Tanner stage [11]. Gestational

data were retrieved as categorical variables by questionnaires answered by the mothers of all subjects. Gestational hypertension was coded as treated and untreated hypertension vs. no hypertension. Gestational smoking was coded as maternal smoking, paternal and maternal smoking vs. no smoking. Maternal alcohol use was coded as 1-4 units/week, 5-9 units/week and 10 or more units per week vs. no alcohol use.

Of all 1988 study participants, 519 received GH treatment. For the aim of the present study, we selected prepubertal short SGA and ISS children. Prepuberty was defined as breast, genital and pubic hair stage ≤ 2, and/or testicular volumes ≤ 4 ml. Of the prepubertal children we excluded the older children by selecting girls ≤ 12 years of age and boys ≤ 14 years of age. In total 371 children were eligible for the analysis of growth during GH treatment. During GH treatment, biosynthetic GH was injected subcutaneously. GH doses of all centers were recalculated into mg/m^2 body surface area/day. IGF-I levels were standardized according to national references (The Netherlands [12]; France: by Diagnostic System Laboratories, Inc; United Kingdom [13]; there were no IGF-I data from Germany). The growth response during GH treatment was corrected for midparental height SDS. Midparental height SDS was calculated by ((maternal height SDS + paternal height SDS)/2), according to the national growth chart [7-10,14].

Statistical analyses

All continuous anthropometric variables were tested for overall differences between the short SGA, SGA catch-up, ISS and control group by univariate analysis of variance. Post-hoc pair wise tests employed a Bonferroni correction for multiple comparisons. Categorical variables were tested for overall differences between the short SGA, SGA catch-up, ISS and control group by chi-square test and differences between the groups were tested by pair wise chi-square test. Measurements during GH treatment were corrected for GH dose and target height SDS by adding these covariates in the univariate analyses. Accumulation of gestational factors was investigated in 1270 subjects who had a complete dataset for all three gestational factors. P-values ≤ 0.05 were considered significant. Statistical tests were performed using SPSS 15.0 package (SPSS Inc., Chicago, IL, USA).

Results

Birth size

The study population consisted of 1988 subjects, 464 short SGA, 336 SGA catch-up, 464 ISS and 724 control subjects (Table 1). All groups had a significantly different birth length SDS ($P < 0.001$). Short SGA subjects had a shorter birth length than SGA catch-up subjects (-2.88 SDS vs. -2.50 SDS, $P< 0.001$), whereas they had a comparable weight at birth (-2.20 SDS vs. -2.26 SDS). Both ISS subjects and controls had a birth size in the normal range. However, ISS subjects had a birth length and weight SDS significantly below the average of 0 SDS (length: -0.59 SDS, weight: -0.49 SDS, both $P < 0.001$). Controls had a weight SDS which was significantly below 0 SDS (-0.20 SDS, $P < 0.001$) in contrast to their length SDS (-0.01 SDS, $P = 0.79$).

Postnatal spontaneous growth

All groups had a significantly different height SDS (Table 1, $P<0.001$). Short SGA subjects were the shortest and leanest of all groups (height: -2.82 SDS, weight: -2.14 SDS). ISS subjects also had a height below the -2 SDS but their weight SDS was in the low-normal range (height: -2.49 SDS, weight: -1.89 SDS). Although SGA catch-up subjects had a height and weight within the normal range, both were significantly below 0 SDS (height and weight: $P<0.001$). Controls were relatively large, with a weight above the average (weight: 0.70 SDS, $P<0.001$).

Growth during GH treatment

262 short SGA and 109 ISS children were prepubertal at start and after 1 year of GH treatment (Table 2). Short SGA children were, compared to ISS children, significantly younger (6.7 vs. 8.7 years, $P<0.001$), received a higher GH dose (1.20 vs. 1.05 mg/m^2/day, $P < 0.001$) and were shorter (-2.96 vs. -2.49 SDS, $P<0.001$) at start of GH treatment. Short SGA children had a 0.3 SDS higher IGF-I level at start of GH treatment compared to ISS children, however, after 1 year of treatment the IGF-I SDS levels were comparable. Weight SDS at start of GH treatment was comparable between the short SGA and ISS subjects.

During one year GH treatment, short SGA subjects had a 0.2 SDS greater increase in height than ISS subjects ($P < 0.001$). The change in weight and IGF-I SDS was comparable between short SGA and ISS subjects.

Gestational factors: short SGA, SGA catch-up, ISS and controls

Mothers of short SGA subjects smoked more frequently during gestation than mothers of ISS and control subjects (35.1% vs. 20.3% and 23.5%, $P < 0.001$). The frequency of maternal

smoking during gestation between short SGA and SGA catch-up subjects was comparable (35.1% vs. 31.7%). Mothers of SGA catch-up subjects had a significantly higher frequency of gestational hypertension than mothers of short SGA, ISS and control subjects (27.9% vs. 16.4%, 6.0% and 12.3%, respectively, P < 0.001, Table 1). Mothers of the short SGA, SGA catch-up and ISS subjects reported a comparable frequency of alcohol use (13.2%, 11.7% and 11.4%, respectively). Maternal alcohol use during gestation was most frequently reported among mothers of controls (25.4%).

Table 1. Birth size, spontaneous postnatal growth and gestational factors in short SGA, SGA catch-up, ISS and control subjects.

	Short SGA	**SGA CU**	**ISS**	**Controls**	**P-value**
Number	464	336	464	724	
Sex (%boys)	53.9%	39.6%	59.5%	45.9%	<0.001
Birth					
Length (SDS)	-2.88 (1.23)	-2.50 (1.26)	-0.59 (0.83)	-0.01 (1.07)	<0.001[a]
Weight (SDS)	-2.20 (0.98)	-2.26 (0.86)	-0.49 (0.88)	-0.20 (1.01)	<0.001[b]
Postnatal					
Age (years)	8.3 (5.8)	15.6 (9.2)	9.7 (6.2)	27.9 (14.2)	<0.001[c]
Height (SDS)	-2.82 (0.69)	-0.49 (1.37)	-2.49 (0.53)	0.05 (1.09)	<0.001[a]
Weight (SDS)	-2.14 (1.14)	-0.29 (1.39)	-1.89 (1.02)	0.70 (1.34)	<0.001[d]
Delta height (SDS)*	0.05 (1.30)	2.01 (1.91)	-1.88 (0.92)	0.11 (1.25)	<0.001[e]
Delta weight (SDS)*	-0.10 (1.28)	1.96 (1.57)	-1.43 (1.29)	0.82 (1.54)	<0.001[a]
Gestational factors					
Hypertension %(n)	16.4 % (72)	27.9% (77)	6.0% (27)	12.3% (26)	<0.001[e]
Maternal smoking %(n)	35.1% (143)	31.7% (83)	20.3% (88)	23.5% (44)	<0.001[d]
Alcohol use %(n)	13.2% (57)	11.7% (32)	11.4% (49)	25.4% (53)	<0.001[f]

*SGA= small for gestational age, CU= catch-up, ISS= idiopathic short stature, SDS= standard deviation score. Continuous variables are presented as mean (SD) and their P-values represent the comparison of the four groups in ANOVA, where after the overall P-value is presented. Categorical variables are presented as percentage (number) and their P-values are determined by chi-square test. [a] All groups differed significantly from each other; [b] except for the short SGA and SGA CU group, [c] except for the short SGA and ISS group, [d] except for the controls and SGA CU, controls and ISS, and the SGA CU and short SGA groups, [e] except for the short SGA and controls, [f] except for the SGA CU and ISS, SGA CU and short SGA, and the ISS and SGA short groups. * Delta SDS= postnatal SDS minus birth SDS.*

Table 2. GH-induced growth in short SGA and ISS subjects.

	Short SGA	ISS	P-value
Number	262	109	
Sex (% boys)	58.4%	56.9%	0.79
Age (years)	6.7 (2.4)	8.7 (2.7)	<0.001
GH dose (mg/m^2/day)	1.20 (0.37)	1.05 (0.23)	<0.001
At start GH:			
Height (SDS)	-2.96 (0.70)	-2.49 (0.54)	<0.001
Weight (SDS)	-1.61 (1.11)	-1.50 (0.72)	0.34
IGF-I (SDS)	-1.27 (1.25)	-1.58 (0.69)	0.04
After 1 year GH*:			
Height (SDS)	-2.18 (0.04)	-1.86 (0.07)	<0.001
Weight (SDS)	-1.26 (0.06)	-1.30 (0.10)	0.72
IGF-I (SDS)	0.54 (0.10)	0.42 (0.24)	0.65
Change*:			
Delta height (SDS)	0.81 (0.02)	0.64 (0.04)	<0.001
Delta weight (SDS)	0.37 (0.03)	0.32 (0.05)	0.39
Delta IGF-I (SDS)	1.77 (0.11)	2.06 (0.29)	0.35
Gestational factors:			
Hypertension %(n)	17.7% (45)	6.5% (7)	0.005
Maternal smoking %(n)	36.8% (85)	26.4% (28)	0.06
Alcohol use %(n)	11.6% (29)	15.7% (17)	0.28

**= corrected for GH dose and midparental height SDS. Presented as mean (SE). All subjects were prepubertal at start and after 1 year of GH treatment.*

Gestational factors: postnatal growth and growth during GH treatment

Short SGA subjects had a significantly greater spontaneous catch-up in height when their mothers had gestational hypertension than short SGA subjects whose mothers did not have gestational hypertension (delta height SDS: 0.64 vs. -0.06, P < 0.001). Gestational hypertension was not related with spontaneous postnatal growth of SGA catch-up, ISS and control subjects. ISS subjects had less postnatal growth retardation when their mothers had smoked during gestation compared to ISS subjects whose mothers did not smoke during gestation (delta

height SDS: -1.65 vs. -1.91, P = 0.02). Maternal alcohol use during gestation did not influence spontaneous postnatal growth of any group.

Gestational hypertension, smoking and alcohol use were not related with GH-induced growth of short SGA subjects. ISS subjects increased significantly more in height during GH therapy when their mothers did smoke during gestation compared to ISS subjects whose mothers did not smoke during gestation (0.77 vs. 0.53 SDS, P = 0.001).

