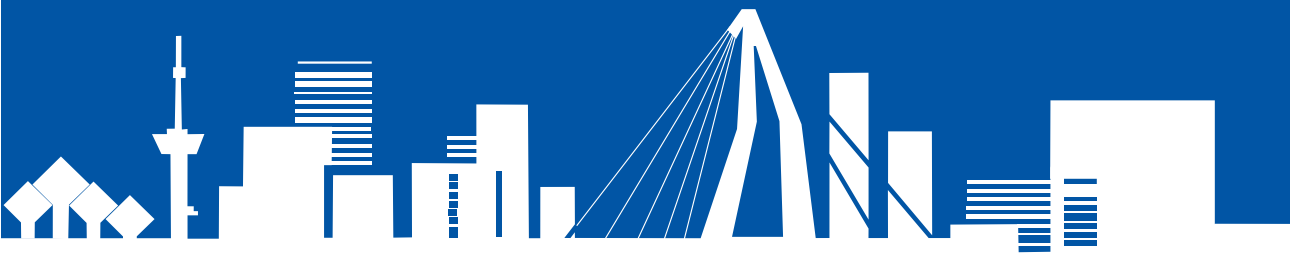

Short Children Born Small for Gestational Age (SGA)

Puberty, Hormonal Profiles, Combined GnRHa and GH Treatment and (Epi)genetics of 2 IGFBP Gene Promoters

Daniëlle C.M. van der Kaay

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Klein geboren kinderen (SGA) met een blijvend kleine lengte

Puberteit, hormoonprofielen, gecombineerde GnRHa en GH Behandeling
en (epi)genetica van 2 IGFBP gen promoters

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam op
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Prof.dr. S.W.J. Lamberts
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Dedicated to my parents and sister

Contents

Chapter 1

General Introduction

This chapter provides definitions and describes prevalence and possible causes of small size at birth (Small for Gestational Age (SGA)), including associated endocrine and genetic factors. The effects of growth hormone (GH) treatment are discussed, with a special emphasis on the effects of GH treatment in short SGA children who present at puberty. Finally, the outline of this thesis, inclusion and exclusion criteria and study designs of the SGA study (appendix A), IUGR-1 and IUGR-2 study (appendix B), IUGR-3 study (appendix C) and PROGRAM study (appendix D) are presented.

1. Small for gestational age (SGA)

1.1 Definitions of SGA

In order to determine whether a child is born SGA the following is required: 1) an accurate knowledge of gestational age, 2) accurate measurements of weight and length at birth, and 3) an appropriate reference population in order to determine a standard deviation score for birth weight and/or birth length [1]. SGA is defined by a birth weight and/or length below -2 standard deviation scores (SDS), adjusted for gestational age [2]. Nowadays, it is increasingly recognized that demographic factors such as maternal age, race, height, weight, ethnicity and parity, and gender of the baby have an influence on size at birth.

SGA only refers to size at birth and does not take fetal growth into account. The term intrauterine growth retardation (IUGR) is used when a fetus suffers from reduced fetal growth, based on two ultrasound measurements. A child born SGA has not necessarily suffered from IUGR, but may have been small from the beginning of fetal life. On the other hand, a child with IUGR late in gestation can have a normal size at birth. These different fetal growth patterns are shown in Figure 1.

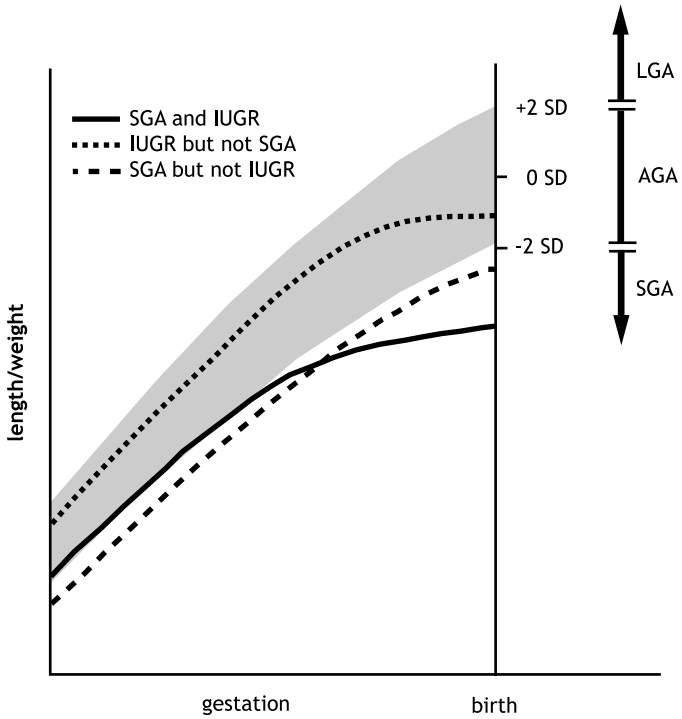

Figure 1. Fetal growth chart showing various growth curves in SGA and IUGR newborns.

1.2 Prevalence and etiology of SGA

Approximately 2.3% of all live-born neonates are born SGA, when SGA is defined as a birth weight or birth length below -2 SDS. Intrauterine growth retardation might be caused by numerous fetal, maternal, placental and environmental factors. It is, however, important to realize that the cause of intrauterine growth retardation remains unidentified in 40% of the cases. Table 1 shows some of the many factors that are associated with IUGR.

Table 1. Factors associated with intrauterine growth retardation.

Fetal factors	
Multiple births	
Congenital malformations	
Chromosomal anomalies	Turner syndrome Down syndrome
Inborn errors of metabolism	
Intrauterine infections	Toxoplasmosis, Other infections, Rubella, Cytomegalovirus, Herpes simplex (TORCH)
Maternal factors	
Medical conditions	Pre-eclampsia Acute or chronic hypertension Severe chronic disease Severe chronic infections Systemic lupus erythematosus Antiphospholipid syndrome Anemia Malignancy Abnormalities of the uterus
Social conditions	Malnutrition Low prepregnancy body mass index Low maternal weight gain Delivery at age <16 or >35 years Low socioeconomic status Drug use (smoking, alcohol, illicit drugs)
Placental factors	
Reduced blood flow	
Reduced area for exchange oxygen and nutrients	Infarcts Hematomas Partial abruption
Environmental factors	
High altitude	
Toxic substances	

Adapted from Bryan and Hindmarch [3].

2. The GH-IGF axis

Fetal and postnatal growth and development are regulated by complex metabolic and endocrine processes, which are under the influence of genetic and environmental factors. The GH-IGF-IGFBP axis (Figure 3) plays a major role in this system and is described in this chapter.

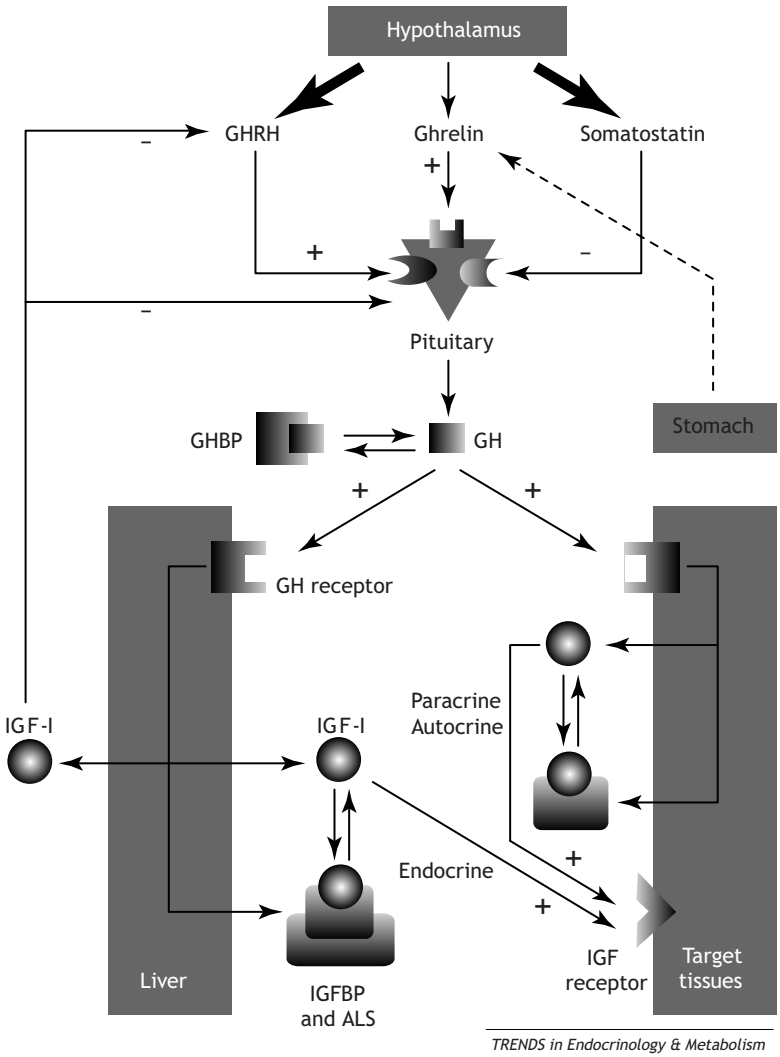

Figure 3. Physiology of the GH-IGF-IGFBP axis. Adapted from Holt [4].

2.1 Growth hormone

GH is secreted by the pituitary gland under the control of the hypothalamic hormones GH-releasing hormone (GHRH) and somatostatin, as well as ghrelin. Ghrelin is predominantly secreted by the stomach, but expression in the hypothalamic arcuate nucleus has also been found [5]. The major effects of GH on growth are mediated via IGF-I expression. GH has also IGF-I independent effects on the growth plate.

Igf-I gene (*Igf1*) knockout mice with increased GH secretion in the absence of the negative feedback of IGF-I, have expansion of the growth-plate germinal zone [6]. Furthermore, GH regulates gene expression of other growth factors important for normal growth [7]. The physiological actions of GH involve multiple organs and physiological systems, amongst which are longitudinal bone growth and bone remodeling, skeletal muscle growth and immunomodulation. Extrapituitary GH production has been reported, suggesting that GH may also play a local paracrine-autocrine role [8].

2.2 Insulin-like growth factors

The Insulin-like Growth Factor (IGF)-family consists of insulin, IGF-I and IGF-II. They show structural similarity by sharing approximately 50% of their aminoacids. The metabolic actions of insulin are mediated through binding to the insulin receptor (IR). The growth-promoting effects of IGF-I and IGF-II are primarily mediated through binding to the IGF-I receptor (IGF-IR). Because of the strong homology between IGFs and insulin and between the IR and IGF-IR, interactions between IGFs and the IR take place [9]. The insulin-like effects of IGF-I are only 5% that of insulin, but in case of excess of free IGF-I, it is capable of lowering glucose levels 50 times more than insulin alone. This is, however, prevented by binding of IGF-I to specific IGF binding proteins (IGFBPs) in the circulation. Between 0.4% and 2% of IGF-I levels circulate as free IGF-I or at least very easily dissociable IGF-I. Acute as well as long-term biological effects of free IGF-I have been described, indicating that free IGF-I is the main biological active fraction [10]. Next to growth, IGFs are important in the development and function of the central nervous system, skeletal muscle and reproductive organs. IGF-I is also locally produced in various tissues, expanding its role to an autocrine-paracrine one, in addition to the classical endocrine role (Figure 4).

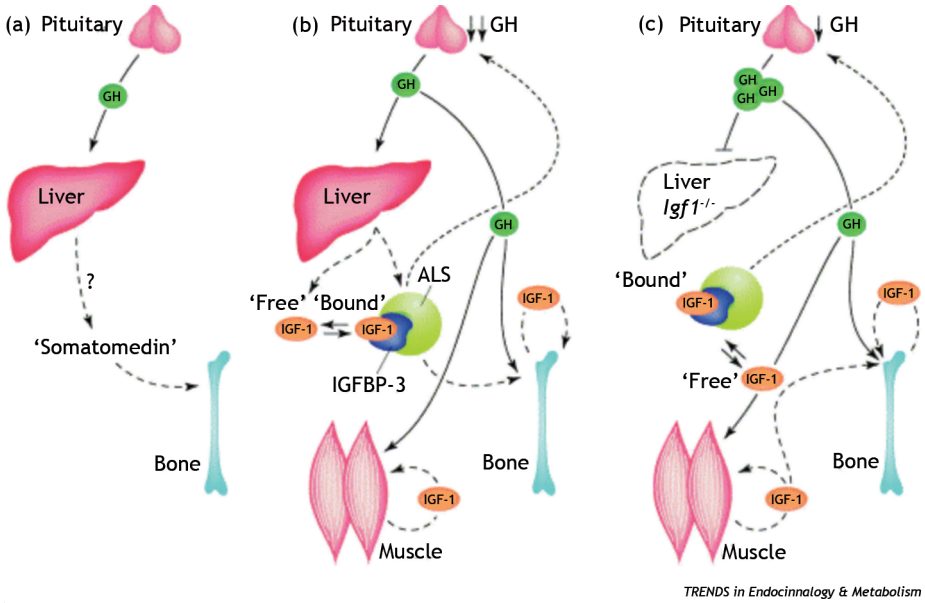

Figure 4. The somatomedin hypothesis. (a) The original hypothesis suggested that effects of GH were mediated by IGF-I, which was derived from the liver. (b) Revision of the original hypothesis, since IGF-I was found to be produced by various other tissues. Furthermore, GH was found to affect circulating IGF-I levels, as well as the IGF-I/IGFBP-3/acid-labile subunit (ALS) complex. In addition, GH was capable of influencing local tissue production of IGF-I. (c) The most recent studies suggest that, at least in mice, liver-derived IGF-I is not essential for postnatal growth and development. Adapted from Le Roith et al [7].

2.3 IGF binding proteins

Six IGFBPs are produced in various tissues and form complexes with IGF-I and -II, ensuring that more than 95% of circulating IGF-I is bound. The majority of IGF-I and -II (75%) is bound in a ternary complex with IGFBP-3 and an acid-labile subunit (ALS). IGFBPs share a strong homology, mostly at the N- and C- terminal regions [11]. Functions of IGFBPs are summarized in Table 2.

Table 2. Functions of the insulin-like growth factor binding proteins.

All IGFBPs	Decrease bioavailability of free IGFs to bind to IGF receptors
IGFBP-1 and -2	Prevent IGF-induced hypoglycemia
IGFBP-1, -2, -3 and -4?	Regulate transport of IGFs between intra- and extravascular spaces
IGFBP-3	Prolong the half-life of IGFs in the circulation
IGFBP-1, -3 and -5	Enhance actions of IGFs by forming a slow-releasing pool of IGFs
IGFBP-1 and -3	Direct cellular effects via own IGFBP receptors

Adapted from Ferry et al [12] and Collet-Solberg et al [13].

IGFBPs regulate the bioavailability of IGFs, but also have IGF-independent effects on growth and metabolism [12,14,15]. Next to their effects in the circulation, IGFBPs have probable autocrine and paracrine effects. Their cellular effects are influenced by several posttranslational modifications: 1) glycosylation affecting cell interaction, 2) phosphorylation affecting IGF binding affinity, 3) susceptibility to proteases, and 4) proteolysis causing decreased affinity for IGFs. These modifications affect both IGF-independent and dependent actions (Figure 5).

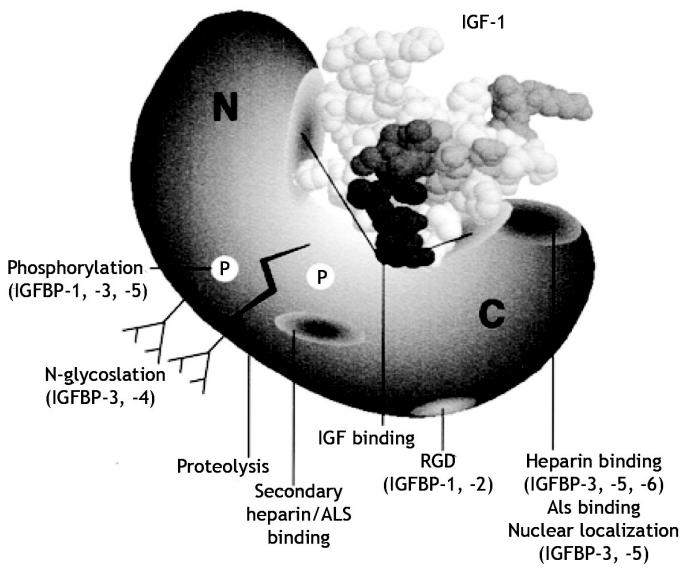

Figure 5. Generalized diagram of IGFBP structure, showing interaction with IGF-I through the N- and C-terminal regions. Functional domains and sites of posttranslational modification are also indicated. RGD=Arg-Gly-Asp integrin-binding motifs. Adapted from Firth and Baxter [14].

Since part of this thesis is focused on IGFBP-1 and IGFBP-3 levels and genetic variability in their respective genes, these binding proteins will be discussed more in detail.

2.3.1 IGFBP-1

IGFBP-1 is produced in the liver, kidneys and decidua. It exists in 2 forms: a highly phosphorylated form (90%) and a small non-phosphorylated form (10%). The phosphorylated form has a 6-fold higher affinity for IGF-I, compared to the non-phosphorylated form. IGFBP-1 binds IGF-I and IGF-II with a greater affinity than their respective receptors and thus prevents IGFs from exerting their actions [16]. Through binding to integrin receptors, IGFBP-1 can act in an IGF-independent fashion stimulating cell migration [12,17]. Production of IGFBP-1 in the liver is suppressed by insulin through binding to at least 2 insulin-response elements in the gene promoter, forming a link between glucose metabolism and the IGF axis [18-20]. Other hormones, such as glucocorticoids and glucagon, stimulate IGFBP-1 production.

2.3.2 IGFBP-3

IGFBP-3 is produced in the liver, as well as in many other tissues. Its production is mainly regulated by GH and the exact mechanism is still under investigation. Three mechanisms have been proposed: 1) a direct effect of GH on Kupffer cells, 2) an indirect effect mediated by IGF-I, and 3) stimulation of non-hepatic tissues [13]. Many other hormones, such as insulin, oestradiol and glucocorticoids, regulate *IGFBP3* expression *in vitro* [12,21]. *In vitro* and *in vivo* studies have demonstrated that IGFBP-3 has IGF-mediated and IGF-independent effects on growth promotion and inhibition [13,22]. The concentration of IGFBP-3 in serum exceeds that of other IGFBPs and the affinity of IGFBP-3 for IGFs is higher than those of most other IGFBPs, reflecting its most important function as a carrier protein for IGFs. IGFBP-3 was found to augment IGF effects [23]. The relative ratio of IGFBP-3 to IGF-I may thus play an important role in the determination whether there is inhibition or stimulation of IGF action and therefore growth.

3. Genes involved in the GH-IGF axis and clinical presentation

3.1 GH and GH receptor gene

Laron syndrome, caused by inactivating mutations affecting the expression or function of the GH receptor and GHBP, was first described in 1966 [24]. Clinical characteristics include severe postnatal growth failure, facial dysmorphism, truncal obesity, delayed puberty, hypoglycemia, elevated GH levels, low IGF-I levels, absent/low or dysfunctional GHBP and resistance to GH.

Polymorphisms in the GH gene have been associated with variability in normal adult height [25]. A common polymorphism in the GH receptor gene was associated with birth size and response to GH treatment in some populations [26-28], although these findings were not reproduced by others [29].

