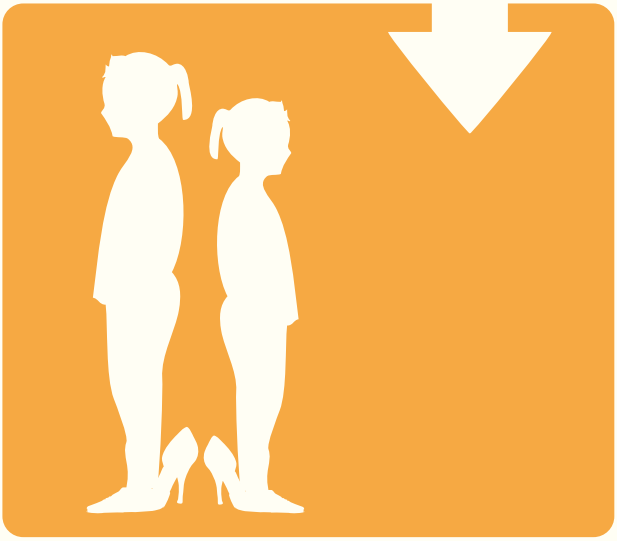
NEW INSIGHTS IN FACTORS INFLUENCING GROWTH IN SHORT CHILDREN BORN SGA



JUDITH S. RENES

New insights in factors influencing growth in children born small for gestational age

Judith S. Renes

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New insights in factors influencing growth in children born small for gestational age

Nieuwe inzichten in factoren die van invloed zijn op de groei in kinderen die SGA geboren zijn

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
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Voor opa

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Chapter 1

Introduction

Since 1988, our research group and others have been investigating children with short stature who were born small for gestational age (SGA). This chapter provides definitions and describes prevalence and etiologies of small size at birth, including endocrine and genetic factors associated with SGA. Also, the effects of growth hormone (GH) treatment in these children are discussed. Finally, the aims of the study and outline of this thesis are described.

Small for gestational age

Small for gestational age (SGA) refers to the size of an infant at birth. It is defined as a birth weight and/or birth length of at least two standard deviation scores (SDS) below the mean for gestational age (1, 2). SGA children can be born full-term or premature. By definition, 2.3% of all liveborn neonates are born SGA. In 2012, 175.457 infants were liveborn in the Netherlands (Central Bureau of Statistics, The Hague, The Netherlands). According to the definition, 4036 of them were born SGA.

In order to determine whether a child is born SGA, accurate information on gestational age, birth weight and birth length is required, as well as an appropriate reference population (1). The term intrauterine growth retardation (IUGR) has also been used to describe infants born SGA. However, IUGR refers to a deceleration of fetal growth and does not always result in SGA birth. For example, a child with IUGR in late gestation may have a normal size at birth. Similarly, being born SGA does not necessarily mean that IUGR occurred, e.g. fetal growth may have been insufficient from the beginning of gestation.

Numerous maternal, fetal, placental and environmental factors are associated with reduced fetal growth (Table 1) (3, 4). Although many factors are known, in 40% of the children no underlying cause can be determined. Nevertheless, it is important to try and identify an underlying cause since this may have consequences for health prognosis and treatment.

Short stature

Short stature is one of the most common medical concerns in childhood, and is defined as a height below the -2 SDS. SGA accounts for approximately 20% of all cases of short stature (5). Most children born SGA show spontaneous catch-up growth during the first two years of life and will reach a height above -2 SDS. Those born prematurely and with more severe degrees of fetal growth retardation may take longer to show complete spontaneous catch-up growth to a normal height. However, 10 to 15% of children born SGA fail to show sufficient catch-up growth and will continue to have a short stature throughout childhood and adolescence (6-8). These children will reach an adult height well below the normal range and/or their target height (TH) range. If a normal height has not been reached by 3 years of age, there is a 7-fold increased risk for short stature in those born with a birth length below the -2 SDS, and a 5-fold increased risk for those born with a birth weight below the -2 SDS (9). Therefore, a child born SGA who is still short at 3 years of age, should be referred to a paediatrician with expertise in endocrinology (1, 2).

The reason for this insufficient catch-up growth and persistent short stature is poorly understood. It has been hypothesized that disturbances in the growth hormone (GH) axis might underlie this failure (10-13).

Table 1. Factors associated with increased incidence of SGA birth (3, 4)

Fetal factors	
Chromosomal disorders	Down syndrome
	Turner syndrome
Genetic disorders	Silver Russell syndrome
	Bloom syndrome
Intrauterine infections	Toxoplasmosis
	Rubella
	Cytomegalovirus
	Herpes Simplex
	Syphilis
	Malaria
Inborn errors of metabolism	
Congenital defects	
Maternal factors	
Medical conditions	Acute or chronic hypertension
	Pre-eclampsia
	Collagen vascular diseases
	Abnormality of the uterus
	Therapeutic drugs (e.g. anticonvulsants)
Social conditions	Maternal nutrition
	Age at delivery <16 or >35 years
	Drug use (smoking, alcohol, illicit drugs)
Placental factors	
Structural abnormalities placenta	Single umbilical artery
	Bilobate placenta
Reduced area for exchange of nutrients and oxygen	Infarcts or local lesions
Reduced blood flow	
Demographic factors	
Maternal and paternal height	
Ethnicity	
Previous delivery of SGA infant	

Growth hormone axis

The GH-axis is the main hormonal axis involved in human growth and is very complex (Figure 1) (14). The secretion of GH sets in motion a cascade involving multiple physiological systems and organs, amongst which are bone growth, skeletal muscle growth and immunomodulation. Also, the factors determining normal growth depend on the child's age. Fetal growth and early postnatal growth until the age of 3 to 6 months, are predominantly insulin dependent. After the first months of life, GH becomes increasingly important in controlling longitudinal growth (15).

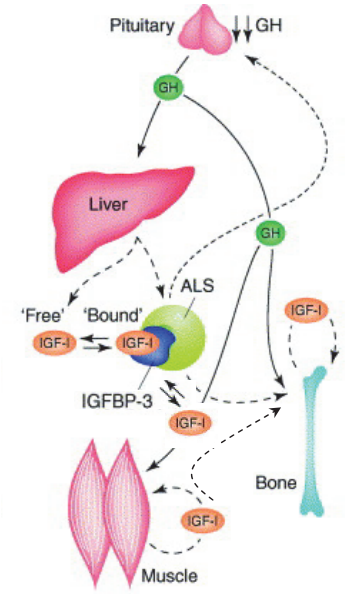

Figure 1. Physiology of the GH-IGF-I axis. Most of the effects of GH are mediated by IGF-I, but GH has also many cellular effects that are independent of IGF-I. GH stimulates IGF-I production, as well as stabilizes circulating IGF-I, via the IGFBP-3-ALS-complex. Adapted from Le Roith et al (14).
ALS = acid-labile subunit; GH = growth hormone; IGF-I = insulin-like growth factor-I; IGFBP-3 = insulin-like growth factor binding protein-3.

Growth hormone

GH is produced by the anterior pituitary gland in a pulsatile pattern, and is usually undetectable in the serum apart from 5-6 nocturnal bursts, which occur in early sleep stages (16). Secretion of GH is under control of the hypothalamic hormones GH-releasing hormone (GHRH) and somatostatin, as well as ghrelin, a hormone mainly produced by the stomach (17). GH promotes growth of many body tissues, amongst which are muscle, cartilage and bone. Some of the effects of GH are direct actions, but much of its effects are mediated through the insulin-like growth factors (IGFs) (14, 18).

Insulin-like growth factors

The IGF family includes three ligands: insulin, IGF-I, and IGF-II, and three closely related membrane bound receptors. The metabolic actions of insulin are mediated through binding to the insulin receptor (IR), and the growth promoting effects of IGF-I and IGF-II are primarily mediated via the IGF-I receptor (IGF1R) (19, 20).

IGF-I and IGF-II are single chain polypeptides of 7.5 kD. In early fetal development, IGF-II plays a dominant role, whereas after organogenesis, IGF-I becomes more important. IGF-II does not play a major role in postnatal growth (21). IGF-I is primarily produced in the liver by hepatocytes, but also by other tissues, including the brain, muscle and bone (Figure 1). IGF-I acts in an endocrine, autocrine, and

paracrine mode (14). IGF-I synthesized in the liver and secreted into the blood is under control of GH, insulin and nutritional status. Autocrine/paracrine IGF-I synthesized in peripheral tissues is controlled by GH and by factors that are locally secreted by surrounding cell types. Next to growth, IGFs together with insulin and GH regulate glucose and lipid metabolism, and body composition (19).

IGF-I and IGF-II show structural and functional similarities with insulin, as do the IR and IGF1R. Because of this homology, interactions between IGFs and the IR take place. Free IGF-I is capable of lowering glucose levels 50 times more than insulin alone. This is, however, prevented because the majority of IGF-I is bound to IGF-binding proteins (IGFBPs) and the acid-labile subunit (ALS) (22, 23). Less than 1% of IGF-I is unbound and circulates in its free form with a half-life of approximately 12 min (24).

IGF binding proteins

IGFBPs play an important role in regulating the bioavailability of IGFs. Six IGFBPs have been identified, known as IGFBP-1 to IGFBP-6. These six binding proteins show similar organization, mostly at the N- and C-terminal regions. Under normal circumstances, IGF-I is mainly sequestered in a ternary complex with IGFBP-3 and the ALS (19, 25).

IGFBP-3 is produced in the liver by Küpffer cells and sinusoidal endothelial cells, as well as in many other tissues (26). It has a molecular weight of approximately 45 kD. Several of the factors that regulate IGF-I production also regulate IGFBP-3 synthesis, such as GH and less by nutritional status (27). The serum concentration of IGFBP-3 exceeds that of the other 5 binding proteins, and the affinity of IGFBP-3 for IGF-I is higher than those of most other binding proteins (28).

Acid-labile subunit

ALS is a member of the leucine-rich repeat (LRR) proteins and contains 20 LRRs (29). It has a molecular weight of 85 kD, and is produced almost exclusively by the hepatocytes in the liver (25). Production of ALS is mostly regulated by GH (30). ALS levels gradually increase with age, with highest values during puberty (31). The main function of ALS is to prolong the half-life of the IGF-I-IGFBP-3 complex by preventing filtration in the glomeruli (23). There has been little evidence of other actions of ALS, although it was recently shown in animals that ALS might be involved in carbohydrate and fat metabolism (32, 33).

Ternary complex formation

In circulation, almost all IGF-I is bound in a 150 kD ternary complex comprising IGF-I, IGFBP-3 (or IGFBP-5) and ALS (22). Free IGF-I has a half-life of approximately 12 min, which in the ternary complex is increased to more than 12 hours (34). Because of its size, the 150 kD complex is retained in the vascular compartment (23, 35). The formation of the ternary complex is specific: ALS has no affinity for free IGF-I or uncomplexed IGFBP-3, therefore IGF-I must bind to IGFBP-3 to which ALS may then associate (36, 37). To efficiently form ternary complexes, there has to be a 2- to 3-fold excess of serum ALS relative to the concentration of IGF-I and IGFBP-3 (38). In the absence of ALS, serum IGF-I and IGFBP-3 levels are markedly reduced, the latter being more affected (39, 40). To assess formation of the 150 kD complex, column chromatography can be performed (Figure 2).

Release of IGF-I from the ternary complex is modulated by proteolysis of IGFBP-3. Proteases are capable of cleaving IGFBP-3 into a form that has a significantly reduced affinity for IGF-I (41, 42). This mechanism was first described in pregnant women, where almost all circulating IGFBP-3 is proteolysed, particularly in the third trimester. It was suggested that the role of this proteolysis is to facilitate the growth of the fetus by increasing IGF bioavailability (43-46).

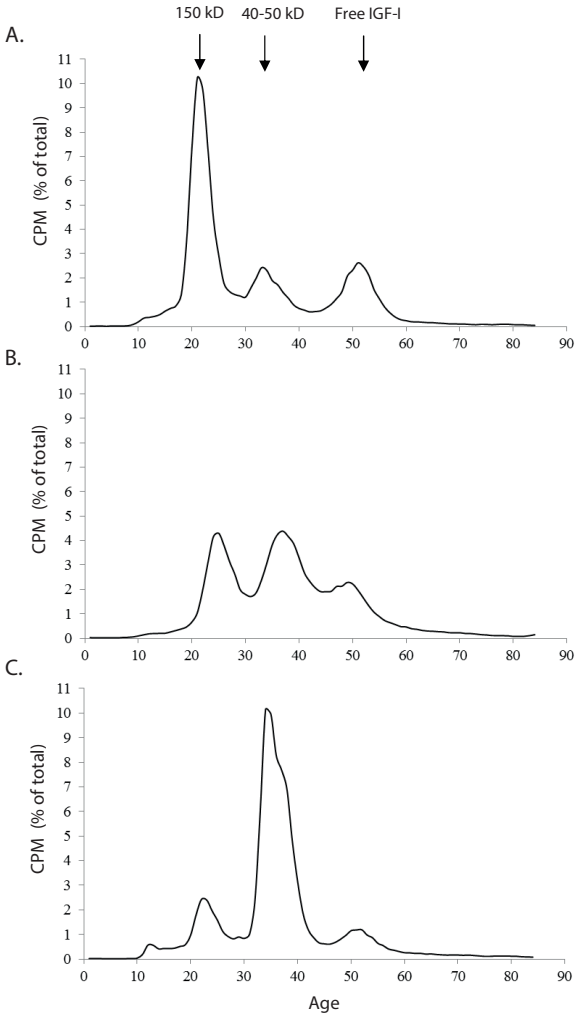

Figure 2. Representative examples of column chromatography. A. normal ternary complex formation; B. reduced ternary complex formation; C. severely reduced ternary complex formation.
CPM = counts per minute; kD = kilodalton; IGF-I = insulin-like growth factor-I

Growth hormone axis in short children born SGA

Several studies have demonstrated that up to 60% of short SGA children show a reduced spontaneous GH secretion measured over 24 hours and/or low GH peaks during provocation tests. Serum IGF-I and IGFBP-3 levels were also reduced when compared with healthy controls. In contrast, some short SGA children have normal or high levels of IGF-I, the latter suggesting a reduced functioning of the IGF-I receptor. Many short children born SGA have a low IGFBP-3 level, a very uncommon finding in other children with short stature without GH deficiency (10-12). There are no data on circulating serum ALS levels in short children born SGA, and there are also no data on ternary complex formation.

Growth hormone treatment

Recombinant GH has been used since 1985 and has replaced GH extracted from human pituitaries. The indications for GH treatment have gradually extended from replacement therapy in children with GH deficiency to conditions in which short stature is not due to GH deficiency. Since 2003, GH treatment for short children born SGA has been licensed by the European Medicines Agency (EMA).

Effects on longitudinal growth

Various clinical trials have shown that most short children born SGA show an improvement of their growth rate during treatment with GH and an improvement of adult height (47-50). A systematic review in 2009 identified 4 high quality trials on adult height in short SGA children treated with GH. Mean height gain was 1.5 SDS in GH-treated versus 0.3 SDS in untreated short children born SGA (51). In Europe, the recommended dose for short children born SGA is 1 mg/m^2/day (The European Agency for the Evaluation of Medicinal Products, 2001).

Effects on IGF-I and binding proteins

GH treatment induces a significant increase in serum IGF-I and IGFBP-3 levels. This increase is dose-dependent and during GH treatment some children display IGF-I and IGFBP-3 values above the normal range (12, 52). Some studies found that IGF-I and IGFBP-3 levels were associated with prostate and premenopausal breast cancers (53, 54). However, no direct causal relationship has been demonstrated (55, 56).

Predicting the growth response

Catch-up growth is most pronounced during the first years of GH treatment, and the attained height SDS is then maintained over the years (12, 50). There is, however, a wide variation in gain in height SDS and in approximately 15% of children adult height is unsatisfactory (50). For that reason, it has been investigated which factors may predict the growth response. Characteristics found to be related to the short-term response were age and height at start of GH treatment and target height (47, 57). For the long-term response, age at start of treatment, GH dose and serum IGFBP-3 levels have been identified (58, 59). Even after accounting for these factors, there remains a wide variation which is difficult to explain. It is therefore important to investigate which other factors can influence the growth response.

One of the factors that could have an impact on the growth response is the use of concomitant medication, such as methylphenidate which is used in the treatment of attention deficit hyperactivity disorder (ADHD). ADHD is a disorder of hyperactivity, inattention and impulsivity that affects approximately 5% of children in the Netherlands (60). Children born SGA might have a higher risk of developing ADHD (61, 62). Use of methylphenidate has been associated with growth deceleration, specifically during the first years of treatment (63-66). In recent years, the use of methylphenidate has substantially increased and more children are being treated with GH and methylphenidate simultaneously (60, 67). There is, however, very little data on the effects of this simultaneous use (68, 69).

Another factor that could have an impact on the long-term growth response is the spontaneous catch-up growth before the start of GH treatment. It is often stated that 10% of the children born SGA show no spontaneous catch-up growth thus remaining short. However, in practice many children do show catch-up growth but not to such an extent that a height within the normal range is reached. It might be that these children have a higher potential of responding to GH treatment since they have been able to show catch-up growth. The influence of spontaneous catch-up growth on the growth response has not been investigated.

In addition, growth during puberty, which is an important determinant of adult height, could be a factor (70). Short children born SGA start puberty at a normal age, however, the age can be relatively early for their actual height (71). Not much is known about growth during puberty in short SGA children treated with long-term GH. In clinical practice there is often a decline in height SDS during puberty resulting in a lower adult height than expected at a younger age. Dahlgren et al. showed that children who were treated for more than 2 years before onset of puberty showed a height loss of 0.2 SDS during puberty (48).

IGFBP-3 at start of treatment was found to be negatively related to adult height SDS (59). Animal data suggest that the presence of ALS in serum is required for maximal effectiveness of exogenous GH (72). It is unknown whether taking into account the baseline serum ALS level, will improve the long-term growth prediction.

Postponement of puberty

The aim of GH treatment is achieving an adult height in the normal range and/or in the TH range. In children with short stature at onset of puberty, the predicted adult height will most likely be poor (<-2.5 SDS). Postponement of puberty using a gonadotropin releasing hormone analogue (GnRHa) could improve adult height (73-76). GnRHa treatment suppresses sexual maturation and skeletal maturation. Administration of GnRHa inhibits the hypothalamic-pituitary-gonadal axis by down-regulating the GnRH receptors in the pituitary gland. Consequently, the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) is inhibited, leading to a decreased production of sex steroids and cessation of pubertal progression. However, reduced growth velocity is an unfavorable phenomenon that may occur during GnRHa treatment (75, 77).

Results on the effect of combined GH/GnRHa treatment in short children born SGA are very scarce, despite its use in clinical practice (49, 78). Studies in patients with idiopathic GH deficiency or in children

with idiopathic short stature showed a beneficial effect on adult height in favor of combined treatment, compared to GH only (73-75). Other studies found no beneficial effect from combined treatment in children with idiopathic short stature (79, 80). It is unknown whether postponement of puberty, in addition to long-term GH treatment, results in an improvement of adult height in short children born SGA.

Genes in the GH-axis involved in short stature

Children born SGA comprise a heterogeneous group with a broad spectrum of clinical characteristics. The genetic nature of short stature in SGA children is still largely unknown despite adult height being one of the most heritable human traits (81). Genome wide association studies have identified a number of genes contributing to variation in height under a polygenic model, however, explaining only mild effects in the general population (82, 83). Only a small number of genetic mutations (<1%) have been found in SGA children with short stature, until now mainly in genes involved in the GH-IGF axis (Figure 3) (84-86). Uncovering the genetic basis of short stature in these children is not only important for diagnosis, but also for health prognosis, genetic counseling and (future) treatment options.