Accumulation of gestational factors

Short SGA and SGA catch-up mothers most frequently reported to have a combination of gestational hypertension and smoking (5.4% vs. 6.5%, Figure 1). Mothers of ISS and control subjects most frequently reported a combination of gestational smoking and alcohol (3.3% vs. 9.2%). A minority of all mothers had a combination of gestational hypertension, smoking and alcohol use (short SGA: 0%, SGA catch-up 1.2%, ISS 0.2% and controls 2.2%).

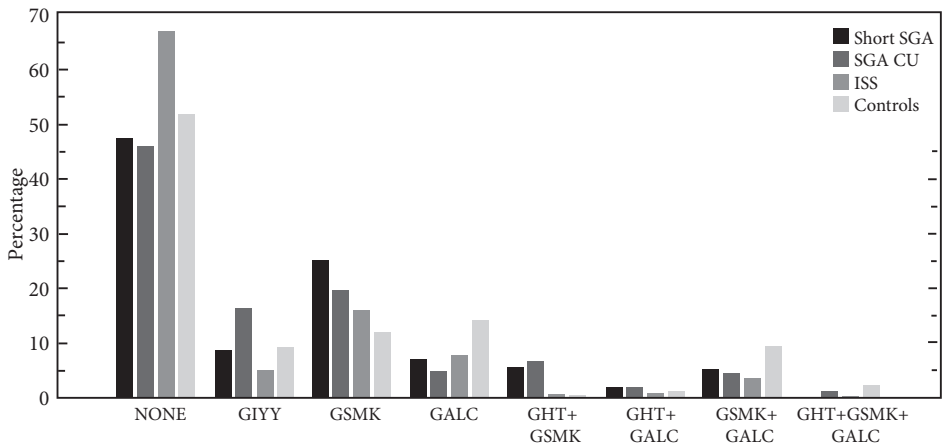

Figure 1. Accumulation of gestational factors in short SGA, SGA catch-up, ISS and control subjects: (1) no gestational hypertension, smoking and alcohol, (2) isolated gestational hypertension, (3) gestational smoking, (4) gestational alcohol, (5) gestational hypertension and smoking, (6) gestational hypertension and alcohol, (7) gestational smoking and alcohol, (8) gestational hypertension, smoking and alcohol.

Table 3. Gestational factors and spontaneous postnatal growth in short SGA, SGA catch-up, ISS and control subjects.

Delta height SDS*	Short SGA	SGA CU	ISS	Controls
Hypertension				
Present	0.64 (1.74)	2.04 (1.35)	-2.02 (1.01)	0.03 (1.36)
Absent	-0.06 (1.18)	1.99 (2.04)	-1.86 (0.89)	0.00 (1.37)
P-value	<0.001	0.87	0.43	0.94
Maternal smoking				
Present	0.14 (1.36)	2.23 (2.53)	-1.65 (0.95)	0.08 (1.40)
Absent	0.01 (1.26)	1.94 (1.47)	-1.91 (0.89)	-0.03 (1.41)
P-value	0.34	0.28	0.02	0.68
Alcohol use				
Present	0.04 (1.31)	1.70 (1.26)	-1.71 (0.88)	-0.21 (1.51)
Absent	0.05 (1.31)	2.06 (1.94)	-1.88 (0.90)	0.07 (1.32)
P-value	0.96	0.35	0.26	0.25

**Delta height SDS is calculated by: postnatal height SDS minus birth length SDS. The P-value represents the ANOVA test result for the difference in height SDS for each gestational factor.*

Discussion

This is the first study in which gestational hypertension, gestational smoking and alcohol use were investigated in relation with spontaneous postnatal and GH-induced growth in short SGA, SGA catch-up, ISS and control subjects in a large international cohort. This study has shown that prenatal growth restriction of SGA subjects is related to gestational hypertension and smoking. Postnatal catch-up growth in height was most pronounced when their mothers had gestational hypertension. Gestational hypertension, smoking and alcohol use were not related to the ISS phenotype although maternal smoking during gestation was associated with less postnatal growth decline and more growth during 1 year of GH treatment.

Although many factors are associated with being born SGA [15], these factors do not fully explain their phenotype. SGA is a clinical definition and not a diagnosis. Our study showed that gestational hypertension and maternal smoking were related to 56% of all SGA subjects on average (short SGA: 16.4% and 35.1%, and SGA catch-up: 27.9% and 31.7%, respectively). This

observation is in line with previous studies which have shown that gestational hypertension and maternal smoking both result in prenatal growth restriction [2,3]. Other support comes from studies which show that although gestational hypertension and maternal smoking have a different mode of action, both would result in maternal endothelial dysfunction and abnormal placentation, which are common features in fetal growth restriction [3,16-18].

Table 4. Gestational factors and growth after 1 year of GH treatment in short SGA and ISS children.

Delta height SDS*	**Short SGA**	**ISS**
Hypertension		
Present	0.84 (0.05)	0.52 (0.13)
Absent	0.81 (0.02)	0.60 (0.03)
P-value	0.60	0.52
Maternal smoking		
Present	0.74 (0.04)	0.77 (0.06)
Absent	0.83 (0.03)	0.53 (0.04)
P-value	0.07	0.001
Alcohol use		
Present	0.73 (0.06)	0.52 (0.08)
Absent	0.82 (0.02)	0.61 (0.04)
P-value	0.18	0.28

**= Delta height SDS is calculated by: height SDS after one year of GH treatment minus height SDS at start of GH treatment. Delta height SDS is adjusted for the GH dose. Means (SE) are presented.*

In this study there was no association between maternal use of alcohol during gestation and postnatal growth. It might well be that mothers of control subjects reported a high frequency of alcohol use during gestation because they had children with a normal growth and may therefore not be hesitant to report alcohol use.

It is unclear which factors determine whether SGA subjects catch-up to the normal range. Many previous studies have shown that 85-90% of all SGA subjects catch-up in height and that 10-15% remain short [19,20]. Although our study showed that gestational hypertension is more frequent in SGA catch-up than in short SGA subjects (27.9% vs. 16.4%, $P < 0.001$), SGA catch-up subjects had no increased spontaneous postnatal growth after gestational hypertension. Thus, it is unlikely that gestational hypertension determines whether postnatal catch-up growth takes place.

Short SGA children might be disadvantaged in their postnatal growth by a genetic predisposition which prevents catch-up growth. Since 10-15% of the SGA populations remain small, this group might have disadvantageous single nucleotide polymorphisms (SNP). These findings merit further study in NESTEGG. It might well be that newly discovered height genes, can explain parts of the postnatally experienced short stature in SGA and ISS subjects.

Recently, many genome wide association (GWA) studies have published SNPs which contribute to small variations in height of the general population. Several gene clusters seem to be involved: Hedgehog signaling genes containing IHH, HHIP, PTCH1, the extracellular matrix genes containing EFEMP1, ADAMTSL3, ACAN, chondrocyte-differentiation gene as GDF5-UQCC, a methyl-DNA-binding transcriptional repressor gene as ZBTB38, cancer-related genes as HMGA2, CDK6, DLEU7 and several others like GPR126, HIST1H1D, TRIP11-ATXN3, LIN28B, DOT1L, CHCHD7-RDHE2, PNPT1, BMP6 and GNA12 [21-25].

We would hypothesize, that the disadvantageous genotypes that result in persistent short stature of short SGA subjects are the opposite genotypes of that in SGA catch-up subjects. Genes which are involved in vasoregulatory processes [26,27] seem good candidates to be associated with catch-up growth as prenatal maternal endothelial dysfunction and abnormal placentation might be a key in the development of the SGA phenotype.

In the interplay between mother, placenta and fetus it should be considered that not only maternal factors as gestational smoking affect the placenta and the fetus, but that the fetal genotype might also affect the maternal status [28,29]. The fetal genotype might induce a rise in maternal blood pressure and glucose levels to provide adequate nutrition of the fetus [29]. This concept might apply to the SGA fetus. However, studies are needed to confirm this hypothesis.

In this study it was observed that gestational smoking was associated with less spontaneous postnatal growth decline and more growth during 1 year of GH treatment in ISS subjects. We do not have an explanation for these findings, although it might be that mothers of ISS subjects who smoked during pregnancy were confounded with another etiological factor leading to a moderate short stature of their child compared to mothers of ISS subjects who did not smoke during gestation having a child with more severe short stature. Further research is needed to confirm and clarify this observation.

Our study has some limitations. Data on gestational hypertension, smoking and alcohol use were obtained by questionnaires, which are vulnerable for underreporting and/ or misclassification. This might give rise to an underestimation of the ultimate effect of gestational factors in pre- and postnatal growth. The anthropometrical measurements were collected in 4 countries and several research centers per country which is prone to systematic measurements errors, however by using standard operating procedures and expressing measurements into standard deviation scores, these errors were minimized.

In conclusion, our study has shown that prenatal growth restriction of SGA subjects is related to gestational hypertension and smoking. Postnatal catch-up growth of short SGA subjects was most pronounced when their mothers had gestational hypertension. Gestational hypertension, smoking and alcohol use were not related to the ISS phenotype although maternal smoking during gestation was associated with less postnatal growth decline and more growth during 1 year of GH treatment.

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Chapter 10

General discussion

General discussion

The present thesis describes genetic and environmental factors which are important in pre- and postnatal growth in children born small for gestational age (SGA), either with short or normal stature. The first part presents a novel subclassification of short SGA subjects. The second part focuses on genetic polymorphisms in the insulin-like growth factor 1 (*IGF1*), the insulin-like growth factor 1 receptor (*IGF1R*) and the insulin (*INS*) genes in relation with growth and blood pressure in short SGA and SGA catch-up subjects. The third part describes the findings of a study to identify relatively large deletions and duplications in 18 growth-related genes in a group of short SGA children. Detailed phenotypic and genotypic data are reported of two short SGA children with an *IGF1R* deletion. Finally, the relation between gestational factors, postnatal growth and phenotypic characteristics is described for a large population consisting of short SGA, SGA catch-up, idiopathic short stature (ISS) and control subjects.