3.2 IGF and IGF receptor genes

Animal knockout studies have demonstrated that IGF-I, IGF-II and their receptors are the most important regulators of fetoplacental growth. *Igf1* knockout mice are 60% smaller than their littermates, without an alteration in placental size [30], whereas *Igf2* knockout mice are also 60% smaller and have reduced placental growth [30,31]. *Igf1* and *Igf2* double knockout mice are 80% smaller than their littermates. LID (Liver IGF-I Deficient) mice, a mouse model where *Igf1* is specifically knocked out in the liver, have normal birth weight and postnatal growth, despite reduced circulating IGF-I and IGFBP-3 levels [32]. These data show that liver-derived IGF-I is the principal source of circulating IGF-I levels, but that normal growth might not only result from circulating free IGF-I but also from local autocrine-paracrine production of IGF-I. Case reports on IGF-I gene deletion in humans demonstrated severe intrauterine growth retardation, severe short stature, mental retardation and sensorineural deafness [33,34].

Twin studies showed that 38-66% of the interindividual variability in IGF-I, IGF-II and IGFBP-3 levels was genetically determined [35,36]. Polymorphisms in *IGF1* have been correlated with IGF-I levels [37,38], head circumference in short SGA children [39], birth weight [38,40] and increased risk of type 2 diabetes and ischemic heart disease [41], although not all studies found similar associations [42,43].

Igf1 and *Igf1 receptor* double knockout mice did not show further decrease in birth weight compared to *Igf1* knockout mice, suggesting that IGF-I only functions through the IGF-IR [44]. On the other hand, *Igf2* and *Igf1 receptor* double knockout

mice showed more severe intrauterine growth retardation, demonstrating that IGF-II not only functions through the IGF-IR. Indeed, it was found that IGF-II also functions via the insulin receptor during early fetal development [44]. Case reports in humans with defects in the *IGF1R* demonstrated pre- and postnatal growth retardation and mental retardation [44,45].

3.3 IGFBP genes

Transgenic mice overexpressing *Igfbp1* had a 15% reduction in birth weight, modest postnatal growth retardation and were glucose intolerant [46,47]. Transgenic mice overexpressing *Igfbp3* had a normal birth weight, modest postnatal growth retardation and a marked reduction in brain size [48,49]. The lower bioavailability of circulating IGF-I is probably the most important explanation behind these phenotypes.

Knockout studies of genes encoding the IGFBPs or ALS demonstrated little effect on fetal growth. Knockout of *Als* resulted in a 60% reduction in IGF-I and IGFBP-3 levels, without an effect on fetal growth, only minor effects on postnatal growth and no mentionable effects on glucose metabolism, questioning the role of circulating IGF-I levels vs. local production of IGF-I [50].

The minor alleles of 4 single nucleotide polymorphisms (SNPs) in *IGFBP1* were associated with a decreased prevalence of diabetic nephropathy in patients with type 2 diabetes [51]. However, no data exist about the influence of these SNPs on circulating IGFBP-1 levels. Ten SNPs in *IGFBP3* have been reported. The -202 A/C SNP has been correlated with circulating IGFBP-3 levels: adults carrying the AA genotype had significantly higher IGFBP-3 levels, compared to adults carrying the CC genotype [52,53]. The impact of this SNP on circulating IGFBP-3 levels and growth in short SGA subjects is still unknown.

4. Epigenetic regulation of gene expression

Gene expression varies greatly among different tissues and is partly established and maintained by epigenetic mechanisms. DNA methylation is an important process in embryonic development, genomic imprinting and X-chromosome inactivation. Methylation occurs at cytosines within cytosine-guanine dinucleotides (CpGs; the "p" representing a phosphate group), converting cytosine to 5-methylcytosine. Methylation regulates gene expression by affecting the binding of methylation-

sensitive DNA-binding proteins. These DNA-binding proteins mediate histone modifications that regulate conformational changes in chromatin, resulting in an altered DNA accessibility for the transcription machinery to that region [54]. DNA methylation and histone modification are mechanistically linked and interdependent, and both mechanisms generally result in reduced transcriptional activation of genes (Figure 6) [55].

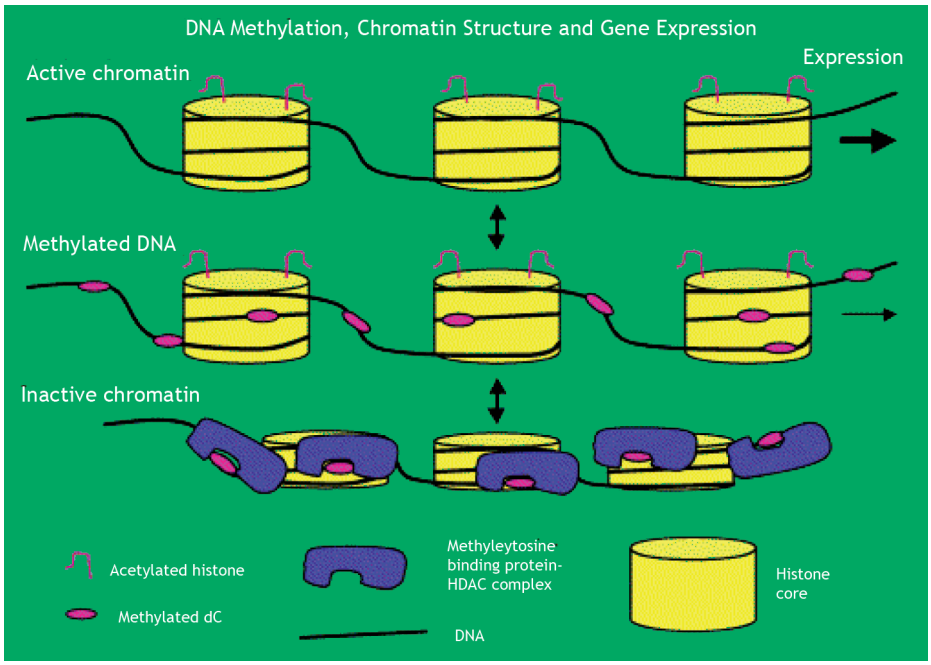

Figure 6. Methylation of DNA sequences permits binding of a chromatin inactivation complex, which deacytelates histones and promotes chromatin condensation. Adapted from Richardson et al [56].

About 80% of CpGs in the genome are methylated. In contrast, CpG islands – short CpG-rich regions containing multiple transcription factor binding sites and often found in gene promoters – remain largely unmethylated. Transcription factors regulate gene expression by binding to recognition sequences (control elements) in gene promoters. Methylation within the gene promoter can disrupt binding of these factors, resulting in reduced transcriptional activation of genes.

Various studies have shown that there is considerable interindividual variability in methylation status, implying a role in the variation of disease risk [57]. Epigenetic regulation is influenced by age [56], genetics and environment. Animal models demonstrated that environmental changes during development caused persistent changes in epigenetic gene regulation [58]. CpG island hypermethylation in the promoter region of tumor suppressor genes, resulting in gene silencing, is the most frequently found epigenetic mechanism associated with cancer [59].

The "developmental origins" hypothesis proposes that during critical periods of pre- and postnatal development, when epigenetic mechanisms are established and undergo major changes, nutrition or other environmental factors influence this development. Intrauterine epigenetic reprogramming may influence postnatal growth and development, inducing permanent changes in metabolism and chronic disease susceptibility [55, 60].

5. Clinical and endocrinological aspects associated with normal and abnormal growth

5.1 Fetal growth

Pituitary GH has a limited impact on late third trimester growth, since infants with congenital absence of the pituitary often have birth weights and birth lengths below the mean, albeit within the normal range [61].

Insulin, IGF-I, IGF-II and their receptors are the most important regulators of fetoplacental growth. All fetal tissues express IGF-I and IGF-II: IGF-II is the principal growth factor during early embryonic growth, whereas IGF-I is more important during later stages of gestation. IGF-I and IGF-II levels are significantly influenced by fetal nutrition and insulin levels. In sheep fetuses, glucose availability and the subsequent increase in fetal insulin levels are the major stimulators of fetal IGF-I production, whereas for IGF-II the effect of glucose was found to be insulin-independent [62]. Fetal pancreatectomy in sheep resulted in low IGF-I levels and severe intrauterine growth retardation. Intrafetal infusion with glucose or insulin increased IGF-I [63]. In human fetuses with intrauterine growth retardation, IGF-I levels are decreased during the second half of gestation [64]. Likewise, IGF-I levels in cord blood were significantly lower in children born SGA, compared to children born appropriate for gestational age (AGA) [65], and levels correlated positively with birth weight and placental weight [66].

All 6 IGFBPs have been found in fetal plasma and tissues. IGFBP-1 is the major IGFBP found in amniotic fluid; it binds IGFs in fetal plasma, increases 20-fold from wk 9 to wk 12, and is the most important regulator of IGF-I bioavailability during pregnancy [67,68]. IGFBP-1 levels are increased in infants born SGA, possibly reflecting the low insulin levels found in fetuses with intrauterine growth retardation [4]. IGFBP-3 levels were significantly lower in newborns born SGA, compared to those born AGA [65].

5.2 Postnatal growth

GH receptor expression is gradually upregulated after birth. Furthermore, GHBP levels remain low during the first 3 months and rise at 6 months of life [69]. Postnatal growth becomes dependent on pulsatile GH secretion and subsequent IGF-I and IGFBP-3 production from about 6 months of life. Several other factors such as sex steroids, nutritional status and liver function also influence serum IGF-I and IGFBP-3 levels.

Catch-up growth is defined as a growth velocity greater than the median for chronological age and gender. Catch-up growth occurs during the first 6 months of life in more than 80% of children born SGA, although premature SGA children may take longer to catch-up [70] (Figure 6).

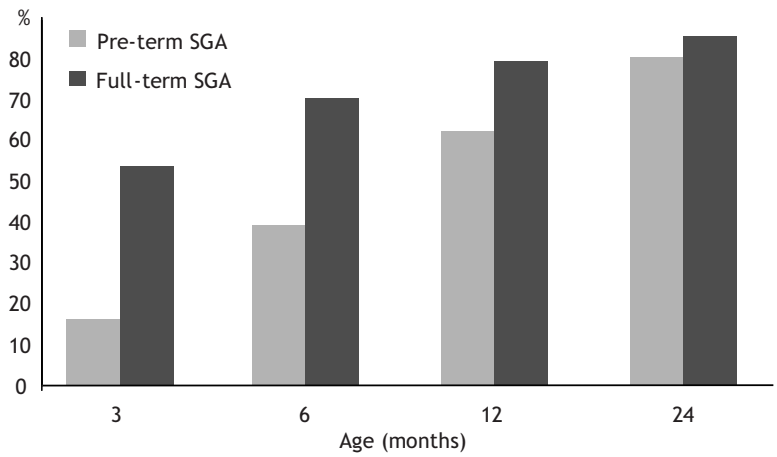

Figure 6. Percentage of pre-term and full-term SGA infants with postnatal catch-up growth. From Hokken-Koelega et al [70].

By the age of 2 years, catch-up is completed in most children born SGA [70,71]. Catch-up growth is associated with a rise in IGF-I and IGFBP-3 levels, as well as higher concentrations of the non-phosphorylated form of IGFBP-1 [72].

Around 10% of children born SGA remain short throughout childhood and into adulthood. In a French cohort, men reached a mean adult height of 161.9 (±8.0) cm and women of 147.6 (±7.0) cm [73)]. Changes in the GH-IGF-IGFBP axis might underlie this failure in catch-up growth in short SGA children. Subnormal to low spontaneous GH levels during overnight GH profiles have been found in prepubertal short children born SGA [74,75]. Likewise, serum levels of IGF-I and IGFBP-3 are significantly lower in children and young adults with persistent short stature, compared to their age-matched controls with normal stature [74,76,77].

5.3 Insulin sensitivity and body composition in short subjects born SGA

Epidemiological studies have shown that the development of diabetes mellitus type 2 and associated disorders such as hypertension, dyslipidemia and cardiovascular disease in adults is associated with low birth weight [78-80]. Reduced insulin sensitivity plays an important role in the pathogenesis of these disorders [81]. Short children born SGA were more insulin resistant, compared to controls born AGA [82,83]. Insulin sensitivity was similar to controls in short young SGA adults, whereas young SGA adults with postnatal catch-up in height and weight had a significantly lower insulin sensitivity [84]. These data indicate that insulin sensitivity is mainly related to the accumulation of fat mass during childhood. The disposition index – a measurement that reflects how well beta cells are able to compensate for a reduction in insulin sensitivity by increasing their insulin secretion – was comparable between short SGA subjects and controls.

IGFBP-1 is the only acute regulator of IGF-I bioavailability and its production in the liver is suppressed by insulin. Reduced IGFBP-1 levels are considered to reflect reduced insulin sensitivity and cardiovascular risk in adults [85,86], women with polycystic ovary syndrome (PCOS) [87] and prepubertal obese children [88]. Limited studies with relatively small numbers of subjects determined IGFBP-1 levels in short children born SGA, with contradictory results [89,90].

Body composition is greatly influenced by gender and height [91] and it is important to adjust for these variables when comparing body composition in SGA and AGA children. Short children born SGA have a significantly decreased fat mass SDS, when adjusted for gender and height [92]. At a young age, their lean mass SDS

adjusted for gender and height was comparable to the population mean. However, it tended to decrease over time during a 3-year follow-up, resulting in significantly lower levels than the population mean in older prepubertal short SGA children [92].

6. Growth hormone treatment in prepubertal short SGA children

6.1 Effects on linear growth

In 1991, the first Dutch multi-center, randomized, double-blind, dose-response (1 mg GH/m^2/day vs 2 mg GH/m^2/day) GH trial was started. Adult height data demonstrated that 85% of children reached a height above -2 SDS and 98% reached a height within the target height range [93] (Figure 7).

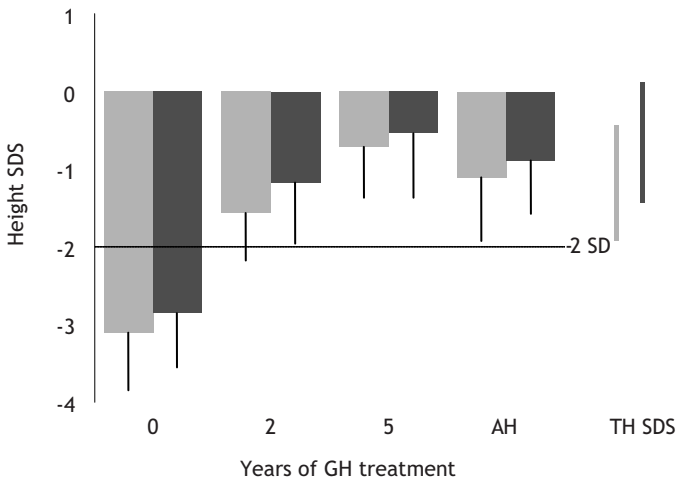

Figure 7. Height SDS (± SD) during GH treatment and at adult height (AH), in relation to target height (TH) SDS. Light grey boxes: 1 mg GH/m^2/day, dark grey boxes: 2 mg GH/m^2/day. From van Pareren et al [93].

Several other studies also showed that GH treatment effectively induces catch-up growth in prepubertal short SGA children [94-96]. Although the GH dose is less important for long-term growth, a dose-dependent effect on growth was found during the first 4-5 years of GH treatment [93,97]. There is, however, considerable variation in the growth response to GH treatment which remains after adjusting for factors

such as age, parental height and duration of treatment [96]. Short children born SGA form a heterogeneous group of patients and genetic variability in growth-related genes and their effect on growth response probably accounts for part of the variation.

6.2 Effects on the GH-IGF-IGFBP axis

Serum GH, IGF-I and IGFBP-3 levels significantly increased during GH treatment and were found to be dose-dependent [98-100]. After 1 year of treatment, mean IGF-I and IGFBP-3 levels increased to respectively 1.2 SDS and 0.2 SDS in children treated with 1 mg GH/m^2/day and to 1.9 SDS and 0.5 SDS in children treated with 2 mg GH/m^2/day. After 3 years of GH treatment, IGF-I and IGFBP-3 levels were comparable in both GH dosage groups [99]. After discontinuation of GH treatment, IGF-I levels were 1.0 SDS and 1.3 SDS, significantly higher than the population mean, in children treated with 1 mg GH/m^2/day and 2 mg GH/m^2/day, respectively. IGFBP-3 levels were -0.8 SDS in children treated with 1 mg GH/m^2/day, which is significantly lower than the population mean, and -0.06 SDS in children treated with 2 mg GH/m^2/day [93]. At 6.5 years after discontinuation of GH treatment, IGF-I (-0.4 SDS) and IGFBP-3 (-1.6 SDS) returned to levels comparable with those found in untreated short individuals born SGA (IGF-I: -0.6 SDS, IGFBP-3: -1.2 SDS), thus significantly lower compared to the population means [101].

6.3 Effects on insulin sensitivity, lipid profile and body composition

Surveillance databases showed that GH treatment is well-tolerated and adverse events are not more common in short children born SGA than in other conditions requiring GH treatment [102].

Monitoring of glucose and insulin levels during GH treatment is necessary, because GH has well-documented insulin-antagonistic effects [2]. Children born SGA had higher fasting insulin levels and relative insulin resistance during GH treatment [103,104], but this increase is largely reversible when treatment is terminated [101, 104]. Van Dijk et al showed that 6.5 years after discontinuation of GH treatment, insulin sensitivity in short children born SGA who were treated with GH was similar to untreated individuals born SGA [101].

GH treatment had positive effects on lipid metabolism and blood pressure in short SGA children and these effects persisted after discontinuation. Serum levels of cholesterol, LDL-cholesterol and HDL-cholesterol as well as and systolic

blood pressure significantly decreased during GH treatment [105]. At 6.5 years after discontinuation of GH treatment, systolic and diastolic blood pressure were significantly lower in GH-treated SGA individuals, compared to untreated SGA individuals [101].

GH has well-documented anabolic effects on muscle mass and lipolytic effects on adipose tissue [106]. During GH treatment, fat mass SDS adjusted for gender and height significantly declined. The greatest reduction was seen during the first treatment year [92]. The increase in lean mass SDS adjusted for gender and height reflected the normal increase as a result of the increase in height. Thus, GH treatment did not lead to an additional increase in lean mass [92].

7. Treatment options in pubertal short children born SGA

Several studies indicated that better growth responses and greater adult height were achieved when children started growth hormone treatment at an early age [99,107, 108]. The age at onset and progression of puberty in short SGA children is comparable to healthy peers [109]. However, some short SGA children only come under medical attention at onset of puberty.

7.1 Postponement of puberty

Postponement of puberty with gonadotropin releasing hormone analogue (GnRHa) treatment was studied in girls and boys with central precocious puberty (CPP) or early puberty [110-113]. Most children reached an adult height within their target height range.

A decline in growth velocity is a well-known phenomenon during GnRHa treatment [114-116]. Some studies with relatively small number of patients found lower stimulated and spontaneous growth hormone levels during GnRHa treatment [117-119]. Other investigators could not replicate these findings [120,121]. It was also suggested that poor growth is directly related to reduced sex steroid levels or growth plate senescence induced by prior oestrogen exposure [122,123]. These studies were performed in children with CPP only. No data exist on spontaneous GH, IGF-I and IGFBP-3 levels in pubertal short children born SGA, either before or during GnRHa treatment.

7.2 LH and FSH profiles before and during pubertal suppression

A classical GnRH stimulation test is often used for evaluation of pubertal suppression during GnRHa treatment. There is, however, no consensus about the best criteria to identify sufficient pubertal suppression: peak LH levels ranging between 1.75 and 5 IU/L during a GnRH stimulation test have been mentioned [124,125]. Various alternatives have been proposed [124,126-128] and the GnRH agonist test (leuprorelide acetate stimulation) is frequently used nowadays [129,130].