GHRH, GHRH receptor and GH gene

GH releasing hormone (GHRH), acting through the GHRH receptor (GHRHR), plays an essential role in the secretion of GH. Mutations in the *GHRH* gene (MIM 139190) itself have not been reported, but mutations and deletions in the *GHRHR* gene (MIM 139191) and the *GH1* gene (MIM 139250) result in GH deficiency. The most important clinical characteristics are short stature, delayed bone maturation, truncal obesity and delayed puberty. GH, IGF-I, and IGFBP-3 levels are severely reduced. Birth weight and birth length are usually within the normal range (84, 87, 88).

GH receptor gene

The effects of GH can only be accomplished in the presence of a normal functioning GH receptor (GHR). The *GHR* gene (MIM 600946) is located on chromosome 5. The classical Laron's syndrome is an autosomal recessive disorder and is caused by a dysfunction of the GHR resulting in a failure to produce IGF-I. Serum concentrations of GH are normal or increased (89, 90). Downstream signaling defects, e.g. *STAT5B* mutations can result in Laron syndrome-like phenotypes.

IGF1 gene

One of the effects of GH is stimulating IGF-I production. The *IGF1* gene (MIM 147440) is located on chromosome 12. There have been a few case-reports describing deletions and point mutations in the *IGF1* gene. These subjects presented with small birth size, short stature, sensorineural deafness, microcephaly, and mental retardation. Serum GH is elevated, IGF-I is severely reduced (deletion) or increased (missense mutation), IGFBP-3 is within the normal range (91, 92).

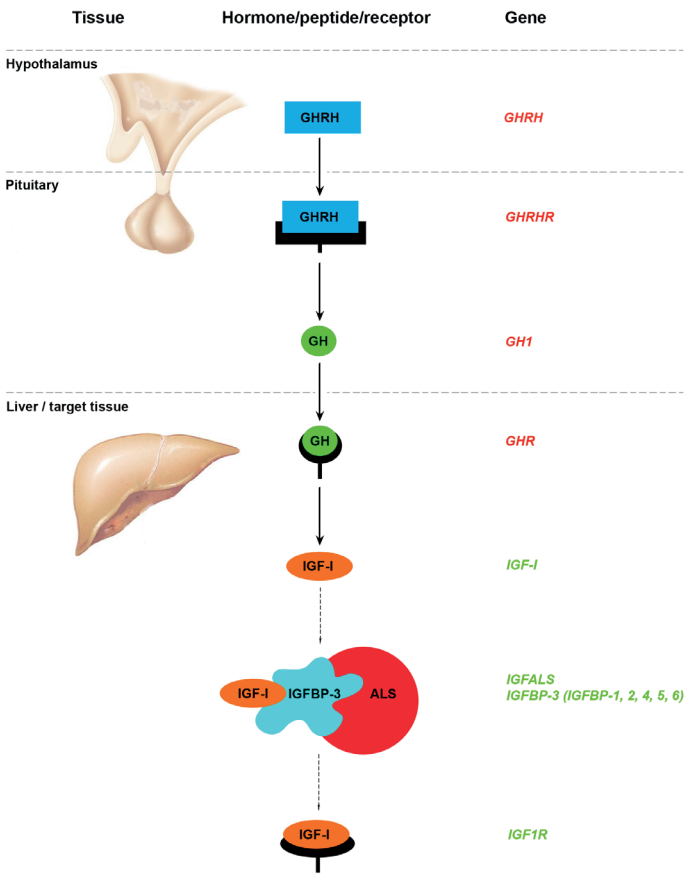

Figure 3. Overview of IGF-I pathway upstream of IGF1R. Genes in red usually do not result in SGA birth.
ALS = acid labile subunit; GH = growth hormone; GHR; = GH receptor; GHRH = GH releasing hormone; GHRHR = GHRH receptor; IGF = insulin-like growth factor; IGF1R = IGF-I receptor; IGFBP = IGF binding protein.

IGFBP-3 gene

The *IGFBP-3* gene (MIM 146732) is located on chromosome 7 and up until now no mutation has been found in the *IGFBP-3* gene in humans (85). Animal knockout studies demonstrated little effect on fetal and postnatal growth (93, 94).

IGFALS gene

The *IGFALS* gene (MIM 601489) is located on chromosome 16. Several case-reports have described deletions, point mutations and compound heterozygous mutations in the *IGFALS* gene. Mutations are mainly present in the LRRs, but also in the amino and carboxy terminal parts of the protein. Subjects present with mild to moderate short stature, delayed onset of puberty, reduced IGF-I levels, and particularly reduced IGFBP-3 levels. Serum GH levels are normal. Very little is known about birth size in

ALS-deficient patients, but small birth size has been reported (95, 96). The *IGFALS* gene has not been studied in a large group of short children born SGA.

IGF1 receptor gene

The *IGF1R* gene (MIM 147370) plays an important role in prenatal and postnatal growth, and is located on chromosome 15. There are several case-reports describing *IGF1R* haploinsufficiency, and the clinical picture is very heterogeneous. The combination of small birth size, short stature, small head circumference, relatively high IGF-I levels, developmental delay and micrognathia is suggestive for children with an *IGF1R* mutation/deletion. Serum GH is normal or increased and IGFBP-3 is within the normal range (86, 97-99).

Syndromes involved with short stature

Children with syndromes are often born SGA. Since GH treatment was licensed for short children born SGA, it has become a frequently applied growth promoting therapy. However, in some specific syndromes, GH treatment is contraindicated, for example in the chromosomal breakage syndromes and DNA repair disorders. These children have a defect in the recognition and/or repair of damage to DNA, and in most cases the genomic instability is associated with a predisposition to develop cancer. The chromosomal breakage syndromes include Fanconi anemia, ataxia-telangiectasia (AT), AT variants, Werner syndrome, and Bloom syndrome.

Fanconi anemia

Fanconi anemia is a clinically and genetically very heterogeneous disease. Fifteen genetic subtypes have been distinguished, but the majority of patients (85%) have a homozygous or compound heterozygous mutation in the *FANCA* gene on chromosome 16 (MIM 607139) (100). Characteristic clinical features include small birth size, short stature, developmental abnormalities in major organ systems, hyper- or hypopigmentation, cafe-au-lait spots, early-onset bone marrow failure, and a predisposition to develop cancer. In 25% of affected individuals no structural abnormalities are present (101).

Ataxia-telangiectasia

AT is an autosomal recessive disorder characterized by short stature, cerebellar ataxia, telangiectases, immune defects, and a predisposition to develop malignancies. Although growth failure may be prenatal in onset, more commonly it becomes evident later in childhood. AT is caused by mutations in the *ATM* gene on chromosome 11 (MIM 607585) (102, 103). AT patients with milder manifestations of the clinical or cellular characteristics of the disease have been reported, and are called AT variants (103, 104).

AT variants

Nijmegen breakage syndrome (AT variant-1) and the phenotypically similar Berlin breakage syndrome (AT variant-2) are caused by mutations in the *NBS1* gene located on chromosome 8 (MIM 602667). They

inherit in autosomal recessive manner. The symptoms in these two syndromes are similar to AT, but neither ataxia nor telangiectasia is present (104, 105).

Werner syndrome

The symptoms in Werner syndrome are reminiscent of premature aging. It is characterised by short stature, scleroderma-like skin changes, cataract, subcutaneous calcification, premature arteriosclerosis, diabetes mellitus, and an increased risk of developing cancer. The majority of patients are small at birth. It is caused by mutations in the *RECQL2* gene on chromosome 8 (MIM 604611). It is usually not diagnosed until young adult life, when symptoms become more apparent (106, 107).

Bloom syndrome

Bloom syndrome is an autosomal recessive disorder caused by a mutation in the *BLM* gene located on chromosome 15 (MIM 210900). Up until now, around 70 mutations in approximately 300 patients have been described (108, 109). This syndrome is characterized by small birth size, short stature, microcephaly, an erythematous and photosensitive facial rash, and a predisposition to develop a wide variety of malignancies at an early age (110-112).

Diagnosing the above described syndromes can be very difficult because they are very rare and hallmark features may vary in severity and age of appearance.

Aims of the study

Growth hormone and methylphenidate treatment

Treatment of ADHD with methylphenidate has greatly increased in recent years, therefore more children are being treated with GH and methylphenidate simultaneously. Some studies have found an association between methylphenidate treatment and growth deceleration. We aimed to explore the effects of methylphenidate treatment on growth in GH-treated short children born SGA.

Growth hormone and spontaneous catch-up growth and growth during puberty

There are few high quality trials on adult height in short children treated with long-term GH. We, therefore, aimed to investigate the efficacy of GH treatment (1 mg/m^2/day) in short children born SGA on adult height. Furthermore, we assessed the influence of spontaneous catch-up growth before the start of GH treatment and growth during puberty on adult height.

ALS levels

Since no data exist on ALS SDS levels in short children born SGA, we aimed to determine ALS levels in a large cohort of short SGA subjects and compared them with controls. We assessed the relationship between ALS levels and several clinical and laboratory characteristics. Also, we investigated whether adding ALS levels to a growth prediction model might improve the long-term growth prediction.

IGFALS gene mutations

In the absence of ALS, serum IGF-I and IGFBP-3 levels are reduced. Many short children born SGA have low or low-normal IGF-I and IGFBP-3 levels. This is also described in children with an *IGFALS* mutation. We determined whether short SGA children with an IGFBP-3 level ≤-1 SDS have mutations in the *IGFALS* gene.

Ternary complex formation

IGF-I is mainly sequestered in a 150 kD ternary complex with IGFBP-3 and ALS. Dissociation of IGF-I from this complex is regulated by proteolysis of IGFBP-3, which then loses its affinity for IGF-I. This results in increased bioavailability of IGF-I to target tissues. Short children born SGA have lower serum IGF-I, IGFBP-3 and ALS levels compared to healthy peers. Data on complex formation and IGFBP-3 proteolysis have not been established. We determined ternary complex formation and IGFBP-3 proteolysis in healthy normal statured children and short SGA children.

Bloom syndrome

Bloom syndrome is a rare chromosomal breakage syndrome characterized by severe pre- and postnatal growth deficiency, a photosensitive facial rash, and a predisposition to develop a wide variety of cancers. GH treatment in these children is contraindicated. Diagnosing this syndrome is difficult because it is very rare and hallmark features may vary in severity. We report two patients with Bloom syndrome illustrating the variety in clinical manifestations and age of appearance.

Outline of this thesis

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

Chapter 2 describes the effects of methylphenidate treatment on growth in GH-treated short SGA children.

Chapter 3 reports on the influence of spontaneous catch-up growth before the start of GH and growth during puberty on adult height in short SGA children.

Chapter 4 describes serum ALS levels and the association with response to growth hormone treatment in short children born small for gestational age

Chapter 5 shows the results of a detailed study on *IGFALS* gene mutations in short children born SGA

Chapter 6 describes complex formation and IGFBP-3 proteolysis in healthy normal statured children and short children born SGA.

Chapter 7 reports the findings in two patients with Bloom syndrome

Chapter 8 discusses our findings in relation to current literature and comments on the clinical implications and conclusions of our study results.

Chapter 9 summarizes our findings in English.

Chapter 10 summarizes our findings in Dutch.

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Chapter 2

Methylphenidate and the response to growth hormone treatment in short children born small for gestational age

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Abstract

Background Growth hormone (GH) treatment has become a frequently applied growth promoting therapy in short children born small for gestational age (SGA). Children born SGA have a higher risk of developing attention deficit hyperactivity disorder (ADHD). Treatment of ADHD with methylphenidate (MP) has greatly increased in recent years, therefore more children are being treated with GH and MP simultaneously. Some studies have found an association between MP treatment and growth deceleration, but data are contradictory.

Objective To explore the effects of MP treatment on growth in GH-treated short SGA children

Methods Anthropometric measurements were performed in 78 GH-treated short SGA children (mean age 10.6 year), 39 of whom were also treated with MP (SGA-GH/MP). The SGA-GH/MP group was compared to 39 SGA-GH treated subjects. They were matched for sex, age and height at start of GH, height SDS at start of MP treatment and target height SDS. Serum insulin-like growth factor-I (IGF-I) and IGF binding protein-3 (IGFBP-3) levels were yearly determined. Growth, serum IGF-I and IGFBP-3 levels during the first three years of treatment were analysed using repeated measures regression analysis.

Results The SGA-GH/MP group had a lower height gain during the first 3 years than the SGA-GH subjects, only significant between 6 and 12 months of MP treatment. After 3 years of MP treatment, the height gain was 0.2 SDS lower in the SGA-GH/MP group (P=0.17). Adult height was not significantly different between the SGA-GH/MP and SGA-GH group (-1.9 SDS and -1.9 SDS respectively, P=0.46). Moreover, during the first 3 years of MP treatment IGF-I and IGFBP-3 measurements were similar in both groups.

Conclusion MP has some negative effect on growth during the first years in short SGA children treated with GH, but adult height is not affected.

Introduction

Short stature persists in approximately 10% of children born small for gestational age (SGA) (1). Growth hormone (GH) treatment has become a frequently applied growth promoting therapy and results in significant catch-up growth (2). Children born SGA have a higher risk of developing attention deficit hyperactivity disorder (ADHD) (3, 4). ADHD is one of the most commonly encountered behavioural problems in childhood. Treatment of ADHD with stimulant drugs, such as methylphenidate (MP), has greatly increased in recent years (5, 6). MP has been associated with growth deceleration, specifically during the first years of treatment (7-14).

An increasing number of children are being treated with both GH and a stimulant drug (15). Data regarding the effect of stimulant medication on growth in children treated with GH are limited. Children with idiopathic GH deficiency treated with GH are reported to have a significant smaller height gain if a stimulant drug is being used simultaneously (15, 16). This suggests that simultaneous use of stimulant medication and GH might not be advised.

The cause of the decelerated growth during MP use has not yet been elucidated. One proposed mechanism is loss of appetite which leads to less weight gain (12). Another possible cause may be the altered dopaminergic pathways in ADHD. These pathways are also involved in GH secretion and MP treatment decreases the re-uptake of dopamine and noradrenalin (17-19). Both proposed mechanisms can lead to less GH secretion and lower serum levels of insulin-like growth factor I (IGF-I) and IGF-binding protein-3 (IGFBP-3), which both play a central role in postnatal growth.

We hypothesized that simultaneous use of GH and MP would negatively affect the growth response to GH therapy, because of lower serum IGF-I and IGFBP-3 levels. To determine the effect of MP on growth we ideally should have randomly assigned MP treatment to two groups of patients. However, since the effectiveness of MP has been established in various studies this was ethically not feasible (20). In this paper we retrospectively report height and weight gain, and adult height in short SGA children treated with GH and MP and compare these data with matched GH-treated SGA subjects without MP. We also describe the effect of MP on serum IGF-I and IGFBP-3 levels.

Patients and methods

Subjects

The study cohort consisted of 427 short children born SGA who participated in four GH trials. The inclusion criteria for these studies have previously been described (21-25). In short, children were included according to the following criteria: 1) birth length and/or birth weight standard deviation score (SDS) below -2.0 for gestational age (26), 2) height SDS for calendar age (CA) below -2.0 according to Dutch standards at start of GH treatment (27), 3) height velocity SDS below zero to exclude children with spontaneous catch-up growth.

For the present study we included 78 subjects, 39 of them received MP for ≥ 6 months (SGA-GH/MP) and 39 GH treated children born SGA (SGA-GH) who had never been exposed to MP treatment. Each subject in the SGA-GH/MP treated group was individually matched to an SGA-GH subject based on sex,

age and height at start of GH treatment, height SDS at start of MP treatment and target height (TH) SDS. The delta accepted for each parameter was 0.5 SDS. Of the 39 SGA-GH/MP subjects, 13 had been on MP treatment before the start of GH treatment. Study protocols were approved by the Medical Ethics Committee of all participating centers (Erasmus Medical Center, Admiraal de Ruyter Hospital, Canisius Hospital, Catharina Hospital, Free University Medical Center, Isala Clinics, Juliana Children's Hospital, Leiden University Medical Center, University Medical Center Groningen, University Medical Center Radboud, Rijnstate Hospital, Walcheren Hospital, Wilhelmina Children's Hospital, and Zaans Medisch Centrum) and written informed consent was obtained from all participants and/or their parents.

Study design

During GH treatment, height, weight and Tanner stage were determined every 3 months as previously described (22). Children were treated with biosynthetic growth hormone approximately 1 mg/m^2/day which was administered once daily at bedtime. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Target height (TH) was calculated as TH = [(maternal height + paternal height + 13) / 2 + 4.5] for boys and TH = [(maternal height + paternal height - 13) / 2 + 4.5] for girls. Height, BMI and TH were expressed in SDS, adjusting for sex and age according to Dutch reference data for children (27). The onset of puberty was defined as breast development stage 2 according to Tanner for girls and testicular volume ≥ 4 ml for boys (28).

Biochemical measurements

Blood samples were taken at baseline and yearly during GH treatment. After centrifugation, all samples were frozen (-80 °C) until assayed. RIA measurements of serum IGF-I and serum IGFBP-3 levels were performed in one laboratory as previously described (29). Levels of IGF-I and IGFBP-3 were expressed as SDS, adjusting for age and gender, using reference values from healthy children of normal stature (30).

Statistical analysis

A paired-sample t-test was used to compare the SGA-GH/MP group with the matched controls in the SGA-GH group. The Wilcoxon signed-ranks test was used to compare age at start of puberty. Height gain SDS was analyzed from start of MP treatment over 3 years. If MP treatment had started before GH treatment we analyzed data from start of GH treatment. The longest consecutive period of MP treatment was used when analyzing the data. The changes over time were analyzed with repeated measures regression analysis to correct for multiple testing and missing data (≥1 year use of MP in 36 subjects, ≥2 years n=26 and ≥3 years n=20). The effect of MP treatment was estimated separately for three time periods, the first 6 months, for 6 to 12 months and for the period after 12 months. Also, the effect for the period after stop of MP treatment was estimated. The analysis of height gain SDS was adjusted for sex, TH SDS, age at start of GH treatment, puberty, GH dose, gonadotropin releasing hormone analogue use (n=8) and duration of GH treatment before start of MP treatment. The analysis of gain in BMI SDS was adjusted for the same variables except for TH SDS. Adult height (AH) was defined as the condition when height velocity dropped <0.5 cm during the previous 6 months. AH SDS was calculated based on

Dutch reference data for age 21 years (27). Corrected AH was calculated by subtracting the TH SDS from the AH SDS. Difference in AH SDS and corrected AH SDS within the matched pairs was analyzed with a repeated measures model correcting for age and height SDS at start of GH treatment, TH SDS, age at start of puberty and GH dose. IGF-I and IGFBP-3 SDS data were adjusted for duration of GH treatment before start of MP treatment, GH dose and puberty. A P-value < 0.05 was considered significant. Analyses were performed using the statistical package SPSS for Windows (version 17.0; SPSS Inc., Chicago, IL). For repeated measures regression analysis SAS 8.2 (SAS Institute Inc., Cary, /nC, USA) was used.

Results

Baseline characteristics

Of the total study group, 39 subjects were treated with MP (~10%). Clinical characteristics of the SGA-GH/MP and SGA-GH subjects are shown in Table 1. MP treatment was more common in boys than in girls (31 boys and 8 girls). There were no significant differences between the SGA-GH/MP and SGA-GH group. Mean (±SD) age at start of MP treatment was 10.6 (±2.2) years and mean duration of MP treatment was 3.7 (±2.5) years. The mean dose of MP was 28.2 (±13.4) mg/day.