Part 1. Subclassification of short SGA children

Subclassification of SGA children with persistent short stature: Growth patterns and response to GH treatment

In a group of short SGA children, we studied SGA_L, SGA_{L+W} and SGA_{L+W+HC} children, who were divided according to their birth length (L), birth weight (W) and head circumference (HC) at birth ≤ -2.00 standard deviation score (SDS) (1). SGA_{L+W+HC} children were the smallest at birth and thus likely to have experienced the most severe growth restriction during pregnancy. Although this might be partly explained by the definition of their group (birth length, birth weight and HC at birth ≤ -2.00 SDS), they had a considerably shorter birth length, weight and head circumference than expected. SGA_{L+W} children showed an intermediate pattern with regard to birth length and birth weight in comparison with SGA_L and SGA_{L+W+HC} children. Notably, SGA_{L+W+HC} had the greatest catch-up in height and HC during early postnatal life, although at the age of 3 years, HC SDS and weight SDS were still significantly lower than in those born SGA_L. The SGA subclassification could not predict a difference in growth response to GH treatment as a similar response to GH was found for all three groups. HC and weight of SGA_{L+W+HC} and SGA_{L+W} children, however, remained significantly below those of SGA_L children after one year of GH treatment.

SGA_L

Whereas SGA_L children had no postnatal increase in height and showed a marked decrease in weight and a decline in HC SDS, short SGA_{L+W} and SGA_{L+W+HC} children had a significant catch-up in height during early postnatal life. These differences might result from different genetic and environmental influences leading to SGA_L as compared to the SGA_{L+W} and SGA_{L+W+HC} children. SGA_L children were mostly born after a vaginal delivery, were less small at birth and showed predominantly a decrease in weight and head circumference SD-scores after birth. In fact, the growth pattern of SGA_L children resembles that of children with idiopathic short stature (ISS). It might well be that subtle skeletal abnormalities play a role in some short SGA_L children. For this group of SGA children it might be interesting to perform genetic research into genes involved in pre- and postnatal bone development. In 2006, Demple et al. reported linkage between ISS subjects and the vitamin D receptor (VDR) gene region [2]. Several other studies reported a sex-specific association between VDR polymorphisms and height in the normal population [3,4]. Although in these studies no gestational-age corrected birth size data were reported, these findings are interesting and a next step might be to investigate tagging SNPs of the VDR gene region in SGA_L children.

SGA_{L+W}

SGA_{L+W} children were characterized by their intermediate birth size and spontaneous postnatal growth in comparison with the SGA_L and SGA_{L+W+HC} children. The mothers of these children had the highest frequency of gestational hypertension which might explain their shorter gestational age and their spared HC at birth. This observation suggests that gestational hypertension might have resulted into fetal growth restriction of SGA_{L+W} children. Our data are in line with a previous report showing that the combination of a low weight and height at birth is most frequent after gestational hypertension [5]. We did however not observe that SGA_{L+W} children were more prone to have cardiovascular risk factors such as a higher catch-up in postnatal weight or BMI in comparison with the other groups [6-8].

SGA_{L+W+HC}

SGA_{L+W+HC} children were the smallest at birth, with regard to length, weight and HC. We expected to find a comparable or somewhat smaller birth length SDS than in the other children, as all groups had by definition a birth length SDS ≤ -2.00. We found, however, that SGA_{L+W+HC} children had a considerably shorter birth length than SGA_L and SGA_{L+W} children, despite of having the highest target height SDS of the groups. Interestingly, SGA_{L+W+HC} children had the greatest postnatal catch-up of all growth parameters (including HC), indicating that SGA_{L+W+HC} children might have had the most severe fetal growth restraint which disappeared after birth. Of note was the finding that 68% of SGA_{L+W+HC} children were delivered by caesarean section in contrast to SGA_L children of whom 76% were born after vaginal delivery. It is likely that the SGA_{L+W+HC} children experienced growth retardation which started early in pregnancy, also affecting head growth. The high frequency of elective caesarean section (CS) might be due to the severe growth retardation. Namely, when serious fetal growth restraint is observed, gynecologists have to consider the fetal and maternal consequences of an elective CS vs. acute CS vs. vaginal delivery. Whereas CS is known to be beneficial for the outcome of the child, vaginal delivery is more favorable for the mother. Thus, often an elective CS will be performed when the condition of the fetus is deteriorating. On the other hand, a Cochrane review on the management of delivery of the small baby, could not draw conclusions about the optimal method of delivery in case of a "small baby", as randomized trials are lacking [9].

Clinical implications and conclusions

We have shown that an SGA subclassification may provide a useful framework to explore the mechanisms underlying differences in the extent of spontaneous postnatal growth in short SGA children. Subclassification might also be useful for elucidating underlying genetic or

environmental causes of SGA and future risk profiles with regard to adult diseases. Whereas SGA_L children appeared to experience the least fetal growth retardation and postnatal height increment, SGA_{L+W} children were most affected by gestational hypertension which might suggest associations with cardiovascular risk profiles in later life. SGA_{L+W+HC} children were born with the smallest HC but experienced a major increase in HC postnatally. Although all three groups of short SGA children similarly increased their head circumference during GH treatment, this intervention seems most indicated for SGA_{L+W+HC} children, having the smallest head size at birth and during postnatal life. Our data merit further studies to evaluate whether interventions such as remedial teaching, to improve cognition during early infancy, or to start GH treatment at a very young age in SGA_{L+W+HC} children are beneficial for this SGA subgroup.

Studies investigating new methods to subclassify the heterogeneous SGA population are very limited. As short SGA children are known for their broad spectrum of pre- and postnatal growth, also during GH treatment, further studies are warranted.

Part 2. Genetic polymorphisms of the *IGF1*, Insulin and *IGF1R* gene

First, literature was investigated on genetic polymorphisms in the *IGF1* and *IGF1R* gene. In a next step, genetic polymorphisms in the *IGF1*, Insulin and *IGF1R* genes were investigated in Dutch SGA patients. By assessing linkage disequilibrium, haplotypes could be constructed to capture genetic variations in the genes. Genetic variations were associated with prenatal, postnatal and GH-induced growth. In addition, the association between the *INS* genetic variations and blood pressure was investigated.

Polymorphisms in the IGF1 and IGF1R gene and children born small for gestational age: Findings of large population studies

Although many *IGF1* gene polymorphisms have been studied, the 192bp CA-repeat in the promoter is the most extensively investigated polymorphism in relation with SGA-related outcomes. The region ranging from the promoter until intron 2 is part of a linkage disequilibrium block which is important in SGA-related outcomes as all association studies in SGA populations report associations with polymorphisms in this area [10,11]. Conflicting results arose especially in general populations and with associations between birth size measures and polymorphisms in this gene region [12-15]. There was only one study evaluating associations between *IGF1R* polymorphisms and SGA-related outcomes, which could not demonstrate any association [16]. Limited gene-gene interaction studies have been performed with SGA-related outcomes and no associations were found [17]. None of these genetic association studies are currently suitable as a diagnostic measure because no therapeutic outcome has been identified yet.

Clinical implications and conclusions

Although several studies reported associations between *IGF1* gene polymorphisms and SGA-related outcomes, results are conflicting [10-12,14,15,17-22]. It is unclear whether this is the result of small sample sizes, population stratification or the different definitions of SGA. In addition, few studies have reported *IGF1R* genetic polymorphisms and SGA-related outcomes. Studies are needed on a methodological, phenotypic and genotypic level. First, an increase in the SGA sample size should be obtained by collaboration within international research consortia. Secondly, further phenotyping of SGA children should be performed, e.g., based on etiological factors, as a starting point for studies into genetic polymorphisms. Thirdly, as the *IGF1* 192bp CA repeat region has shown to be closely linked to the SGA phenotype, studies into the effect of this polymorphism on protein level are needed. At last, these studies into the *IGF1* and *IGF1R* gene have shown to explain limited aspects of the SGA phenotyope, so studies are needed to

identify other candidate genes, e.g. by a genome wide approach, to identify additional, important genes related to the SGA phenotype.

IGF1 gene

Genetic polymorphisms in the IGF1 gene and head size of short SGA children

Short SGA children were investigated for four *IGF1* gene polymorphisms in the promoter (-G1245A, -841(CA)$_n$), intron 2 (+3703(CT)$_n$) and 3UTR (+A1830G). The well-known -841(CA)$_n$/192bp polymorphism in the *IGF1* gene promoter, has been previously associated with birth size, cardiovascular disease and IGF-I levels, and is in linkage disequilibrium with the -G1245A SNP (rs35767). Our study showed that the -1245 A-allele was a marker-allele for the well-known -841(CA)$_n$/ non192bp-allele. The -1245 A-allele was not associated with head circumference at birth, but was associated with a postnatal 0.3 SDS smaller head circumference at 1 and 2 years of age. The -1245 A-allele was also associated with a 1 week shorter gestational age. No associations were found with gestational age-adjusted birth size, height and weight SDS during postnatal life and with growth during GH treatment. These results are in line with previous studies showing an association between the *IGF1* gene promoter and the SGA phenotype, and an association between an *IGF1* gene polymorphism and postnatal head size (10, 11). Other studies could not demonstrate an association between the -841(CA)$_n$ polymorphism and head size, however, these studies have predominantly been executed in normal and diabetic populations [12,15,23].

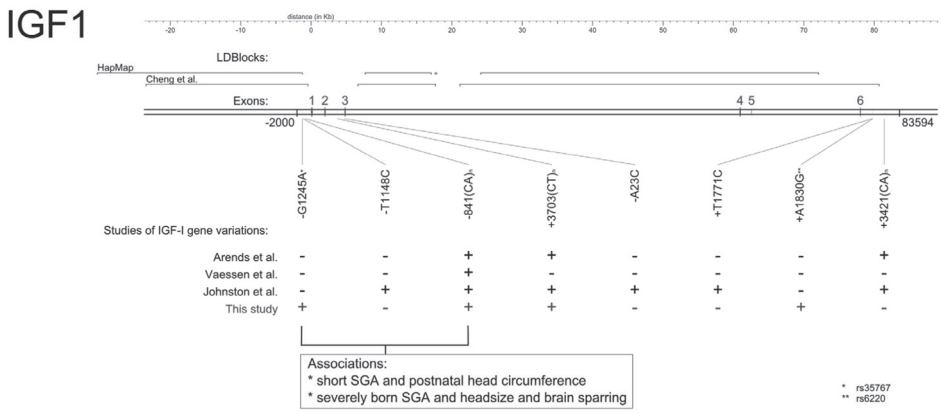

Figure 1. Associations between *IGF1* gene variations and head size in our SGA populations.