Spontaneous luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels, measured with highly sensitive assays in healthy children, showed that prepubertal children have very discrete diurnal pulsatile patterns of gonadotropins [131-134]. At onset of puberty (Tanner stage 2), LH and FSH are secreted in a regular pattern during daytime with a further amplification during sleep. Increasing pulse frequency and pulse amplitude with an obvious night-day rhythm were found from early puberty onwards [131-138]. Data concerning spontaneous overnight LH and FSH profile patterns during GnRHa treatment are very limited and there are no data on LH and FSH profiles, both before and during GnRHa treatment, in pubertal short children born SGA.

7.3 GnRHa and GH treatment

Postponement of puberty in combination with GH treatment has been investigated in specific groups of patients. Studies in patients with idiopathic growth hormone deficiency (IGHD) and in children with idiopathic short stature (ISS) showed a beneficial effect on adult height in favor of combined treatment, compared to GH treatment alone [139-141]. Other studies found no benefit from combined treatment in children with ISS [142,143]. It is still unknown whether postponement of puberty, in addition to treatment with GH, is beneficial for adult height improvement in short children born SGA.

Treatment with different GH dosages resulted in a dose-dependent rise in serum GH levels in GH-deficient patients and children with Turner syndrome [144,145]. In prepubertal short children born SGA, treatment with 2 mg GH/m^2/day resulted in significantly higher levels of serum GH, IGF-I and IGFBP-3 compared to children treated with 1 mg GH/m^2/day [99,100]. No data are available on the effect of treatment with 2 different GH dosages in combination with a GnRH analogue on GH, IGF-I and IGFBP-3 levels in pubertal short children born SGA.

7.4 Effects of GnRHa treatment on body composition, insulin sensitivity and lipid metabolism

There is a physiological decrease in insulin sensitivity with pubertal progression, which resolves by the end of puberty [146,147]. Since both GH and IGF-I levels significantly increase during puberty, the GH-IGF axis is thought to be an important contributor to pubertal insulin resistance [148]. A brief period of GnRHa treatment in young, healthy women did not result in changes in insulin secretion [149]. The effect of long-term GnRHa treatment on insulin sensitivity in children is, however, unknown.

In children with CPP, some studies reported an increase in fat mass or BMI SDS during GnRHa treatment with a return to values comparable to those at baseline after discontinuation [150], whereas others reported no changes [110], or even a decreased BMI SDS during GnRHa treatment [151]. Lean body mass adjusted for gender and age significantly decreased during GnRHa treatment [150]. No data are available on lipid parameters during GnRHa treatment in children. Furthermore, the effects of combined treatment with GnRHa and different GH dosages on body composition, insulin sensitivity and lipid profile is also unknown.

8. Aims of the study

This doctoral thesis describes the results of various studies performed in (I) pubertal short children born SGA treated with a combination of a GnRH analogue and either 1 or 2 mg GH/m^2/day in the fourth Dutch GH trial (SGA study), and in (II) prepubertal short children born SGA treated with GH in the first, second, third and fourth Dutch GH trials (IUGR-1, IUGR-2, IUGR-3 and SGA studies), as well as in (III) short untreated young adults born SGA and age-matched controls with normal stature included in the PROGRAM study. The SGA study aimed to investigate (I) the spontaneous overnight LH and FSH secretion in pubertal short SGA children, (II) the effect of GnRHa treatment on overnight serum LH and FSH levels, and (III) the effect of GnRHa treatment on the GH-IGF-IGFBP axis. Furthermore, the SGA study aimed to investigate the effect of combined treatment with a GnRH analogue and either 1 or 2 mg GH/m^2/day on (III) the GH-IGF-IGFBP axis and first year growth response, and (IV) body composition, insulin sensitivity and lipid profile in pubertal short SGA children. In addition, we aimed (V) to determine IGFBP-1 levels in short SGA subjects in comparison to levels in controls with normal stature, and (VI) to investigate the impact of an *IGFBP1* promoter SNP on circulating IGFBP-1 levels. We also assessed (VII) the relationship between *IGFBP3* promoter SNPs, IGFBP-3

levels, spontaneous growth and growth response to GH treatment in prepubertal short SGA children, and (VIII) promoter methylation status in a subgroup of short SGA subjects and controls.

8.1 Effects on LH and FSH secretion

We investigated serum LH and FSH levels during an overnight profile in pubertal short SGA children, before and after 3 months of GnRHa treatment. We also performed a GnRH agonist test in all children. A peak LH level below 3 IU/L with prepubertal oestradiol levels (below 50 pmol/l) in girls and prepubertal testosterone levels (below 1 nmol/l) in boys are routinely used as cut-off levels for sufficient pubertal suppression in the Netherlands [130,152,153]. We evaluated how many children had sufficient pubertal suppression according to the overnight LH profiles and according to the GnRH agonist test.

8.2 Effects on the GH-IGF-IGFBP axis and first year growth response

We performed overnight GH profiles and measured fasting levels of IGF-I and IGFBP-3 in pubertal short SGA children. To investigate the effect of GnRHa treatment, we compared GH, IGF-I and IGFBP-3 levels after 3 months of GnRHa treatment with those at baseline. We also compared GH levels in pubertal short SGA girls with those found in prepubertal short SGA girls. To determine the effect of treatment with a GnRH analogue in combination with either 1 or 2 mg GH/m^2/day on GH levels and growth, we subsequently measured GH, IGF-I and IGFBP-3 levels after 1 year of combined GnRHa and GH treatment and compared levels with those found at start of GH treatment. Furthermore, we investigated associations between GH profile characteristics, IGF-I and IGFBP-3 levels and first year growth response.

8.3 Effects on body composition, insulin sensitivity and lipid parameters

We evaluated changes in body composition and fat distribution, measured by Dual Energy X-ray Absorptiometry (DXA), in pubertal short SGA children. We evaluated changes in insulin sensitivity, acute insulin response and disposition index, measured with a frequently sampled intravenous glucose tolerance test (FSIGT) including administration of tolbutamide. Likewise, changes in lipid profile were assessed. We investigated the effect of GnRHa treatment and the effect of combined treatment of a GnRH analogue with either 1 or 2 mg GH/m^2/day on these parameters.

8.4 Serum IGFBP-1 levels, associations with clinical and laboratory parameters and genetic variability in the *IGFBP1* promoter

Since limited and contradictory data exist on circulating IGFBP-1 levels in short SGA subjects, we determined IGFBP-1 levels in a large group of prepubertal and pubertal short children, and short young adults born SGA. We compared levels to those in age-matched controls with normal stature. In addition, we assessed the relationship between IGFBP-1 levels and various clinical and laboratory parameters.

Thirty-six percent of the interindividual variability in circulating IGFBP-1 levels is genetically determined [36]. The minor allele of a relative frequent promoter SNP has been associated with a decreased prevalence of diabetic nephropathy in patients with type 2 diabetes [51], but no data exist on the impact of this SNP on circulating IGFBP-1 levels. We assessed genotype frequency and determined its impact on circulating IGFBP-1 levels in short SGA individuals.

8.5 Genetic and epigenetic variability in the *IGFBP3* promoter: correlations with serum levels and growth

Twin studies have shown that 60% of the interindividual variability in circulating IGFBP-3 levels is genetically determined [35]. We assessed the relationship between two *IGFBP3* promoter SNPs, serum IGFBP-3 levels and growth. In addition, we analyzed if the *IGFBP3* promoter SNPs are associated with GH treatment-related rises in IGFBP-3 levels and may be useful for predicting growth response to GH treatment.

Methylation is one of the predominant epigenetic modifications of DNA in mammalian genomes, leading to alterations in the binding affinity of transcription factors to DNA binding sites and subsequent reduced gene expression. We investigated *IGFBP3* promoter methylation status in a subgroup of short SGA individuals and compared results with the methylation status in controls with normal stature.

9. Outline of the thesis

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

Chapter 2 describes the overnight LH and FSH profiles before and after 3 months of GnRHa treatment in pubertal short SGA girls.

Chapter 3 describes the overnight LH, FSH and GH profiles and serum levels of IGF-I and IGFBP-3 before and after 3 months of GnRHa treatment in pubertal short SGA boys.

Chapter 4 describes the overnight GH profiles and serum levels of IGF-I and IGFBP-3

before and after 3 months of GnRHa treatment in pubertal short SGA girls.

Chapter 5 reports the effects of combined treatment with a GnRH analogue and either 1 or 2 mg GH/m^2/day on overnight GH profiles, serum levels of IGF-I and IGFBP-3 and first year growth response in pubertal short SGA children.

Chapter 6 reports the effects of 2 years of combined treatment with a GnRH analogue and either 1 or 2 mg GH/m^2/day on body composition, insulin sensitivity and ß-cell function, and lipid parameters.

Chapter 7 describes serum IGFBP-1 levels in a large cohort of short SGA individuals, in comparison with those found in a large cohort of age-matched controls with normal stature, and describes the impact of an *IGFBP1* promoter SNP on IGFBP-1 levels.

Chapter 8 describes the genetic and epigenetic variability in the *IGFBP3* gene in relation to serum IGFBP-3 levels, spontaneous growth and growth response to GH treatment in a large cohort of short SGA individuals.

Chapter 9 discusses and concludes on our findings in relation to the current literature and comments on clinical implications of our study results.

Chapter 10 summarizes our findings in English.

Chapter 11 presents a Dutch summary.

Appendix A

SGA study

Inclusion criteria:

1. Birth length and/or birth weight <-2 SD for gestational age [154].
2. Short stature defined in prepubertal children as height SDS below -2.5 [155] or a predicted adult height <-2.5 SDS, calculated as the height at start of puberty +30 cm for boys and +20 cm for girls.
3. Height velocity (cm/year) for chronological age ≤ P50 in prepubertal children [155].
4. Chronological age at start of treatment: 8 years or older (boys and girls).
5. Well-documented growth data from birth up to 2 years and at least 1 year before the start of the study.
6. Informed consent.

Exclusion criteria:

1. Turner syndrome in girls, known syndromes and serious dysmorphic symptoms suggestive for a syndrome that has not yet been described, except for Silver-Russell Syndrome.
2. Severe asphyxia (Apgar score <3 after 5 minutes), and no serious diseases such as long-term artificial ventilation and oxygen supply, bronchopulmonary dysplasia or other chronic lung disease.
3. Celiac disease and other chronic or serious diseases of the gastrointestinal tract, heart, genito-urinary tract, liver, lungs, skeleton or central nervous system, or chronic or recurrent major infectious diseases, nutritional and/or vitamin deficiencies.
4. Any endocrine or metabolic disorder such as diabetes mellitus, diabetes insipidus, hypothyroidism, or inborn errors of metabolism.
5. Medications or interventions during the previous 6 months that might have interfered with growth, such as corticosteroids (including high dose of corticosteroid inhalation), sex steroids, growth hormone, or major surgery (particularly of the spine or extremities).
6. Use of medication that might interfere with growth during GH treatment, such as corticosteroids, sex steroids, GnRH analogue.

7. Active or treated malignancy or increased risk of leukemia.
8. Serious suspicion of psychosocial dwarfism (emotional deprivation).
9. Expected non-compliance.

Design

The SGA study started in 2003. It is a multi-center, randomized, open-labeled, dose-response GH trial. Prepubertal children are treated with GH 1 mg/m^2/day. At start of puberty, children were stratified for gender, pubertal stage (Tanner stage 2 or 3) and parental height SDS (one parent with height SDS below -2 SDS or both parents with height SDS within the normal range). Children with a height < 140cm at start of puberty are treated with a GnRH analogue for 2 years and 1 mg GH/m^2 body surface area/day (~ 33 µg/kg/day) until adult height or a GnRH analogue for 2 years and 2 mg GH/m^2/day (~ 66 µg/kg/day) until adult height. Children with a height > 140cm at start of puberty are treated with 1 mg GH/m^2/day or 2 mg GH/m^2/day until adult height. Biosynthetic GH (Genotropin® (Somatropin)) is administered subcutaneously once daily. Three-monthly, the GH dose is adjusted to the calculated body surface area.

Appendix B

IUGR-1 and IUGR-2 studies

Inclusion criteria:

1. Birth length SDS below -1.88 (comparable to 3rd percentile) for gestational age [154].
2. An uncomplicated neonatal period, without signs of severe asphyxia (defined as Apgar score below 3 after 5 minutes), sepsis or long-term complications of respiratory ventilation such as bronchopulmonary dysplasia.
3. Chronological age (CA) between 3.00 and 7.99 years at start of the study.
4. Height SDS for age below -1.88 according to Dutch standards [155].
5. Height velocity SDS for age below zero to exclude children with spontaneous catch-up growth [155].
6. Prepubertal, defined as Tanner stage 1 or a testicular volume <4 ml [156].
7. Normal liver, kidney and thyroid functions.

Exclusion criteria:

1. Any endocrine or metabolic disorder such as diabetes mellitus, diabetes insipidus, hypothyroidism, or inborn errors of metabolism, except GHD.
2. Disorders of major organs.
3. Chromosomal abnormalities or signs of a syndrome, except Silver-Russell Syndrome.
4. Chrondrodysplasia.
5. Hydrocephalus.
6. Active malignancy or increased risk of leukemia.
7. Serious suspicion of psychosocial dwarfism (emotional deprivation).
8. Previous anabolic sex steroid or GH treatment.

Design of IUGR-1 study

The IUGR-I study started in 1991. Before entering the study, children underwent a 24 hour plasma GH profile. To stratify for spontaneous GH secretion during the 24 hour GH profile, the total group of 79 children was divided into 3 groups: “normal profile”, “GH insufficient profile” (area under the curve <90 µg/l) and “no profile performed”. After stratification for spontaneous GH secretion during the 24 GH

profile and age, all children were randomly and blindly assigned to one of two GH dosage groups: group A, 1 mg GH/m^2/day or group B, 2 mg GH/m^2/day. Biosynthetic GH (r-hGH Norditropin®, Novo Nordisk A/S, Denmark) was given subcutaneously once daily at bedtime. Three-monthly, the GH dose was adjusted to the calculated body surface area. The study was kept double-blind until the end of the study in 2003 by using an equal volume of a reconstituted preparation.

Design of IUGR-2 study

The IUGR-2 study started in 1996. The study design was an open-labeled, multi-center study with a randomized control group. Before entering the study, the GH status was evaluated in all children using GH stimulation tests (arginine and/or clonidine). Children with GH deficiency (GHD), which was defined as a GH peak <10 µg/l during two stimulation tests, were not randomized but started treatment at a dose of 1 mg GH/m^2/day (GHD-group). The non-GHD children were stratified according to age (3.00-5.50 vs 5.50-7.99 years) and height of the parents (height of both parents above -1.88 SDS vs. height of at least one parent below -1.88 SDS). After stratification, children were randomly assigned to either the GH-group (2/3 of children) or the control group (1/3 of children). The GH-group started immediately with treatment at a dose of 1 mg GH/m^2/day. The control group remained untreated for 3 years and received subsequently the same GH treatment as the GH-group. Biosynthetic GH (r-hGH Norditropin®, Novo Nordisk A/S, Denmark) is given subcutaneously once daily at bedtime. Three-monthly, the GH dose is adjusted to the calculated body surface area.

Appendix C

IUGR-3 study

Inclusion criteria:

1. Birth length and/or birth weight SDS below -2 for gestational age [154].
2. Uncomplicated neonatal period, without signs of severe asphyxia (Apgar score >3 after 5 minutes) or long-term complications of respiratory ventilation such as bronchopulmonary dysplasia.
3. Short stature, defined as height SDS below -2.5 [155].
4. Height velocity SDS below zero to exclude children with spontaneous catch-up growth [155].
5. Age between 3 and 8 years at start of the study.
6. Prepubertal stage, defined as Tanner breast stage 1 for girls and testicular volume less than 4 ml for boys [156].

Exclusion criteria:

1. Chromosomal disorders and known syndromes, except for Silver-Russell syndrome.
2. Disorders of major organs.
3. Endocrine or metabolic disorders, such as diabetes mellitus, diabetes insipidus, hypothyroidism, or inborn errors of metabolism, except for GHD.
4. Chrondrodysplasia.
5. Hydrocephalus.
6. Active malignancy or increased risk of leukemia.
7. Emotional deprivation.
8. Previous anabolic sex steroid or GH treatment.

Design

The IUGR-3 study started in 2002. It is an open-labeled, randomized, multi-center study. After stratification for gender, GH-status (maximum serum GH levels between 20-30 mU/L vs serum GH levels >30 mU/L during a GH stimulation test) and BMI (<-1 SDS vs >1 SDS), children were randomized into 2 different groups. During 6 months, children in group A received treatment with a dose of 1 mg GH/m^2/day and children in group B received a dose of 2 mg GH/m^2/day. Subsequently, all children received

a dose of 1 mg GH/m^2/day. Biosynthetic GH (r-hGH Norditropin®, Novo Nordisk A/S, Denmark) is given subcutaneously once daily at bedtime using the Nordipen™ 15. Three-monthly, the GH dose is adjusted to the calculated body surface area.

Appendix D

PROGRAM study

Inclusion criteria:

1. Gestational age of 36 weeks or more.
2. Born singleton.
3. Caucasian origin.
4. Age between 18 and 24 years.

Exclusion criteria:

1. A complicated neonatal period with signs of severe asphyxia (defined as Apgar score <3 after 5 minutes), sepsis or long-term complications of respiratory ventilation such as bronchopulmonary dysplasia.
2. Previous or present treatment known to interfere with growth (e.g. growth hormone treatment, treatment with glucocorticoids, radiotherapy).
3. Endocrine or metabolic disorders such as growth hormone deficiency, severe chronic illness, emotional deprivation.
4. Chromosomal defects, syndromes, or serious dysmorphic symptoms suggestive for a yet unknown syndrome.

Design

Individuals aged 18-23 years, were randomly selected from hospitals in the Netherlands, where they had been registered because of being small at birth (SGA), showing short stature (after being born SGA or appropriate for gestational age (AGA). Normal controls were randomly invited to participate. Based on birth length SDS and adult height SDS, individuals were assigned to one of the four subgroups:

- Born SGA (<-2 SDS) [154] with a short adult height (<-2 SDS) [155].
- Born SGA (<-2 SDS) with spontaneous catch-up growth resulting in a normal adult height (>-1 SDS).
- Born AGA (>-1 SDS) with postnatal growth retardation resulting in short adult height (<- 2 SDS).
- Born AGA (>-1 SDS) and normal adult height (>-1 SDS).

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Chapter 2

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Overnight Luteinizing and Follicle Stimulating Hormone profiles during GnRHa Treatment in Short Girls Born Small for Gestational Age

Abstract

Background: Since puberty starting at a height less than 140cm might reduce adult height, postponement of puberty was studied in pubertal short girls born SGA. Data on overnight LH and FSH profiles during GnRHa treatment are very limited.

Objectives: To evaluate if 3 months of GnRHa treatment results in sufficient suppression of pubertal LH and FSH profile patterns. To evaluate if girls show sufficient pubertal suppression according to a consensus-based peak LH cut-off level of 3 IU/L during a GnRH agonist test.

Subjects: Twenty-one pubertal short girls born SGA.

Intervention: After baseline LH and FSH profiles, children received leuprorelide acetate depots of 3.75 mg subcutaneously, every 4 weeks.

Results: At baseline, amplitude and frequency of LH and FSH pulsatility were higher in girls with breast stage 3, compared to girls with breast stage 2. After 3 months of GnRHa treatment, all girls showed clinical arrest of puberty and their LH and FSH levels during overnight profiles had significantly decreased to prepubertal levels. In contrast, peak LH during the GnRH agonist test indicated insufficient pubertal suppression in 33% of girls. No differences in LH and FSH profiles were found between girls with a peak LH above or below 3 IU/L.

Conclusion: After 3 months of GnRHa treatment, central puberty was adequately suppressed in all girls, as shown by the prepubertal LH and FSH profiles. The GnRH agonist falsely indicated insufficient pubertal suppression in 33% of these girls.