Table 1. Patient characteristics

	SGA-GH/MP	SGA-GH	P-value
Male/female	31 / 8	31 / 8	1.00
Age at start GH (years)	8.7 (2.9)	8.7 (2.8)	0.93
Height SDS at start of GH	-2.7 (0.9)	-2.8 (0.5)	0.60
Target height SDS	-0.4 (0.7)	-0.4 (0.7)	0.72
Weight SDS at start of MP	-2.1 (1.2)	-1.8 (1.2)	0.08
BMI SDS at start of MP	-1.0 (1.0)	-0.7 (1.1)	0.18
Age at start puberty (years), male	12.5 (10.2 to 14.7)	12.4 (10.0 to 12.4)	0.70
Age at start puberty (years), female	11.0 (9.9 to 11.8)	10.9 (9.8 to 11.9)	0.89

Values are expressed as mean (SD). Age at start puberty is expressed as median (interquartile range).
BMI = body mass index; GH = growth hormone; MP = methylphenidate; SDS = standard deviation score; SGA = small for gestational age.

Growth

Figure 1 shows the height gain SDS in the two groups. Height gain was not significantly different between the two groups during the first 6 months of MP treatment (0.005 SDS, P=0.85), but between 6 and 12 months, the SGA-GH/MP group had a significantly smaller height gain SDS than the SGA-GH group (-0.05 SDS, P=0.03). Between 1 and 3 years of MP treatment, the height gain SDS was also smaller in the SGA-GH/MP group but not significantly, with a difference of -0.04 SDS (P=0.06) in every six months. After 3 years, the difference between the two groups amounted to 0.2 (± 0.1) SDS (P=0.17).

Adult height was reached in 25 of the 39 SGA-GH/MP subjects (64%) and in 28 of the 39 SGA-GH subjects (72%). Mean age at reaching AH was 17.3 (±1.2) years for boys and 16.0 (±0.9) years for girls in the SGA-GH/MP group and 16.9 (±1.2) years for boys and 15.6 (±0.5) years for girls in the SGA-GH group

(boys P=0.26 and girls P=0.41). In the SGA-GH/MP group, the mean duration of MP treatment was 4.6 (±2.6) years and of GH treatment was 8.1 (±2.5) years. In the SGA-GH group the duration of GH treatment was 7.8 (±2.3) years. Mean AH was -1.9 (±0.7) SDS in the SGA-GH/MP and -1.9 (±0.6) SDS in the SGA-GH group. AH between the groups was not significantly different (adjusted difference 0.07 SDS, P=0.57). Corrected AH was -1.4 (±0.8) SDS in the SGA-GH/MP and -1.4 (±0.7) in the SGA-GH group, this was not significantly different (adjusted difference 0.09 SDS, P=0.44).

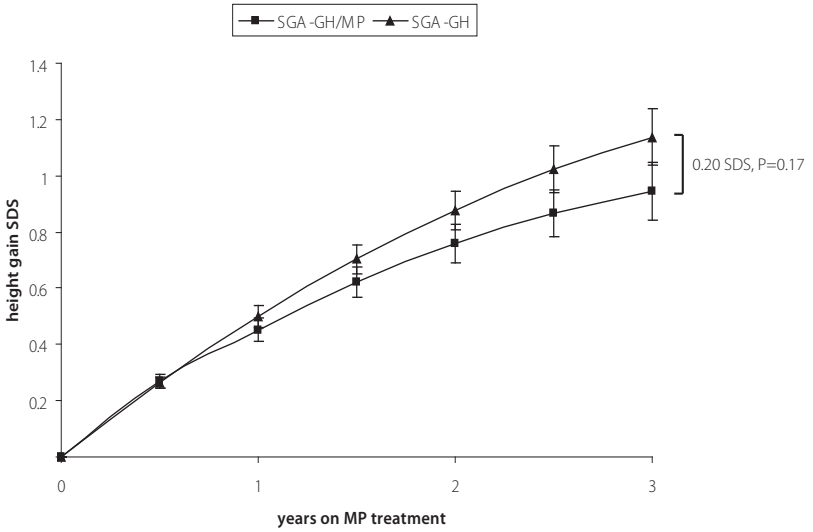

Figure 1. Height gain SDS during 3 years of MP treatment. Data are expressed as model estimates (standard error), ≥1 year use of MP in 36 subjects, ≥2 years n=26 and ≥3 years n=20.
GH = growth hormone; MP = methylphenidate; SGA = small for gestational age; SDS = standard deviation score.

At start of MP treatment, the BMI SDS was -1.0 (±1.0) SDS in the SGA-GH/MP group and -0.7 (±1.1) SDS in the SGA-GH subjects. During the first 6 months of MP treatment, the gain in BMI SDS was not significantly different between the two groups (-0.09 SDS, P=0.13). However, between 6 and 12 months the SGA-GH/MP group had a significantly larger gain in BMI SDS than the SGA-GH group (0.13 SDS, P=0.02). Between 1 and 3 years, the six-monthly BMI gain SDS was not significantly different between the two groups (0.02 SDS, P=0.52).

After cessation of MP treatment, there was no acceleration of growth after six months (height gain SDS 0.03 SDS, P=0.36) compared to the controls. There was also no acceleration in BMI SDS gain, six months after discontinuation of MP (0.02 SDS, P=0.74).

IGF-I and IGFBP-3 levels

Serum IGF-I and IGFBP-3 SDS levels are shown in Figure 2. The gain in IGF-I SDS was not significantly different between the SGA-GH/MP and SGA-GH group during three years of MP treatment (-0.07 SDS, P=0.75).

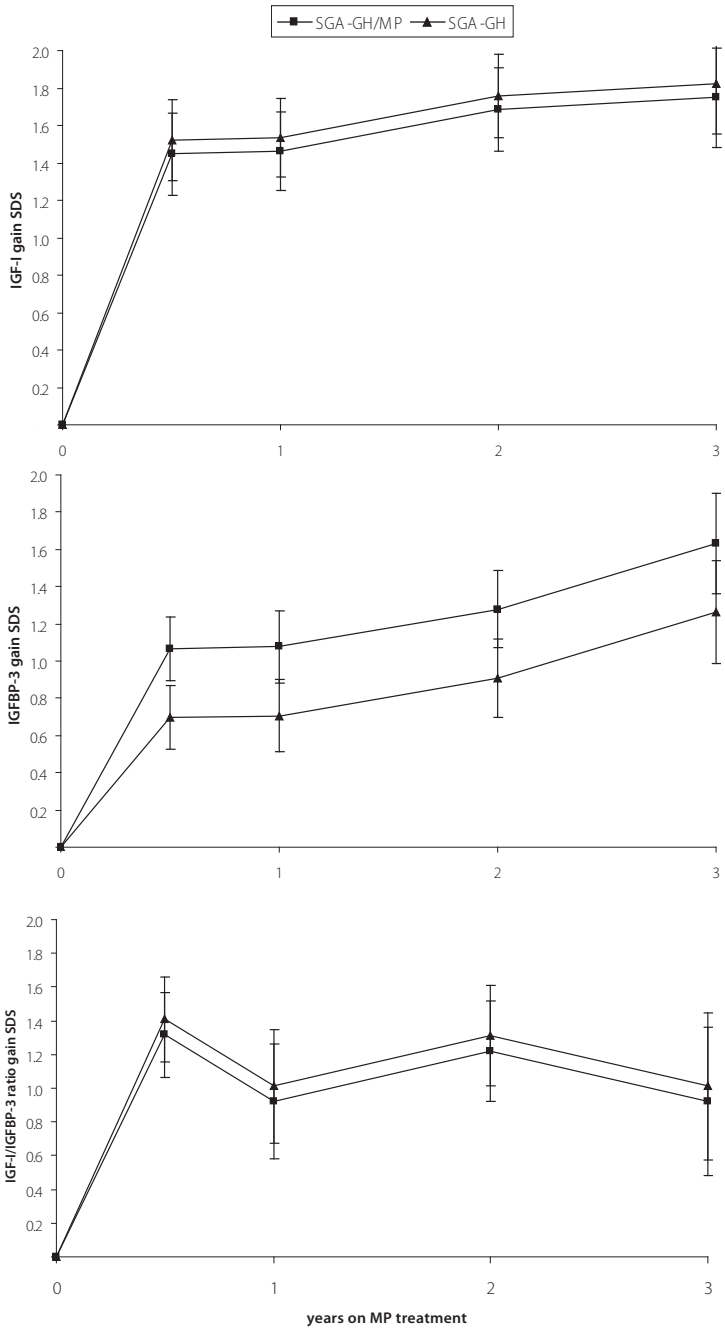

Figure 2. IGF-I, IGFBP-3 and IGF-I / IGFBP-3 ratio gain SDS values during 3 years of MP treatment. Data are expressed as model estimates (standard error).
IGF-I = insulin-like growth factor-I; IGFBP-3 = insulin-like growth factor binding protein-3; MP = methylphenidate; SGA = small for gestational age; SDS = standard deviation score.

The gain in serum IGFBP-3 SDS was higher in the SGA-GH/MP group, but not significantly so (0.37 SDS, P=0.06). The IGF-I / IGFBP-3 ratio SDS was also not significantly different between the SGA-GH/MP and SGA-GH group (-0.09 SDS, P=0.76).

Discussion

In this study we investigated the effect of methylphenidate (MP) treatment on growth in short children born SGA treated with GH. We analysed growth during the first 3 years of MP in addition to GH treatment and also adult height. We showed that the 3-year height gain was 0.2 SDS smaller in children treated with MP than in non-MP-treated children, but not significant. Reassuringly, there was no difference in adult height between the SGA-GH/MP group and the SGA-GH group. The gain in BMI SDS was not negatively affected by MP treatment and IGF-I and IGFBP-3 levels were also not significantly different between the two groups.

ADHD is a disorder of hyperactivity, inattention and impulsivity that affects approximately 5% of children in the Netherlands (6). The most frequently prescribed drugs in the treatment of ADHD are stimulant drugs, such as MP (5, 6). Since the use of stimulant drugs has increased substantially in recent years more children are being treated with GH and MP simultaneously (5, 6, 15). In our cohort of short SGA children, approximately 10% were being treated with MP, which is considerably higher than the reported 5%, indicating that ADHD seems to occur more frequently in children born SGA. This is in line with data reported by van der Reijden-Lakeman and Groen-Blokhuis et al. (4, 31).

Simultaneous use of MP during GH treatment has been associated with growth deceleration, specifically during the first years of treatment (15, 16). We showed that after 3 years of GH treatment, the difference between the SGA-GH/MP and SGA-GH group was 0.2 SDS. These results are in line with Rao et al., who reported a negative effect on height gain of 0.17 SDS during 3 years of MP treatment (16). It demonstrates that there is a small but distinct risk of diminished growth during the first years of MP treatment in GH-treated short SGA children.

Data on adult height are very limited. To our knowledge, there are no reports on adult height in subjects treated with both GH and MP. Our study shows no significant difference in adult height SDS between the SGA-GH/MP and SGA-GH group (0.07 SDS, p=0.46) which suggests that the initial loss of height gain has no long-term adverse effect on adult height. Earlier reports investigating adult height in children treated with only MP did not find any effects either, despite significant effects on height and weight during the first years of treatment (32-35). A possible explanation might be catch-up growth after cessation of MP treatment. However, we found no increase in height gain SDS 6 months after discontinuation of MP (0.03 SDS, P = 0.36), despite reports that cessation of MP treatment results in catch-up growth (36-38).

The mechanism by which MP affects growth is not completely understood. Decreased appetite leading to reduced weight gain has been proposed as a cause of growth reduction (12). We did not find that MP negatively influenced gain in BMI SDS during three years of treatment. In fact, during the second half of the first year of MP treatment gain in BMI SDS was higher in the SGA-GH/MP group. This might

be explained by the low weight SDS at the start of MP treatment (-2.1 SDS). Parents are perhaps being made more aware of reduced appetite as possible side effect of MP and tend to "overfeed" their children to prevent a reduction in weight. Furthermore, the negative effect of MP on weight gain appears to be more pronounced in children who are overweight, which is not the case in short SGA children (10, 39).

Another cause of the decelerated growth may be that MP has a negative influence on the re-uptake of dopamine, a hormone that is involved in the regulation of GH secretion. The effects of GH are mediated by IGF-I. Although gain in serum IGF-I levels was lower in the SGA-GH/MP group, this difference was not significant. Gain in serum IGFBP-3 levels was higher in the SGA-GH/MP group, but again not significantly so. The lower serum IGF-I and higher serum IGFBP-3 levels could result in reduced free IGF-I levels. However, the gain in IGF-I / IGFBP-3 ratio SDS was not significantly lower in the SGA-GH/MP group. Other studies on the effect of MP on GH, serum IGF-I and IGFBP-3 levels are contradictory. Bereket et al. showed that MP caused a transient decrease in IGF-I and IGFBP-3 levels (19). On the other hand a study by Toren et al. found no difference in GH, GH binding protein and IGF-I levels (40). Our results suggest that the cause of growth deceleration does not seem to be the result of significantly lower IGF-I and IGFBP-3 levels.

In this study we measured height and weight every 3 months enabling us to accurately assess growth patterns. In this study thirteen subjects had been treated with MP before the start of GH. Since the negative effects on growth are most pronounced during the first years of MP treatment, it is possible that we have underestimated the decrease in height gain. Furthermore, although our study followed the growth of short SGA children treated with both GH and MP, we did not follow a stimulant-untreated ADHD control group. Ideally the start of stimulant medication should be randomized. However, since the efficacy of MP treatment has been well established such a design was considered unethical (20).

In conclusion, physicians should be aware that height gain can be somewhat reduced during the first years of MP treatment in children treated with GH. It is however reassuring that adult height does not seem to be affected. Growth deceleration during the first years of MP treatment does not seem to be mediated through a reduced BMI gain or a reduction in IGF-I and IGFBP-3 levels.

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Chapter 3

New insights in factors influencing adult height in short SGA children: Results of a large multicenter growth hormone trial

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Chapter 4

ALS levels and the association with response to growth hormone treatment in short children born small for gestational age

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Abstract

Aims To determine ALS levels in short SGA children and to assess the relationship between ALS levels and several clinical and laboratory characteristics. Also, to assess whether adding ALS levels to a growth prediction model might improve the long-term growth prediction.

Design/methods ALS levels were measured in 312 short SGA children at start of GH treatment

Results Median (IQR) ALS of all subjects was -0.5 SDS, significantly below the 0 SDS (P<0.001). In 34 children (11%), ALS levels were ≤-2 SDS. ALS SDS correlated significantly with height SDS (r=0.24, P<0.001), weight SDS (r=0.30, P<0.001), BMI SDS (r=0.20, P=0.001), IGF-I SDS (r=0.56, P<0.001) and IGFBP-3 SDS (r=0.67, P<0.001). ALS SDS was also positively correlated with fasting insulin (r=0.41, P<0.001) and glucose levels (r=0.33, P<0.001), and HOMA-IR (r=0.35, P<0.001). Baseline ALS levels contributed to the long-term growth prediction of GH treatment (5%, P<0.001).

Conclusion Short SGA children tend to have lower ALS levels compared to controls, albeit less reduced than IGF-I and IGFBP-3 levels. Our data suggest that ALS may be involved in glucose homeostasis. Determination of ALS levels before the start of GH treatment in short SGA children contributes moderately to a more accurate prediction of the growth response to GH treatment.

Introduction

Persistent short stature occurs in approximately 10% of children born small for gestational age (SGA) (1). Short children born SGA have on average low-normal serum insulin-like growth factor-I (IGF-I) levels and particularly low insulin-like growth factor-binding protein-3 (IGFBP-3) levels (2, 3). It has been hypothesized that abnormalities in the growth hormone (GH)-IGF-IGFBP axis might underlie the failure in complete catch-up growth in children born SGA with persistent short stature (3).

IGF-I and IGFBP-3 play a central role in postnatal growth. Under normal circumstances, IGF-I circulates in the serum in a ternary complex with mainly IGFBP-3 and the acid-labile subunit (ALS) (4). ALS is secreted under the influence of GH, prolongs the half-life of the IGF-IGFBP-3 complexes and plays a role in the modulation of IGF-I availability (5). In the absence of ALS, serum IGF-I and in particular IGFBP-3 levels are markedly reduced (6). GH, IGF-I and its binding proteins play a role in glucose metabolism and homeostasis. In animals, ablation of the gene appeared to increase insulin sensitivity (7). Interestingly, in some severely ALS deficient patients, reduced insulin sensitivity has been described (8). Patient and animal data suggests that ALS might also play a role in fat metabolism (9).

Short SGA children can be effectively treated with GH. There are prediction models to optimize and individualize treatment and to provide useful information about the expected long-term growth. Baseline IGFBP-3 is a determinant related to height SDS at onset of puberty in GH-treated short children born SGA (10). Animal data suggest that the presence of ALS in serum is required for maximal effectiveness of exogenous GH (11). Also, patients with low ALS levels due to an mutation respond insufficiently to GH treatment (8).

Given the on average reduced IGF-I and IGFBP-3 levels in the SGA population, we hypothesized that the average ALS level would also be reduced. Thus, the first aim was to determine serum ALS levels in a large cohort of short SGA subjects, to compare these levels to those of age-matched controls, and to assess the relationship between ALS levels and clinical and laboratory parameters. The second aim was to assess whether adding ALS levels to a growth prediction model might improve the long-term growth prediction.

Subjects and methods

Subjects

The study cohort comprised 312 short SGA children, 258 prepubertal and 54 pubertal. These children participated in three GH trials (12-14). In short, the studies included children with 1) birth length and/or birth weight <-2.0 standard deviation score (SDS) (15); 2) height SDS for calendar age (CA) <-2.0 according to Dutch standards (16) and 3) height velocity SDS for CA below zero to exclude children with spontaneous catch-up growth (17). Children with growth failure caused by GH deficiency, defined as a maximum serum GH level <20 mU/L during a stimulation test, a chromosomal disorder or another disorder or syndrome were excluded.

Reference values for serum ALS levels were established using blood samples from 159 children (81 girls, 78 boys), aged 10 months to 18 years. Samples were obtained from healthy children from several

primary schools in the Netherlands, patients undergoing minor elective surgery or healthy siblings. In addition, samples of adolescents and adults (176 females, 181 males) ranging from 18 to 78 years were obtained from the Red Cross Blood Bank Utrecht (Utrecht, The Netherlands), and from healthy volunteers working in the endocrine department of the University Medical Center Utrecht. None of these subjects were suffering from malnutrition or showed signs of acute disease or endocrine abnormalities (18, 19). This study was performed according to the Helsinki Declaration and approved by the Medical Ethics Committees of all participating centers. Written informed consent was obtained from all participants and/or their parents.

Study design

Children were treated with biosynthetic GH 1 mg/m^2/day (n = 276) or 2 mg/m^2/day (n = 36), depending on the study design. Height and weight were determined every three months as previously described (3). Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Target height (TH) was calculated as TH = [(maternal height + paternal height + 13) / 2 + 4.5] for boys and TH = [(maternal height + paternal height - 13) / 2 + 4.5] for girls. Height, weight, BMI and TH were expressed in SDS, adjusting for sex and age according to Dutch reference data (16). The onset of puberty was defined as a testicular volume ≥4 ml for boys and breast development stage 2 according to Tanner for girls (17). Age at start of puberty was available in 233 out of 312 children (15 were lost to follow-up, 10 children were still prepubertal (7 boys and 3 girls, age < 12 years) and 54 children were pubertal at start of GH treatment).