The –G1245A IGF1 polymorphism relates with small head size and less brain sparing in short SGA and SGA catch-up children

We investigated whether the –G1245A SNP (rs35767) was associated with head circumference and brain sparing in a large cohort of 439 short SGA and 196 SGA catch-up subjects. All SGA subjects had a postnatal head size below the population mean. Whereas SGA catch-up subjects had a head size which was in proportion with their height, short SGA subjects displayed extensive brain sparing. The most severely SGA born subjects had a smaller postnatal head size and less brain sparing when carrying the -1245 A-allele, in contrast to G-allele carriers.

Previous studies have shown that the *IGF1* gene plays an important role in brain growth [24,25]. Lee et al]24[demonstrated that transgenic mice overexpressing *IGF1* could ameliorate brain growth retardation caused by undernutrition, which resulted in a comparable brain growth as the well-fed control mice. A reduced *IGF1* gene expression has also been associated with a reduced brain size in mice whereas *IGF1* overexpression gave rise to increased brain growth, although the IGF-I level in brain tissue was among the lowest one measured in all body tissues [26, 27]. This might also be applicable to short SGA subjects having the -1245 A-allele which might result in less local IGF-I expression in the brain and a smaller head size. Altogether these observations suggest that some short SGA subjects may experience a differential, tissue-dependent *IGF1* gene expression which influence their head size.

Clinical implications and conclusions

Previous studies demonstrated that short stature and a small head size are important predictors for subnormal intellectual performance [28-30]. We have shown that a small birth size in combination with the -1245 A-allele in the *IGF1* gene promoter, was asso-ciated with the smallest head circumference and less brain sparing in SGA subjects, and especially in short SGA subjects. These data implicate that the -G1245A SNP might be an additional factor determining subnormal intellectual performance in these short SGA children. Studies are needed to investigate the association between the -1245 A-allele and intellectual performance in SGA children. Our findings warrant further research into this gene region. The -G1245A SNP is an easy to determine gene variant by Taqman assay compared to the -841$(CA)_n$/192bp VNTR which is mainly determined by a more laborious technique to analyze VNTRs (Genescan).

***IGF1R* gene**

Genetic polymorphisms in the IGF1R gene and postnatal weight

The insulin-like growth factor 1 receptor (*IGF1R)* gene plays an important role in pre- and postnatal growth and its promoter has multiple transcription factor binding sites affecting *IGF1R*

protein expression. We investigated whether the rs4965425 and rs1319868 polymorphisms in the *IGF1R* gene promoter were associated with postnatal growth in a large population of 635 SGA subjects. Short SGA subjects with the rs4965425 C-allele had a lower weight and BMI due to postnatal catch-down in weight compared to non-carriers. This was most pronounced in those who were born with both a birth weight and birth length < -2.00 SDS. Our data show that the *IGF1R* rs4965425 promoter SNP might have a role in modifying postnatal weight, thereby potentially influencing the risk for cardiovascular disease. Our study was the first association study investigating *IGF1R* promoter polymorphisms in SGA subjects.

Clinical implications and conclusions

Our findings demonstrate that genetic variations in the *IGF1R* gene promoter associate with postnatal loss in weight of short SGA subjects. This was most pronounced in those who were born with both a birth weight and birth length < -2.00 SDS. These data stimulate further research into the identification of predictors of postnatal weight development in SGA children.

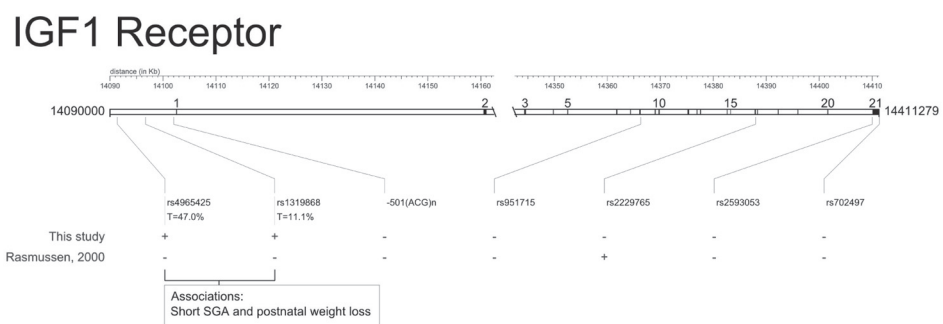

Figure 2. Associations between *IGF1R* gene variations and weight development in our SGA populations.

***INS* gene region**

Genetic polymorphisms in the INS gene region and blood pressure

The *INS* gene region is an important region with regard to fetal growth, coronary heart disease and hypertension [31-39], although also conflicting results have been reported [40-43]. Three polymorphisms in the insulin gene region were studied, the rs7924316 SNP, the rs3842748 SNP and the insulin VNTR (rs689). Our data show that SGA subjects have a higher blood pressure when carrying the class I *INS* VNTR and especially, the rs7924316 G-allele which is the major allele. Although rapid postnatal growth is a well-known factor associated with a higher blood pressure in later life [44,45], our study showed that there is also a polymorphism in the *INS* gene region which has an independent influence on blood pressure in SGA subjects, next to

rapid postnatal growth. Our results are in line with previous studies showing that children with a postnatal catch-up in weight (> 0.67 SDS), had a higher BMI at age 7 when having the class I-allele vs. the class III/III genotype [37].

Clinical implications and conclusions

Our data showed that the rs7924316 SNP is an independent contributing factor in blood pressure regulation of subjects born SGA, particularly in those with postnatal weight gain. This study shows, for the first time, that there is a polymorphism in the *INS* gene region which influences blood pressure in SGA subjects. Further studies are needed to investigate the influence of the rs7924316 G-allele on protein expression, before a final conclusion can be drawn and clinical implications can be addressed.

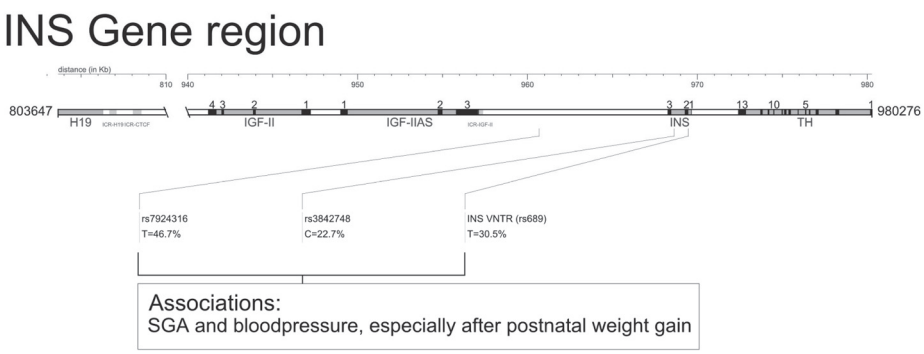

Figure 3. Associations between *INS* gene region variations and blood pressure in our SGA populations.

Part 3. Copy number variants of growth-related genes in short SGA subjects

Relatively large deletions and duplications in 18 growth-related genes

We investigated whether copy number variations in growth-related genes (*SHOX, GH1, GHR, IGF1, IGF1R, IGF2, IGFBP1-6, NSD1, GRB10, STAT5B, ALS, SOCS2, SOCS3*) were present in a group of 100 children born SGA with persistent short stature. Our data showed that *IGF1R* haploinsufficiency was present in 2 out of 100 short SGA children and that no other copy number variants were found in the 17 other growth-associated genes in the rest of the population.

Short SGA children with IGF1R haploinsufficiency

One patient had a heterozygous deletion of the complete *IGF1R* gene (15q26.3, exon 1-21) including distally flanking sequences. The other patient was heterozygous for a deletion comprising exon 3-21 of the *IGF1R* gene which extended into the telomeric region. Both children moderately increased their height during GH treatment. They both had a relatively mild phenotype compared to other patients who were reported with a terminal *IGF1R* deletion. The latter patients had lung hypoplasia, atrial and/or ventricular septum defects, hypoplastic left atrial or ventricular heart, dextrocardia and diaphragmatic hernia [46-52]. We believe that these clinical signs are primarily linked to other genes in the area, as in children with an *IGF1R* mutation a much smaller number of additional characteristics have been observed [53-56]. Poot et al indicated *IGF1R* flanking genes which might be responsible for the phenotypic variation in children with an *IGF1R* deletion [47].

Clinical implications and conclusions

Our study showed that *IGF1R* haploinsufficiency was present in 2 out of 100 short SGA children. The combination of a small birth size, short stature, small head size, a relatively high IGF-I level, developmental delay and micrognathia is suggestive for an *IGF1R* deletion in SGA children. We recommend that children with these characteristics are tested for an *IGF1R* deletion, especially because GH therapy has shown to have a beneficial effect on their height. Multiplex ligation-dependent probe amplification (MLPA) has shown to be a valuable tool in identifying these deletions.

Part 4. Phenotypic characteristics of short SGA, SGA catch-up and ISS subjects

Spontaneous and GH-induced growth of short SGA, SGA catch-up and ISS subjects: relation with gestational hypertension, smoking and alcohol. The Network of Studies into Genes in Growth (NESTEGG)

The network of studies into genes in growth (NESTEGG) is a growth genomics project in which subjects born small for gestational age (SGA) with and without a postnatal short stature, subjects with idiopathic short stature (ISS) and controls are investigated. Our study showed that birth length and postnatal height were significantly different between short SGA, SGA catch-up, ISS and control subjects. Short SGA subjects increased 0.2 SDS more in height than ISS subjects during 1 year of GH treatment. Postnatal catch-up growth of short SGA subjects was most pronounced when their mothers had gestational hypertension. Gestational hypertension, smoking and alcohol use were not related to the ISS phenotype although maternal smoking during gestation was associated with less postnatal growth decline and more growth during 1 year of GH treatment. These observations are in line with previous studies which have shown that gestational hypertension and maternal smoking both result in prenatal growth restriction [57,58]. Other support comes from studies which show that although gestational hypertension and maternal smoking have a different mode of action, both would result in maternal endothelial dysfunction and abnormal placentation, which are common features in fetal growth restriction [58-61].