Introduction

About 10% of children born small for gestational age (SGA) fail to show catch-up growth to a normal height [1,2]. Since many short children present at a pubertal age, several therapeutic approaches have been proposed for short children at the onset of puberty [3-5]. Postponement of puberty was studied in girls with central precocious puberty and most of these girls reached an adult height within their target height range after gonadotropin releasing hormone analogue (GnRHa) treatment [6,7].

Spontaneous luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels, measured with highly sensitive assays in healthy children, showed that prepubertal children have very discrete diurnal pulsatile patterns of gonadotropins [8]. At onset of puberty (B2), LH and FSH are secreted in a regular pattern during daytime with a further amplification during sleep. Increasing pulse frequency and pulse amplitude with an obvious night-day rhythm were found from early puberty onwards [8-11].

Data concerning spontaneous overnight LH and FSH profile patterns during GnRHa treatment are very limited. Therefore, our primary objective was to evaluate if treatment with leuprorelide acetate depots of 3.75 mg subcutaneously results in sufficient suppression of pubertal LH and FSH profile patterns.

Since admitting children to a hospital in order to perform overnight LH and FSH profiles is not suitable for daily practice, we also performed a GnRH agonist test in all girls. A peak LH level below 3 IU/L (peak LH_{GnRH}; P95 of prepubertal peak LH response) with prepubertal oestradiol levels (below 50 pmol/l (=13.6 pg/ml)) is used as a cut-off level for sufficient pubertal suppression in the Netherlands [12-14]. Our second objective was to evaluate in how many girls sufficient pubertal suppression is identified by the GnRH agonist test.

Materials and methods

Subjects

The study group consisted of 21 short girls born SGA who were at the beginning of puberty. They were included in a clinical trial investigating combined treatment of GnRHa and growth hormone (GH). All girls started GH treatment after 3 months of GnRHa treatment. All children were included according to the following criteria at

start of the study: 1) birth length and/or birth weight standard deviation score (SDS) below -2 for gestational age [15], 2) chronological age of 8 years or older at start of the study, 3) current height SDS below -2.5 or a predicted adult height < -2.5 SDS (calculated as height at start of puberty plus 20 cm, according to Dutch references [16]), 4) early pubertal stage, defined as Tanner breast stage 2 (B2) or 3 (B3) [17] and a GnRH agonist test (leuprorelide acetate stimulation) with a peak LH of 10 IU/L or more, indicating central puberty. Exclusion criteria were: 1) a complicated neonatal period with signs of severe asphyxia (defined as Apgar score < 3 after 5 minutes), 2) long-term complications of respiratory ventilation such as bronchopulmonary dysplasia, 3) endocrine (including idiopathic growth hormone deficiency) or metabolic disorders, chromosomal defects, growth failures caused by other disorders (such as emotional deprivation, severe chronic illness, chondroplasia) or syndromes (except for Silver-Russell syndrome), 4) previous or present medication that could interfere with growth or growth hormone treatment. The study was approved by the Medical Ethics Committee of the participating centers and written informed consent was obtained from the parents or custodians and from the children aged 12 years or older.

Study design

Overnight LH and FSH profiles were performed in all girls before and after 3 months of GnRHa treatment (leuprorelide acetate depots of 3.75 mg subcutaneously every 4 weeks, with an interval of 14 days between the first 2 injections). Children were admitted to the hospital and an indwelling venous catheter was inserted in the anticubital vein. For a period of 12 hours (19.00-7.20 h.), blood was taken every 20 minutes for determination of serum LH and FSH levels. All girls followed their normal eating and sleeping pattern. Height was measured using a Harpenden stadiometer, 3 measurements per visit were taken and the mean was used for analysis.

Height was expressed as SDS for calendar age [16]. The same investigator (DvdK) assessed pubertal stage according to Tanner during both visits [17]. Bone age was assessed by one investigator (DvdK), using the segmented Greulich and Pyle reference [18]. The inhibition of gonadotropin secretion was evaluated the morning after the second overnight LH and FSH profile by a GnRH agonist test: 0.5 mg (=0.5 ml) leuprorelide acetate was injected subcutaneously and after 3 hours a blood sample was taken for determination of peak LH, peak FSH and baseline oestradiol levels [13,19].

Hormonal assays

LH and FSH levels were measured by chemoluminescence-based immunometric methods (Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA) using WHO-preparations 80/552 and 78/549 as standards. Detection limits for both assays were 0.1 IU/l and values lower than 0.1 IU/L were assigned as 0.1 IU/L. Intra- and interassay coefficients of variation were below 4% and 7% for LH and below 3% and 6% for FSH. Oestradiol levels were measured using coated tube radioimmunoassays obtained from Diagnostic Products Corporation. The detection limit was 10 pmol/l and values below 10 pmol/l were assigned as 10 pmol/l. Intra- and interassay coefficients of variation were below 8% and 9%.

Reported LH and FSH profiles for prepubertal girls

In previous studies performed in healthy prepubertal girls, maximum LH levels were between 0.2 and 1.2 IU/L and maximum FSH levels were between 1.2 and 5 IU/L [8-10].

Calculations

The area under the curve above zero (AUC_0) of LH and FSH levels was calculated by the trapezoidal method.

Statistics

Because of a non-Gaussian shaped distribution, data were expressed as median (interquartile range). The Mann-Whitney test was used for differences between groups. The Wilcoxon signed rank test was used to determine differences between points in time within groups. Spearman's correlation coefficient was used for correlations. A P-value < 0.05 was considered significant. Analyses were performed using the computer statistical package SPSS (version 11) for Windows.

Results

Baseline

Clinical characteristics

Table 1 shows the baseline clinical data.

Table 1. Baseline clinical data at start of GnRHa treatment.

Number of girls	21
Gestational age (weeks)	37.5 (36.0 to 39.8)
Birth weight SDS	-2.1 (-2.6 to -1.4)
Birth length SDS	-2.6 (-3.3 to -2.0)
At start of GnRHa treatment	
Age (yrs)	11.9 (11.2 to 12.6)
Bone age (yrs)	11.6 (11.0 to 12.4)
Height SDS	-2.9 (-3.5 to -2.5)
Weight SDS	-2.2 (-2.9 to -1.9)
Breast stage 2	15
Breast stage 3	6

Data are expressed as median (interquartile range)

Overnight LH and FSH profiles

The characteristics of the baseline overnight LH and FSH profiles are depicted in Table 2. Girls with breast stage 2 had a low-frequency and low-amplitude pulsatile pattern during the night and early morning. In girls with breast stage 3, both the amplitude and frequency of LH and FSH pulsatility were higher, with pulses found from the evening until the early morning (Figures 1 and 2).

Correlations between LH profiles, clinical characteristics and the GnRH agonist test

Correlations between various parameters are shown in Table 3. At baseline, positive correlations were found between mean and maximum LH levels during the LH profile and bone age (r=0.48, P=0.03 and r=0.57, P=0.007, respectively). Maximum LH levels during the LH profile were also significantly correlated with oestradiol levels (r=0.50, P=0.03).

After 3 months of GnRHa treatment

Clinical characteristics

After 3 months of GnRHa treatment, none of the girls had clinical progression of puberty and 8 girls (5 girls with B2 and 3 girls with B3) had clinical regression of puberty.

Overnight LH and FSH profiles

The characteristics of the overnight LH and FSH profiles after 3 months of GnRHa treatment are shown in Table 2. AUC_0, mean and maximum LH and FSH levels had significantly decreased to very low levels. Mean LH levels were 0.31 IU/L and maximum LH levels 0.46 IU/L, whereas mean FSH levels were 0.97 IU/L and maximum FSH levels 1.22 IU/L. Thus, all girls had a prepubertal profile (Figures 1 and 2). No significant differences in LH and FSH profiles were found between girls who showed clinical regression of puberty, compared to those who showed clinical arrest of puberty.

Table 2. Characteristics of overnight LH and FSH profiles and the GnRH agonist test, at baseline and after 3 months of GnRHa treatment.

	Baseline	3 months	P-value[a]
Overnight LH profiles			
AUC_0 LH (IU/L*12h)	32.4 (9.84-43.7)	4.0 (2.89-4.67)	< 0.0001
Mean LH (IU/L)	2.53 (0.77-3.39)	0.31 (0.23-0.36)	< 0.0001
Max LH (IU/L)	8.75 (4.79-12.0)	0.46 (0.37-0.53)	< 0.0001
Overnight FSH profiles			
AUC_0 FSH (IU/L*12h)	56.5 (39.9-70.4)	12.1 (7.80-14.2)	< 0.0001
Mean FSH (IU/L)	4.43 (3.15-5.53)	0.97 (0.63-1.15)	< 0.0001
Max FSH (IU/L)	7.35 (4.71-8.36)	1.22 (0.81-1.33)	< 0.0001
GnRH agonist test			
Peak LH (IU/L)	24.0 (14.9-32.4)	2.4 (1.4-3.4)	< 0.0001
Peak FSH (IU/L)	18.4 (12.0-27.6)	2.9 (1.9-5.2)	< 0.0001
Oestradiol (pmol/l)	57.0 (40.0-80.0)	19 (13.0-25.0)	0.002

Data are expressed as median (interquartile range)
[a] compared to baseline

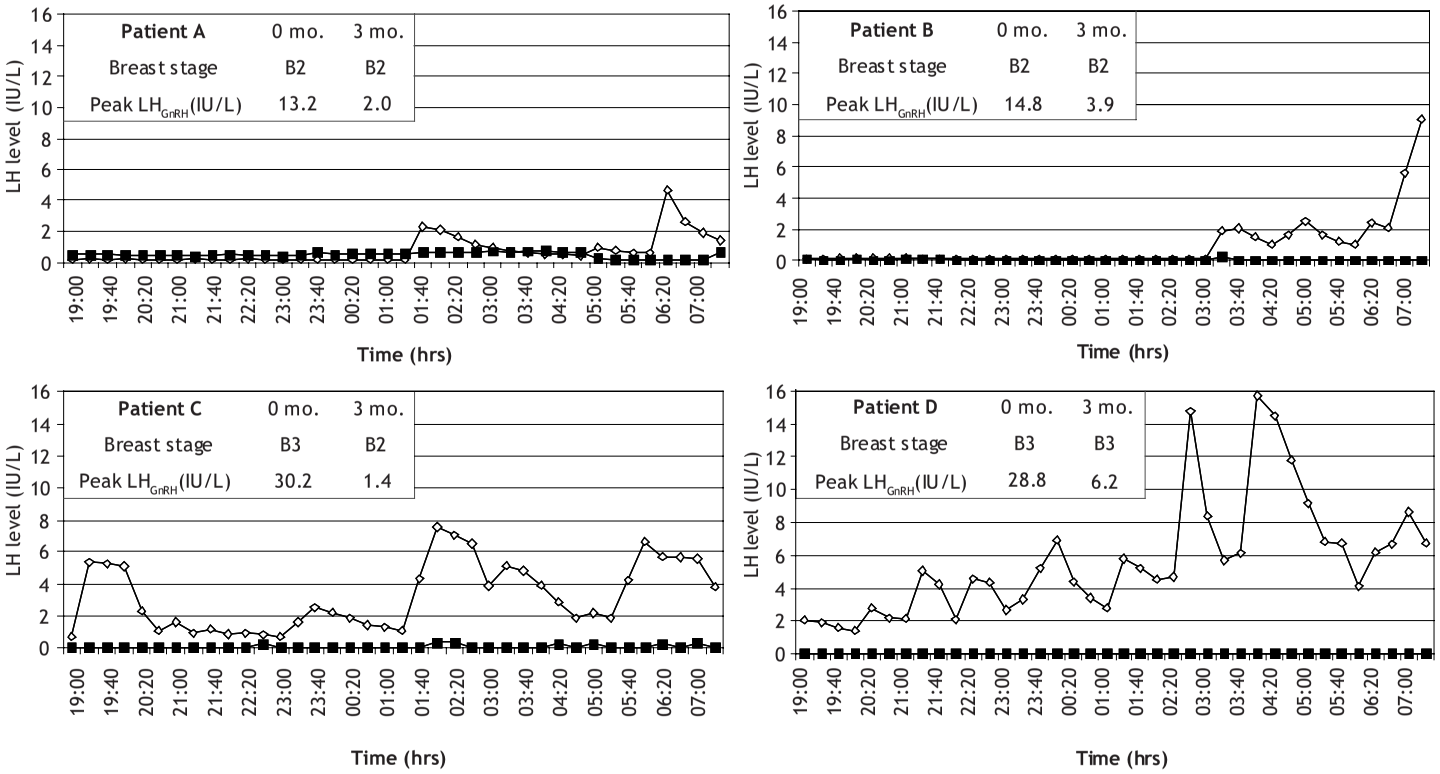

Figure 1. Representative examples of individual overnight LH profiles performed at baseline (open diamonds) and after 3 months of GnRHa treatment (solid squares). Tanner stage and peak LH_{GnRH} at baseline and after 3 months of GnRHa treatment are depicted in the figures.

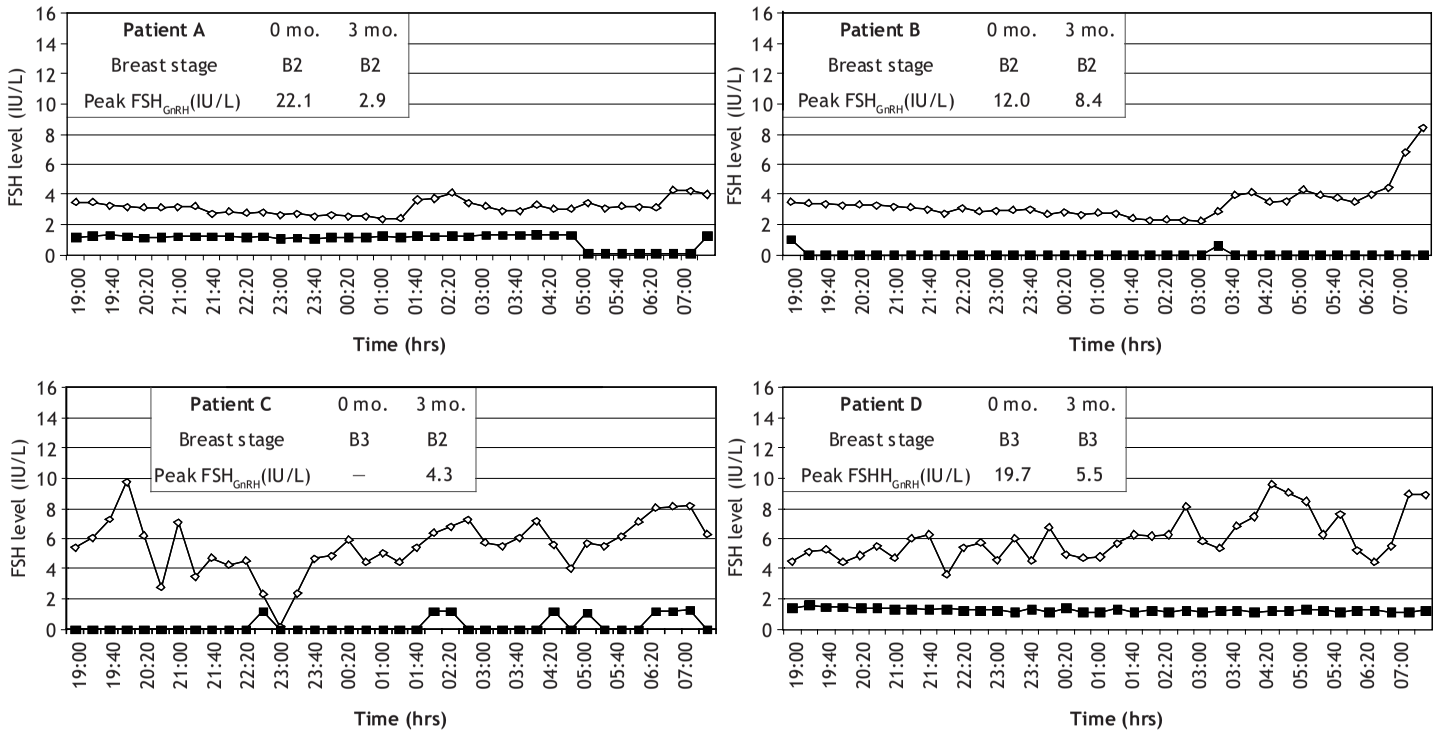

Figure 2. Representative examples of individual overnight FSH profiles performed at baseline (open diamonds) and after 3 months of GnRHa treatment (solid squares) in the same 4 girls. Tanner stage and peak FSH_{GnRH} at baseline and after 3 months of GnRHa treatment are depicted in the figures.

Notably, all girls had similar LH profiles, regardless of their breast stage at baseline. In contrast, the FSH levels were significantly lower in girls with breast stage 2 at baseline, compared to girls with breast stage 3 at baseline (median (IQR): AUC_0 (B2: 9.93 (7.68 to 12.0) IU/L and B3: 15.8 (10.4 to 27.0) IU/L, P=0.008), mean (B2: 0.79 (0.61 to 0.95) IU/L and B3: 1.28 (0.88 to 2.14) IU/L, P=0.007) and maximum (B2: 0.96 (0.71 to 1.24) IU/L and B3: 1.49 (1.03 to 2.86) IU/L, P=0.01)).

GnRH agonist test

After 3 months of GnRHa treatment, peak LH_{GnRH} was below 3 IU/L in 14 out of 21 girls. Oestradiol levels were below 50 pmol/l in 13 out of 14 girls and was 63 pmol/l in one girl. Peak LH_{GnRH} was between 3.1 and 7.3 IU/L in 7 girls (33%), with peak LH_{GnRH} between 3 and 4 IU/L in 4 out of 7 girls. Oestradiol levels were below 50 pmol/l in 6 out of 7 girls and was 58 pmol/l in one girl.

No significant differences in LH and FSH profiles were found between girls with a peak LH_{GnRH} above or below 3 IU/L. Furthermore, no significant differences in peak LH_{GnRH} and peak FSH_{GnRH} were found between girls who showed clinical regression, compared to girls who showed clinical arrest of puberty.

Correlations between LH-profiles, clinical characteristics and the GnRH agonist test

Correlations between various parameters are shown in Table 3. After 3 months of GnRHa treatment, a positive correlation was found between maximum LH levels during the LH profile and peak LH_{GnRH} (r=0.51, P=0.02).

No correlations were found between oestradiol, peak LH and peak FSH levels during the GnRH agonist test at baseline versus levels found during the GnRH agonist test after 3 months of GnRHa treatment.

Table 3. Correlations between overnight LH profiles, clinical characteristics and the GnRH agonist test; at baseline and after 3 months of GnRHa treatment.

	Overnight LH profiles			
	Baseline		After 3 months of GnRHa	
	Mean LH (IU/L)	Max LH (IU/L)	Mean LH (IU/L)	Max LH (IU/L)
Age (years)	0.09	0.14	–	–
Bone age (years)	0.48[a]	0.57[b]	–	–
Pubertal stage	0.30	0.21	–	–
Pubertal regression	-0.23	-0.44[a]	0.03	-0.06
GnRH agonist test at baseline				
Peak LH (IU/L)	0.31	0.28	–	–
Oestradiol (pmol/l)	0.29	0.50[a]	–	–
GnRH agonist test after 3 months				
Peak LH (IU/L)	–	–	0.33	0.51[a]
Oestradiol (pmol/l)	–	–	-0.28	-0.07

Pubertal regression was defined as 0 = pubertal arrest and 1 = pubertal regression
[a] $P < 0.05$
[b] $P < 0.01$

Discussion

Our study shows that treatment with subcutaneous leuprorelide acetate depots of 3.75 mg every 4 weeks results in adequate pubertal suppression in all girls, as demonstrated by the prepubertal overnight LH and FSH profiles. Furthermore, all girls showed clinical arrest of puberty after 3 months of GnRHa treatment. In contrast, the GnRH agonist test performed after 3 months of GnRHa treatment falsely indicated insufficient pubertal suppression in 33% of the girls.