Biochemical measurements

Blood samples were taken at start of GH treatment. After centrifugation, all samples were kept frozen (-80 °C) until assayed. Serum ALS levels were determined using the ELISA kit of Mediagnost (Reutlingen, Germany). Intra-assay variations were 6.55 and 6.84 % at mean levels of 911 and 1338 mU/ml (n=16), respectively. Inter-assay variations were 9, 8 and 8 % at mean levels of 931, 1061 and 1926 mU/ml (n=10), respectively. The time between collection of blood samples and measurement of ALS (= age of samples) differed between short SGA subjects (range 2.9 to 15.0 yr). In samples stored ≥5 years, ALS (mg/L) was negatively correlated with age of the sample, r=-0.15, P=0.008. In a multiple regression analysis, a decrease of 0.3 per year was estimated. ALS (mg/l) levels were adjusted as ALS mg/L = ALS mg/L + 0.3 × (age samples - 5). Smoothed references for ALS levels were constructed by the LMS method using data of our healthy controls (20). RIA measurements of serum IGF-I and IGFBP-3 were performed in one laboratory as previously described (21). Levels of serum IGF-I and IGFBP-3 were expressed in SDS, adjusting for age and gender (22). Fasting insulin was measured by chemoluminescent assay on an Immulite 2000 analyzer and fasting glucose was measured on a Hitachi 917 analyzer (Diagnostic Product Corporation, Los Angeles, CA). HOMA insulin resistance index (HOMA-IR) was calculated (23). Total cholesterol (TC), LDL-cholesterol (LDL-c), HDL-cholesterol (HDL-c) and triglycerides were determined as previously described (24).

Statistical analysis

Baseline data of the total study group are presented as median (interquartile range). Pearson's correlation coefficient was used to test for correlations. Insulin and triglyceride levels were ln-transformed before analysis because of a skewed distribution. Using the prediction model described by de Ridder et al., we tested baseline ALS SDS as a contributing factor in the prediction model for height SDS at onset of puberty (10). Firstly, the predicted height SDS at onset of puberty was calculated (=PREDICT) using the formula 3.10 + 0.70 * height SDS at start GH + 0.13 * TH SDS – 0.004 * IGFBP-3 SDS + 0.16 * GH dose – 0.28 * GH dose * IGFBP-3 SDS + 0.070 * (CA-BA) – 0.34 * Gender – 0.27 * age at start GH (10). Secondly, the variables PREDICT and ALS SDS were entered in a multiple regression model to determine if baseline ALS SDS improved the prediction of height SDS at onset of puberty. A P-value <0.05 was considered significant. Analyses were performed using the statistical package SPSS (version 20.0; IBM SPSS Inc., Chicago, IL) for Windows.

Results

Clinical and laboratory data

Baseline clinical and laboratory data are shown in Table 1. Median (interquartile range) age of the study population was 7.7 (5.4 to 10.9) years.

Table 1. Baseline clinical and laboratory characteristics

		Total group
Boys/girls	312	164/148
Gestational age (weeks)	312	37.6 (33.5 to 39.7)
Birth length SDS	206	-2.7 (-3.8 to -2.1)
Birth weight SDS	307	-2.1 (-3.0 to -1.5)
Target height SDS	300	-0.5 (-1.2 to 0.0)
At start of GH treatment		
CA (years)	312	7.7 (5.4 to 10.9)
Height SDS	312	-3.0 (-3.3 to -2.5)
Weight SDS	312	-2.9 (-3.4 to -2.2)
BMI SDS	312	-1.2 (-2.0 to -0.5)
IGF-I SDS	312	-1.2 (-2.0 to -0.3)
IGFBP-3 SDS	312	-1.2 (-1.7 to -0.5)
ALS SDS	312	-0.5 (-1.5 to 0.7)
Insulin (mU/L)	117	3.7 (2.0 to 6.3)
Glucose (mmol/L)	160	4.9 (4.6 to 5.1)
HOMA-IR	98	0.9 (0.5 to 1.6)
Total cholesterol (mmol/L)	235	4.2 (3.7 to 4.7)
LDL-cholesterol (mmol/L)	230	2.3 (2.0 to 2.8)
HDL-cholesterol (mmol/L)	233	1.5 (1.2 to 1.7)
Triglycerides (mmol/L)	234	0.7 (0.6 to 1.0)

Data are expressed as median (interquartile range).
ALS = acid labile subunit; BMI = body mass index; CA = calendar age; GH = growth hormone; HDL = high-density lipoprotein; HOMA-IR = HOMA insulin resistance index; IQR = interquartile range; IGF-I = insulin-like growth factor-I; IGFBP-3 = insulin-like growth factor binding protein-3; LDL = low-density lipoprotein; SDS = standard deviation score.

In both short SGA children and controls, serum ALS levels significantly increased with age (r=0.43, P<0.001 and r=0.52, P<0.001, respectively) (Figure 1). Compared with the population mean (0 SDS), ALS SDS levels in short SGA children were significantly lower (P=0.001).

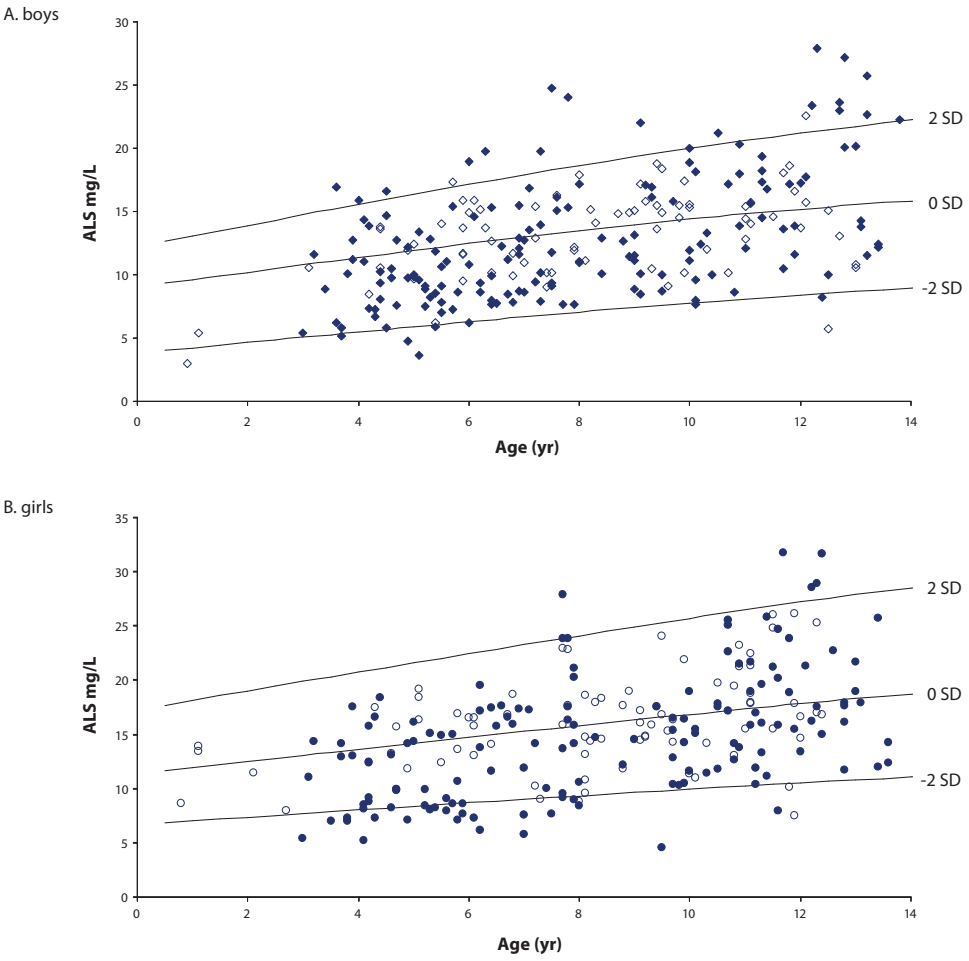

Figure 1. ALS levels (mg/L) in healthy controls (◊ and o) and short SGA children at start of GH treatment (♦ and •).

In 34 short SGA children (11%), serum ALS levels were ≤-2 SDS, which occurred significantly (P<0.001) more frequently in girls than in boys. We sequenced the gene in these patients and found no gene mutation. ALS levels ≥2 SDS were found in 26 children, and occurred more in boys (P<0.001). ALS SDS levels were significantly higher in pubertal than in prepubertal children (0.5 (-0.6 to 1.6) SDS vs. -0.7 (-1.6 to 0.4) SDS, P<0.001). Median fasting insulin, glucose and HOMA-IR levels were within the normal range, as were lipid levels.

Correlations between ALS and clinical parameters

Table 2 shows correlations between serum ALS SDS values and several clinical and laboratory parameters in the total group of short SGA children. ALS SDS levels were positively correlated with height SDS, weight SDS and BMI SDS. There was no significant correlation between serum ALS levels and age at start of puberty. In 54 children puberty started shortly before the start of GH treatment, at a median age of 12.5 (11.6 to 13.1) years. In this subgroup there was also no correlation between ALS SDS levels and age at start of puberty (r=0.16, P=0.22).

Strong positive correlations were observed between ALS SDS levels and IGF-I SDS (r=0.56, P<0.001) and IGFBP-3 SDS levels (r=0.67, P<0.001). ALS SDS levels were also positively correlated with fasting insulin (r=0.41, P<0.001) and glucose (r=0.33, P<0.001) levels, and HOMA-IR (r=0.35, P<0.001). ALS SDS levels remained significantly correlated with fasting insulin and glucose, and HOMA-IR after additional correction for age. ALS SDS levels were not correlated with total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides. After additional correction for age, only triglycerides were positively correlated with ALS SDS (r=0.20, P=0.003).

Table 2. Correlations between ALS SDS and various clinical and laboratory parameters

	ALS SDS[a]	
	R	**P-value**
Gestational age (weeks)	0.05	0.35
Birth length SDS	-0.02	0.80
Birth weight SDS	0.05	0.35
Height SDS	0.24	**<0.001**
Weight SDS	0.30	**<0.001**
BMI SDS	0.20	**0.001**
IGF-I SDS	0.56	**<0.001**
IGFBP-3 SDS	0.67	**<0.001**
Fasting insulin (mU/L)	0.41	**<0.001**
Fasting glucose (mmol/L)	0.33	**<0.001**
HOMA-IR	0.35	**<0.001**
Total cholesterol (mmol/L)	0.09	0.17
LDL-cholesterol (mmol/L)	0.03	0.67
HDL-cholesterol (mmol/L)	0.04	0.57
Triglycerides (mmol/L)	0.12	0.07

a. variables at start of growth hormone treatment
ALS = acid labile subunit; BMI = body mass index; HDL = high-density lipoprotein; HOMA-IR = HOMA insulin resistance index; IGF-1 = insulin-like growth factor-I; IGFBP-3 = insulin-like growth factor binding protein-3; LDL = low-density lipoprotein; SDS = standard deviation score

Contribution of baseline serum ALS SDS to growth prediction

Variables contributing to the prediction of height SDS at onset of puberty in GH-treated short SGA children were described by de Ridder et al. and included baseline height SDS, TH SDS, IGFBP-3 SDS, GH

dose, gender, age, CA-BA and the interaction term IGFBP-3 · GH dose (10). Baseline ALS SDS before start of GH treatment was positively related to height SDS at onset of puberty (β=0.14, P<0.001). The final model, including baseline ALS SDS, explained 74% of the variance in height SDS at onset of puberty and without ALS SDS the model explained 69% (P<0.001). Fifty-three children were used in both the study by de Ridder et al. and in the present analysis. Excluding these children did not change the results (data not shown). Analyzing the above described variables as independent factors in a multiple regression model, and replacing IGFBP-3 SDS and the interaction term IGFBP-3 · GH dose with ALS SDS and ALS · GH dose did not further improve the explained variance (data not shown).

Discussion

In the present study, we investigated serum ALS levels in a cohort of 312 short children born SGA and compared these levels with those of a control population of similar age. We also evaluated whether adding ALS levels to a growth prediction model could improve the long-term growth prediction in short SGA children treated with GH.

Our data show that serum ALS levels in short SGA children gradually increase with age, with highest values during puberty. This is in line with data described in children with normal stature and parallels the simultaneous rise in serum IGF-I and IGFBP-3 levels (25, 26). Under normal circumstances, IGF-I is mainly complexed with IGFBP-3 and ALS (4, 27). We found that serum ALS levels were closely related to IGF-I and in particular IGFBP-3 levels. The closer correlation with IGFBP-3 levels may reflect that most of the IGFBP-3 is bound to ALS because its unbound form is rapidly cleared from the circulation (25).

We found low ALS levels (≤-2 SDS) in 11% of the short SGA subjects. To efficiently form ternary complexes there has to be a 2- to 3-fold excess of serum ALS relative to the concentration of IGF-I (28). We speculate that subjects with ALS levels ≤-2 SDS form ternary complexes less adequately which results in lower IGF-I and IGFBP-3 levels. Lower levels might contribute to the insufficient catch-up growth of these 11% of short SGA subjects. This is in line with data described in patients with a heterozygous *IGFALS* mutation, who also show lower ALS, IGF-I and IGFBP-3 SDS levels, and, as a result, also reduced ALS-IGF-IGFBP-3 complex formation (29). Compared to wild type individuals, height was reduced by approximately 1 SD (29, 30).

In 8% of the short SGA children, ALS levels were ≥2 SDS. These children were older and more frequently pubertal at time of ALS measurement. Production of ALS is predominantly stimulated by GH, and during puberty spontaneous GH secretion is increased (5). The high serum ALS levels might be a reflection of this increase. Furthermore, ALS levels ≥2 SDS occurred more frequently in boys. The GH secretion during puberty is gender-specific (e.g. high amplitudes at an earlier pubertal stage in boys) and this might explain why high ALS levels are more frequently seen in boys (31). Another explanation might be the limited number of healthy controls during puberty. However, our data are in line with Yu et al. who determined serum ALS levels in approximately 1200 children (18).

A low birth weight for gestational age has been described in children with an *IGFALS* mutation, but we did not find a correlation between birth length, birth weight and ALS levels (32, 33). In *IGFALS*

knockout mice birth weight was also normal (28). A recent publication showed significantly lower cord blood levels of ALS in SGA newborns (34). That study, however, reported only serum levels and no standard deviation scores, so no correction was made for gender and gestational age.

Serum IGF-I and IGFBP-3 levels are regulated by multiple variables. Several of these variables, such as nutritional status, will possibly also regulate ALS synthesis and secretion. We found that subjects with a lower weight and BMI had lower serum ALS levels. Others have also demonstrated lower ALS levels after calorie restriction and in patients with anorexia nervosa (35). These results indicate that nutritional status plays a role in the regulation of serum ALS levels. A study in rats showed that insulin deficiency may be the primary defect causing decreased serum ALS levels during calorie restriction (36). Indeed, we also found that lower insulin levels were associated with lower ALS SDS levels. Other data suggest that ALS is involved in carbohydrate metabolism (9). Ablation of the *IGFALS* gene in rodents showed an increase in insulin sensitivity (7). We found that serum ALS levels were positively correlated with HOMA-IR values, indicating that low ALS levels increase insulin sensitivity, which is in line with data in rodents. This might be explained by the fact that IGFs in the ternary complex cannot cross the vascular endothelium and are therefore unable to regulate GH secretion. In the absence of ALS, IGF-I is mainly found in a binary complex which can cross the capillary barrier and thus control GH secretion (7).

In our cohort of non-GH deficient short SGA subjects, median serum ALS levels were significantly lower than the population mean (-0.5 SDS), albeit still in the normal range, whilst IGF-I and IGFBP-3 levels were <-1 SDS. This is in line with recent data on ALS levels in children with idiopathic short stature (37). The reduced IGF-I and IGFBP-3 levels in short SGA subjects might be due to the reduced spontaneous GH secretion (38). However, since GH is the only known major hormonal determinant of serum ALS levels, we would then also expect similarly low serum ALS levels. A possible explanation might be that GH levels are not reduced to such an extent that ALS production is affected. Another cause might be that there are other, unknown, mechanisms involved in the secretion of ALS.

GH-treatment of short SGA children results in catch-up growth, however, the variability is high (39). Age at start of treatment, GH dose and IGFBP-3 levels are important determinants (10). Even after accounting for these factors, there remains a wide variation which is difficult to explain. We demonstrate that ALS SDS levels at start of GH treatment are positively correlated with height SDS at onset of puberty. Furthermore, adding ALS to the prediction model described by M. de Ridder et al. improved the model by 5% to 74% (10). As the growth response in short SGA children is highly variable, further research is needed, to optimize individual GH treatment. By using prediction models it will become possible to decide on an individual dose. As with all characteristics (e.g. age start of GH treatment, GH dose, and IGFBP-3) the contribution of ALS to the prediction model is modest. However, our results suggest that determination of serum ALS at start of GH treatment may contribute to a more accurate prediction of the growth response to GH treatment.

In conclusion, serum ALS levels are lower in short SGA subjects compared to controls, but within the normal range. Furthermore, ALS levels are less reduced than IGF-I and IGFBP-3 levels. Our data suggest that ALS may be involved in glucose homeostasis. Determination of serum ALS levels before the start of GH treatment modestly improves the long-term prediction of height SDS with 5%.

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Chapter 5

Heterozygous *IGFALS* gene mutations in short children born small for gestational age

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Chapter 6

Ternary complex formation and IGFBP-3 proteolytic activity during childhood: Age-dependent changes

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Chapter 7

Bloom syndrome in short children born small for gestational age: a challenging diagnosis

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Abstract

Background Growth hormone (GH) treatment has become a frequently applied growth promoting therapy in short children born small for gestational age (SGA). In some disorders GH treatment is contraindicated, e.g. chromosomal breakage syndromes. Bloom syndrome is a rare chromosomal breakage syndrome characterized by severe pre- and postnatal growth deficiency, a photosensitive facial erythema, immunodeficiency, mental retardation or learning disabilities, endocrinopathies and a predisposition to develop a wide variety of cancers.

Objective We report two patients with Bloom syndrome illustrating the variety in clinical manifestations. They were initially diagnosed with short stature after SGA birth and Silver Russell syndrome and treated with GH.

Cases Both patients presented with pre- and postnatal growth failure, but no clear other characteristic features associated with Bloom syndrome. Photosensitive skin lesions developed only at a pubertal age and were minimal. Also, both children showed normal immunoglobulin levels, normal development and no signs of endocrinopathies at start of GH. Dysmorphic features resembling Silver Russell syndrome were observed in both patients. Remarkably, during GH treatment IGF-I levels increased to values greater than 3.5 SDS, with normal IGFBP-3 levels.

Conclusion Short children born SGA comprise a heterogeneous group. Bloom syndrome should be tested for in children with consanguineous parents, dysmorphic features (particularly resembling Silver Russell syndrome), skin abnormalities, and/or IGF-I levels greater than 2.5 SDS during standard GH treatment with normal IGFBP-3 levels.

Introduction

Small for gestational age (SGA) refers to size at birth, and is defined as a birth weight and/or birth length ≤2 standard deviation scores (SDS) below the mean for gestational age (1). Children born SGA comprise a heterogeneous group with a broad spectrum of clinical characteristics. Approximately 10% of children born SGA fail to show sufficient catch-up growth and remain short (2). Since growth hormone (GH) treatment was licensed for short children born SGA, it has become a frequently applied growth promoting therapy (3).

The start of GH treatment in short SGA children should be preceded by a thorough diagnostic work-up to identify an underlying cause (1). The causes of pre- and postnatal growth restriction are numerous. Children with genetic disorders are often born SGA. In some specific disorders and syndromes, GH treatment is contraindicated, for example, in DNA repair disorders and the chromosomal breakage syndromes. The latter include Fanconi anemia, ataxia-telangiectasia (AT), AT-like syndrome, Werner syndrome, Nijmegen breakage syndrome, and Bloom syndrome. Because increasing numbers of short SGA children are treated with GH, there is an increasing risk of treating children with such a syndrome.