Clinical implications and conclusions

Our study has shown that prenatal growth restriction of SGA subjects is related to gestational hypertension and smoking. Furthermore, we have described for the first time the growth-phenotype of short SGA, SGA catch-up and ISS subjects in a large, European study. Hereby, it is in a next step, possible to identify genes that contribute to foetal or postnatal growth. These genetic factors may allow for a more specific diagnosis and may be the base of more targeted treatment possibilities. In addition, novel genetic factors affecting growth might give rise to new treatment modalities.

General conclusions

The studies described in this thesis were performed to obtain insight in genetic and environmental factors which are important in pre- and postnatal growth disorders and specifically in children born small for gestational age (SGA) with or without postnatal catch-up growth. We studied frequent genetic polymorphisms, and low frequent copy number variants. Our data show that these polymorphisms had limited relations with the SGA-related phenotype. The study into relatively large deletions and duplications in 18 growth-related genes, illustrated that only 2 out of 100 short SGA children had such a copy number variant. Their phenotypes were not related to extreme fetal and postnatal growth. Our study concerning gestational factors showed that gestational hypertension and smoking were related to both short SGA and SGA catch-up children. The extent of postnatal catch-up growth was partially related to these gestational factors. In conclusion, the studies in this thesis have shown that diverse genetic and environmental factors play a role in SGA and postnatal catch-up growth, making them so-called complex genetic phenomena.

In the first part of this thesis, we showed that subclassification of SGA based on birth length, birth weight and head circumference at birth, may provide a useful framework to explore the mechanisms underlying differences in the extent of spontaneous postnatal growth that are observed in a population of short SGA children. Such a subclassification of short SGA children might also be useful for elucidating underlying genetic or environmental causes of SGA and future risk profiles with regard to adult diseases.

The second part of this thesis demonstrated that a small birth size in combination with the -1245 A-allele of the *IGF1* promoter polymorphism, was associated with the smallest head circumference and less brain sparing in the group of SGA subjects, and especially in short SGA subjects (Figure 1). In addition, the C-allele of the rs4965425 SNP in the *IGF1R* gene promoter, associated with postnatal catch-down in weight development of short SGA subjects. This was most pronounced in those who were born with both a birth weight and birth length < -2.00 SDS (Figure 2). The *INS* rs7924316 SNP turned out to be a contributing factor in blood pressure regulation of subjects born SGA, particularly in those with postnatal catch-up in weight (Figure 3).

The third part of this thesis reports that *IGF1R* haploinsufficiency was present in only 2 out of 100 short SGA children. None had deletions or duplications in the other 17 genes. The combination of a small birth size, short stature, small head size, a relatively high IGF-I level, developmental delay and micrognathia was suggestive for an *IGF1R* deletion. This is especially relevant as our study showed that GH treatment had in contrast to our expectation, a beneficial

effect on the linear growth of these children. Our study also showed that SGA children with a telomeric *IGF1R* deletion display a heterogeneous phenotype.

Finally, our study showed that prenatal growth restriction of SGA subjects is related to gestational hypertension and smoking. Postnatal catch-up growth of short SGA subjects was most pronounced when their mothers had gestational hypertension. Gestational hypertension, smoking and alcohol use were not related to the ISS phenotype.

Considerations and directions for future research

Subgrouping of SGA children would increase our knowledge of the variability of the SGA phenotype, helping to thereby identify potential predictors which may finally lead to improved patient care. It is unknown whether SGA subgroups, either based on dysmorphic features, intelligence or response to GH treatment, indicate the etiology of the initial growth disorder.

Genetic studies into polymorphisms in genes of SGA children can be performed by candidate-gene and genome-wide approaches. Currently, candidate-gene approaches have not been able to find important genetic variations in the genes which may serve as predictors for postnatal growth and metabolism. This might be due to the very heterogeneous phenotype of subjects born SGA. It is likely that when genome-wide approaches are used, the same problem will occur. Therefore we believe that a very detailed phenotypic description is mandatory to perform focused genetic research in SGA subjects. Both candidate-gene and genome-wide approaches might be tools to identify genes which are specific for subgroups of SGA children.

Genetic studies into mutations and large copy number variants may follow a different approach. Our study on copy number variants has shown that the unrestricted inclusion of short SGA children was beneficial, as the children with an *IGF1R* deletion did not appear to have an aberrant growth pattern compared to other short SGA children.

Pharmacogenetic studies might be interesting to perform in order to clarify the genetic background of the wide variation in growth response to GH therapy in these children. Both quantitative and qualitative genome-wide approaches can provide new insights in this phenomenon. A quantitative approach, like gene expression profiling, requires however clarity on the choice which tissue type and which time moment in growth should be taken, to investigate the response to GH therapy. These methodological issues should first be clarified before further steps can be taken.

Epigenetic studies have been previously performed in the *H19-IGF2-INS* gene region in children with Silver-Russell syndrome as well as in short SGA children [62,63]. It might well

be that detailed phenotypic studies regarding dysmorphic features in short SGA children will identify Silver-Russell-like phenotypes in the short SGA population. A second approach would be to identify differential methylation of the *H19-IGF2-INS* gene region, which may be related to the various Silver-Russell-like phenotypes in the short SGA population.

In conclusion, the studies described in this thesis aimed to investigate differences in phenotype, growth, genetic variations and deletions, in children born small for gestational age (SGA), either with or without short stature. These and future studies can improve our knowledge regarding the etiology of SGA and might generate predictors of spontaneous and GH-induced growth. This will eventually lead to better counseling and treatment strategies.

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Chapter 11

Summary

Chapter 1

This chapter gives an overview of the definitions, prevalence and etiology of SGA born children with and without postnatal catch-up growth. In addition, the *IGF1*, *IGF1R* and *INS* gene region are described. Several clinical and endocrinological aspects associated with SGA are pointed out, including the GH-IGF-IGFBP axis, fetal and postnatal growth, short stature and cardiovascular risk factors. It also presents the Dutch genetic study and the Network of European Studies in Genes in Growth (NESTEGG). Than, the investigated genetic variations, strategy and techniques in the Dutch study are summarized. At the end of this chapter, the aims and outline of the thesis are presented.

Chapter 2

Short SGA children appear to benefit from growth hormone (GH) treatment in terms of height increment. However, even after accounting for differences in parental height, age and duration of treatment, there appears to be a wide variation in the response to GH treatment that has been difficult to explain. We investigated whether subclassification of short SGA children according to their birth anthropometrics could delineate differences in gestation, type of delivery, postnatal growth, response to GH treatment and parental height.

SGA_{L+W+HC} children were born after the shortest gestational age and more often by caesarean section than SGA_{L} children (36.3 vs. 38.1 weeks, 68.4 vs. 24.4%). SGA_{L+W} children had an intermediate pattern and experienced most gestational hypertension ($P = 0.01$). At birth, SGA_{L+W+HC} children were shorter than SGA_{L} or SGA_{L+W} (-4.12 vs. -2.67 and -3.72 SDS, $P \leq 0.001$). During the first 3 years of life, SGA_{L+W+HC} children exhibited a greater catch-up in height (0.98 SDS) and HC (1.28 SDS) compared to SGA_{L} (height, -0.06 SDS; HC, -0.30 SDS) and SGA_{L+W} (height, 0.62 SDS; HC, -0.31 SDS). However, HC SDS remained smaller for SGA_{L+W+HC} than the other groups at age 3. The groups did not differ in growth response during GH treatment. SGA_{L} children tended to have shorter parents and target height than SGA_{L+W+HC} children.

Our study suggests that subclassification of SGA children with persistent short stature according to their birth length, weight and HC, may provide a useful framework to explore the mechanisms underlying differences in the extent of spontaneous postnatal growth. Such a subclassification of short SGA children might also be useful for elucidating underlying genetic or environmental causes of SGA and future risk profiles with regard to adult diseases.

Chapter 3

In this review, we performed a literature search into genetic association studies of the *IGF1* and *IGF1R* gene and SGA-related outcomes. Also, linkage disequilibrium and haplotype studies in the *IGF1* and *IGF1R* gene and gene-gene interaction studies were investigated.

This review showed that many *IGF1* polymorphisms have been studied but that the 192bp CA-repeat in the *IGF1* promoter was most likely to associate with SGA-related outcomes. The region ranging from the promoter until intron 2 is part of a linkage disequilibrium block which is important in SGA-related outcomes as all association studies in SGA populations report associations with polymorphisms in this area. Conflicting results arise especially in "normal" cohorts and with associations between birth size measures and polymorphisms in this gene region. There was only one study which evaluated associations between *IGF1R* polymorphisms and SGA-related outcomes such as birth size and insulin sensitivity. In this study, no associations could be demonstrated. Limited gene-gene interaction studies have been performed with SGA-related outcomes and no associations were found. None of these genetic association studies are currently suitable as a diagnostic measure because no therapeutic outcome has been identified yet.

Chapter 4

It was unknown whether the *IGF1* gene 192bp polymorphism associated with gestational age, birth head circumference and postnatal growth in short SGA children. Also it was unknown whether the 192bp allele associated with the response to GH treatment and whether nearby genetic variants might function as a genetic marker for the 192bp allele. We evaluated whether anthropometric measurements at birth, during postnatal growth, and during GH treatment, were associated with *IGF1* gene polymorphisms and haplotypes in a population of 201 short SGA children. The -1245 A-allele was identified as a marker-allele for the well-known -841(CA)$_n$/ non192bp-allele, both part of Haplotype 2. The -1245 A-allele was not associated with head circumference at birth, but was associated with a postnatal 0.3 SDS smaller head circumference at age 1-3. The -1245 A-allele was also associated with a 1 week shorter gestational age which explained the association with a smaller absolute birth size. No associations were found with gestational age-adjusted birth size, height and weight SDS during postnatal life and with growth during GH treatment. In conclusion, the -1245 A-allele in the *IGF1* promoter was the only polymorphism which associated with a smaller head circumference SDS during spontaneous postnatal growth and a shorter gestational age. No other associations were found with birth size and growth, neither spontaneously, nor during GH treatment. The –G1245A SNP appeared to be a marker for the well-known -841(CA)$_n$/192bp polymorphism.