After 3 months of GnRHa treatment, AUC_0, mean and maximum LH and FSH levels had significantly decreased to very low levels during the overnight profile. The pattern of LH and FSH secretion profiles after 3 months of GnRHa treatment in pubertal short girls born SGA was similar to prepubertal profiles found in healthy girls [8-10].

Overnight LH and FSH profiles were performed for research purpose since admitting children to a hospital in order to perform overnight LH and FSH profiles

is not feasible in daily clinical care. After 3 months of GnRHa treatment, LH levels were above the cut-off level of 3 IU/L in 33% of the girls. LH and FSH profiles were comparable between girls with a peak LH_{GnRH} above or below 3 IU/L and oestradiol levels were very low. Our results are in line with the only comparable study in 6 children with CPP, performed by Cook et al [20]. They found that 3 children who were clinically sufficiently suppressed had overnight LH levels similar to prepubertal children. Three children who showed clinical progression of pubertal development had increased overnight LH levels, similar to pubertal children.

Peak LH_{GnRH} was known before LH levels during the overnight profiles were available. According to the Dutch consensus guideline, the schedule of leuprorelide acetate depot injections was changed to every 3 weeks instead of every 4 weeks in girls who had a peak LH_{GnRH} above 3 IU/L. However, this adjustment to a higher frequency of injections proved to be unnecessary once we received the results of the overnight LH profiles.

Nowadays, the GnRH agonist test is frequently used as an alternative for the classical GnRH stimulation test to assess sufficient pubertal suppression during GnRHa treatment [13,19]. There is, however, no consensus about the peak LH cut-off level to identify sufficient pubertal suppression, thus one may consider the cut-off level of 3 IU/L as rather arbitrary [21-24]. Our results show that the peak LH cut-off level of 3 IU/L might be too low for the GnRH agonist test.

Since 100% of the girls had prepubertal LH profiles after 3 months of GnRHa treatment, we could not determine a new peak LH cut-off level during the GnRH agonist test, based on the LH profiles. However, based on our results, future research in a larger and more heterogeneous group of children - including children who are sufficiently and insufficiently suppressed according to their LH profiles - is warranted in order to determine a new peak LH_{GnRH} cut-off level.

After 3 months of GnRHa treatment, mean FSH levels were significantly higher than mean LH levels, indicating that GnRHa treatment results in a greater suppression of LH secretion, compared to FSH secretion. Over the past several decades, the existence of one or more GnRH-independent mechanisms of FSH secretion has been suggested [25-27]. Pulsatile secretion of LH and FSH was found to be discordant during 75-87% of time in healthy women [25]. Furthermore, after administration of a GnRH antagonist, LH release was suppressed by 60%, whereas FSH levels decreased only minimally [27,28]. The clinical importance of the higher FSH levels is however debatable, since mean and maximum FSH levels were within the prepubertal range [8,9].

In conclusion, treatment with subcutaneous leuprorelide acetate depots of 3.75 mg every 4 weeks results in an effective inhibition of central puberty, as shown by the prepubertal overnight LH and FSH secretion patterns and clinical signs of pubertal arrest in short pubertal girls born SGA. The GnRH agonist test falsely indicated insufficient pubertal suppression in 33% of these girls, which resulted in unnecessary adjustments in the frequency of depots injections.

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Chapter 3

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Overnight levels of Luteinizing Hormone, Follicle Stimulating Hormone and Growth Hormone before and during GnRHa Treatment in Short Boys Born Small for Gestational Age

Abstract

Aims: To evaluate if 3 months of GnRHa treatment results in sufficient suppression of pubertal LH and FSH profile patterns in short pubertal SGA boys. To compare GH profiles and fasting IGF-I and IGFBP-3 levels after 3 months of GnRHa treatment with those at baseline.

Methods: After baseline overnight profiles and IGF-I and IGFBP-3 levels, fourteen pubertal short SGA boys received leuprorelide acetate depots of 3.75 mg subcutaneously, every 4 weeks.

Results: At baseline, mean GH levels were comparable with controls, whereas IGF-I and IGFBP-3 SDS were significantly lower than zero SDS. After 3 months of GnRHa treatment, all boys showed clinical arrest of puberty. AUC_0, mean and maximum LH and FSH had significantly decreased to prepubertal levels. Peak LH during the GnRH agonist test, however, indicated insufficient pubertal suppression in 43% of boys. Overnight GH profile characteristics, IGF-I and IGFBP-3 levels did not significantly change.

Conclusion: Puberty was sufficiently suppressed, as shown by the prepubertal LH and FSH profiles. After 3 months of GnRHa treatment, overnight GH profile characteristics had not significantly changed, reflecting that GH levels are comparable for prepubertal and early pubertal boys.

Introduction

Although catch-up growth occurs in most children born small for gestational age (SGA), about 10% of infants remain short throughout childhood and adulthood [1,2]. Persistent changes in the GH/IGF/IGFBP axis might underlie this failure in catch-up growth [3-7].

In some short SGA children puberty started at a relatively early age for their short stature, thereby compromising adult height [8,9]. Postponement of puberty with gonadotropin releasing hormone analogue (GnRHa) treatment was studied in boys with central precocious puberty (CPP) and most of these boys reached an adult height in the range of their genetic height potential [10].

In healthy, older prepubertal boys, a very discrete day and night pulsatile secretion pattern of luteinizing hormone (LH) and follicle stimulating hormone (FSH) is detectable [11-13]. At onset of puberty (G2), LH and FSH are secreted in a regular pattern during daytime with a further amplification during sleep. From early puberty onwards, increasing pulse frequency and pulse amplitude with an obvious night-day rhythm was found [11-14].

Data concerning spontaneous overnight LH and FSH profile patterns during GnRHa treatment are scarce. Therefore, our primary objective was to evaluate if GnRHa treatment results in sufficient suppression of pubertal LH and FSH profile patterns. Admitting children to a hospital in order to perform overnight LH and FSH profiles is not feasible in routine care. Therefore, all boys also underwent a GnRH agonist test during the morning after the second overnight profile. In the Netherlands, a consensus-based peak LH level below 3 IU/L (peak LH_{GnRH}; P95 of prepubertal peak LH response) with testosterone levels below 1 nmol/l (upper limit of prepubertal values) during a GnRH agonist test are used as cut-off levels for sufficient pubertal suppression [15]. Our second objective was to evaluate in how many boys sufficient pubertal suppression is identified by the GnRH agonist test.

A decrease in growth velocity is a well-known phenomenon during GnRHa treatment [16-19]. There are only limited data on spontaneous GH, IGF-I and IGFBP-3 levels during GnRHa treatment [20-22]. Notably, no data are available in pubertal short boys born SGA, either before or during GnRHa treatment. Therefore, our third objective was to determine overnight GH profiles and fasting levels of IGF-I and IGFBP-3, before and after 3 months of GnRHa treatment.

Materials and methods

Subjects

The study group comprised short boys born SGA who were at the beginning of puberty. They were included in a clinical trial investigating combined treatment of GnRHa and GH. All boys started GH treatment after 3 months of GnRHa treatment. Children who met the following criteria were included: 1) birth length and/or birth weight standard deviation score (SDS) below -2 for gestational age [23], 2) chronological age of 8 years or older at start of the study, 3) current height SDS below -2.5 or a predicted adult height < -2.5 SDS (calculated as height at start of puberty plus 30 cm, according to Dutch references [24]), 4) early pubertal stage defined as a testicular volume of 4 ml or more, Tanner genital stage 2 or 3 [25] and a GnRH agonist test result with a peak LH of 10 IU/L or more, indicating central puberty [26]. Children were excluded if they met one of the following criteria: 1) a complicated neonatal period with signs of severe asphyxia (defined as an Apgar score < 3 after 5 minutes), 2) long-term complications of respiratory ventilation such as bronchopulmonary dysplasia, 3) endocrine (including idiopathic growth hormone deficiency) or metabolic disorders, chromosomal defects, growth failures caused by other disorders (such as emotional deprivation, severe chronic illness or chondroplasia) or syndromes (except for Silver-Russell syndrome), 4) previous or present medication that could interfere with growth or growth hormone treatment. The study was approved by the Medical Ethics Committee of the participating centers and written informed consent was obtained from parents or custodians and from children if aged 12 years or older.

Study design

Overnight LH, FSH and GH profiles were performed in 10 boys, both before and after 3 months of GnRHa treatment (leuprorelide acetate depots of 3.75 mg subcutaneously, every 4 weeks, with an interval of 14 days between the first 2 injections). Children were admitted to the hospital and an indwelling venous catheter was inserted in an anticubital vein. For a period of 12 hours (19.00-7.20 h.) blood was taken every 20 minutes for determination of serum LH, FSH and GH levels. Children followed their normal eating and sleeping pattern. Sleep during both admissions was recorded by the same investigator (DvdK). All children went to bed at 22.30h, slept before 23.30h and woke-up around 7.00h the next morning. The time awake was comparable between

both admissions. The next morning a fasting blood sample was taken for measurement of IGF-I and IGFBP-3 levels.

Height was measured using a Harpenden stadiometer and expressed as SDS for calendar age [24]. The same investigator (DvdK) assessed pubertal stage according to Tanner during both visits, using an orchidometer [25]. Bone age was assessed by one investigator (DvdK), using the segmented Greulich and Pyle reference [27]. Fat mass for height at baseline was measured by Dual Energy X-ray Absorptiometry (DXA) [28].

The inhibition of gonadotropin secretion was checked the morning after the second overnight profile by a GnRH agonist test: 0.5 mg (=0.5 ml) leuprorelide acetate was injected subcutaneously and after 3 hours a blood sample was taken for determination of LH, FSH and testosterone levels [29,30]. In the 4 boys in whom overnight profiles were not performed, fasting IGF-I and IGFBP-3 levels and a GnRH agonist test were performed during the second visit, after 3 months of GnRHa treatment. We compared our overnight GH profile results with those found by Rose et al, who performed overnight GH profiles in healthy boys with normal stature and similar pubertal stage [31].

Hormone assays

Overnight LH and FSH levels were measured by chemoluminescence-based immunometric methods (Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA) using WHO-preparations 80/552 and 78/549 as standards. Detection limits for both assays were 0.1 IU/l and values below 0.1 IU/L were assigned as 0.1 IU/L. Intra- and interassay CVs were below 4% and 7% for LH and below 3% and 6% for FSH. Testosterone levels were measured using coated tube radioimmunoassays obtained from Diagnostic Products Corporation. The detection limit for this assay is 0.1 nmol/l. Intra- and interassay CVs were below 6% and 8%.

Overnight GH levels were measured by an immunometric assay (Immulite 2000) with a lower detection limit of 0.13 mU/L. Values lower than 0.13 mU/L were assigned as 0.13 mU/L. The results of low, medium and high standards (mean ± intra-assay SD and ± interassay SD, respectively) were 6.8±0.23 and ±0.44; 14.0±0.47 and ±0.75; 44±1.87 and ±2.94 mU/L. Intra- and interassay coefficients of variation (CVs) were respectively 3.5% and 6.5% at a level of 6.8 mU/L, 3.4% and 5.5% at a level of 14.0 mU/L and 4.2% and 6.6% at a level of 44 mU/L. Based on standard samples, our assay was comparable with the assay used by Rose et al who measured GH levels by

polyclonal RIA with a detection limit of 0.5 µg/l [20]. Their results (mean ± SD) of low, medium and high standards were 1.1±0.1, 3.6±0.4 and 16.9±2.0 µg/l. Intra- and interassay CVs were respectively 9.1% and 18.7% at a level of 1.2 µg/l, 3.7% and 16.6% at a level of 5.1 µg/l and 3.1% and 9.0% at a level of 18.5 µg/l. A conversion factor of 2.6 was used to transform data from µg/l to mU/L [32].

Serum IGF-I and IGFBP-3 levels were measured in one laboratory using a specific RIA [33]. Serum levels were expressed as SDS to adjust for age and sex [34]. The intra- and interassay CVs were 4% and 6%.

Reported LH profiles for prepubertal boys

Prepubertal boys with a testicular volume of 1-2 ml have a very discrete nighttime pulsatile pattern of gonadotropin secretion with mean (± SEM) LH levels of 0.27 ±0.15 IU/L and maximum LH levels of 1.06 ±0.24 IU/L. Mean and maximum LH levels increased to 1.34 ±0.42 IU/L and 3.70 ±1.21 IU/L in prepubertal boys with a testicular volume of 3 ml [12].

Calculations

The area under the curve above zero (AUC_0) of LH and FSH profile patterns was calculated by the trapezoidal method. Overnight GH profiles were analyzed using the Pulsar program [33,35].

Statistics

Because of a non-Gaussian shaped distribution, data were expressed as median (interquartile range). The Mann-Whitney test was used for differences between groups. The Wilcoxon signed rank test was used to determine differences between points in time within groups. SD-scores were compared with zero SDS using Chi-Square tests. To analyze night-to-night variation, correlation coefficients were calculated between individual time points within 1 child. The calculated coefficients were analyzed using Chi-Square tests. Spearman's correlation coefficient was used for correlations. A P-value <0.05 was considered significant. Analyses were performed using the computer statistical package SPSS (version 11) for Windows.

Results

Clinical characteristics

Table 1 shows clinical data of all subjects. After 3 months of GnRHa treatment, none of the boys showed clinical progression of puberty, 2 boys showed clinical regression of puberty.

Table 1. Clinical characteristics before start of GnRHa treatment.

Number of boys	14
Gestational age (weeks)	39.0 (37.4-40.0)
Birth weight SDS	-1.9 (-2.2 to -1.6)
Birth length SDS	-2.4 (-2.8 to -2.1)
At start of GnRHa treatment	
Genital stage 2	12
Genital stage 3	2
Age (yrs)	12.7 (12.2-12.9)
Bone age (yrs)	12.1 (11.2-12.6)
Height SDS	-2.5 (-3.4 to -2.2)
Weight SDS	-2.7 (-3.4 to -1.3)
Fat mass SDS	-0.5 (-1.4 to -0.08)

Data are expressed as median (interquartile range)

Overnight LH and FSH profiles

Baseline

Characteristics of baseline overnight LH and FSH profiles are shown in Table 2.

After 3 months of GnRHa treatment

Characteristics of overnight LH and FSH profiles after 3 months of GnRHa treatment are shown in Table 2. AUC_0, mean and maximum LH and FSH levels had significantly decreased to very low levels. Mean LH levels were 0.41 IU/L and maximum LH levels 0.72 IU/L, whereas mean FSH levels were 0.21 IU/L and maximum FSH levels 0.38 IU/L. Thus, none of the boys had a pubertal pulsatile pattern (Figures 1 and 2).

Table 2. Characteristics of overnight LH and FSH profiles, at baseline and after 3 months of GnRHa treatment.

	Baseline	3 months	P-value[a]
Overnight LH profiles			
AUC_0 LH (IU/L*12h)	36.0 (18.1-52.1)	5.2 (3.7-8.3)	0.005
Mean LH (IU/L) [0.27-1.3 IU/L]	2.8 (1.4-4.1)	0.41 (0.29-0.66)	0.005
Max LH (IU/L) [1.1-3.7 IU/L]	7.3(4.3-9.7)	0.72 (0.43-0.92)	0.005
Overnight FSH profiles			
AUC_0 FSH (IU/L*12h)	21.0 (11.3-63.7)	2.7 (1.5-5.3)	0.005
Mean FSH (IU/L)	1.7 (0.89-5.0)	0.21 (0.12-0.42)	0.005
Max FSH (IU/L)	4.0 (1.6-6.3)	0.38 (0.22-0.93)	0.005
GnRH agonist test			
Peak LH (IU/L)	32.4 (14.1-43.1)	2.8 (2.1-4.0)	0.001
Peak FSH (IU/L)	7.2 (4.3-11.8)	0.65 (0.40-1.1)	0.001
Testosterone (nmol/l)	6.5 (1.3-9.9)	0.25 (0.10-0.53)	0.001

Data are expressed as median (interquartile range)
Reported mean prepubertal values for mean and maximum LH levels are shown between brackets
[a] compared to baseline

GnRH agonist test

After 3 months of GnRHa treatment, peak LH_{GnRH} was below 3 IU/L with testosterone levels below 1 nmol/l in 8 out of 14 boys. Peak LH_{GnRH} varied between 3.3 and 5.2 IU/L in 6 boys (43%). Testosterone levels were below 1 nmol/l in all 6 boys and none of these boys showed clinical progression. No significant differences in LH and FSH profiles were found between boys with a peak LH_{GnRH} above or below 3 IU/L.

Testosterone levels had significantly decreased from 6.5 (1.3-9.9) nmol/l to 0.25 (0.10-0.53) nmol/l (P=0.001) after 3 months of GnRHa treatment. No significant differences in testosterone levels were found between boys with a peak LH_{GnRH} above or below 3 IU/L.

Correlations between LH-profiles and the GnRH agonist test

At baseline, mean LH levels during the LH profile correlated positively with testosterone levels during the GnRH agonist test (r=0.67, P=0.03). Maximum LH levels during the LH profile correlated positively with peak LH_{GnRH} (r=0.69, P=0.03).

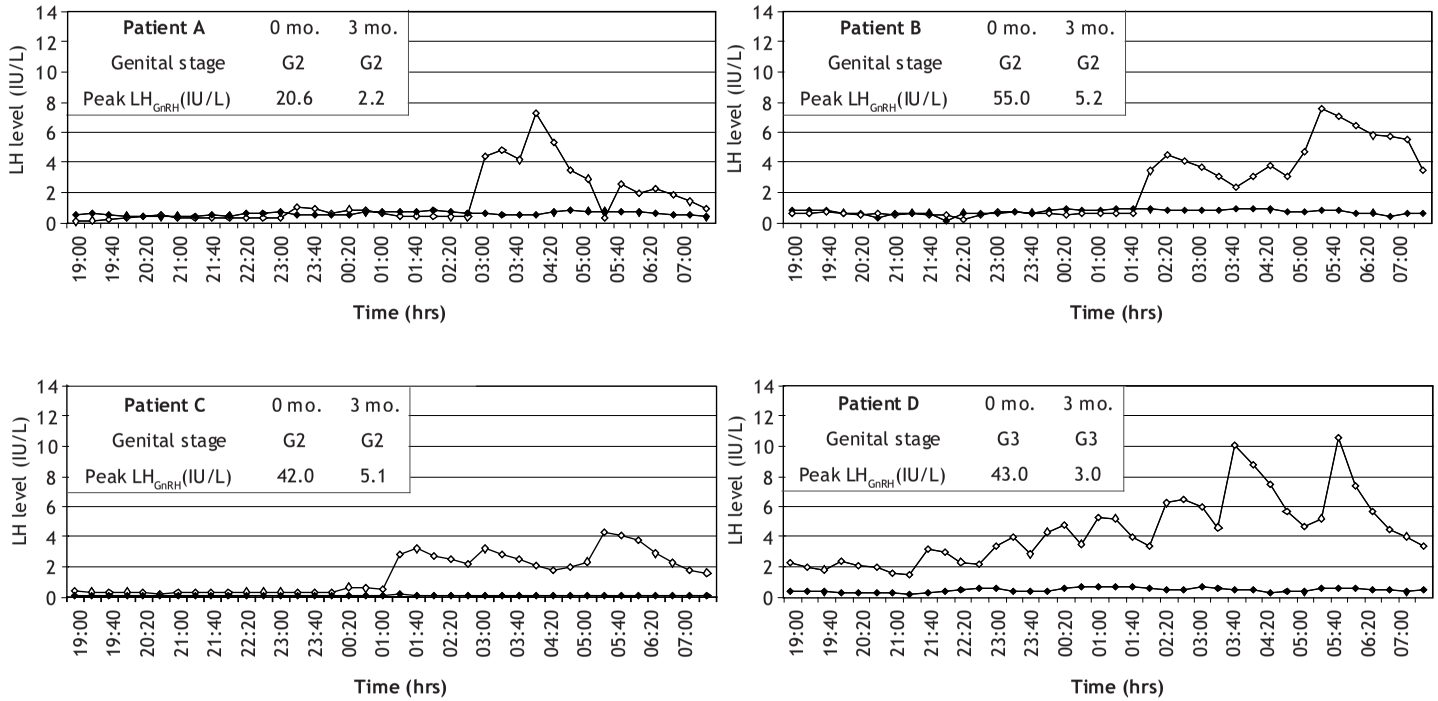

Figure 1. Representative examples of individual overnight LH profiles in 4 boys, performed at baseline (open diamonds) and after 3 months of GnRHa treatment (solid diamonds). Subjects A, B and C had genital stage 2, subject D had genital stage 3. The results of the GnRH agonist test performed after 3 months of GnRHa treatment are shown in the figures.