Bloom syndrome is a rare chromosomal breakage syndrome with fewer than 300 patients known to the Bloom's Syndrome Registry (4). It is characterized by severe pre- and postnatal growth deficiency, an erythematous and photosensitive facial rash, dysmorphic features such as microcephaly and malar hypoplasia, immunodeficiency, and a predisposition to develop a wide variety of malignancies at an early age (5-8). Diagnosing this syndrome is difficult because it is very rare and hallmark features may vary in severity.

We present two patients illustrating the variety in clinical manifestations and the difficulty to diagnose this syndrome. Also, we provide a new insight which can lead to diagnosing this syndrome in short SGA children treated with GH.

Clinical presentation

Patient 1

This girl was born spontaneously after 37 weeks of gestation as the first child of consanguineous Turkish parents. Pregnancy and delivery were uneventful. Her birth weight was 1760 g (-3.0 SDS), her birth length was unknown. During her first year, she exhibited poor appetite, feeding difficulties, and failure to thrive. After extensive investigations, no underlying cause was determined besides mild delayed development of oral and motor skills. She had a somewhat triangular face and patent ears, and Silver Russell syndrome was considered. However, in 1994 genetic testing was not available. Physical therapy was started and during the next years her intake improved, but growth remained well below the -2.5 SDS (Figure 1).

At 5 years of age, she was referred to our clinic. Her height was 95.9 cm (-3.7 SDS), weight 11.4 kg (-3.2 SDS weight for height), sitting height to height ratio 0.58 (2.1 SDS), and head circumference 46.3 cm (-2.7 SDS). The height of her father was 169 cm (-0.9 SDS), and of her mother 162 cm (-0.2 SDS), based on Turkish reference data). Neurological, cardiovascular, respiratory, and abdominal examinations were all normal.

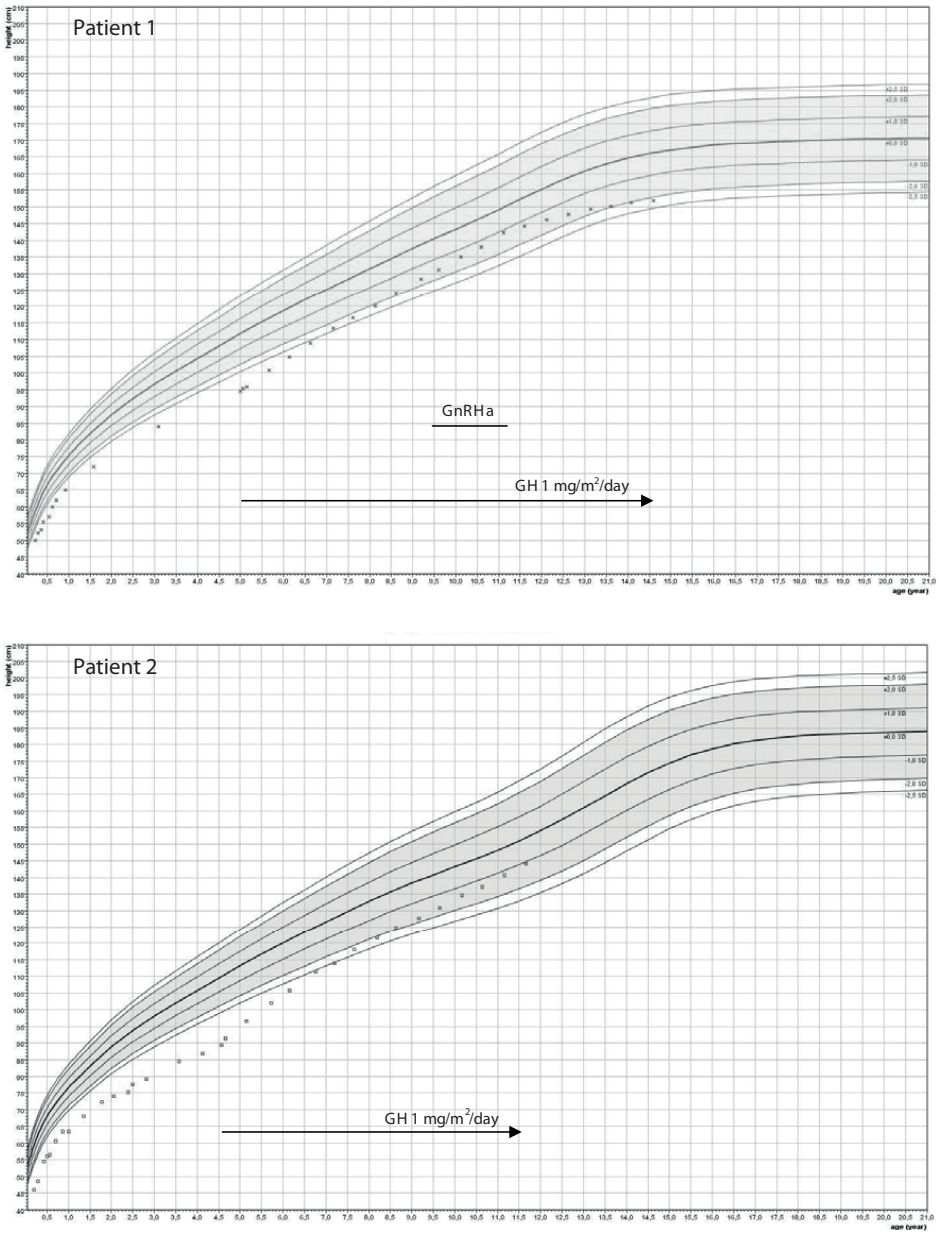

Figure 1. Growth charts of the two children with Bloom syndrome. In patient 1, puberty was postponed for 2 years using a GnRHa.
GH = growth hormone; GnRHa = gonadotrophin releasing hormone analogue.
Growth chart according to Growth Analyser 4.0

Psychomotor development was now normal, and she went to a regular primary school. Overall blood examination showed no abnormalities. An arginine stimulation test was performed with a maximum GH response of 13.5 µg/L (35 mU/L). Insulin like growth factor-I (IGF-I) was 223 ng/mL (1.8 SDS) and IGF binding protein 3 (IGFBP-3) was 2.1 mg/L (0 SDS). Thyroid function was normal. Bone age was not delayed, and skeletal survey was normal. Genetic testing showed a normal karyotype (46, XX) and uniparental disomy (UPD) 7 was not present. Because no underlying cause was found, she was diagnosed with short stature after SGA birth.

GH treatment was initiated at a dose of 1 mg/m^2/day (0.033 mg/kg/day) (Figure 1). During treatment, serum IGF-I levels increased substantially and after 2 years, IGF-I was 642 ng/mL (4.0 SDS). Because we suspected IGF-I insensitivity, we sequenced the *IGF1R* gene, which in the end turned out to be normal. Over the years, IGF-I fluctuated around 3 SDS, despite treatment with the standard dose of 1 mg/m2/day. IGFBP-3 levels remained well within the normal range. She was 9.2 years at start of puberty with a height of 128 cm (-1.7 SDS). During treatment, overall blood examination and carbohydrate and lipid parameters were normal. At 10 years of age, she was included in a study to identify genetic variations in children with short stature in relation to their phenotype. As her mild dysmorphic features had become less subtle over the years, and showed a long, narrow and somewhat triangular face, broad nasal bridge, full lips, micrognathia, low set ears, a low implanted first digit and mild clinodactyly (Figure 2). However, some of these features were also present in her parents.

At 11 years of age, she developed a mild photosensitive facial rash (Figure 3). After extensive testing by several dermatologists, including skin biopsies, and a rheumatologist, she was diagnosed with cutaneous lupus erythematosus. She was 13-years-old when TSH levels increased to 9.7 mU/L, free T_4 was normal (14.4 pmol/L (112 pg/dL)), antithyroperoxidase antibodies and anti-thyroglobulin antibodies were negative, and anti-TSH receptor antibodies were normal. She went to a regular secondary school. At 14.8 years of age, she presented with difficulty swallowing, a cough, and weight loss. A chest X-ray film showed a mediastinal mass, a B cell non-Hodgkin lymphoma. Fanconi anemia was considered, and although chromosomal breakage was increased, it was not the type found in Fanconi patients. Subsequent analysis showed an increased sister chromatid exchange, confirming the diagnosis of Bloom syndrome. DNA testing showed a homozygous mutation in the *BLM* gene c.2643G>A (p.Thr881X). GH treatment was discontinued, and because of Bloom syndrome, she was treated with a modified chemotherapy regime (without cyclophosphamide and adriamycin). She did respond to chemotherapy, but at 16 years of age, she died due to a sepsis.

Patient 2

Patient 2 is a boy, born as the first child of consanguineous Dutch parents. Intrauterine growth retardation was observed during the third trimester. At 33 weeks, a caesarian section was performed due to fetal stress. His birth weight was 1025 g (-3.6 SDS); birth length was unknown. Cranial ultrasound showed multiple small hemorrhages, and eye examination showed chorioretinitis caused by a perinatal toxoplasmosis infection. He was treated with antibiotics for one year. During his first year, he exhibited poor appetite and gastroesophageal reflux, and his height remained at the -4.0 SDS (Figure 1).

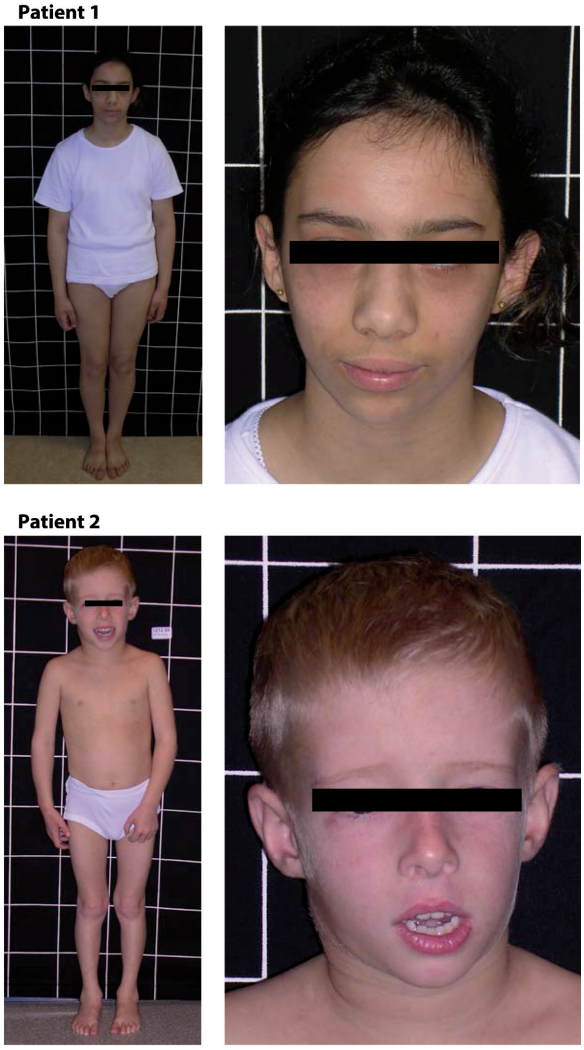

Figure 2. Physical characteristics of patient 1, at 10 years of age, and patient 2 at 4 years of age. Parents gave permission for publication of these photographs.

The height of his father was 185 cm (0.2 SDS) and of his mother 168 cm (-0.4 SDS). He had frequent mild upper airway infections and a dry eczematous skin; both were attributed to an atopic constitution.

At 4 years of age, he was referred to our clinic. His height was 91.4 cm (-4.5 SDS), weight 10.3 kg (-3.6 SDS weight for height), sitting height to height ratio 0.56 (0.9 SDS), and head circumference 45.9 cm (-3.2 SDS). Neurological, cardiovascular, respiratory, and abdominal examinations were all normal. Psychomotor development was normal, and he went to a regular primary school. Overall blood examination showed no abnormalities. An arginine stimulation test was performed with a maximum GH response of 21.5 µg/L (56 mU/L). His IGF-I was 65 ng/mL (-0.5 SDS) and IGFBP-3 was 1.3 mg/L (-1.2 SDS). Thyroid function was normal. IgA was 0.2 g/L (normal 0.1-1.0 g/L), IgG 6.4 g/L (normal 3.3-11.6 g/L) and IgM 0.5 g/L (normal 0.4-1.7 g/L). His bone age was 1 year behind. After examination by several clinical geneticists, he was diagnosed with Silver Russell syndrome, although DNA testing could not confirm UPD 7. Genetic testing showed a normal karyotype (46, XY) and Nijmegen breakage syndrome was not present.

At age 4, GH treatment was initiated at a dose of 1 mg/m^2/day (0.033 mg/kg/day) (Figure 1). During treatment, serum IGF-I levels increased substantially and after 4 years, IGF-I was 510 ng/mL (3.2 SDS). We suspected IGF-I insensitivity, but genetic testing of the *IGF1R* gene showed no abnormalities. The GH dose was reduced to 0.7 mg/m^2/day (0.023 mg/kg/day), but IGF-I levels remained around 3 SDS. IGFBP-3 levels were well within the normal range and overall blood examination, carbohydrate and lipid parameters were normal. Over the years, his dysmorphic features became more prominent (Figure 2 and 3). At the age of 7 years, he was reexamined by a clinical geneticist who reported hypertelorism, mild down slant, a somewhat pointed nose with a wide base, full lips, clinodactyly of the hand and feet and an eczematous skin .

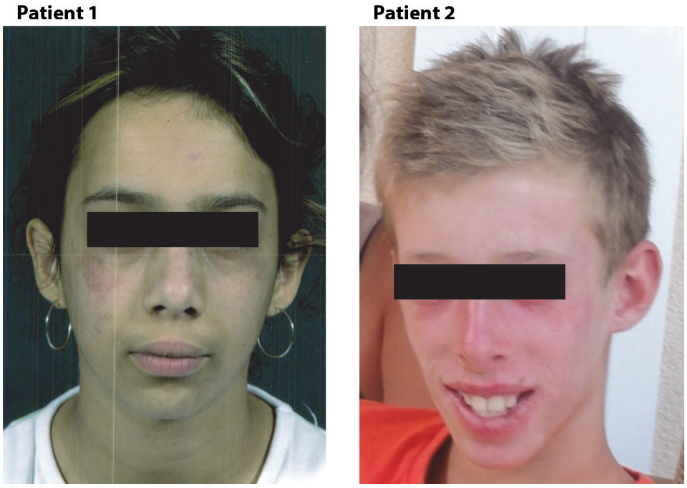

Figure 3. Facial erythema in patient 1, at 13 years of age, and of patient 2 at 11 years of age. Both patients developed a sunsensitive facial erythema at an older age. Parents gave permission for publication of these photographs.

At 9 years of age, he developed a photosensitive facial rash (Figure 3). He was referred to a dermatologist, who diagnosed him with hemorrhagic polymorphous light eruption. We also referred him to a geneticist for testing of Bloom syndrome. This was confirmed, based on a homozygous mutation in the *BLM* gene (c.1933C>T (p.Gln645X)). GH treatment was discontinued, and he is now screened regularly. Two years after the diagnosis, he is doing well. Despite low immunoglobulin levels, serious infections have not yet occurred.

Discussion

Growth hormone (GH) treatment has become a frequently applied growth promoting therapy in short children born small for gestational age (SGA) (3). These children comprise a heterogeneous group, and before GH treatment is started, an extensive diagnostic work-up should be performed to find an underlying cause (1). GH treatment is contraindicated in several disorders, such as the chromosomal breakage syndromes. However, diagnosing these syndromes can be challenging as illustrated by the two patients described here.

Bloom syndrome is one of the chromosomal breakage syndromes and was first described in 1954 (5). It is caused by a mutation in the *BLM* gene that encodes a protein called BLM and is a member of the RecQ helicase family (9). The function of BLM is to maintain genomic stability during DNA replication and repair, and without this protein the number of chromatid exchanges is greatly increased (9, 10). Bloom syndrome is confirmed by finding excessive numbers of sister chromatid exchanges or pathogenic mutations in the *BLM* gene. This gene is located on chromosome 15 (band 15q26.1) (11). Up until now, approximately seventy mutations in around 300 patients have been described (MIM 210900) (12, 13).

Hallmark features of Bloom syndrome are pre- and postnatal growth failure, photosensitive erythematous skin lesions, and a predisposition to develop a wide variety of cancers at an early age (5, 7, 8). The skin lesions usually develop on the nose and cheeks during the first two years of life (5, 7). Other features frequently described are feeding difficulties, immunodeficiency, mental retardation or learning disabilities, and endocrinopathies such as glucose intolerance and abnormal thyroid function (Table 1) (7, 14-16). However, these features can vary considerably and can be subtle as illustrated in both our patients. In the two patients reported here, skin lesions developed only at an older, pubertal age and were minimal, particularly in the girl in whom they resembled cutaneous lupus erythematosus (17). Also, at start of GH treatment, both children showed a normal appetite, normal immunoglobulin levels, normal development, and no signs of endocrinopathies.

Dysmorphological features often described in Bloom syndrome are a small head circumference, a long and somewhat narrow face, malar hypoplasia, nasal prominence, micrognathia, and low-set ears (4, 7). Both our patients had full lips. Although not officially described, this is frequently seen in case reports of patients with Bloom syndrome (6, 7, 18-20). In both our patients, dysmorphic features developed with age: from very subtle at a young age to slowly becoming more apparent. Particularly at a young age, there were striking similarities with Silver Russell syndrome, and in both patients this syndrome was first suspected. However, because most patients with Silver Russell syndrome have a normal head circumference, this may help to differentiate between these diagnoses.

Table 1. Overview of characteristics of Bloom syndrome in children

	Bloom syndrome	Index patients at start of GH
Clinical parameters		
	Birth weight <<-2 SDS	2/2
	Birth length <<-2 SDS	2/2
	Height <<-2 SDS	2/2
	Sun sensitive skin lesions	0/2
	Feeding difficulties	2/2
	Gastroesophageal reflux	1/2
	Frequent (upper) airway infections	1/2
Laboratory parameters		
	Immunodeficiency	0/2
	Hypothyroidism	0/2
	Insulin resistance	0/2
Dysmorphic facial features		
	Long and narrow face	1/2
	Triangular face	2/2
	Ophthalmological abnormalities	1/2
	Hypertelorism	1/2
	Nasal prominence, broad nasal bridge	2/2
	Micrognathia	1/2
	Malar hypoplasia	0/2
	Protuberant ears	Low set ears
		Full lips
Other dysmorphic features		
	Clinodactyly	2/2
	Short stubby fingers	0/2
	Café au lait spots	0/2
Central nervous system		
	Microcephaly	2/2
	Mental retardation	0/2
	Learning disabilities	0/2
	Speech delay	0/2

Since an extensive diagnostic work-up showed no clear abnormalities, one patient was diagnosed with short stature after SGA birth and one with Silver Russell syndrome. Both started GH treatment with a standard dose and responded, increasing their height by 0.7 and 1.1 SDS, respectively, resulting in a height of approximately -1 SDS in the following years. Their first year growth response was comparable to that of other short SGA children (~0.8 SDS) (21). During GH treatment, serum IGF-I increased to levels well above the normal range in both patients, whereas IGFBP-3 levels remained within the normal range. We suspected IGF-I insensitivity and investigated therefore the *IGF1R* gene, but in the end no mutations were found. To our knowledge, high IGF-I levels during standard or low dose GH treatment has not been

described in children with Bloom syndrome. Our data show that this phenomenon can be an indication for diagnosing Bloom syndrome in short SGA children treated with GH.