Chapter 5

It was unknown whether the *IGF1* 192bp gene polymorphism was related to head size and brain sparing in short SGA versus SGA catch-up subjects. In addition, it was unknown whether SGA severity modifies the relation between brain sparing and this genetic polymorphism. We investigated 635 SGA subjects of which 439 remained short and 196 had a postnatal height > -2.00 SDS for the association between the -G1245A SNP *IGF1* gene polymorphism and head size. All SGA subjects had a postnatal head size below the population mean (-1.01 SDS, P<0.001). Whereas SGA catch-up subjects had a head size which was in proportion with their height, short SGA subjects displayed extensive brain sparing (HC SDS minus height SDS: SGA CU: 0.01 vs. short SGA: 1.75, P<0.001). The most severely SGA born subjects had a 0.4 SDS smaller postnatal head size and 0.6 SDS less brain sparing when carrying the -1245 A-allele in contrast to G-allele carriers (P = 0.03). The association between the -G1245A SNP and head size remained significant after correction for birth weight SDS and postnatal height SDS (P = 0.03). Birth weight SDS, birth length SDS and postnatal height SDS were not related with the -G1245A SNP. In conclusion, we observed that the most severely SGA born subjects had a 0.4 SDS smaller head size and 0.6 SDS less brain sparing when carrying the -1245 A-allele in contrast to G-allele carriers, which might be of clinical relevance. Our study has shown for the first time that the -G1245A SNP is related to postnatal head size in SGA subjects regardless of their postnatal growth.

Chapter 6

It was unknown whether *IGF1R* gene polymorphisms in the promoter were associated with postnatal growth of SGA subjects. We investigated whether haplotypes of two single nucleotide polymorphisms (SNPs) in the *IGF1R* gene promoter, rs4965425 and rs1319868, were associated with postnatal growth in 635 SGA subjects (439 short SGA, 196 catch-up SGA). The rs4965425 and rs1319868 SNPs were in complete linkage disequilibrium (D' = 1.00, R^2 = 0.14). The minor allele frequency of the rs1319868 SNP was significantly higher in the SGA population compared to controls. Haplotype 1, consisting of the rs4965425 C-allele and rs1319868 G-allele, was associated with a 0.4 SDS lower weight, BMI and catch-down in weight in short SGA subjects. Short SGA subjects carrying Haplotype 1 and with a birth length and -weight < -2.00 SDS, had a 0.8 SDS lower postnatal weight and BMI, and the largest postnatal catch-down in weight compared to non-carriers (P = 0.002, P = 0.002 and P = 0.02, respectively). The rs4965425 C-allele drove these associations. Growth of SGA catch-up subjects was not associated with any SNP. In conclusion, short SGA subjects with the rs4965425 C-allele have a lower weight and BMI due to postnatal catch-down in weight compared to non-carriers. This was most pronounced

in those who were born with both a birth weight and birth length < -2.00 SDS. Our findings suggest that genetic variations in the IGF1R gene promoter contribute to weight development of short SGA subjects. Studies are warranted to identify the risk for cardiovascular disease in short SGA rs4965425 T-allele carriers.

Chapter 7

The INS gene region has been recognized as an important region in relation to diseases in later life. The insulin minisatellite (INS VNTR, rs689) class III, has been associated with variations in birth size, obesity, type I and type II diabetes mellitus and polycystic ovary syndrome. However, other studies could not demonstrate that the INS VNTR was associated with accelerated weight gain, reduced beta cell function or adiposity. We determined whether haplotypes of three polymorphisms in the insulin gene region, rs7924316, rs3842748 and the INS VNTR, associate with blood pressure in 635 SGA subjects (439 short SGA and 196 SGA catch-up subjects).

All polymorphisms were in high linkage disequilibrium (D' = 96-100, R^2 = 32-68). SGA catch-up subjects had a significantly higher rs7924316 T-allele (P = 0.007) and rs689 T-allele (P = 0.03) frequency than short SGA subjects. The association between the rs7924316 and the *INS* VNTR and systolic blood pressure was most pronounced in SGA catch-up subjects (P=0.003 and P = 0.007). These associations were not seen in short SGA subjects. SGA catch-up subjects with Haplotype 1, consisting of the rs7924316 G-allele, rs3842748 G-allele and the class I *INS* VNTR, had a 0.6 SDS higher systolic and 0.5 SDS higher diastolic blood pressure. The rs7924316 G-allele drove these associations. The rs7924316 and the *INS* VNTR were associated with systolic blood pressure in the total SGA population (P = 0.05 and P = 0.007) and these associations remained significant after correction for change in weight and age. The association of the rs7924316 and the *INS* VNTR with blood pressure, was most pronounced in subjects with postnatal weight gain (> 0.67 SDS: P = 0.006 and P =0 .01). Our study has shown that the rs7924316 SNP is a contributing factor in blood pressure development of subjects born SGA, particularly in those with postnatal weight gain. This study shows for the first time that there is a polymorphism in the *INS* gene region which independently influences blood pressure in SGA subjects.

Chapter 8

Genetic causes have only been found in a very small proportion of short SGA children, including point-mutations and deletions in the *IGF1* and *IGF1R* genes. The availability of the complete sequence of the human genome and the introduction of high throughput DNA scanning techniques provides us with novel tools to investigate the genetic basis of short stature. In this

study we used MLPA to rapidly investigate whether copy number variations in growth-related genes (*SHOX, GH1, GHR, IGF1, IGF1R, IGF2, IGFBP1-6, NSD1, GRB10, STAT5B, ALS, SOCS2, SOCS3*) were present in a group of 100 children born SGA with persistent short stature. We found two short SGA children with a *de novo IGF1R* deletion. No deletions or insertions were found in the other genes. Patient A had a heterozygous deletion of the complete *IGF1R* gene (15q26.3, exon 1-21) including distally flanking sequences. Surprisingly, she also had a low IGF-I level (-2.78 SDS), probably because of a co-existing growth hormone (GH) deficiency. In the DNA of patient B, heterozygosity for a deletion comprising exon 3-21 of the *IGF1R* gene was detected which extended further into the telomeric region. Both children increased their height during GH treatment, with a GH dose of 1 mg/m^2/day (Patient A: after 4 yrs GH: delta height +1.65 SD; Patient B: after 4 yrs GH: delta height +1.69 SD) and continue to receive GH until their adult height will be attained.

We have shown that *IGF1R* haploinsufficiency was present in 2 out of 100 short SGA children. This study also illustrated that the heterogeneity in phenotype in case of *IGF1R* haploinsufficiency. The combination of a small birth size, short stature, small head size, relatively high IGF-I level, developmental delay and micrognathia is suggestive for an *IGF1R* deletion. Since GH therapy leads to a moderate catch-up growth of ~1.0 SD in the first year, we recommend that an *IGF1R* deletion is ruled out in SGA children with a persistent short stature. Particularly, if small birth size, short stature, small head size, relatively high IGF-I level, developmental delay and micrognathia are present. The multiplex ligation-dependent probe amplification (MLPA) has shown to be a valuable tool in rapidly identifying these relatively large deletions in short SGA children.

Chapter 9

Previous studies have shown that gestational hypertension, maternal smoking and gestational alcohol influence pre- and postnatal growth. However, it is unknown to what extent these gestational factors influence spontaneous postnatal and GH-induced growth in short SGA, SGA catch-up, ISS and control subjects. To determine postnatal and GH-induced growth and their relation with maternal hypertension, smoking and alcohol during gestation, 464 short SGA, 336 SGA catch-up, 464 ISS and 724 control subjects were investigated. GH treatment data were available of 262 short SGA and 109 ISS prepubertal subjects. Birth length and postnatal height were significantly different between short SGA, SGA catch-up, ISS and control subjects (short SGA: -2.88 vs. -2.82 SDS, SGA catch-up: -2.50 vs. -0.49 SDS, ISS: -0.59 vs. -2.49 SDS and controls: -0.01 vs. 0.05 SDS, respectively). Short SGA subjects increased 0.2 SDS more in height than ISS subjects during 1 year of GH treatment ($P < 0.001$). Mothers of SGA catch-

up subjects had more gestational hypertension than mothers of short SGA, ISS and control subjects (27.9% vs. 16.4%, 6.0% and 12.3%, P < 0.001). Mothers of short SGA subjects smoked more during gestation than mothers of ISS and control subjects (35.1% vs. 20.3% and 23.5%, P<0.001). Short SGA subjects increased more in height when their mothers had gestational hypertension compared to short SGA subjects without (spontaneous growth: 0.64 SDS vs. -0.06 SDS, P < 0.001).

In conclusion, our study has shown that prenatal growth restriction and postnatal growth of SGA subjects are related to gestational hypertension and smoking. Postnatal catch-up growth of short SGA subjects was most pronounced when their mothers had gestational hypertension. Gestational hypertension, smoking and alcohol use were not related to the ISS phenotype although maternal smoking during gestation was associated with less postnatal growth decline and more growth during 1 year of GH treatment.

Chapter 10

In the general discussion, we discuss our findings in relation to the current literature. We end this chapter with general conclusions and suggestions for future research.

Chapter 12

Summary in Dutch

Samenvatting

Hoofdstuk 1

Dit hoofdstuk beschrijft de prevalentie van kinderen die met een te kleine lengte en/of laag gewicht ten opzichte van de zwangerschapsduur werden geboren (SGA) en die daarna ofwel te klein zijn gebleven ofwel een lengte binnen het "normale" bereik kregen. Tevens worden de definities en mogelijke oorzaken van SGA geboorte beschreven. Vervolgens worden het insuline-achtige groeifactor1 gen (IGF1), het IGF1-receptor gen (IGF1R) en de insuline gen regio besproken, zo ook klinische en endocrinologische aspecten die met SGA samenhangen zoals, de GH-IGF-IGFBP as, foetale en postnatale groei, een te kleine lengte en risicofactoren voor hart- en vaatziekten. Ook de Nederlandse en de Europese studie (NESTEGG) naar genen en groei worden gepresenteerd. Tenslotte wordt een overzicht gegeven van de doelstellingen van dit proefschrift en wordt de opzet van de Nederlandse genetische studie en de NESTEGG studie besproken, zo ook de belangrijkste genetische technieken die in de studies werden toegepast.