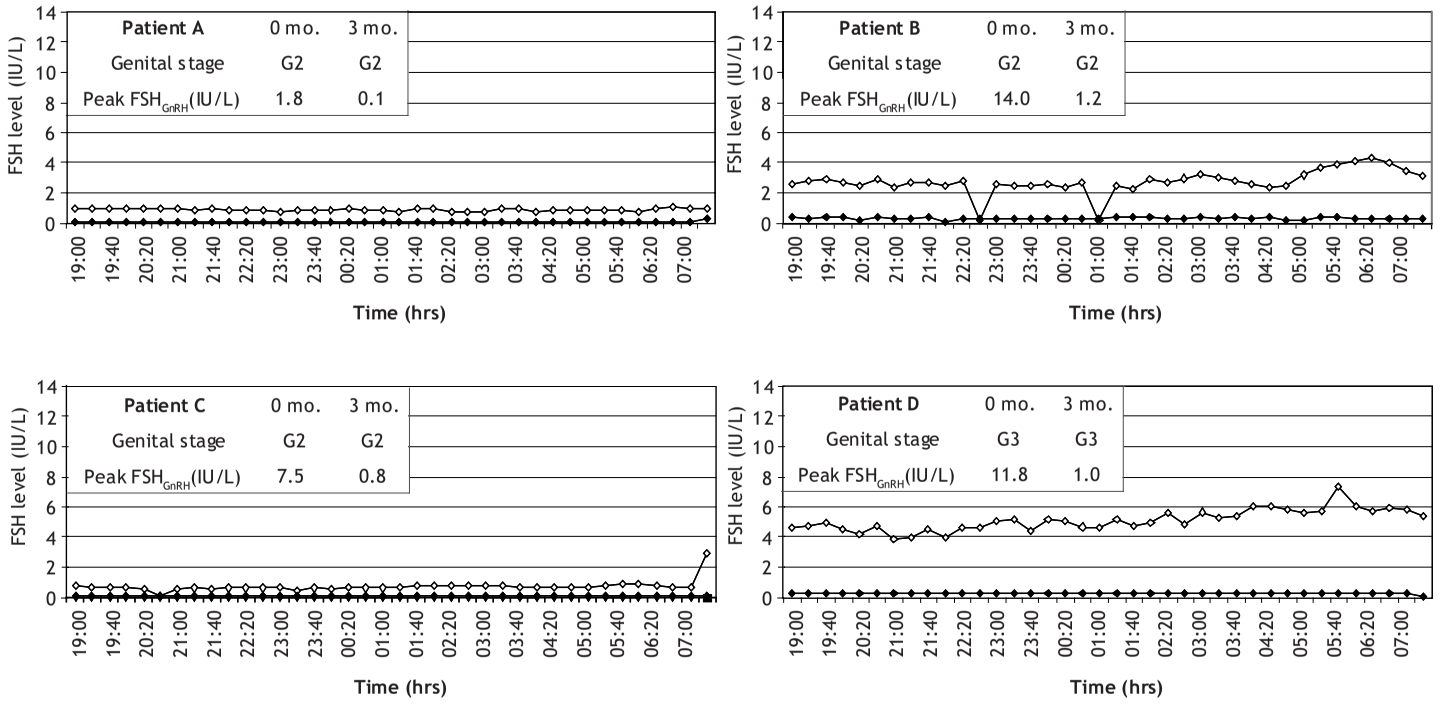

Figure 2. Representative examples of individual overnight FSH profiles in the same 4 boys as shown in Figure 1, performed at baseline (open diamonds) and after 3 months of GnRHa treatment (solid diamonds). Subjects A, B and C had genital stage 2, subject D had genital stage 3. The results of the GnRH agonist test performed after 3 months of GnRHa treatment are shown in the figures.

After 3 months of GnRHa treatment, no correlations were found between characteristics of the LH profiles and peak LH or testosterone levels during the GnRH agonist test.

Peak LH_{GnRH} and peak FSH_{GnRH} at baseline correlated respectively with peak LH_{GnRH} and peak FSH_{GnRH} after 3 months of GnRHa treatment (r=0.73, P=0.003 and r=0.70, P=0.006, respectively).

Overnight GH-profiles, IGF-I and IGFBP-3 levels

Baseline

Characteristics of the overnight GH profiles and IGF-I and IGFBP-3 levels at baseline are listed in Table 3. Mean GH levels in boys with genital stage 2 were comparable with mean GH levels found in boys with normal stature and similar genital stage. Since only 2 boys had genital stage 3, a comparison with boys with normal stature and similar pubertal stage was not feasible.

IGF-I and IGFBP-3 levels were significantly lower than zero SDS (P=0.03 and P=0.001, respectively).

After 3 months of GnRHa treatment

Characteristics of overnight GH profiles, IGF-I and IGFBP-3 levels after 3 months of GnRHa treatment are listed in Table 3. No significant differences were found between GH profile characteristics at baseline and after 3 months of GnRHa treatment. Mean GH levels remained comparable with those found in controls. Figure 3 shows that there was a wide interindividual variation in mean serum GH levels, both at baseline and after 3 months of GnRHa treatment.

IGF-I and IGFBP-3 levels did not significantly change and SDS values remained significantly lower than zero SDS (P=0.03 and P<0.001, respectively).

In all boys, there was a significant correlation between the timing of GH peaks at baseline and after 3 months of GnRHa treatment (r=0.4, P=0.01).

Correlations between GH-profiles, clinical characteristics and the GnRH agonist test

Neither at baseline, nor after 3 months of GnRHa treatment were correlations found between characteristics of the overnight GH profiles and age, height SDS, peak LH_{GnRH}, bone age (at baseline) or fat mass SDS (at baseline).

Table 3. Characteristics of overnight GH levels, IGF-I and IGFPB-3 levels, at baseline and after 3 months of GnRHa treatment.

	Baseline	3 months	P value[a]
AUC_0 (mU/L*12h)	135 (92.1-263)	130 (70.0-238)	0.6
Mean GH (mU/L)	10.8 (7.3-20.8)	10.4 (5.6-18.8)	0.6
Max GH (mU/L)	46.2 (32.8-137.8)	55.3 (30.9-89.7)	0.4
Pulse amplitude (mU/L)	16.4 (13.7-32.5)	22.8 (11.6-32.7)	0.4
No. of GH peaks > 10 mU/L	3.5 (2.8-5.0)	4.0 (3.0-4.0)	1.0
IGF-I SDS	-0.8 (-1.5 to -0.04)[b]	-0.9 (-1.4 to -0.2)[b]	0.6
IGFBP-3 SDS	-1.0 (-1.5 to -0.6)[c]	-1.2 (-1.7 to -0.7)[c]	0.1

Data are expressed as median (interquartile range)
[a] at 3 months compared to baseline
[b] P≤0.05 compared to zero SDS
[c] P≤0.001 compared to zero SDS

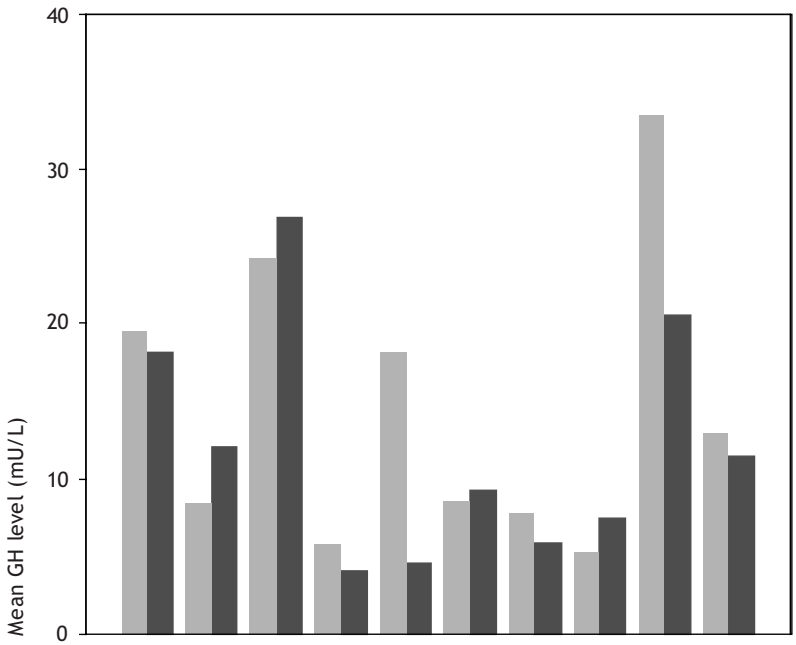

Figure 3. Mean GH levels for each individual during overnight GH profiles, at baseline (light grey bars) and after 3 months of GnRHa treatment (dark grey bars).

Correlations between GH-profiles and IGF-I and IGFBP-3 levels

At baseline, mean GH levels correlated significantly with IGF-I SDS (r=0.72, P=0.02). After 3 months of GnRHa treatment, mean GH levels correlated significantly with IGF-I SDS (r=0.76, P=0.01) and IGFBP-3 SDS (r=0.64, P=0.048). Maximum GH levels correlated significantly with IGF-I SDS (r=0.69, P=0.03).

Discussion

In the present study, LH and FSH levels had significantly decreased to prepubertal levels in all boys, showing that treatment with leuprorelide acetate depots of 3.75 mg subcutaneously every 4 weeks resulted in an adequate suppression of puberty. The GnRH agonist test performed after 3 months of GnRHa treatment falsely indicated insufficient pubertal suppression in 43% of the boys. No significant changes in overnight GH profile characteristics and IGF-I and IGFBP-3 SDS were found after 3 months of GnRHa treatment, compared to baseline.

Overnight LH and FSH profiles

After 3 months of GnRHa treatment, all boys had clinical arrest of puberty and 2 boys had clinical regression of puberty. AUC_0, mean and maximum LH and FSH levels had significantly decreased to very low levels. The pattern of LH and FSH profiles after 3 months of GnRHa treatment in short boys born SGA was similar to prepubertal profiles found in healthy boys [12].

Overnight LH and FSH profiles were performed for research purpose since admitting children to a hospital in order to perform overnight profiles is not suitable for routine clinical care. After 3 months of GnRHa treatment, peak LH_{GnRH} was above the cut-off level of 3 IU/L in 43% of the boys [15]. Notably, all boys had prepubertal testosterone levels (below 1 nmol/l). Furthermore, no significant differences in LH and FSH profiles were found between boys with a peak LH_{GnRH} above or below 3 IU/L. Our findings are in agreement with the only comparable study, performed in 6 children (2 boys) with CPP [36]. In this study, children who were clinically well suppressed had overnight LH levels similar to prepubertal children.

Peak LH_{GnRH} was known before LH levels during the overnight profiles were available. The schedule of leuprorelide acetate depot injections was changed to every 3 weeks instead of every 4 weeks in boys with a peak LH_{GnRH} above 3 IU/L,

according to Dutch consensus guidelines. This adjustment seemed to be unnecessary once we received the results of the overnight LH profiles.

Although we found prepubertal LH profiles in all boys after 3 months of GnRHa treatment, LH and FSH profiles were determined in a rather small group. In order to determine which peak LH_{GnRH} cut-off level indicates sufficient pubertal suppression, we recommend future research in a larger study group, including boys and girls with different pubertal stages and with sufficient and insufficient pubertal suppression according to their LH profiles.

Overnight GH profiles, IGF-I and IGFBP-3 levels

To our knowledge, this is the first study describing baseline GH profiles in pubertal short boys born SGA. We found normal mean GH levels, compared to boys with normal stature and similar pubertal stage [31]. Overnight GH profiles have been performed in prepubertal short SGA children with conflicting results. Some authors reported significantly lower levels in short children born SGA, compared to healthy children born appropriate for gestational age [3,4], whereas others found comparable mean GH levels [37]. The wide variability in – and overlap between – GH secretion seen in SGA cohorts and control populations are consistent phenomenona [3,4,20,37]. Within the heterogeneous SGA population, this probably reflects a continuum in GH secretion, ranging from GH deficiency to normal GH secretion. Alterations in the GH/IGF/IGFBP pathway and genetic variations found in genes involved in this pathway [38,39] might in part explain this continuum.

Baseline IGF-I and IGFBP-3 SDS were significantly lower than zero SDS, which is in line with previous studies describing low IGF-I and IGFBP-3 levels throughout childhood in subjects born SGA [6,40,41]. IGF-I SDS was significantly correlated with mean GH levels, both at baseline and after 3 months of GnRHa treatment, and IGFBP-3 SDS was significantly correlated with mean GH levels after 3 months of GnRHa treatment. IGF-I and IGFBP-3 levels were found to reflect GH secretion in healthy children [42]. Our study shows that this reflection is also applicable for short boys born SGA.

After 3 months of GnRHa treatment, we found no significant changes in AUC_0, mean and maximum GH levels, compared to baseline. Likewise, IGF-I and IGFBP-3 levels had not significantly changed. Rose et al have shown that mean GH levels in healthy boys with testicular volumes between 5 to 10 ml remained near prepubertal levels, whereas a significant increase in spontaneous GH and IGF-I levels was found when Tanner stage 3 was reached [31]. In our study, all boys in whom overnight GH profiles were performed,

had testicular volumes between 4 and 8 ml. Thus, this could well explain why we did not find significant changes in GH and IGF-I levels after 3 months of GnRHa treatment.

We found a significant trend in the timing of GH peaks within the same individual. The reproducibility of measurements of overnight GH secretion was reported to be superior to that of provocative tests [32]. The intra-individual reproducibility was found to be less profound [43]. Our results indicate the existence of an intrinsic rhythm regulating endogenous growth hormone secretion in individual subjects.

In conclusion, treatment with leuprorelide acetate depots of 3.75 mg every 4 weeks results in an effective inhibition of central puberty, as shown by prepubertal overnight LH and FSH secretion patterns and clinical signs of pubertal arrest. The GnRH agonist test falsely indicated insufficient pubertal suppression in almost half of the boys, resulting in unnecessary adjustments in the frequency of depot injections. Low IGF-I and IGFBP-3 levels were found at the start of puberty, although mean GH levels were normal for pubertal stage. Parameters of the overnight GH profile did not significantly change after 3 months of GnRHa treatment, consistent with GH levels being comparable for prepubertal and early pubertal boys.

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Chapter 4

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Reduced levels of Growth Hormone during GnRH Analogue Treatment in Pubertal Short Girls Born Small for Gestational Age

Abstract

Context: Several studies showed a decrease in height velocity during GnRHa treatment. No information is available on growth hormone levels during GnRHa treatment in short SGA girls.

Objective: To study overnight GH profiles and IGF-I and IGFBP-3 levels in girls with Tanner stage 2 and stage 3, before and after 3 months of GnRHa treatment, and to compare levels with those found in prepubertal short SGA girls.

Patients: Twenty-four pubertal and 16 prepubertal short SGA girls.

Intervention: After baseline overnight GH profiles, pubertal girls received leuprorelide acetate depots of 3.75 mg subcutaneously every 4 weeks.

Outcome measures: GH, IGF-I and IGFBP-3 levels.

Results: At baseline, mean GH levels were comparable to levels found in prepubertal short SGA girls and IGF-I and IGFBP-3 SDS were significantly below the population mean. After 3 months of GnRHa treatment, AUC_0 (P=0.02), mean (P=0.02) and maximum GH levels (P=0.008) had significantly decreased. Mean GH levels were significantly lower than in prepubertal short SGA girls (P=0.03). Eight girls with a more than 40% decrease in mean GH levels also had a significantly greater decrease in IGF-I and IGFBP-3 levels. Mean and maximum GH levels at baseline correlated significantly with those after 3 months of GnRHa treatment.

Conclusion: Short SGA girls lack the normal increase in GH levels seen in puberty and have reduced IGF-I and IGFBP-3 levels, which might explain their reduced pubertal growth spurt. GnRHa treatment led to a significant reduction in GH levels. Therefore, combining GnRHa treatment with GH treatment might improve adult height of short SGA girls.

Introduction

Children with persistent short stature after being born small for gestational age (SGA) comprise a specific group within the field of pediatric patients. Postnatal catch-up growth occurs in most SGA infants [1,2], whereas about 10% of these children fail to show catch-up growth in height above the -2 standard deviation score (SDS). Changes in the growth hormone (GH) and insulin-like growth factor (IGF) axis in short SGA children have been described [3-6].

The age at onset and progression of puberty in short SGA children is comparable to healthy peers. Some children will, however, have a compromised adult height due to the relatively early start for their short stature [7].

Various studies have evaluated whether postponement of puberty would increase adult height. These studies were mainly performed in girls with central precocious or early puberty [8,9]. Most of them reached an adult height within their target height range, after gonadotropin releasing hormone analogue (GnRHa) treatment.

Some studies showed that height velocity significantly decreased during GnRHa treatment, even to levels below the age-appropriate normal range in some patients [10-12]. Limited data are available about stimulated and spontaneous GH and IGF-I levels during GnRHa treatment [12-14]. These studies were performed in children with central precocious puberty only and relatively small numbers of patients were included. Other studies suggested that poor growth is directly related to reduced sex steroid levels or growth plate senescence induced by prior estrogen exposure [15,16].

No data are available on spontaneous GH, IGF-I and IGFBP-3 levels in pubertal short girls born SGA and it is unknown if GnRHa treatment has an effect on GH, IGF-I and IGFBP-3 levels. We therefore studied GH levels during a 12-hour overnight GH profile and measured fasting serum levels of IGF-I and IGFBP-3, both before and after 3 months of GnRHa treatment. In addition, we compared GH profile characteristics with those found in prepubertal short SGA children.

Materials and methods

Subjects

The study group consisted of 24 short girls born SGA who were at the beginning of puberty. They were included in a clinical trial investigating combined treatment with GnRHa and GH. All girls started with GH treatment after 3 months of GnRHa treatment. They fulfilled the following inclusion criteria at start of the study: 1) birth length and/or birth weight SDS below -2 for gestational age [17], 2) chronological age of 8 years or older at start of the study, 3) current height SDS below -2.5 or a predicted adult height <-2.5 SDS (calculated as height at start of puberty plus 20 cm, according to Dutch references [18], 4) Tanner pubertal stage 2 or stage 3 [19] and a GnRH agonist test indicating central puberty [20]. Exclusion criteria were: 1) a complicated neonatal period with signs of severe asphyxia (defined as Apgar score <3 after 5 minutes), 2) long-term complications of respiratory ventilation such as bronchopulmonary dysplasia, 3) endocrine or metabolic disorders, chromosomal defects, growth failures caused by other disorders (such as emotional deprivation, severe chronic illness, chondroplasia) or syndromes (except for Silver-Russell syndrome), 4) previous or present medication that could interfere with growth or GH treatment. The study was approved by the Medical Ethics Committee of the participating centers. Written informed consent was obtained from parents or custodians and from girls who were 12 years or older.