In retrospect, we regret that we did not lower the GH dose in patient 1 as was done in patient 2. However, this girl started GH treatment in 1998, when IGF-I insensitivity was suspected to cause or contribute to the persistent short stature in short SGA children. At that time, treatment with a higher GH dose was advised to stimulate IGF-I production to overcome IGF-I resistance in short SGA children (22-24). High IGF-I levels are not unusual in short SGA children treated with GH (21). Fall et al. also showed that children with a low birth weight developed higher IGF-I levels than expected for their height and weight, and that these relatively high IGF-I concentrations may reflect a degree of IGF resistance (25). Short SGA subjects with subnormal IGF-I levels during GH therapy show slower growth, while IGF-I levels close to +2 SDS support catch-up growth (22). When the IGF-I levels in patient 1 became high despite a standard GH dose of 1 mg/m^2/day, we suspected severe IGF-I insensitivity, and for that reason we sequenced the *IGF1R* gene. A decade ago, such genetic analyses took considerable time. At the end, no *IGF1R* mutation or deletion was found. With the present knowledge, we advise to lower the GH dose or even stop GH treatment when a short SGA child has recurrent IGF-I levels greater than 2.5 SDS and no *IGF1R* mutation or deletion, awaiting the results of testing for Bloom syndrome.

Based on our data, one might consider to exclude Bloom syndrome in very short SGA children with consanguineous parents, microcephaly and dysmorphic features (particularly resembling Silver Russell syndrome) before starting GH treatment (Table 1). During GH treatment, increased IGF-I levels might also be suggestive for Bloom syndrome (Figure 4).

IGF-I has mitogenic and antiapoptotic properties, so in theory GH treatment might affect cancer risk (26). The role of GH in cancer development is not clear. However, epidemiologic studies have not demonstrated a significantly increased risk in GH-treated patients, with or without a history of cancer (27, 28). Currently available data do not indicate any increase in cancer risk during or after GH treatment and, at this point, we do not believe that GH is causative in the development of cancer in patient 1. Nonetheless, GH treatment is contraindicated in children with Bloom syndrome since many patients develop cancer at a young age. If cancer is present, GH may stimulate malignant cell growth (29).

Management of patients with Bloom syndrome consists of surveillance and treatment of complications. One complication in patients with Bloom syndrome is diabetes mellitus (DM) type 2 (19). There is a high frequency of impaired glucose tolerance and insulin resistance in children, and approximately 15% of patients develop DM (4, 14). In the two patients described here, there were no signs of an altered carbohydrate or lipid metabolism before or during GH treatment. The most frequent long-term complication is cancer, with a mean age at diagnosis of 26 years (range <1 – 49) (8). There is a wide variation in sites and types of malignancies, but patients younger than 20 years are prone to develop leukemia or lymphomas and patients older than 20 years are more prone to develop carcinomas, particularly gastrointestinal and skin carcinomas (8). Patients with Bloom syndrome often develop more than one primary malignancy (8).

There is very little evidence regarding an appropriate screening regimen in children. Yearly evaluation of glucose metabolism, thyroid function and immunoglobulins has been suggested in addition to a

regular hematological examination (14, 19). However, whether early diagnosis of, for example, leukemia improves prognosis is unknown, and frequent examination might also increase the risk of psychological morbidity (8, 19).

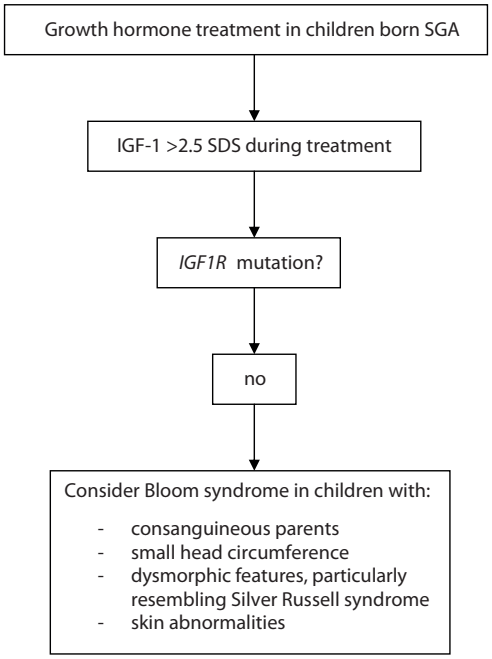

Figure 4. Flowchart increased IGF-I levels during GH treatment

In conclusion, we present two patients with Bloom syndrome who were initially diagnosed with short stature after SGA birth and Silver Russell syndrome and started on GH treatment. Based on our data, we suggest that very short SGA children with consanguineous parents, a small head circumference, dysmorphological features (particularly resembling Silver Russell syndrome), skin abnormalities, and/or IGF-I levels greater than 2.5 SDS during standard GH treatment with normal IGFBP-3 levels are tested for Bloom syndrome.

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Chapter 8

General discussion

Short stature is one of the most common medical concerns in childhood, and SGA accounts for approximately 20% of all cases of short stature (1, 2). In 2003, GH treatment was licensed for short children born SGA, and since then it has become a frequently applied growth promoting therapy. There is, however, a wide variation in growth response and in approximately 15% of children adult height is unsatisfactory (3-7). Even after adjustment for predictive variables, there remains a wide variation which is difficult to explain (8). It is therefore important to investigate new factors which can influence the response to GH. We therefore assessed the effects of long-term GH treatment, and studied the following factors influencing the response to GH treatment: use of methylphenidate, spontaneous catch-up growth before start of GH treatment, pubertal growth, and serum ALS levels. In addition, we determined ternary complex formation in healthy normal statured and GH-treated short SGA children. Furthermore, underlying causes of short stature following SGA birth, such as *IGFALS* gene mutations and Bloom syndrome were investigated. This is important since these may have consequences for growth response and health prognosis.

In this chapter, results from the studies described in this thesis are discussed in view of current literature. The clinical implications of these data, as well as directions for future research are given.

Methylphenidate

Methylphenidate is a frequently prescribed drug used in treating attention deficit hyperactivity disorder (ADHD) (9, 10). Its use has been associated with growth deceleration, suggesting that simultaneous use of GH and methylphenidate might not be advised (11-18). We evaluated whether methylphenidate would negatively affect the response to GH treatment (Chapter 2).

Growth

Our study showed that in the cohort of GH-treated short SGA children, approximately 10% of children were also treated with methylphenidate. This is considerably higher than the reported 5% in The Netherlands, indicating that ADHD occurs more frequently in children born SGA (9). This is line with studies showing that children born SGA have an increased risk of developing ADHD (19-21).

Children treated with GH and methylphenidate had a lower height gain during the first 3 years of combined treatment compared to children treated with GH only. After 3 years of combined GH and methylphenidate treatment, height was 0.2 SDS lower compared to children who received only GH. However, adult height was not significantly different between the two groups. Our short-term data are in line with two comparable studies performed up until now, but we are the first to report adult height in children treated with GH and methylphenidate (22, 23).

Clinical implications and conclusions

The use of methylphenidate has substantially increased in recent years, and more children are being treated with GH and methylphenidate simultaneously. Our data indicate that physicians should be aware that the growth response can be somewhat reduced during the first years of methylphenidate

treatment in children treated with GH. However, it is reassuring that adult height does not seem to be affected. Since this is the first study to report adult height data, future clinical research is needed to confirm our results.

Spontaneous catch-up growth and pubertal growth

For short children born SGA, GH is effective in improving growth and adult height (4-7). In Chapter 3 we evaluated adult height results of 170 GH-treated short children born SGA. We also assessed the influence of spontaneous catch-up growth after birth and growth during puberty on the total height gain SDS to adult height.

GH treatment and adult height in short children born SGA

Children started GH treatment at a median age of 7.1 years. GH treatment significantly improved height SDS, bringing 73% of the children in the normal AH range and/or their target height range. Total height gain was 1.1 SDS, varying between 0.6 to 1.5 SDS. These results are in line with data described by Van Pareren and Dahlgren (5, 7, 24). It has been shown that a younger age at start of GH treatment is positively associated with the growth response (8, 25). However, there is a group of short statured children that only come to medical attention at an older age. In 34% of the study group, age at start of GH treatment was ≥8 years. GH treatment in our older group of children resulted in a median total height gain of 0.8 SDS, in line with the 0.9 SD increase reported by Dahlgren et al (5).

Spontaneous catch-up growth

In 42% of the children, spontaneous catch-up growth after birth was ≥0.5 SDS. We hypothesized that these children would have a higher potential of responding to GH treatment since they had already been able to show catch-up growth. In contrast to our hypothesis, we found that children with greater spontaneous catch-up growth after birth showed a lower total height gain SDS. This might be explained by the fact that children with a greater spontaneous catch-up growth reached a height closer to their target height trajectory, because in our total study group spontaneous catch-up growth was negatively correlated with the distance to target height at start of GH.

Although children with a greater spontaneous catch-up growth showed a smaller total height gain SDS, these children were taller at reaching adult height. This is probably the result of a greater height SDS at start of GH treatment because children with a greater spontaneous catch-up growth were taller at start of GH.

Pubertal growth during GH treatment

Median height gain until onset of puberty was 1.6 SDS in boys, and 1.5 SDS in girls. However, during puberty, height SDS declined with -0.4 SDS in boys and -0.5 SDS in girls, resulting in a lower total height gain SDS. Particularly children who started GH at a younger age showed a decline in height SDS during puberty. Dahlgren et al. also described that children treated for more than 2 years before onset of puberty, showed a loss in height SDS during puberty (-0.2 SDS) (5).

We investigated possible explanations for this decline in height gain SDS during puberty in the group of children who started GH before 8 years of age. Possible explanations might be an early onset of puberty, a reduced pubertal height gain (cm), an acceleration of bone maturation and/or a different pubertal growth pattern (26-29). Our GH-treated SGA children did, however, not start puberty at a younger age compared to normal statured Dutch children born AGA. In fact boys and girls started their puberty even significantly later: 12.1 vs. 11.5 years in boys and 11.4 vs. 10.7 years in girls (30). We found that the median pubertal height gain in cm was indeed reduced compared to the median pubertal height gain of AGA children. This might be due to the later onset of puberty, because children with delayed pubertal development also demonstrate a lower height velocity during their growth spurt (31-33). Furthermore, although there was no acceleration in bone maturation during puberty, BA at onset of puberty was moderately advanced. These findings together might contribute to the early growth deceleration occurring from mid-puberty. This phenomenon has also been described in untreated short SGA children (27).

Although IGF-I SDS levels at onset of puberty and at reaching adult height were the same, relatively low IGF-I levels during puberty could also contribute to the decline in height SDS during puberty. We have previously shown that boys treated with GnRHa and GH 2 mg/m^2/day showed a higher adult height compared to children who received GH 1 mg/m2/day. So doubling the GH dose might help in improving pubertal height gain (6).

Combined GH/GnRHa treatment

When children are short at start of puberty, their adult height is expected to be poor. In 30% of the children, height at onset of puberty was below 140 cm and in these children puberty was postponed for 2 years by adding GnRHa to GH treatment. We found that children with combined GH and GnRHa treatment attained a similar adult height as those who were above 140 cm and treated with GH only. Combined GH/GnRHa treatment resulted in a longer duration of puberty and a higher pubertal height gain compared to children who received GH only. Although our present study was not designed to investigate pubertal postponement by GnRHa versus no postponement in a randomized design, it indicates a beneficial effect of GnRHa treatment probably as a result of delayed BA development. This is in line with recently published data of our group investigating combined GH/GnRHa treatment (6).

Clinical implications and conclusions

We conclude that GH treatment with the standard dose of 1 mg/m^2/day is effective in improving adult height, even when started at an older age. However, this should not lead to postponement of starting GH treatment until an older age, because a younger age at start results in a better adult height despite some height loss during puberty (8, 25). In addition, a normal height during childhood has important advantages (34, 35).

Children with greater spontaneous catch-up growth before start of GH treatment had a lower total height gain SDS than previously expected. Height SDS declined from mid-puberty onwards, due to a marked early deceleration of growth velocity. These findings indicate that height SD score before onset

of puberty is not a reliable predictor of adult height. For that reason physicians should be careful in extrapolating prepubertal SD scores to predict adult height because this can result in an overestimation. Future studies are needed to increase our understanding of the different pubertal growth patterns of short SGA children. Determining the optimal GH dose during puberty in individual children may help in optimizing height gain.

When GH-treated short SGA children are still short at onset of puberty, they might benefit from combined GH/GnRHa treatment. However, results of combined GH/GnRHa treatment in short SGA children are scarce, despite its widespread use in clinical practice. Randomized, long-term and adequately powered trials are needed to fully evaluate the effect of combined GH and GnRHa treatment compared to GH treatment only.

Acid-labile subunit

Short children born SGA have on average low-normal serum IGF-I levels (approximately -1 SDS) and particularly low IGFBP-3 levels (approximately -1.5 SDS) (36). One component of the GH-IGF axis that has received relatively little attention is the ALS. We investigated serum ALS levels in a cohort of 312 short children born SGA, and also assessed whether adding ALS levels to a growth prediction model might improve the long-term growth prediction (Chapter 4).

Acid-labile subunit levels in children born SGA

Short SGA subjects had significantly lower ALS levels (-0.5 SDS) compared to controls with normal stature, albeit within the normal range. Also, ALS levels were less reduced than IGF-I and IGFBP-3 levels. A recent study performed in children with idiopathic short stature also found moderately reduced ALS levels, whilst IGF-I and IGFBP-3 levels were below the -1 SDS (37). Possible explanations might be that GH levels are not reduced to such an extent that ALS production is affected, or IGF-I production is selectively inhibited, or it might be that there are other, unknown, mechanisms involved in the secretion of ALS.

We found that subjects with a lower weight SDS and BMI SDS had lower ALS levels. Others demonstrated lower ALS levels after calorie restriction and in patients with anorexia nervosa (38). These results indicate that nutritional status plays a role in the regulation of ALS levels. Animal data suggest that the primary defect causing decreased ALS levels during calorie restriction may be lower insulin secretion (39). We also found that ALS levels were positively correlated with insulin levels. Furthermore, low ALS levels were associated with increased insulin sensitivity measured by HOMA-IR. This is in line with data in rodents, and might be explained by the fact that IGF-I in the ternary complex cannot cross the vascular endothelium and is therefore unable to regulate GH secretion. However, in the absence of ALS, IGF-I is mainly found in a binary complex which can cross the capillary barrier and can thus control GH secretion (40).

Prediction model

Animal data suggest that the presence of sufficient ALS in serum is required for maximal effectiveness of exogenous GH (41). We found that ALS SDS levels at start of GH treatment are positively correlated

with height SDS at onset of puberty. Using the prediction model described by de Ridder et al., we tested baseline ALS SDS as a contributing factor in the prediction model for height SDS at onset of puberty (8). Adding ALS modestly improved the R^2 of the model by 5% to 74%.

Clinical implications and conclusions

From our study we conclude that short SGA children tend to have lower ALS levels compared to controls, albeit less reduced than IGF-I and IGFBP-3 levels. Our data suggest that ALS may be involved in glucose homeostasis. Prediction models might be used to individualize the GH treatment. The contribution of ALS to the prediction model is modest, as with all predictive variables (e.g. age start of GH treatment and IGFBP-3). However, our results suggest that determination of serum ALS at start of GH treatment may contribute to a more accurate prediction of the growth response to GH treatment. Further research is needed to investigate the role of ALS in glucose homeostasis during and after long-term GH treatment.

IGFALS gene

Several characteristics of short SGA children are also described in children with an *IGFALS* gene mutation. For example, many short children born SGA have low IGFBP-3 levels (36). Low IGFBP-3 levels are very uncommon in children with short stature without GH deficiency. This characteristic is only found in short SGA children and children with an *IGFALS* gene mutation (36, 42). We therefore sequenced the *IGFALS* gene in 214 short children born SGA, of which 79% had an IGFBP-3 level ≤-1 SDS (Chapter 5).

IGFALS gene mutations in children born SGA

We found 4 novel heterozygous missense and 1 novel heterozygous synonymous mutation, only in the group with an IGFBP-3 level below -1 SDS. These children had a median height of -3.0 SDS at start of GH treatment. In 4 out of 5 children, ALS, IGF-I and IGFBP-3 levels were reduced. In addition, 5 heterozygous missense SNPs were found, also only in children with an IGFBP-3 ≤-1 SDS. These children had a median height of -2.3 SDS at start of GH treatment. ALS, IGF-I and IGFBP-3 levels were in the low to low-normal range in 4 out of 5 subjects. Eight out of 10 children with either an *IGFALS* mutation or SNP responded well to GH treatment with an increase in height ≥0.5 SDS during the first year.

We also performed family-analysis which allowed us to perform genotype-phenotype analysis. The clinical picture presented by the patients was heterogeneous and there were some discrepancies between results in one family. However, our data are in agreement with reported cases on heterozygous mutations in the *IGFALS* gene (43-45). In recent years it has become clear that individuals who carry the same disease-causing mutations can exhibit different symptoms. This results in a large variability which cannot always be explained.

Ternary complex formation was also assessed in children and their parents, because reduced formation of the ternary complex could indicate a disturbance in the spatial arrangement of the ALS protein. We found that ternary complex formation improved with increasing age. The age-dependency of complex formation could be an explanation for our finding that results between children and parents

were not always consistent despite similar serum ALS, IGF-I and IGFBP-3 levels. Since normal reference values of ternary complex formation were not available at time of the study, it was difficult to interpret our results. We concluded that further studies were required to determine reference values for ternary complex formation from birth to adulthood.

Clinical implications and conclusions

Genetic mutations in the GH-IGF axis have only been found in 1% of short SGA children (46, 47). Thus, our finding that 6% of the investigated short SGA children with serum IGFBP-3 ≤-1 SDS carry a possibly harmful variation in the *IGFALS* gene indicates that this type of variation occurs relatively frequent. Based on our data, we advise clinicians to consider to test for an *IGFALS* mutation when short SGA children have an IGFBP-3 level ≤-1 SDS, a reduced IGF-I level, signs of insulin resistance, and a head circumference ≤-1 SDS. In children with these characteristics, gene analysis is most likely to detect a pathogenic *IGFALS* variant.

Ternary complex formation and IGFBP-3 protease activity

It is generally accepted that in healthy individuals the majority of IGF-I does not circulate in its free form but is sequestered in a 150 kD ternary complex with IGFBP-3 and ALS (48, 49). Dissociation of IGF-I from the ternary complex is modulated by proteolysis of IGFBP-3. Proteases are capable of cleaving IGFBP-3 into a 29 kD fragment which has a significantly reduced affinity for IGF-I. Presumably, this results in destabilization of the 150 kD ternary complex and an increased bioavailability of IGF-I to target tissues (50, 51).

Column chromatography with ^{125}I-IGF-I can be used as a functional test for assessing ternary complex formation in patients with an *IGFALS* gene deletion or mutation (45). Our previous study on *IGFALS* gene mutations in short SGA children indicated that ternary complex formation improved with increasing age. However, normal reference values of ternary complex formation were not available. Using ^{125}I-hIGF-I column chromatography, we assessed ternary complex formation in healthy controls from birth to adulthood and short SGA children. We also assessed the relationship between ^{125}I-hIGF-I ternary complex formation and clinical and laboratory parameters, including IGFBP-3 proteolysis (Chapter 6).