Hoofdstuk 2

SGA geboren kinderen met een te kleine lengte (short SGA) werden geclassificeerd aan de hand van de geboortelengte, het geboortegewicht en de hoofdomtrek bij de geboorte. De samenhang tussen deze classificatie en factoren als: de zwangerschapsduur, de bevalling, de postnatale groei, de groei tijdens groeihormoon (GH) behandeling en de lengte van de ouders, werden onderzocht. Kinderen met zowel een geboortelengte, geboortegewicht als hoofdomtrek bij de geboorte onder de -2,0 standaard deviatie score (SDS) (SGA_{L+W+HC}), werden na een kortere zwangerschapsduur geboren en vaker na een keizersnede dan kinderen die alleen kort waren bij de geboorte (36,3 weken versus 38,1 weken en 68,4% versus 24,4%) (SGA_{L}). Kinderen met zowel een korte geboortelengte als een laag geboortegewicht (SGA_{L+W}) toonden een tussenliggend patroon en hun moeder had het meest frequent zwangerschapshypertensie (P=0,01). Bij de geboorte waren SGA_{L+W+HC} kinderen korter dan de SGA_{L} en SGA_{L+W} kinderen (-4,12 versus -2,67 en -3,72 SDS, $P \leq 0,001$). Tijdens de eerste drie levensjaren, hadden SGA_{L+W+HC} kinderen een betere groei in lengte (0.98 SDS) en hoofdomtrek (1,28 SDS) in vergelijking met SGA_{L} (lengte, -0,06 SDS; hoofdomtrek, -0,30 SDS) en SGA_{L+W} (lengte, 0,62 SDS; hoofdomtrek, -0,31 SDS) kinderen. Desalniettemin bleef de hoofdomtrek van SGA_{L+W+HC} kinderen kleiner dan die van de andere groepen op de leeftijd van 3 jaar. De groepen verschilden niet in hun groeirespons tijdens GH-behandeling. SGA_{L} kinderen hadden niet-significant kleinere ouders en een kleinere streeflengte dan SGA_{L+W+HC} kinderen.

Een onderverdeling van kleine SGA kinderen op basis van de geboortelengte, het geboortegewicht en de hoofdomtrek bij de geboorte, is een methode om mechanismen die verschillen in spontane groei veroorzaken, te onderzoeken. Deze onderverdeling van kleine

SGA kinderen maakt het mogelijk om onderliggende erfelijke- en omgevingsfactoren, die een rol spelen bij het SGA geboren worden, te bestuderen. Dit betreft ook eventuele risico's die deze kinderen hebben op het ontstaan van ziekten in het volwassen leven.

Hoofdstuk 3

In dit hoofdstuk wordt een literatuuronderzoek beschreven naar genetische associatie studies van het *IGF1* en *IGF1R* gen en SGA-gerelateerde uitkomsten. Dit betrof ook resultaten van "linkage disequilibrium" (LD) en haplotypes van deze genen, alsmede gen-gen interacties. Het literatuuronderzoek laat zien dat de zogenaamde "192bp CA-repeat" in de *IGF1* promoter frequent is geassocieerd met SGA-gerelateerde uitkomsten. De promoter regio tot aan intron 2 is onderdeel van een LD-blok waarmee frequent associaties met groei-gerelateerde uitkomsten worden beschreven. Tegenstrijdige resultaten worden met name bij "normale" onderzoekspopulaties gezien en bij associaties met het absolute gewicht bij de geboorte. Associaties tussen genetische polymorfismen in het *IGF1R* gen en SGA-gerelateerde uitkomsten werden slechts eenmaal onderzocht, deze konden toen niet worden aangetoond.

Hoofdstuk 4

Het was onbekend of het zogenaamde "192bp CA-repeat" polymorfisme in de *IGF1* promoter, geassocieerd is met de zwangerschapsduur, geboorte hoofdomtrek en postnatale groei van kleine SGA kinderen. Het was ook onbekend of het zogenaamde "192bp CA-repeat" polymorfisme, geassocieerd was met de groeirespons tijdens GH-behandeling, en of genetische polymorfismen die nabij gelegen zijn, als een marker voor dit polymorfisme kunnen dienen. Wij onderzochten of de lengte, het gewicht en de hoofdomtrek bij de geboorte, de spontane postnatale groei en de groei tijdens GH-behandeling geassocieerd waren met *IGF1* gen polymorpfismen en haplotypes in 201 te kleine SGA kinderen. Het A-allel van de –G1245A SNP bleek een marker-allel te zijn voor het "192bp CA-repeat" polymorfisme, en beiden waren onderdeel van Haplotype 2. Het -1245 A-allel was niet geassocieerd met de hoofdomtrek bij de geboorte maar wel met een 0,3 SDS kleinere hoofdomtrek op de leeftijd van 1-3 jaar. Het -1245 A-allel was ook geassocieerd met absolute geboortemetingen, hetgeen verklaard kon worden door een associatie tussen het -1245 A-allel en een één week kortere zwangerschapsduur. Er werden geen associaties gevonden met de geboorte metingen die waren gecorrigeerd voor de zwangerschapsduur, met de lengte en gewicht SDS tijdens het postnatale leven en met de groei tijdens GH-behandeling. Concluderend was het -1245 A-allel in de *IGF1* gen promoter het enige polymorfisme dat geassocieerd was met een kleinere hoofdomtrek tijdens de spontane postnatale groei en met een kortere zwangerschapsduur. Er werden geen andere associaties

gevonden met metingen bij de geboorte, de spontane postnatale groei en de groei tijdens GH-behandeling. De –G1245 SNP bleek een marker te zijn voor het bekende "192bp CA-repeat" polymorfisme.

Hoofdstuk 5

Het was onbekend of het zogenaamde "192bp CA-repeat" polymorfisme in de *IGF1* promoter, gerelateerd was aan de hoofdomtrek en de mate waarin het hoofd gespaard blijft van SGA geboren kinderen met een te kleine lengte (short SGA) of zonder een te kleine lengte (SGA catch-up). Een gespaard hoofd houdt in dat ondanks de verminderde (prenatale) groei van het lichaam, het hoofd een normale groei heeft weten te behouden waardoor het ook relatief groter is dan de rest van het lichaam is. Ook was het onbekend of de ernst van het SGA geboren zijn, een relatie had met de associatie tussen het genetische polymorfisme en de mate waarin het hoofd gespaard blijft. Wij onderzochten 635 SGA kinderen waarvan 439 een te kleine lengte hadden en 196 een inhaalgroei hadden tot een lengte boven de -2,0 SDS. Alle SGA kinderen hadden een postnatale hoofdomtrek onder het gemiddelde (-1,01 SDS, P<0,001). Terwijl SGA catch-up kinderen met een normale lengte na de geboorte een hoofdomtrek hadden die in proportie was met hun lengte, hadden SGA kinderen met een te kleine lengte een gespaard hoofd (hoofdomtrek-lengte: SGA catch-up: 0,01 versus short SGA: 1,75 SDS, P<0,001). De meest ernstig SGA geboren kinderen hadden een 0,4 SDS kleinere postnatale hoofdomtrek en een 0,6 SDS minder gespaard hoofd als ze het -1245 A-allel in plaats van het G-allel hadden (P=0,03). De associatie tussen het –G1245A polymorfisme en de hoofdomtrek bleef statistisch significant na correctie voor het geboortegewicht en de postnatale lengte SDS (P<0,001). De lengte en het gewicht bij de geboorte, en de postnatale lengte SDS bleken niet gerelateerd aan de –G1245A SNP.

Concluderend, de meest ernstig geboren SGA kinderen bleken een 0,4 SDS kleinere hoofdomtrek en 0,6 SDS minder gespaard hoofd te hebben als ze het -1245 A-allel hadden in tegenstelling tot -1245 G-allel dragers hetgeen klinische relevant is. Deze studie laat ook voor de eerste keer zien dat het –G1245A polymorfisme gerelateerd is aan de postnatale hoofdomtrek vam de totale groep SGA kinderen, onafhankelijk van de mate van postnatale lengtegroei.

Hoofdstuk 6

Het was onbekend of polymorfismen in de promoter van het *IGF1R* gen geassocieerd waren met de postnatale groei van SGA kinderen. We onderzochten of haplotypes van twee polymorfismen, de rs4965425 en rs1319868, in de *IGF1R* gen promoter geassocieerd waren met de postnatale

groei van 635 SGA kinderen waarvan 439 een te kleine lengte hadden en 196 een inhaalgroei hadden tot een lengte boven de -2,0 SDS. De rs4965425 en rs1319868 polymorfismen waren in compleet "linkage disequilibrium" (D'=1,00, R^2= 0,14). De frequentie van het minst voorkomende allel van rs1319868, was significant hoger in de totale SGA populatie dan in controles. Het rs4965425 C-allel was geassocieerd met een 0,3 SDS lager gewicht en "body mass index" (BMI) en lagere postnatale gewichtstoename in te kleine SGA kinderen, vergeleken met de kinderen die het T-allel hadden. Er waren geen associaties met rs1319868. De combinatie van het rs4965425 C-allel en het rs1319868 G-allel (Haplotype 1), was geassocieerd met een 0,4 SDS lager gewicht, BMI en een lagere postnatale gewichtstoename in te kleine SGA kinderen. Te kleine SGA kinderen met een geboortelengte en geboortegewicht ≤ -2,0 SDS, hadden een 0,8 SDS lager postnataal gewicht, BMI en lagere postnatale gewichtstoename, in vergelijking met niet-dragers (P= 0,002, P=0,002 en P=0,02, respectievelijk). Het rs4965425 C-allel dreef deze associaties. De groei van SGA kinderen met een normale lengte was niet geassocieerd met dit polymorfisme. Concluderend, te kleine SGA kinderen die drager zijn van het rs4965425 C-allel, hebben een lager gewicht en BMI door een lagere postnatale gewichtstoename vergeleken met niet-dragers. Dit was het meest uitgesproken in de kinderen die zowel een geboortelengte als een geboortegewicht onder de -2,0 SDS hadden. Onze bevindingen suggereren dat genetische variaties in de *IGF1R* gen promoter een rol hebben bij de ontwikkeling van het lichaamsgewicht van te kleine SGA kinderen. Onze studie laat zien dat onderzoek naar het risico op hart- en vaatziekten bij te kleine SGA kinderen met het rs4965425 T-allel, geïndiceerd is.