We compared GH profile characteristics with those found by van Dijk et al, who assessed overnight GH profiles in prepubertal short girls born SGA (n=16). Inclusion and exclusion criteria have been described [21].

Study design

At baseline and after 3 months of GnRHa treatment, complete overnight GH profiles were performed in 22 children. GH profiles were incomplete in 2 girls, in 1 at baseline and in the other after 3 months of GnRHa treatment. Children were admitted to the hospital and an indwelling venous catheter was inserted in the anticubital vein. For a period of 12 hours (19.00-7.20 h.) blood for determination of serum GH levels was taken every 20 minutes. Children followed their normal eating and sleeping pattern. The next morning a fasting blood sample was taken for measurement of IGF-I and IGFBP-3 levels. Height was measured using a Harpenden stadiometer and expressed as the SDS for calendar age [18]. The same investigator (DvdK) assessed pubertal stage according to

Tanner during both visits [19]. Bone age was assessed by one investigator (DvdK), using the segmented Greulich and Pyle reference [22]. At baseline, fat mass was measured by DXA [23]. We previously demonstrated that all girls had clinical arrest of puberty and prepubertal overnight LH profiles, indicating sufficient suppression of central puberty [24,25].

Hormone assays

GH levels were measured by IMMULITE 2000 (Diagnostic Products Corporation, L.A. CA 90045-5597 USA) with a lower detection limit of 0.13 mU/L. Values lower than 0.13 mU/L were assigned as 0.13 mU/L. The results of different standards (mean ± intra-assay SD and ± inter-assay SD, respectively) were 6.8±0.23 and ±0.44, 14.0±0.47 and 0.75, 44±1.87 and ±2.94 mU/L. Intra- and interassay coefficients of variation (CVs) were respectively 3.5% and 6.5% at a level of 6.8 mU/L, 3.4% and 5.5% at a level of 14.0 mU/L, 4.2% and 6.6% at a level of 44 mU/L. GH levels in pubertal and prepubertal short SGA girls were measured in the same laboratory using the same assay.

Serum IGF-I and IGFBP-3 levels were measured in one laboratory using an automated chemi-luminescence immunometric assay (Immulite-1000 systems, Siemens Healthcare Diagnostics, Tarrytown NY, USA). The intra-assay and interassay CVs were <4% and <10%. Serum levels were expressed as SDS to adjust for age and sex, using reference data from a healthy Dutch population [26].

LH levels were measured by chemoluminescence-based immunometric methods (Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA). The detection limit for this assay was 0.1 IU/l and values lower than 0.1 IU/L were assigned as 0.1 IU/L. Intra- and interassay CVs were below 4% and 7%. Oestradiol levels were measured using coated tube radioimmunoassays obtained from Diagnostic Products Corporation. The detection limit was 10 pmol/l and values below 10 pmol/l were assigned as 10 pmol/l. Intra- and interassay CVs were below 8% and 9%.

Calculations

The GH profiles were analyzed using the Pulsar program [27,28]. From this program, the following characteristics were derived: area under the curve above zero (AUC_0), mean and maximum GH levels.

Statistics

Because of a non-Gaussian shaped distribution, data were expressed as median (interquartile range (IQR)). IGF-I and IGFBP-3 SDS were compared with the population means using one-sample T-Tests. The Mann-Whitney test was used for differences between groups. The Wilcoxon signed rank test was used to determine differences between points in time within groups. To analyze night-to-night variation, correlation coefficients were calculated between individual time points within one child. The calculated coefficients were analyzed using Chi-Square Tests. Spearman's correlation coefficient was used for correlations. A P-value <0.05 was considered significant. Analyses were performed using the computer statistical package SPSS (version 11) for Windows.

Results

Baseline

Clinical characteristics

Table 1 shows the baseline clinical data in pubertal and prepubertal short SGA girls. Median (IQR) oestradiol and LH levels were 57 (40-75) pmol/l and 18.9 (14.2-33.0) IU/L, respectively. Bone age was significantly higher in girls with Tanner stage 3, compared to girls with stage 2. Compared to pubertal short SGA girls, prepubertal short SGA girls were significantly younger and had a significantly lower fat mass SDS.

Table 1. Baseline clinical characteristics in pubertal and prepubertal short SGA girls at start of GnRHa treatment.

	Girls Tanner stage 2 (n=17)	Girls Tanner stage 3 (n=7)	Girls Prepubertal (n=16)
Gestational age (weeks)	38.0 (36.0 to 39.7)	36.6 (31.3 to 39.9)	38.0 (33.0 to 40.3)
Birth weight SDS	-2.0 (-2.4 to -1.4)	-2.8 (-3.5 to -2.1)	-1.8 (-2.5 to -1.3)
Birth length SDS	-2.9 (-3.3 to -1.8)	-3.1 (-3.7 to -2.7)	-3.1 (-3.7 to -2.2)
Age at start GnRHa (yrs)	11.8 (11.1 to 12.6)	11.5 (11.2 to 13.1)	7.1 (6.0 to 7.9)***
Bone age at start GnRHa (yrs)	11.2 (11.0 to 11.8)	12.4 (11.9 to 12.8)*	5.8 (4.4 to 6.1)***
Height SDS at start GnRHa	-3.0 (-3.7 to -2.4)	-2.8 (-3.4 to -1.8)	-3.1 (-3.4 to -2.9)
Fat SDS at start GnRHa	-0.6 (-1.4 to 0.07)	-0.1 (-0.5 to 0.2)	-1.7 (-2.8 to -1.0)**

Data are expressed as median (interquartile range)
* P<0.05; ** P<0.01; *** P<0.001

Overnight GH-profiles, IGF-I and IGFBP-3 levels

Characteristics of baseline overnight GH profiles and IGF-I and IGFBP-3 levels are listed in Table 2. AUC_0, mean and maximum GH levels were comparable between girls with Tanner stage 2 and stage 3. GH levels were similar to those found in prepubertal short SGA children [pubertal vs prepubertal children: AUC_0: 130 (81.5-178) vs 130 (112-151) mU/L; mean: 10.4 (6.4-14.1) vs. 10.8 (9.4-12.5) mU/L and maximum: 47.9 (37.2-64.3) vs. 50.8 (39.8-58.2) mU/L].

IGF-I and IGFBP-3 levels were comparable between girls with Tanner stage 2 and stage 3 and levels were significantly below the population means (P=0.01 and P<0.001, respectively).

After 3 months of GnRHa treatment

Overnight GH-profiles, IGF-I and IGFBP-3 levels

Characteristics of the overnight GH profiles and IGF-I and IGFBP-3 levels after 3 months of GnRHa treatment are listed in Table 2. Overnight GH profile characteristics and IGF-I and IGFBP-3 levels were comparable between girls with Tanner stage 2 and stage 3 at baseline. After 3 months of GnRHa treatment, AUC_0, mean and maximum GH levels had significantly decreased, compared to baseline values.

Figure 1 shows individual mean GH levels at baseline and after 3 months of GnRHa treatment. There was a wide interindividual variability in response to GnRHa treatment: 8 girls had a more than 40% decrease in mean GH levels, whereas 3 girls showed an increase in mean GH levels. These 3 girls had prepubertal LH and FSH levels during the overnight profiles and peak LH_{GnRH} was below 3 IU/L, indicating that puberty was sufficiently suppressed after 3 months of GnRHa treatment. Bone age, Tanner stage and fat mass SDS at baseline, as well as oestradiol and LH levels, and IGF-I and IGFBP-3 SDS at baseline and after 3 months of GnRHa treatment were comparable between girls who showed a more than 40% decrease, a decrease between 0% and 40% and an increase in mean GH levels.

Table 2. Characteristics of overnight GH profiles, IGF-I and IGFBP-3 levels, at baseline and after 3 months of GnRHa treatment.

	Baseline	3 months	P value[a]
AUC_0 GH(mU/L*12h)	130 (81.5 to 178)	108 (68.4 to 133)	0.02
Mean GH (mU/L)	10.4 (6.4 to 14.1)	8.6 (5.4 to 10.5)	0.02
Max GH (mU/L)	47.9 (37.2 to 64.3)	43.2 (29.0 to 50.3)	0.008
IGF-I (ng/ml)	215 (135-291)	202 (157-287)	0.5
IGF-I SDS	-0.7 (-1.7 to 0.1)[b]	-0.8 (-1.6 to 0.08)[c]	0.1
IGFBP-3 (ng/ml)	2.08 (1.77-2.39)	2.01 (1.80-2.39)	0.8
IGFBP-3 SDS	-1.0 (-1.6 to -0.7)[c]	-1.3 (-1.6 to -0.6)[c]	0.5

Data are expressed as median (interquartile range)
[a] at 3 months compared to baseline
[b] P≤0.01 compared to zero SDS
[c] P≤0.001 compared to zero SDS

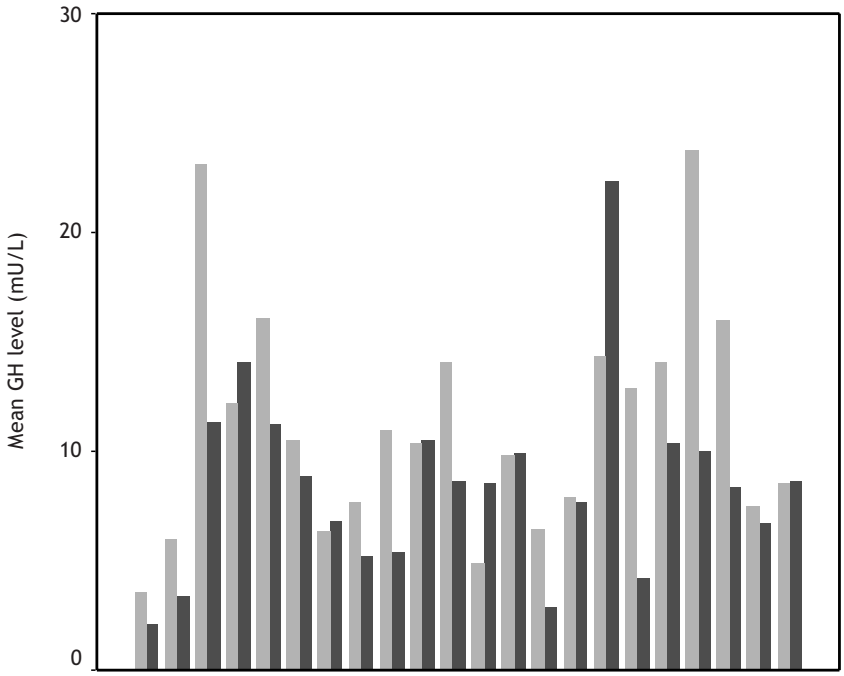

Figure 1. Mean GH levels for each individual during the overnight GH profile at baseline (light grey bars) and after 3 months of GnRHa treatment (dark grey bars).

AUC_0 and mean GH levels were significantly lower in pubertal short SGA children, compared to prepubertal short SGA children [AUC_0 (108 (68.4-133) vs 130 (112-151) mU/L, P=0.04) and mean (8.6 (5.4-10.5) vs 10.8 (9.4-12.5) mU/L, P=0.03]. Maximum GH levels showed a trend towards lower levels in short pubertal SGA children, compared to short prepubertal SGA children [43.2 (29.0-50.3) vs. 50.8 (39.8-58.2) mU/L, P=0.09].

No significant changes in IGF-I and IGFBP-3 levels were found after 3 months of GnRHa treatment, thus levels remained significantly below the population means (P=0.001 and P<0.001, respectively). However, girls with a more than 40% decrease in mean GH levels had a significantly greater decrease in IGF-I and IGFBP-3 levels, compared to girls with a decrease in mean GH levels between 0% and 40% (Δ IGF-I SDS: -0.76 (-0.90 to -0.10) vs -0.03 (-0.39 to 0.29) SDS, P=0.02; and Δ IGFBP-3 SDS: -0.24 (-0.44 to -0.08) vs -0.05 (-0.16 to 0.23) SDS, P=0.02).

Figure 2 shows a representative example of an individual's overnight GH profile. There was a significant correlation between the timing of growth hormone peaks at baseline and after 3 months of GnRHa treatment in each child (r=0.4, P<0.001).

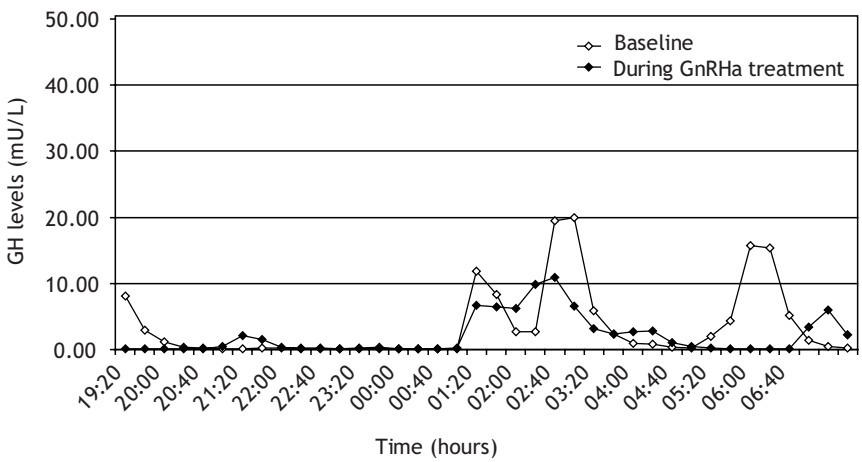

Figure 2. A representative individual overnight GH profile in a girl with Tanner stage 2, performed at baseline (open diamonds) and after 3 months of GnRHa treatment (solid diamonds).

Correlations between GH-profiles and IGF-I and IGFBP-3 levels

Both at baseline and after 3 months of GnRHa treatment, IGF-I SDS correlated significantly with IGFBP-3 SDS (r=0.64, P=0.001 and r=0.61, P=0.002, respectively). No significant correlations were found between GH profile characteristics and IGF-I or IGFBP-3 levels.

The decrease in mean GH levels during GnRHa treatment correlated significantly with the decrease in IGF-I SDS (r=0.45, P=0.04) and IGFBP-3 SDS (r=0.50, P=0.02).

Mean and maximum GH levels at baseline correlated significantly with mean and maximum GH levels after 3 months of GnRHa treatment (r=0.67, P=0.001 and r=0.60, P=0.003).

Correlations between GH-profiles and clinical and laboratory characteristics

At baseline, mean GH levels correlated significantly with fat mass SDS (r=-0.56, P=0.006). No correlations were found between mean or maximum GH levels and age, bone age, oestradiol levels and mean or maximum LH levels during the overnight LH profile.

No correlations were found between mean or maximum GH levels and age and bone age at baseline, oestradiol levels and the overnight LH profile after 3 months of GnRHa treatment. The same held true for the decrease in mean or maximum GH levels. IGFBP-3 SDS correlated significantly with mean and maximum LH levels during the overnight profile after 3 months of GnRHa treatment (r=0.57, P=0.008 and r=0.50, P=0.02).

Discussion

In the present study, overnight GH profiles were performed in short girls born SGA at start of puberty. In addition, we determined the effect of GnRHa treatment on GH, IGF-I and IGFBP-3 levels and compared our results with those found in short prepubertal girls born SGA. At start of puberty, GH levels were similar to prepubertal short SGA girls and IGF-I and IGFBP-3 SDS were significantly lower than the population means. GnRHa treatment led to a significant reduction of serum GH levels.

To our knowledge, this is the first study describing baseline GH profiles at start of puberty in short girls born SGA. No significant differences in GH profile characteristics were found between girls with Tanner stage 2 and Tanner stage 3, which is in line

with findings in healthy controls [29,30]. We found similar GH levels compared to short prepubertal girls born SGA. In girls with normal stature, GH secretion increases during puberty, with the highest levels found at Tanner stage 3 and stage 4 [29]. At baseline, oestradiol levels in our study group were comparable to those in pubertal girls with normal stature, in whom a positive association between oestradiol levels and height velocity was demonstrated [31]. The lack of a rise in GH levels in short pubertal SGA girls with Tanner stage 2-3 might play a role in the less intense pubertal growth spurt found in short SGA children who do not receive GH treatment [32,33].

As reported in prepubertal short children born SGA [6,34], baseline IGF-I and IGFBP-3 SDS in pubertal short girls were also significantly lower than the respective population means. Girls with Tanner stage 2 and stage 3 had comparable levels. Our findings are in line with Carel et al who found low IGF-I levels in 89% of short pubertal SGA children [35]. Together, our results indicate that these SGA girls might benefit from GH treatment, particularly during puberty.

At baseline, a significant inverse relationship was found between mean GH levels and fat mass SDS. In pubertal girls with normal stature, spontaneous GH levels correlated with BMI SDS or weight for height SDS [29,30]. Thus, the inverse association between fat mass and GH levels also exists in short pubertal SGA girls.

After 3 months of GnRHa treatment, AUC_0, mean and maximum GH levels had significantly decreased. Furthermore, mean GH levels were significantly lower compared to prepubertal short SGA girls, whereas there was a trend towards lower maximum GH levels. This is in line with results found in the few GH profile studies performed in patients with precocious puberty [12-14]. Our results indicate that reduced GH levels might result in reduced growth during GnRHa treatment, at least in short pubertal girls born SGA. From that perspective, GH treatment might be beneficial to improve growth during GnRHa treatment in short SGA girls. Baseline mean and maximum GH levels correlated positively with those after 3 months of GnRHa treatment, suggesting that girls who have higher GH levels at baseline have a smaller reduction in GH secretion during GnRHa treatment.

There was a wide interindividual variability in GH secretion in response to GnRHa treatment: 8 girls had a more than 40% decrease in mean GH levels, whereas 3 girls actually had an increase in mean GH levels. No correlations were found between mean GH levels and oestradiol levels, and between mean GH levels and mean and maximum LH levels during the overnight LH profile. This implies that girls with the same degree of pubertal suppression had different GH levels during GnRHa treatment. This is an interesting finding, which demands further research.

IGF-I and IGFBP-3 levels were already low at baseline and remained so after 3 months of GnRHa treatment. Children who had a larger reduction in GH levels also had a larger reduction in IGF-I and IGFBP-3 levels. Several studies demonstrated a significantly decreased height velocity during GnRHa treatment [10-12]. Since all girls started with GH treatment after 3 months of GnRHa treatment, it was not possible to reliably determine height velocity in our study group. Our results nonetheless indicate that alterations in the GH-IGF-IGFBP axis during GnRHa treatment might be responsible for poor growth. The decrease in mean GH levels during 3 months of GnRHa treatment correlated significantly with the decrease in IGF-I and IGFBP-3 levels. Thus, IGF-I and IGFBP-3 levels do not only reflect spontaneous GH secretion in healthy children [36], but also in short girls born SGA.

Our study demonstrates that the intra-individual variation in timing of growth hormone peaks is small. The reproducibility of overnight GH profiles is superior to provocative tests [37]. A marked reproducibility of 24-hour GH profiles was reported in healthy prepubertal children, both in terms of pattern and total secretion [38]. However, these authors found a less profound intra-individual reproducibility. This could be due to the fact that these children were on average 9.5 years during the first profile and 11 years during the second profile and alterations in the pulsatile GH pattern in girls already occur before clinical appearance of puberty [29]. We found a significant trend in the timing of GH peaks, indicating an intrinsic rhythm of endogenous GH secretion in individual children, regardless of pubertal suppression.

The optimal GH dose for short children born SGA during puberty and whether postponement of puberty will improve adult height is still unknown. Our study shows that short girls born SGA do not have the usual increase in GH levels during Tanner stage 2 and stage 3, as found in girls with normal stature. Mean GH levels significantly decreased during 3 months of GnRHa treatment to levels lower than those found in prepubertal short SGA girls. Combining GnRHa treatment with GH treatment might therefore improve adult height in short girls born SGA.