Reference values ternary complex formation

We found that in healthy controls, formation of the ^{125}I-IGF-I 150 kD ternary complex showed an age-specific pattern, and we are the first to present age-specific reference ranges. This age-dependency could be partly explained by the age- and puberty-related increase in IGF-I, IGF-II, IGFBP-3, and ALS levels, and decrease in IGFBP-1 levels. Indeed, children with higher IGF-I levels compared to IGFBP-3 levels were more likely to form ^{125}I-hIGF-I 150 kD ternary complexes. Higher IGFBP-1 and IGF-II levels resulted in increased 40-50 kD binary complex formation, which consequently resulted in lower 150 kD complex formation.

The method we used to determine ternary complex formation is an *ex-vivo* method and might not represent *in-vivo* complex formation. We therefore also performed neutral chromatography which

revealed that most of IGF-I was present in the 150 kD ternary complex, even in those with severely reduced ^{125}I-hIGF-I 150 kD complex formation. Subsequent investigations showed that young healthy children had higher levels of the proteolysed 29 kD IGFBP-3 fragment, which cannot bind ^{125}I-hIGF-I thus resulting in reduced ^{125}I-hIGF-I 150 kD complex formation. This is a similar phenomenon as previously described in serum from pregnant woman (52).

IGFBP-3 proteolytic activity

IGFBP-3 proteases are capable of cleaving intact 42-39 kD IGFBP-3 into a 29 kD IGFBP-3 fragment which has a significantly reduced affinity for IGF-I. Presumably this results in an increased bioavailability of IGF-I to target tissues. Our results showed that young healthy children showed an increased presence of the 29 kD IGFBP-3 fragment and thus considerable IGFBP-3 proteolytic activity which declined with age. Proteolysis of IGFBP-3 has been observed in newborns, GH-deficient patients, and children with intrauterine growth retardation (53-55). It has been suggested that proteolysis of IGFBP-3 might be related to growth velocity (53). The few studies that have been performed have not found an age-dependent decline in proteolyzed IGFBP-3, but this is likely caused by the small sample size and the absence of healthy controls (54, 55). Although growth velocity is highest during the first few years of life and during puberty, we only found increased IGFBP-3 proteolytic activity during early childhood. This is in line with the data described by Hasegawa et al. in 2 infants and 5 pubertal children, and with the data described by Koedam et al. in mice (55, 56). A possible explanation might be the absolute serum levels of IGF-I during childhood and puberty. Serum IGF-I levels are low during early childhood and increase over time, with a typical pubertal peak between 13 and 17 years of age due to a marked increase in GH secretion (57, 58). Our findings suggest that the IGFBP-3 proteolytic activity during early childhood is increased to ensure the bioavailability of IGF-I to target tissues to stimulate growth despite the relatively low IGF-I levels. During puberty, however, the IGF-I levels are high enough to stimulate growth, without the need to increase IGFBP-3 proteolytic activity (55).

Ternary complex formation in short children born SGA

Compared to controls, short SGA children showed similar ^{125}I-IGF-I 150 kD ternary complex formation, however, 40-50 kD binary complex formation was increased. This may be the result of the higher IGF-II and IGFBP-1 levels found in our short SGA children. Also, short SGA children showed a higher amount of the 29 kD IGFBP-3 fragment compared to our healthy controls (35.1% vs. 12.2%), indicating more IGFBP-3 proteolytic activity. A similar phenomenon was described in GH deficient children (54). It has been suggested that in GH deficiency a protease system is present resulting in proteolysis of IGFBP-3 which presumably leads to the release of IGF-I from the 150 kD complex, thereby increasing the availability of IGF-I for binding to the IGF-I receptor (56). Although none of the SGA children were GH deficient, several studies have demonstrated that up to 60% of short SGA children show a reduced spontaneous GH secretion (36, 59). Serum IGF-I levels in our SGA children were reduced compared to healthy controls.

We also assessed the IGFBP-3 proteolytic activity in short SGA children after 1 year of GH treatment. Both growth velocity and serum IGF-I levels increased significantly after start of GH but the presence

of the 29 kD IGFBP-3 fragment did not change. These findings are in line with those during puberty, indicating that the GH treatment induced high enough serum IGF-I levels to stimulate growth.

Clinical implications and conclusions

Our results show increased IGFBP-3 proteolytic activity during early childhood and in short SGA children, probably to ensure bioavailability of IGF-I to stimulate growth despite relatively low serum IGF-I levels. During puberty and GH treatment, serum IGF-I levels are high enough to stimulate growth without proteolysis. Age and changes in IGFs, IGFBPs and IGFBP-3 proteolytic activity must be taken into account when interpreting results of ^{125}I-hIGF-I column chromatography. A decrease in IGF-I, and an increase in IGF-II, IGFBP-1 and IGFBP-3 proteolytic activity results in reduced ^{125}I-hIGF-I ternary complex formation.

Bloom syndrome

Children born SGA comprise a heterogeneous group, and before GH is started, we always perform an extensive diagnostic work-up to find an underlying cause (60). In Chapter 7, we reported two patients with Bloom syndrome, initially diagnosed with short stature after SGA birth and Silver Russell syndrome, and therefore treated with long-term GH.

Underlying cause of SGA birth and short stature

Bloom syndrome is a rare chromosomal breakage syndrome, and our two patients illustrate the variety in clinical manifestations (61). Both patients presented with pre- and postnatal growth failure, but no clear other characteristic features associated with Bloom syndrome. One of the hallmark features of Bloom syndrome is the development of sun-sensitive skin lesions during the first two years of life (Figure 1) (62, 63). However, in our two patients, skin lesions developed only at a pubertal age and were minimal.

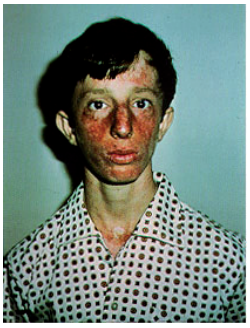

Figure 1. Patient with classical phenotype of Bloom syndrome (70)

Other features frequently described are immunodeficiency, mental retardation or learning disabilities, and endocrinopathies such as an abnormal thyroid function (63-65). None of these features were present and both patients started GH treatment 1 mg/m^2/day. Height increased by 0.7 and 1.1 SDS after 1 year of treatment, resulting in a height of approximately -1 SDS in the following years. Remarkably,

during GH treatment, IGF-I levels increased to values well above the normal range, while IGFBP-3 levels remained normal. We investigated the *IGF1R* gene, but no mutations were found. In patient 2, the GH dose was lowered to 0.7 mg/m^2/day. Because IGF-I has mitogenic and anti-apoptotic properties, GH treatment might in theory affect cancer risk (66). The role of GH in cancer development is not clear but currently available data do not indicate any increase in cancer risk during or after GH treatment (67, 68). Nonetheless, GH treatment is contraindicated in children with Bloom syndrome since many patients develop cancer at a young age (69).

Clinical implications and conclusions

Our data show that features associated with a syndrome are often not yet present at a young age but develop over time. We therefore advice clinicians to be aware of uncommon underlying genetic conditions resulting in short stature after SGA birth, and to frequently re-evaluate the original diagnosis. The combination of a small size at birth, short stature, consanguineous parents, dysmorphic features (particularly resembling Silver Russell syndrome), and skin abnormalities is suggestive for Bloom syndrome. We recommend that children with these characteristics are tested for Bloom syndrome before start of GH treatment. IGF-I levels >2.5 SDS during treatment with GH 1 mg/m^2/day are suggestive for an *IGF1R* gene deletion, but also for Bloom syndrome.

General conclusions

Children born SGA comprise a heterogeneous group with a broad spectrum of clinical characteristics. In these children, GH treatment has become a frequently applied growth promoting therapy. The few high quality clinical trials on the effects of GH treatment have shown that most short SGA children show an improvement of their growth rate during treatment with GH and an improvement of adult height. However, the growth response shows a dramatically wide variation and a satisfactory growth is not always achieved. In the present thesis we investigated new factors which can influence the response to GH treatment. Also, uncovering the cause of short stature in short SGA children is not only important for diagnosis, but also for treatment options and expectations regarding the growth response. In some specific disorders and syndromes the response to GH treatment is poor, or GH treatment is even contraindicated.

We showed that the simultaneous use of GH and methylphenidate can somewhat reduce the short-term growth response to GH, however, adult height was not affected. The results of our GH trial demonstrated that GH treatment is effective in improving height SDS, bringing 73% of the children in the normal AH range and/or their target height range. Children with greater spontaneous catch-up growth after birth showed a smaller pubertal height gain SDS, which resulted in a lower total height gain SDS. Height SDS declined from mid-puberty, due to a marked early deceleration of growth velocity. In addition, we showed that determination of serum ALS at start of GH treatment modestly contributes to a more accurate prediction of the long-term growth response to GH treatment. We investigated the *IGFALS* gene, and found that the combination of a small size at birth, short stature, an IGFBP-3 level ≤-1

SDS, a reduced IGF-I level, signs of insulin resistance, and a head circumference ≤-1 SDS is suggestive for an *IGFALS* mutation. Almost all children with an *IGFALS* mutation responded well to GH treatment. We presented age-specific reference ranges for ^{125}I-hIGF-I 150 kD ternary complex formation. In conditions where IGF-I levels are low, such as in short SGA children, IGFBP-3 proteolytic activity is increased to ensure IGF-I bioavailability. Finally, we described two patients with Bloom syndrome illustrating the variety in clinical manifestations. The features associated with this syndrome developed over time. Both children showed a good growth response during GH treatment. Bloom syndrome should be tested for in children with consanguineous parents, dysmorphic features, skin abnormalities, and/or IGF-I levels >2.5 SDS during standard GH treatment with normal IGFBP-3 levels.

Directions for future research

Because of rising healthcare costs it becomes increasingly important to identify short SGA children before start of GH treatment who will not benefit from long-term GH treatment. Increasing our knowledge of the variability of the SGA phenotype would help in identifying factors that can predict the response to GH treatment. Pharmacogenetic studies could perhaps clarify some of the wide variation in growth response.

There is a necessity to optimize the GH dosage since this will help in improving growth results. By using advanced growth prediction models it might become possible to decide on an individual GH dosage from start of treatment, thereby aiming for the best treatment options for each individual child. To investigate this, a study should be set up where patients are randomized to receive the standard GH dose versus the individualized GH dose. Also, future studies are needed to increase our understanding of the different pubertal growth pattern of short SGA children. Determining the optimal GH dose during puberty in individual children may help in optimizing growth results. Also, long-term and adequately powered trials are needed to fully evaluate the effect of combined GH and GnRHa treatment.

Up until now genetic mutations in the GH-IGF axis have only been found in a small percentage of short SGA children. The lack of a clear identified cause of short stature in this group of children renders an etiopathological approach to therapy difficult. The availability of the complete sequence of the human genome and the introduction of high throughput DNA scanning techniques, such as SNP arrays and whole exome sequencing, will provide us with novel tools to investigate the genetic basis of human stature.

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Chapter 9

Summary
Samenvatting

Summary

Chapter 1

This chapter provides an overview of definitions, prevalence and possible causes of SGA birth. Clinical and endocrinological aspects associated with SGA are described, such as short stature and the GH-IGF-IGFBP axis. Reported effects of GH treatment, also in combination with GnRHa treatment are discussed, as well as possible factors influencing the response to GH treatment. Furthermore, genes in the GH-IGF-IGFBP axis and syndromes involved in short stature are briefly described. Finally, the aims and outline of this thesis are presented.

Chapter 2

GH treatment has become a frequently applied growth promoting therapy in short children born SGA. One factor that could have an impact on the response to GH treatment is the use of methylphenidate which is a frequently prescribed drug in the treatment of attention deficit hyperactivity disorder. Some studies found an association between methylphenidate treatment and growth deceleration, suggesting that simultaneous use of GH and methylphenidate might not be advised. We evaluated whether methylphenidate negatively affects the response to GH on the short-term and on the long-term. We therefore performed anthropometric measurements in 78 short SGA children treated with GH (mean age 10.6 years), 39 of whom were also treated with methylphenidate.

Children treated with GH and methylphenidate had a lower height gain during the first 3 years of combined treatment compared to children treated with GH only. The 3-year height gain was 0.2 SDS smaller in children treated with GH and methylphenidate compared to children treated with GH only. However, adult height was not significantly different between the two groups.

In conclusion, physicians should be aware that the growth response can be somewhat reduced during the first years of methylphenidate treatment in children treated with GH. However, it is reassuring that adult height does not seem to be affected.

Chapter 3

The response to GH treatment shows a wide variation and approximately 15% of the children reach an unsatisfactory adult height. It is therefore important to investigate new factors influencing the growth response to GH treatment. We performed a longitudinal, multicentre GH trial in 170 children, and investigated the efficacy of GH treatment (1 mg/m^2/day) on adult height, and the relation between spontaneous catch-up growth after birth, and growth during puberty on the total height gain SDS to adult height.

In our study, short SGA children started treatment at an average age of 7.1 years. Height increased significantly to 1.8 SDS (TH-corrected AH 1.1 SDS) in boys, and -1.9 SDS (TH-corrected AH 1.3 SDS) in girls. GH treatment in short SGA children who started GH >8 years of age resulted in a median total

height gain of 0.8 SDS. In 42% of the children spontaneous catch-up growth after birth was ≥0.5 SDS which was, in contrast to our hypothesis, negatively correlated with total height gain SDS. During puberty, height declined with -0.4 SDS in boys and -0.5 SDS in girls. Short SGA boys and girls started their puberty significantly later compared to Dutch appropriate for gestational age (AGA) children. Also, pubertal height gain was significantly lower and bone age was moderately advanced compared to AGA children. These findings together might contribute to the early growth deceleration occurring from mid-puberty.

In conclusion, GH treatment improves adult height in short SGA children, even when started after the age of 8 years. Children with greater spontaneous catch-up growth after birth show a lower total height gain SDS during GH. Furthermore, height SDS declines from mid-puberty, due to a marked early deceleration of growth velocity.

Chapter 4

IGF-I and IGFBP-3 play a central role in postnatal growth. Acid-Labile Subunit (ALS) forms a ternary complex with IGF-I and IGFBP-3. Short children born SGA have on average low-normal serum IGF-I levels and particularly low IGFBP-3 levels compared to controls. There were no data on circulating serum ALS levels in short children born SGA. We investigated serum ALS levels in 312 short SGA children, and also assessed whether adding ALS levels to a growth prediction model might improve the long-term growth prediction.

ALS levels were significantly lower (-0.5 SDS) compared to controls, albeit well within the normal range. Also, ALS levels were less reduced than IGF-I and IGFBP-3 levels. Subjects with a lower weight and BMI had lower ALS levels, indicating that nutrition plays a role in the regulation of ALS levels. We also found that ALS levels were positively correlated with insulin levels and negatively with insulin sensitivity. Furthermore, determination of ALS levels before start of GH treatment contributed to the long-term growth prediction (5%).

In conclusion, short children born SGA tend to have lower serum ALS levels compared to healthy controls, albeit less reduced than IGF-I and IGFBP-3 levels. Our data suggest that ALS may be involved in glucose homeostasis. Determination of ALS levels before the start of GH treatment modestly improves the long-term prediction of the growth response.

Chapter 5

Several characteristics of short children born SGA are also described in children with an *IGFALS* gene mutation. For example, many short children born SGA have low IGFBP-3 levels which are very uncommon in children with short stature without GH deficiency. We therefore sequenced the *IGFALS* gene in 214 short children born SGA, of which 79% had an IGFBP-3 level ≤-1 SDS.

We found 4 novel heterozygous missense and 1 novel heterozygous synonymous mutation in the group with an IGFBP-3 level below -1 SDS. These children had a median height of -3.0 SDS. In 4/5 children, ALS, IGF-I and IGFBP-3 levels were reduced. In addition, 5 heterozygous missense single

nucleotide polymorphisms (SNPs) were found. These children had a median height of -2.3 SDS. ALS, IGF-I and IGFBP-3 levels were in the low to low-normal range in 4/5 subjects. In 8/10 children complex formation was reduced. We also performed family-analysis which allowed us to perform genotype-phenotype associations. The clinical picture presented by the patients was heterogeneous and there were some discrepancies between results in one family. However, our data were in agreement with reported cases on heterozygous mutations in the *IGFALS* gene. No mutations or missense SNPs were found in the children with an IGFBP-3 >-1 SDS.

In conclusion, 6% of the investigated short SGA children with serum IGFBP-3 ≤-1 SDS carry a variation in the *IGFALS* gene with a possibly negative influence on growth. Based on our data, we advise clinicians to consider to test for an *IGFALS* mutation when short SGA children have an IGFBP-3 level ≤-1 SDS, a reduced IGF-I level, signs of insulin resistance, and a head circumference ≤-1 SDS. In children with these characteristics, gene analysis is most likely to detect a pathogenic *IGFALS* variant.

Chapter 6

IGF-I is mainly sequestered in a 150 kD ternary complex with IGFBP-3 and ALS. Dissociation of IGF-I from this complex is in part regulated by proteolysis of IGFBP-3. Proteases are capable of cleaving IGFBP-3 into a 29 kD fragment which has a significantly reduced affinity for IGF-I. This results in destabilization of the 150 kD ternary complex and an increased bioavailability of IGF-I to target tissues. Data on ternary complex formation and IGFBP-3 proteolytic activity in healthy children and short SGA subjects have not been established. Complex formation was assessed using ^{125}I-hIGF-I column chromatography in 70 controls and 40 short SGA children. IGFBP-3 was determined by Western immunoblotting.

^{125}I-hIGF-I complex formation showed an age-specific pattern in healthy controls. Variables positively influencing ternary complex formation were higher serum IGF-I levels compared to IGFBP-3 levels, and lower serum IGF-II and IGFBP-1 levels. In addition, a higher presence of proteolyzed IGFBP-3 negatively influenced 150 kD complex formation. At a young age, healthy children showed considerable IGFBP-3 proteolytic activity which declined with aging. IGFBP-3 proteolytic activity was negatively correlated with IGF-I levels. Compared to healthy controls, short SGA children showed reduced IGF-I levels and increased proteolyzed 29 kD IGFBP-3.

In conclusion, our results show increased IGFBP-3 proteolytic activity during early childhood and in short SGA children, probably to ensure bioavailability of IGF-I to stimulate growth despite relatively low serum IGF-I levels. During puberty and GH treatment, serum IGF-I levels are high enough to stimulate growth without proteolysis. Age-specific reference ranges for ^{125}I-hIGF-I 150 kD ternary complex formation are presented. A decrease in IGF-I, and an increase in IGF-II, IGFBP-1 and IGFBP-3 proteolytic activity results in reduced ^{125}I-hIGF-I ternary complex formation.

Chapter 7

Children born SGA comprise a heterogeneous group, and before GH is started a thorough diagnostic work-up should be performed to find an underlying cause. In some specific disorders and syndromes GH treatment is contraindicated, such as the chromosomal breakage syndromes. Bloom syndrome is a

rare chromosomal breakage syndrome and diagnosing this syndrome can be challenging. We report two patients illustrating the variety in clinical manifestations.

Both patients presented with pre- and postnatal growth failure, but no clear other characteristic features associated with Bloom syndrome. One of the hallmark features of Bloom syndrome is the development of sun-sensitive skin lesions which our two patients only developed at a pubertal age and were minimal. Also, none of the other features frequently described in Bloom syndrome, such as immunodeficiency, mental retardation or learning disabilities, and endocrinopathies, were present at start of GH. In both patients, dysmorphic features developed with age and only features resembling Silver Russell syndrome were observed. Remarkably, during GH treatment IGF-I levels increased to values >3.0 SDS, with normal IGFBP-3 levels.

In conclusion, our data show that features associated with a syndrome are often not yet present at a young age but develop over time. The combination of a small size at birth, short stature, consanguineous parents, dysmorphic features (particularly resembling Silver Russell syndrome), and skin abnormalities is suggestive for Bloom syndrome. We recommend that children with these characteristics are tested for Bloom syndrome before start of GH treatment. IGF-I levels >2.5 SDS during treatment with GH 1 mg/m^2/day are suggestive for an *IGF1R* gene deletion, but also for Bloom syndrome.