Hoofdstuk 7

De insuline gen regio is een belangrijke regio met betrekking tot genetische variaties en associaties met ziekten in het volwassen leven. De insuline minisatelliet *(INS* VNTR, rs689) klasse III is geassocieerd met geboorte antropometrie, obesitas, type I and type II diabetes mellitus en polycysteus ovarium syndroom. Desalniettemin konden andere studies geen relatie aantonen tussen de *INS* VNTR en een versnelde gewichtstoename, verminderde beta-cel functie of adipositas. Wij onderzochten of haplotypes van de drie polymorfismen in de insuline gen regio, rs7924316, rs3842748 and the *INS* VNTR, geassocieerd waren met de bloeddruk van 635 SGA kinderen waarvan 439 een te kleine lengte hadden en 196 een inhaalgroei hadden tot boven de -2,0 SDS. Alle polymorfismen waren in compleet "linkage disequilibrium" (D'= 96-100, R^2= 32-68). SGA kinderen met inhaalgroei tot boven de -2,0 SDS hadden een significant hogere rs7924316 T-allel (P=0,007) en rs689 T-allel (P=0,03) frequentie dan SGA kinderen met een te kleine lengte. De associatie tussen de rs7924316, de *INS* VNTR en systolische bloeddruk, was het meest uitgesproken in SGA kinderen met inhaalgroei tot boven de -2,0 SDS (P=0,003

en P=0,007). Deze associaties waren niet aanwezig bij de SGA kinderen met een te kleine lengte. SGA kinderen met een inhaalgroei tot boven de -2,0 SDS en Haplotype 1, hadden een 0,6 SDS hogere systolische en 0,5 SDS hogere diastolische bloeddruk. Het rs7924316 G-allel dreef deze associaties. Rs7924316 en de *INS* VNTR waren ook geassocieerd met de systolische bloeddruk in de totale SGA populatie (P= 0,05 en P= 0,007, respectievelijk), en deze associaties bleven significant na correctie voor de gewichtsverandering en de leeftijd. De associatie tussen de bloeddruk, rs7924316 en de *INS* VNTR, was het meest uitgesproken in kinderen met een postnatale gewichtstoename (>0,67 SDS: P=0,006 en P=0,01). Onze studie heeft laten zien dat het rs7924316 polymorfisme bijdraagt aan bloeddruk regulatie van kinderen die SGA zijn geboren en met name bij kinderen met een grotere postnatale gewichtstoename. Deze studie heeft voor het eerst laten zien dat er een polymorfisme in de insuline gen regio is dat de bloeddruk van SGA kinderen beïnvloedt.

Hoofdstuk 8

Erfelijke oorzaken van SGA geboorte zijn in een beperkt deel van de kinderen met een te kleine lengte vastgesteld. Dit betreft onder meer puntmutaties en deleties in het *IGF1* en *IGF1R* gen. De beschikbaarheid van de complete menselijke DNA sequentie en de introductie van "high throughput" DNA technieken, hebben nieuwe middelen geleverd om de genetische oorzaken van een te kleine lengte te onderzoeken. In deze studie is de "multiplex-dependent probe amplification" (MLPA) techniek gebruikt om snel te onderzoeken of er veranderingen in het aantal genkopieën aanwezig waren in een groep van 100 SGA geboren kinderen met een te kleine lengte. We onderzochten 18 groeigerelateerde genen: *SHOX, GH1, GHR, IGF1, IGF1R, IGF2, IGFBP1-6, NSD1, GRB10, STAT5B, ALS, SOCS2, SOCS3*. We vonden twee kinderen met een nieuw-ontstane deletie in het *IGF1R* gen. Er werden geen veranderingen in de andere groeigerelateerde genen gezien. Patiënt A had een heterozygote deletie van het complete *IGF1R* gen (15q26.3, exon 1-21) inclusief de distale sequentie. Tot onze verrassing bleek Patiënt A een laag IGF-I gehalte in het bloed te hebben (-2,78 SDS), waarschijnlijk door een gelijktijdige groeihormoon deficiëntie. In het DNA van patiënt B werd een heterozygote deletie gezien van exon 3-21 van het *IGF1R* gen ook inclusief de distale sequentie. Beide kinderen hadden een versnelde lengtegroei tijdens GH-behandeling, bij een GH dosis van 1 mg/m^2/dag (Patiënt A: na 4 jaar GH: delta lengte +1,65 SDS; Patiënt B: na 4 jaar GH: delta lengte +1,69 SDS). Onze studie heeft laten zien dat *IGF1R* haploinsufficiëntie aanwezig was bij twee van de honderd SGA kinderen met een te kleine lengte. Deze studie liet zien dat de combinatie van SGA geboren zijn, een te kleine lengte, een te kleine hoofdomtrek, relatief hoge IGF-I niveaus, ontwikkelingsachterstand, een kleine onderkaak en laaggeïmplanteerde

duimen, suggestief is voor een deletie van het *IGF1R* gen. Aangezien GH-behandeling leidt tot een redelijke inhaalgroei van ~1,0 SDS in het eerste jaar van behandeling, adviseren wij om SGA kinderen met een te kleine lengte te testen op een *IGF1R* deletie, zeker als de genoemde kenmerken aanwezig zijn. De MLPA-techniek blijkt een waardevolle methode te zijn om snel veranderingen in het aantal kopieën van genen te onderzoeken in SGA kinderen met een te kleine lengte.

Hoofdstuk 9

Eerdere studies hebben laten zien dat zwangerschapshypertensie alsmede roken en alcoholgebruik tijdens de zwangerschap, de pre- en postnatale groei beïnvloeden. Desalniettemin is onbekend in hoeverre deze invloeden tijdens de zwangerschap, de spontane postnatale groei en de GH-geïnduceerde groei beïnvloeden van kinderen met en zonder een te kleine lengte (short SGA versus SGA catch-up), kinderen met een idiopathisch te kleine lengte (ISS) en controles.
In dit hoofdstuk worden de eerste resultaten van het Netwerk van Europese Studies naar Genen in Groei (NESTEGG) beschreven. Deze studie vond plaats in Nederland, Frankrijk, Duitsland en het Verenigd Koninkrijk. Deze studie is opgezet om erfelijke- en omgevingsfactoren die de pre- en postnatale groei beïnvloeden van te kleine SGA, SGA catch-up, ISS en controle kinderen, te bestuderen. Om de postnatale en de GH-geïnduceerde groei te bepalen en de relatie met zwangerschapshypertensie en roken en alcoholgebruik tijdens de zwangerschap, werden 464 te kleine SGA, 336 SGA catch-up, 464 ISS en 724 controle kinderen onderzocht. Gegevens over GH-behandeling waren beschikbaar van 262 short SGA en 109 ISS kinderen. De geboortelengte en de postnatale lengte waren significant verschillend tussen de vier groepen (short SGA: -2,88 en -2,82 SDS, SGA catch-up: -2,50 en -0,49 SDS, ISS: -0,59 en -2,49 SDS en controles: -0,01 en 0,05 SDS, respectievelijk). Short SGA kinderen hadden 0,2 SDS meer lengte-groei tijdens 1 jaar GH-behandeling dan ISS kinderen (P<0,001). Moeders van SGA catch-up kinderen hadden frequenter zwangerschapshypertensie dan moeders van short SGA, ISS of controle kinderen (27,9% versus 16,4%, 6,0% en 12,3%, P<0,001). Moeders van short SGA kinderen rookte frequenter tijdens de zwangerschap dan moeders van ISS en controle kinderen (35,1% versus 20,3% en 23,5%, P<0,001). Short SGA kinderen hadden een betere postnatale lengte groei als hun moeders zwangerschapshypertensie hadden doorgemaakt, in vergelijking met short SGA kinderen waarvan de moeders geen zwangerschapshypertensie hadden doorgemaakt (spontane groei: 0,64 SDS versus -0,06 SDS, P<0,001). Concluderend, onze studie heeft aangetoond dat SGA geboorte gerelateerd is aan zwangerschapshypertensie en roken tijdens de zwangerschap. Postnatale inhaalgroei van te kleine SGA kinderen is het meest uitgesproken als hun moeders zwangerschapshypertensie hebben doorgemaakt. Zwangerschapshypertensie, roken tijdens de

zwangerschap en alcoholgebruik tijdens de zwangerschap waren niet gerelateerd aan het ISS fenotype.

Hoofdstuk 10

In de discussie worden onze bevindingen besproken in relatie met de huidige literatuur. Het hoofdstuk wordt afgesloten met algemene overwegingen en suggesties voor toekomstig onderzoek.

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Curriculum vitae

The author of this thesis was born on March 1st 1975 in Wageningen, the Netherlands. She attended secondary school at "De Brug" in Lelystad and passed her V.W.O. exam in 1993. From 1993-1997 she studied Medical Biology at the University of Amsterdam. During her study she spend four months at the Western General Hospital, Edinburgh, UK, performing a research project on glucocorticoid receptor gene expression and postnatal stress (head: Prof. J.R. Seckl). In 1997 she obtained her Master's degree. From 1996-2002 she studied Medicine at the University of Amsterdam. During her study she has spent three months at the Diakonessenhuis, Paramaribo, Suriname performing a paediatric rotation (supervision: Dr. Zijlstra). She worked for one month at the "Centre for Health and Allied Themes" (CEHAT), Mumbai, India, performing a research project on domestic violence in slum area's (supervision: Dr. Jesani). In 2000, she performed a research project on genetic polymorphisms in the glucocorticoid receptor, IGF1 and ApoE gene (head: Prof. Lamberts), at the Erasmus University, Rotterdam. In 2002 she obtained her Medical degree. After working six months as a pediatric resident at the Academical Medical Center in Amsterdam, she worked for five months as a research-physician for the IUGR2-study in which SGA born children are treated with biosynthetic growth hormone (head: Prof. A.C.S. Hokken-Koelega). In september 2003 she started her PhD research which resulted in this thesis at the Erasmus MC- Sophia Children's Hospital, Rotterdam. During this period she obtained a Master's degree in Genetic Epidemiology (NIHES, Erasmus University, Rotterdam). During her PhD research she was actively involved in the Dutch coordination of the "Network of European Studies in Genes in Growth" (NESTEGG) in which she is still active. Since January 2008, she started her training in Clinical Genetics at the Leiden University Medical Center (head: Prof. M.H. Breuning and Dr. S.G. Kant).

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