Chapter 8

In the general discussion, we discuss our findings in a broader context. We emphasize clinical implications and give suggestions for further research.

Samenvatting

Hoofdstuk 1

Dit hoofdstuk beschrijft de definities, prevalentie en mogelijke oorzaken van een kleine lengte en/of laag gewicht bij de geboorte (SGA, small for gestational age). Klinische en endocrinologische aspecten die samenhangen met SGA worden besproken, zoals een te kleine lichaamslengte en de GH-IGF-IGFBP as. Eerder beschreven effecten van groeihormoonbehandeling en factoren die de respons op groeihormoon kunnen beïnvloeden worden samengevat, ook in combinatie met uitstel van de puberteit. Vervolgens worden genen betrokken bij de GH-IGF-IGFBP-as besproken, evenals syndromen die kunnen leiden tot een kleine lichaamslengte. Aan het eind van dit hoofdstuk worden de doelstellingen van de studies en de indeling van dit proefschrift besproken.

Hoofdstuk 2

SGA geboren kinderen met een blijvend kleine lengte hebben baat bij groeihormoon (GH)-behandeling, maar de groeirespons is variabel. Eén van de factoren die de groeirespons kan beïnvloeden is het gebruik van methylfenidaat, een medicijn dat wordt voorgeschreven bij ADHD. Een aantal studies heeft laten zien dat behandeling met methylfenidaat geassocieerd is met groeivertraging. Dit suggereert dat gelijktijdige behandeling met GH en methylfenidaat niet aan te raden is. Wij onderzochten of methylfenidaat de groeirespons negatief beïnvloedt, zowel op de korte als de lange termijn. De groei van 78 te kleine, SGA geboren kinderen (gemiddelde leeftijd 10,6 jaar) die behandeld werden met GH werd geanalyseerd, 39 kinderen werden ook behandeld met methylfenidaat.

Kinderen die behandeld werden met GH en methylfenidaat hadden een lagere groeirespons gedurende de eerste drie jaar van de gecombineerde behandeling in vergelijking met de kinderen die alleen behandeld werden met GH. Na 3 jaar was de groeirespons 0,2 SDS lager in kinderen die behandeld werden met GH en methylfenidaat in vergelijking met de kinderen die alleen behandeld werden met GH. De volwassen lengte was niet verschillend tussen de twee groepen.

Wij concluderen dat methylfenidaat de groeirespons gedurende de eerste jaren negatief kan beïnvloeden in te kleine, SGA geboren kinderen die behandeld worden met GH. Het is echter geruststellend dat de volwassen lengte niet negatief beïnvloed lijkt te worden.

Hoofdstuk 3

Kinderen die SGA geboren zijn met een persisterend kleine lengte kunnen behandeld worden met GH. De groeirespons laat echter een grote variatie zien en ongeveer 15% van de kinderen reageert niet adequaat, resulterend in een kleine volwassen lengte. Het is daarom belangrijk om nieuwe factoren te onderzoeken die de groeirespons kunnen beïnvloeden. Wij evalueerden de effectiviteit van GH-behandeling met een dosering van 1 mg/m^2/dag op de volwassen lengte in een longitudinale, multicenter GH studie in 170 te kleine, SGA geboren kinderen. Daarnaast onderzochten wij of de spontane inhaalgroei na geboorte en de groei tijdens de puberteit gerelateerd is aan de totale lengtewinst SD-score.

In onze studie was de leeftijd bij start van de behandeling gemiddeld 7,1 jaar. De lengte verbeterde significant naar -1,8 SDS bij volwassen lengte in jongens (-1,1 SDS gecorrigeerd voor de doellengte) en -1,9 SDS bij volwassen lengte in meisjes (-1,3 SDS gecorrigeerd voor de doellengte). De gemiddelde totale lengtewinst van de kinderen die ouder dan 8 jaar waren bij start van behandeling was 0,8 SDS. In 42% van de kinderen was de spontane inhaalgroei na de geboorte ≥0.5 SDS. In tegenstelling tot onze hypothese was de spontane inhaalgroei na de geboorte negatief gecorreleerd aan de totale lengtewinst SDS. Tijdens de puberteit nam de lengte met 0,4 SDS af in jongens en met 0,5 SDS in meisjes. Te kleine, SGA geboren jongens en meisjes starten significant later met de puberteit in vergelijking met Nederlandse kinderen met een normale grootte bij de geboorte (AGA, appropriate for gestational age). Daarnaast was de groei tijdens de puberteit significant lager en de botleeftijd mild voorlopend in vergelijking met AGA-controles. Deze bevindingen zorgen samen mogelijk voor de vroege afname van de groeisnelheid vanaf de tweede helft van de puberteit.

Concluderend, GH-behandeling resulteert in een betere volwassen lengte in te kleine, SGA geboren kinderen, zelfs wanneer gestart wordt op een leeftijd boven de 8 jaar. Kinderen met een grotere spontane inhaalgroei na de geboorte laten een lagere totale lengtewinst SDS zien. Verder neemt de lengte SDS af in de tweede helft van de puberteit, ten gevolge van een snelle afname van de groeisnelheid.

Hoofdstuk 4

IGF-I en IGFBP-3 spelen een centrale rol in postnatale groei. Acid-labile subunit (ALS) vormt samen met IGF-I en IGFBP-3 een stabiel tertiair complex dat circuleert in de bloedsomloop. Te kleine, SGA geboren kinderen hebben gemiddeld laag-normale serum IGF-I spiegels en met name lage IGFBP-3 spiegels in vergelijking met controles. Er waren nog geen data beschikbaar over circulerende serum ALS spiegels in te kleine, SGA geboren kinderen. Wij bepaalden serum ALS spiegels in 312 te kleine, SGA geboren kinderen. Daarnaast onderzochten wij of het toevoegen van ALS resultaten aan een groei predictiemodel de lange termijn predictie verbetert.

Serum ALS spiegels waren significant lager (-0,5 SDS) in vergelijking met controles, ofschoon de spiegels ruim binnen de normaalwaarde was. ALS spiegels waren ook minder verlaagd in vergelijking met IGF-I en IGFBP-3 spiegels. Kinderen met een lager gewicht en BMI hadden lagere ALS spiegels, hetgeen er op wijst dat voeding een rol speelt in de regulatie van ALS spiegels. We vonden ook dat ALS spiegels positief gecorreleerd waren met insuline spiegels en negatief gecorreleerd met de insuline gevoeligheid. Het bepalen van ALS spiegels voor start van de behandeling kan bijdragen aan een betere groeipredictie op de lange termijn (5%).

Wij concluderen dat te kleine, SGA geboren kinderen lagere ALS spiegels hebben in vergelijking met controles, maar dat deze minder verlaagd zijn dan de IGF-I en IGFBP-3 spiegels. Onze data suggereren dat ALS mogelijk betrokken is bij de glucose homeostase. Het bepalen van ALS spiegels voor de start van de GH-behandeling geeft een bescheiden verbetering van de lange termijn groeipredictie.

Hoofdstuk 5

Verschillende karakteristieken die beschreven worden in te kleine, SGA geboren kinderen worden ook beschreven in kinderen met een *IGFALS* mutatie, zoals bijvoorbeeld lage serum IGFBP-3 spiegels. Lage IGFBP-3 spiegels worden veelal niet gevonden in niet-SGA geboren kinderen met een te kleine lengte zonder GH deficiëntie. Wij hebben daarom het *IGFALS* gen gesequenced in 214 te kleine, SGA geboren kinderen waarvan 79% een IGFBP-3 ≤-1 SDS had.

We hebben 4 nieuwe heterozygote missense en 1 nieuwe heterozygote synonymous mutatie gevonden in de groep kinderen met een serum IGFBP-3 ≤-1 SDS. Deze kinderen hadden een gemiddelde lengte van -3,0 SDS. In 4 van de 5 kinderen vonden we lage serum ALS, IGF-I en IGFBP-3 spiegels. We vonden bovendien 5 heterozygote missense single nucleotide polymorphismen (SNPs). Deze kinderen hadden een gemiddelde lengte van -2,3 SDS. Bij hen vonden we lage ALS, IGF-I en IGFBP-3 spiegels in 4 van de 5 kinderen. In 8 van de 10 kinderen was de tertiaire complexvorming verminderd. We hebben ook de verschillende familieleden onderzocht waardoor we genotype-fenotype vergelijkingen konden uitvoeren. Het klinische beeld was erg heterogeen en er waren enige discrepanties tussen de resultaten in één familie. Onze data zijn echter wel in overeenstemming met eerder gepubliceerde gegevens over kinderen en volwassenen met een heterozygote *IGFALS* mutatie. We vonden geen mutaties of missense SNPs in de kinderen met een serum IGFBP-3 >-1 SDS.

Concluderend, 6% van de onderzochte te kleine, SGA geboren kinderen met een serum IGFBP-3 ≤-1 SDS dragen een variatie in het *IGFALS* gen dat mogelijk een negatieve invloed heeft op de lengtegroei. Gebaseerd op onze data adviseren wij te overwegen om te kleine, SGA geboren kinderen met een IGFBP-3 ≤-1 SDS, lage IGF-I spiegels, een hoofdomtrek ≤-1 SDS en tekenen van insuline resistentie te testen op een *IGFALS* mutatie. In kinderen met deze karakteristieken is de kans het grootst om met genetisch onderzoek een pathogene *IGFALS* variant te vinden.

Hoofdstuk 6

IGF-I is voornamelijk gebonden in een tertiair complex van 150 kD met IGFBP-3 en ALS. Dissociatie van IGF-I van dit complex wordt deels gereguleerd door proteolyse van IGFBP-3. Proteasen kunnen IGFBP-3 afbreken tot een 29 kD fragment. Dit fragment heeft een sterk gereduceerde affiniteit met IGF-I. Het gevolg hiervan is destabilisatie van het150 kD complex en een verhoogde beschikbaarheid van IGF-I voor de weefsels. Er zijn geen gegevens beschikbaar van tertiaire complexvorming en IGFBP-3 proteolyse in gezonde kinderen en te kleine, SGA geboren kinderen. Wij hebben daarom de complexvorming onderzocht door middel van ^{125}I-hIGF-I kolomchromatografie in 70 gezonde kinderen en 40 te kleine, SGA geboren kinderen. IGFBP-3 werd onderzocht door middel van Western Immunoblots.

^{125}I-hIGF-I kolomchromatografie vertoonde een leeftijdsspecifiek patroon in gezonde controles. Complexvorming werd positief beïnvloed door hogere serum IGF-I spiegels in vergelijking tot IGFBP-3 spiegels en lagere serum IGF-II en IGFBP-1 spiegels. De150 kD complexvorming werd negatief beïnvloed door een hogere aanwezigheid van geproteolytiseerd IGFBP-3. Gezonde, jonge kinderen vertoonden aanzienlijke IGFBP-3 proteolyse, dat daalde naarmate ze ouder werden. IGFBP-3 proteolyse was negatief

gecorreleerd met de IGF-I spiegels. Te kleine, SGA geboren kinderen vertoonden in vergelijking met controles lagere serum IGF-I spiegels en een toegenomen aanwezigheid van 29 kD IGFBP-3.

Concluderend tonen onze resultaten een verhoogde IGFBP-3 proteolyse tijdens de vroege kinderjaren en in te kleine, SGA geboren kinderen. Dit zorgt er waarschijnlijk voor dat de beschikbaarheid van IGF-I wordt gewaarborgd om de groei te kunnen stimuleren ondanks relatief lage serum IGF-I spiegels. Tijdens de puberteit en GH-behandeling zijn de serum IGF-I spiegels hoog genoeg om de groei te stimuleren en is IGFBP-3 proteolyse niet noodzakelijk. Verder presenteren we leeftijdsspecifieke referentiewaarden voor ^{125}I-hIGF-I complex vorming. ^{125}I-hIGF-I complexvorming wordt negatief beïnvloed door lagere IGF-I spiegels en hogere IGF-II , IGFBP-1 spiegels, alsmede IGFBP-3 proteolyse.

Hoofdstuk 7

SGA geboren kinderen vormen een heterogene groep en GH-behandeling moet vooraf worden gegaan door een zorgvuldig diagnostisch traject om zo een eventuele onderliggende oorzaak aan te tonen. In sommige specifieke ziekten en syndromen is GH-behandeling gecontra-indiceerd, zoals bijvoorbeeld in geval van chromosomale breuksyndromen. Eén van deze zeldzame syndromen is het Bloom syndroom dat zeer moeilijk te diagnosticeren kan zijn. Wij presenteren twee patiënten die de variabiliteit van het klinische beeld illustreren.

Beide patiënten presenteerden zich met pre- en postnatale groeivertraging, maar geen duidelijke andere karakteristieken die vaak beschreven zijn in patiënten met het Bloom syndroom. Eén van de karakteristieke kenmerken van het Bloom syndroom is het ontwikkelen van een ernstige zonovergevoeligheid op zeer jonge leeftijd. Onze twee patiënten ontwikkelden dit echter pas rond de puberteit en bovendien waren de klachten minimaal. Daarnaast waren bij het starten van de GH-behandeling geen andere kenmerken aanwezig die frequent beschreven worden in patiënten met het Bloom syndroom, zoals immunodeficiëntie, mentale retardatie of leerstoornissen en endocrinopathieën. Met het ouder worden ontwikkelden beide patiënten meer dysmorfe kenmerken vergelijkbaar met het Silver Russell syndroom. Opmerkelijk was dat tijdens de GH-behandeling, de serum IGF-I spiegels stegen tot >3,5 SDS, terwijl de IGFBP-3 spiegels normaal bleven.

Concluderend laten onze data zien dat de kenmerken die geassocieerd zijn met een syndroom op jonge leeftijd vaak nog niet aanwezig zijn en zich ontwikkelen met de tijd. De combinatie van SGA geboorte, te kleine lengte, consanguine ouders, dysmorfe kenmerken (vooral indien lijkend op Silver Russell syndroom) en huidafwijkingen in het gelaat is suggestief voor het Bloom syndroom. Wij raden aan om kinderen met deze kenmerken te testen op Bloom syndroom voordat de GH-behandeling wordt gestart. IGF-I spiegels >2,5 SDS tijdens GH-behandeling zijn niet alleen suggestief voor een *IGF1R* deletie, maar dus ook voor het Bloom syndroom.

Hoofdstuk 8

In de algemene discussie worden de resultaten van de verschillende studies besproken in een bredere context. Wij sluiten dit hoofdstuk af met algemene overwegingen en suggesties voor toekomstig onderzoek.

Chapter 10

List of abbreviations

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List of Abbreviations

ADHD	attention deficit hyperactivity disorder
AGA	appropriate for gestational age
AH	adult height
ALS	acid-labile subunit
BMI	body mass index
CPM	counts per minute
GH	growth hormone
GnRHa	gonadotropin releasing hormone analogue
HOMA-IR	homeostasis model assessment insulin resistance index
IGF	insulin-like growth factor
IGF-IR	insulin-like growth factor-I receptor
IGFBP	insulin-like growth factor binding protein
IQR	interquartile range
IR	insulin receptor
IUGR	intra uterine growth retardation
kD	kilodalton
LRR	leucine-rich repeat
MP	methylphenidate
SDS	standard deviation score
SGA	small for gestational age
SNP	single nucleotide polymorphism
TCF	ternary complex formation
TH	target height
WIB	western immunoblot

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Curriculum Vitae

Judith Renes was born on the 29th of July 1983 in Naarden, The Netherlands. She grew up in Huizen, where she graduated from secondary school in 2001 at the Erfgooiers College. That same year, she started her medical training at the VU University. In this period she became interested in paediatrics, and from her third year onward she participated in the graduation program Child & Youth. During her final elective internship she became involved in a research project at the Research Center Linnaeus Institute of the Spaarne Hospital, Hoofddorp. After obtaining her medical degree in December 2007, she worked for 1 year as a paediatric resident (ANIOS) at the Diakonessenhuis, Utrecht. In March 2009, she started working at the Neonatal Intensive Care Unit of the Erasmus Medical Center – Sophia Children's Hospital in Rotterdam. After 7 months she started working on a clinical research project (IUGR-3 study) at the department of Paediatric Endocrinology of the Erasmus Medical Center – Sophia Children's Hospital (supervisor Prof.dr. A.C.S. Hokken-Koelega), which has resulted in this thesis. Part of her research project was performed at the Laboratory of Endocrinology, University Medical Center Utrecht in Utrecht. In January 2014 she will start her paediatric residency in training (AIOS) at the Erasmus Medical Center – Sophia Children's Hospital in Rotterdam (heads: Prof.dr. A.J. van der Heijden and Prof.dr. M. de Hoog).

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PhD Portfolio

Summary of PhD training and teaching activities

Erasmus MC Department:	Pediatrics, Subdivision of Endocrinology
Research School:	Molecular Medicine Postgraduate School (MolMed)
PhD period:	October 2009 – December 2013
Promotor:	Prof.dr. A.C.S. Hokken-Koelega

1. PhD training

	Year	ECTS
General courses		
Presenting skills course, MolMed, Erasmus MC	2013	1
Integrity in scientific research, Erasmus MC	2012	2
Biomedical English writing and communication, MolMed, Erasmus MC	2011	4
Classical methods for data-analysis, NIHES, Erasmus MC	2010	5.7
Methodology of research and preparing grant applications, Erasmus MC	2010	0.5
Good clinical practice, Erasmus MC	2010	1
Specific courses		
Basic human genetics course, MolMed, Erasmus MC	2011	0.5
SNPs and Human Disease, MolMed, Erasmus MC	2010	1.4
Pubmed and Endnote, Medical Library, Erasmus MC	2009	0.3
Seminars and workshops		
Pediatric research day, Erasmus MC	2013	0.3
Young investigator day, TULIPS / NVK	2012	0.9
Indesign CS5, MolMed, Erasmus MC	2012	0.2
Photoshop and Illustrator CS5, MolMed, Erasmus MC	2011	0.3
Molecular diagnostics for medical doctors, MolMed, Erasmus MC	2010	0.3
Annual Lof der Geneeskunst, Erasmus MC	2009-2011	0.5
Annual PhD day	2010-2012	0.9
Weekly Pediatric Endocrinology meetings, Erasmus MC	2009-2013	4
Conferences		
International		
9th Joint Meeting LWPES/ESPE, Milan, Italy (oral presentation)	2013	1
Meeting "10 years SGA treatment experience insights & future perspectives", Madrid, Spain (oral presentation)	2013	1

	Year	ECTS
51st Annual Meeting of the ESPE, Leipzig, Germany (poster presentation)	2012	1
50th Annual Meeting of the ESPE, Glasgow, United Kingdom(oral presentation)	2011	1
49th Annual Meeting of the ESPE, Prague, Czech Republic (poster presentation)	2010	1
National		
Congress Dutch society for paediatrics (NVK) (oral presentation)	2013	1
Master class Paediatric Endocrinology II: SGA (small for gestational age) (oral presentation)	2011	1

2. Teaching activities

Lecturing

Lecture Symposium "Short adolescents born SGA"	2012	0.5
Lecture Annual SGA day (SGA platform)	2012	0.5
Educational lecture Novo Nordisk	2012	1
Educational lecture minor students	2011	1

Advising

Medical advisor SGA platform	2012-2013	0.5

3. Other activities

Board of PhD students (Sophia Onderzoekers Vertegenwoordiging)	2011-2013	4
Peer review of articles for international scientific journals	2012-2013	0.5

'Feeling gratitude and not expressing it is like wrapping a present and not giving it'

Dankwoord

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