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Chapter

5

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Submitted

Randomized GH Trial with 2 Different Dosages in Combination with a GnRH Analogue in SGA Children: Effects on Serum Growth Hormone, IGF-I and IGFBP-3 Levels

Abstract

Context: Postponement of puberty in combination with growth hormone (GH) treatment has been proposed to increase adult height. However, the effect of combined treatment with a gonadotropin releasing hormone analogue (GnRHa) and GH in 2 different dosages on serum GH levels, growth factors and short-term growth in short SGA children is unknown.

Objective: To assess overnight GH profiles, fasting levels of IGF-I and IGFBP-3 and growth after 1 year of combined treatment with GnRHa and GH.

Patients: Thirty-four pubertal short SGA children at the beginning of puberty, median (IQR) age: 12.0 (11.3-12.6) years.

Intervention: Children received leuprorelide acetate depot 3.75 mg subcutaneously every 4 weeks, and were randomly assigned to receive 1 mg (group A) or 2 mg (Group B) GH/m^2/day.

Outcome measures: GH profiles, IGF-I and IGFBP-3 levels and growth.

Results: After 1 year of combined treatment, GH, IGF-I and IGFBP-3 levels had significantly increased in both GH dosage groups. GH and IGF-I levels and height velocity SDS were significantly higher in group B compared to group A. Children with Tanner stage 2 at start of GnRHa treatment had a significantly greater height velocity SDS during combined treatment than children with Tanner stage 3.

Conclusion: Pubertal short SGA children treated with GnRHa and either 1 or 2 mg GH/m^2/day show a dose-dependent increase in GH and IGF-I levels and 1st-year growth response. Our results suggest that GnRHa treatment in combination with GH 2 mg/m^2/day might be more effective than with GH 1 mg/m^2/day, but adult height data need to be awaited before definitive conclusions can be drawn.

Introduction

Growth hormone (GH) treatment effectively and safely induces catch-up growth in short prepubertal children who were born SGA [1,2]. Some studies indicated that better growth responses and greater adult height were achieved when children were treated with growth hormone for at least 2 years prior to onset of puberty [3,4]. However, several short SGA children only come under medical attention at onset of puberty.

Postponement of puberty in combination with GH treatment has been investigated in specific groups of patients. Studies in patients with idiopathic growth hormone deficiency and in children with idiopathic short stature (ISS) showed beneficial effects in favor of combined treatment compared to GH treatment alone [5-8]. Other studies found no benefit from combined treatment in children with ISS [9,10]. Whether postponement of puberty is beneficial for adult height improvement in persistently short children born SGA is still unknown.

Treatment with GH results in a dose-dependent rise in serum GH levels in GH-deficient adolescents and children with Turner syndrome [11,12]. In prepubertal short children born SGA, GH treatment with 2 mg/m^2/day resulted in significantly higher levels of serum GH, IGF-I and IGFBP-3 compared to children treated with 1 mg/m^2/day [13,14].

No data are available on the effect of treatment with a GnRH analogue in combination with different GH dosages on GH levels in pubertal short children born SGA. We therefore performed a randomized trial investigating the effect of 2 dosages of GH (1 mg vs. 2 mg GH/m^2/day) on overnight GH profiles and serum IGF-I and IGFBP-3 levels at start of GH treatment and after 1 year of combined treatment with GnRHa and GH. Furthermore, we investigated whether GH profile characteristics, IGF-I and IGFBP-3 levels were associated with the first year growth response.

Materials and methods

Subjects

The study group consisted of 34 short pubertal children (24 girls) born SGA who were at the beginning of puberty. They were included in a randomized trial investigating 2 dosages of GH in combination with GnRHa treatment (leuprorelide acetate depots 3.75 mg subcutaneously every 4 weeks). Children who met the following inclusion criteria were included in the study: 1) birth length and/or birth weight SDS below -2 for gestational age [15], 2) chronological age of 8 years or older at start of the study, 3) current height SDS below -2.5 or a predicted adult height below -2.5 SDS (calculated as height at start of puberty plus 20 cm for girls and plus 30 cm for boys, according to Dutch references [16]), 4) early pubertal stage, defined as Tanner stage 2 or 3 [17] and a GnRH agonist test result indicating central puberty [18]. Exclusion criteria were: 1) a complicated neonatal period, with signs of severe asphyxia (defined as Apgar score <3 after 5 minutes), 2) long-term complications of respiratory ventilation such as bronchopulmonary dysplasia, 3) endocrine or metabolic disorders, chromosomal defects, growth failure caused by other disorders (such as emotional deprivation, severe chronic illness and chondroplasia) or syndromes (except for Silver-Russell syndrome), 4) previous or present medication that could interfere with growth or GH treatment. The study was approved by the Medical Ethics Committee of the participating centers and written informed consent was obtained from parents or custodians. Written informed assent was obtained from children aged 12 years or older.

Study design

After 3 months of GnRHa treatment, children were randomized in two GH dosage groups; after stratification for gender, pubertal stage (Tanner stage 2 or 3) and parental height SDS (one parent with height SDS below -2 or both parents with height SDS within the normal range) (Figure 1). Children were assigned to group A receiving 1 mg GH/m^2/day (~0.033 mg/kg/day) or to group B receiving 2 mg GH/m^2/day (~0.067 mg/kg/day) Genotropin® (Somatropin). GH was administered subcutaneously once daily at bedtime. Three-monthly, the GH dose was adjusted to the calculated body surface area.

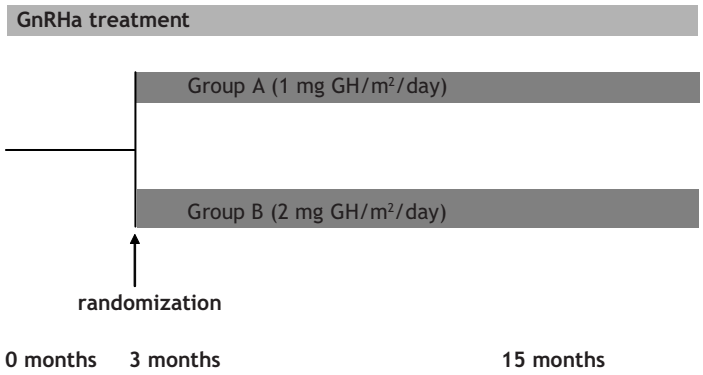

Figure 1. Schematic representation of the study design.

Complete overnight GH profiles were available in 33 children, both at start of GH treatment and after approximately 1 year of combined treatment with GnRHa and GH. Children were admitted to the hospital and an indwelling venous catheter was inserted in the anticubital vein. For a period of 12 hours (19:00-7:20 h) blood for determination of serum GH levels was taken every 20 minutes. Children followed their normal eating and sleeping pattern. The next morning a fasting blood sample was taken for measurement of IGF-I and IGFBP-3 levels.

Pubertal stage according to Tanner was assessed by the same investigator (DvdK) during all visits [17]. Height was measured using a Harpenden stadiometer and expressed as SDS for calendar age and sex [16]. We previously demonstrated that all children had clinical arrest of puberty and prepubertal overnight LH and FSH profiles, indicating sufficient suppression of central puberty [19,20]. Since all children could be considered as prepubertal due to successful GnRHa treatment, height velocity SDS was calculated, based on prepubertal height velocity references adapted for a wide age range [21]. Bone age at start of GnRHa treatment and after 1 year of combined treatment was assessed using the segmented Greulich and Pyle reference [22]. Fat mass at start of GnRHa treatment was measured by Dual Energy X-ray Absorptiometry scans on one machine (DXA; type Lunar Prodigy, GE Healthcare, Chalfont St Giles, UK). All scans were performed by the same investigator (DvdK). Fat mass was expressed as SDS to adjust for gender and height [23].

Hormone assays

GH levels were measured by IMMULITE 2000 (Diagnostic Products Corporation, L.A. CA 90045-5597 USA) with a lower detection limit of 0.13 mU/L. Values lower than 0.13 mU/L were assigned as 0.13 mU/L. The results of different standards (mean ± intra-assay SD and ± inter-assay SD, respectively) were 6.8 ±0.23 and ±0.44, 14.0 ±0.47 and 0.75, 44 ±1.87 and ±2.94 mU/L. Intra- and interassay coefficients of variation (CVs) were respectively 3.5% and 6.5% at a level of 6.8 mU/L, 3.4% and 5.5% at a level of 14.0 mU/L, 4.2% and 6.6% at a level of 44 mU/L. Serum IGF-I and IGFBP-3 levels were measured in one laboratory using an automated chemi-luminescence immunometric assay (Immulite-1000 systems, Siemens Healthcare Diagnostics, Tarrytown NY, USA). The intra-assay and interassay CVs were <4% and <10%. Serum levels were expressed as SDS to adjust for age and sex, using reference data from a healthy Dutch population [24].

LH and FSH levels were measured by chemoluminescence-based immunometric methods (Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA) using WHO-preparations 80/552 and 78/549 as standards. Detection limits for both assays were 0.1 IU/l and values lower than 0.1 IU/L were assigned as 0.1 IU/L. Intra- and interassay CVs were below 4% and 7% for LH and below 3% and 6% for FSH. Oestradiol levels were measured using coated tube radioimmunoassays obtained from Diagnostic Products Corporation. The detection limit was 10 pmol/l and values below 10 pmol/l were assigned as 10 pmol/l. Intra- and interassay CVs were below 8% and 9%. Testosterone levels were measured using coated tube radioimmunoassays obtained from Diagnostic Products Corporation. The detection limit for this assay is 0.1 nmol/l. Intra- and interassay CVs were below 6% and 8%.

Calculations

GH profiles were analyzed using the Pulsar program [25,26]. Mean and maximum GH levels were derived from this program.

Statistics

Results are expressed as median (interquartile range (IQR)). The Mann-Whitney test was used for differences between groups. The Wilcoxon signed rank test was used to determine differences between points in time within groups. To test for linear relationships, partial correlations were determined for group A and B together, with adjustment for GH dosage. Multiple linear regression analysis was used to test

multivariate relationships. Only results of the best fitting model (in terms of R-squared) are shown. A P-value <0.05 was considered significant. Analyses were performed with the computer statistical package SPSS (version 11) for Windows.

Results

Clinical characteristics

Table 1 lists the clinical data. At start of GnRHa treatment, children with Tanner stage 2 had a significantly lower birth weight than children with Tanner stage 3 (-2.82 (-3.51 to -2.13) SDS vs. -1.97 (-2.27 to -1.59) SDS, P=0.03) and a significantly higher bone age (12.5 (12.0-13.0) vs. 11.2 (11.0-12.0) years, P=0.009).

At start of GH treatment, all children had clinical suppression of puberty and prepubertal overnight LH and FSH profile patterns. No significant differences were found between groups A and B or between boys and girls.

Table 1. Baseline characteristics of both GH dosage groups.

	Group A 1 mg GH/m^2/day	Group B 2 mg GH/m^2/day
Number (female)	16 (11)	18 (13)
Gestational age (wks)	38 (36.6-39.4)	38.6 (33.1-40.0)
Birth weight SDS	-2.0 (-2.5 to -1.4)	-2.2 (-2.6 to -1.7)
Birth length SDS	-2.6 (-3.3 to -1.5)	-2.7 (-3.2 to -2.3)
At start of GnRHa treatment		
Age (yrs)	11.9 (11.2-12.6)	12.2 (11.3-12.7)
Bone age (yrs)	11.3 (11.0-12.3)	11.9 (11.0-12.4)
Height SDS	-2.9 (-3.5 to -2.3)	-2.7 (-3.4 to -2.4)
Target height SDS	-0.42 (-1.5 to -0.3)	-0.45 (-1.4 to 0.05)
Fat SDS	-0.65 (-1.3 to 0.07)	-0.28 (-1.5 to 0.25)
Tanner stage 2	14	12
Tanner stage 3	2	6
At start of GH treatment		
Age (yrs)	12.1 (11.5-12.8)	12.3 (11.5-12.9)
Height SDS	-2.9 (-3.5 to -2.4)	-2.7 (-3.5 to -2.3)
Mean LH (IU/L)*	0.28 (0.22-0.36)	0.36 (0.30-0.52)
Mean FSH (IU/L)*	0.79 (0.30-1.02)	0.62 (0.32-1.13)
Oestradiol (pmol/l) in girls	19.0 (10.0-27.0)	18.5 (14.5-26.3)
Testosterone (nmol/l) in boys	0.40 (0.30-0.70)	0.20 (0.10-0.20)

Data are expressed as median (interquartile range)
* measured during 12-hour overnight LH and FSH profiles [19,20]

Table 2. Characteristics of overnight GH profiles, IGF-I and IGFBP-3 levels and growth, at start of GH treatment and after 1 year of combined treatment with GnRHa and GH.

	Group A (1 mg GH/m^2/day) n=16		P-value[1]	Group B (2 mg GH/m^2/day) n=18		P-value[2]	P-value[3]
	At start of GH treatment	During combined treatment		At start of GH treatment	During combined treatment		
Mean GH (mU/L)	8.5 (4.7-11.3)	25.2 (19.7-31.4)	P=0.001	9.6 (6.5-11.5)	53.4 (45.5-63.3)	P<0.0001	P<0.0001
Max GH (mU/L)	39.3 (26.4-49.4)	60.0 (44.3-83.6)	P=0.001	49.0 (35.9-61.9)	109 (96.8-145)	P=0.001	P<0.0001
GH>40 mU/L (hrs)	-	2.8 (0.67-3.9)	-		7.2 (6.3-8.8)	-	P<0.0001
GH>20 mU/L (hrs)	-	7.8 (5.4 to 8.3)	-		10.8 (8.8 to 11.3)	-	P<0.0001
IGF-I SDS	-0.93 (-2.2 to -0.11)	0.81 (0.55 to 1.1)	P<0.0001	-0.58 (-1.5 to 0.38)	1.5 (1.2 to 2.5)	P<0.0001	P=0.001
IGFBP-3 SDS	-1.5 (-2.0 to -0.83)	-0.55 (-0.74 to -0.14)	P=0.001	-0.83 (-1.4 to -0.46)	0.26 (-0.04 to 0.77)	P<0.0001	P=0.001
Δ IGFBP-3 SDS	-	1.0 (0.32-1.4)	-	-	1.1 (0.89-1.6)	-	NS
Height velocity SDS	-	4.3 (2.8-5.5)	-		6.9 (4.0-7.4)	-	P=0.03

Data are expressed as median (interquartile range)
[1] Group A: during combined treatment, compared to start of GH
[2] Group B: during combined treatment, compared to start of GH
[3] Group B vs. group A: after 1 year of combined treatment with GnRH and GH

Overnight GH profiles

At start of GH treatment

Characteristics of overnight GH profiles for groups A and B are depicted in Table 2. At start of GH treatment, mean and maximum GH levels were comparable for groups A and B and for boys and girls.

After 1 year of combined treatment with GnRHa and GH

Characteristics of overnight GH profiles during combined treatment are depicted in Table 2. Mean and maximum GH levels significantly increased in both GH dosage groups and levels were significantly higher in group B, compared to group A. Following the sc. GH injection at 20.00h, GH levels remained significantly longer above 40 mU/L and 20 mU/L in group B, compared to group A ($P<0.0001$) (Figure 2). As shown in Figure 3, there was a wide interindividual variability in mean GH levels. No correlations were found between GH profile characteristics and age, bone age, gender, pubertal stage, peak LH level during a GnRH agonist test and fat mass SDS.

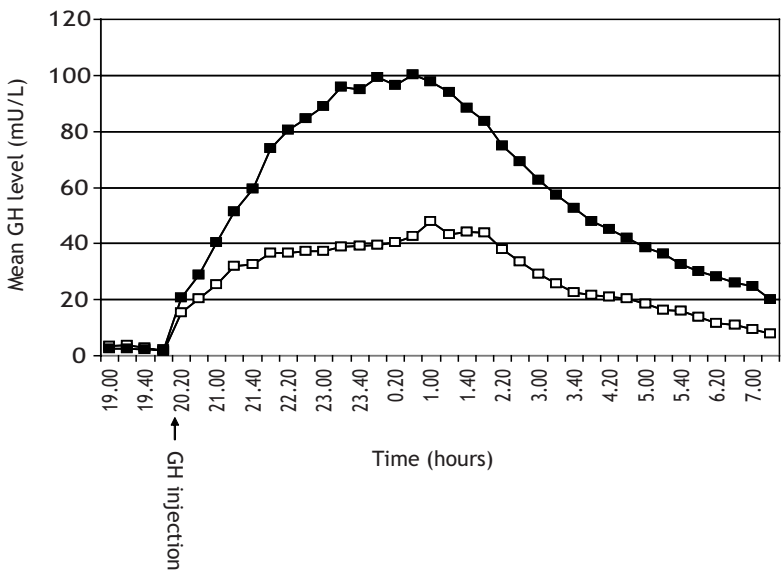

Figure 2. Mean GH levels for each time point during the overnight GH profile after 1 year of combined treatment with GnRHa and GH. Open squares: group A and solid squares: group B.

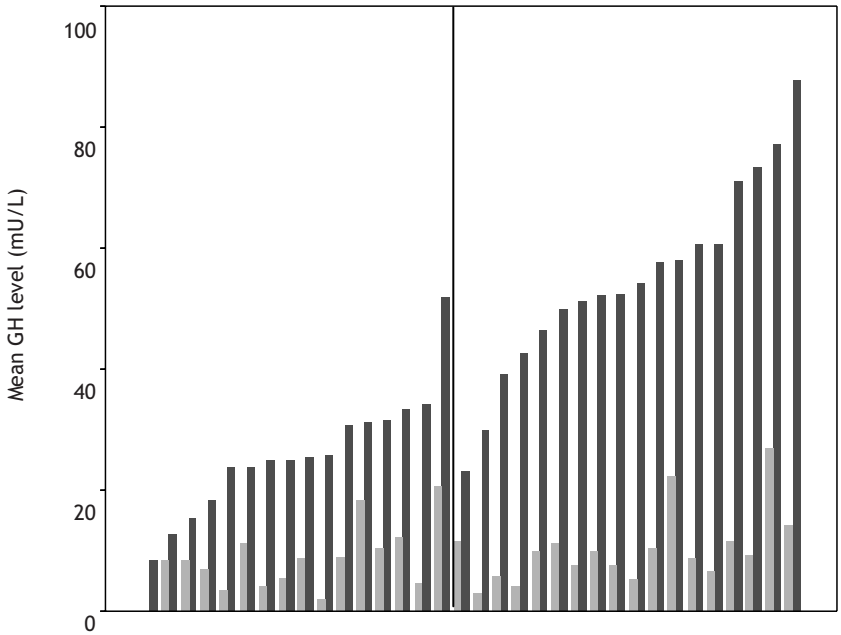

Figure 3. Mean GH levels for each individual during both overnight GH profiles (light grey boxes: at start of GH treatment and dark grey boxes: after 1 year of combined treatment with GnRHa and GH). Left panel: group A and right panel: group B.

IGF-I and IGFBP-3 levels

At start of GH treatment

IGF-I and IGFBP-3 levels were significantly lower than the respective population means (P<0.0001) (Table 2). IGF-I levels were comparable for groups A and B and for boys and girls.

IGFBP-3 levels were significantly lower in group A, compared to group B, but comparable for boys and girls.

IGF-I levels correlated with IGFBP-3 levels (r=0.62, P<0.0001) and mean GH levels (r=0.36, P=0.04). IGFBP-3 levels correlated with mean GH levels (r=0.39, P=0.03).

After 1 year of combined treatment with GnRHa and GH

During combined treatment, IGF-I levels increased to levels significantly higher than the population mean (P<0.0001) in both GH dosage groups. IGF-I levels were significantly higher in group B compared to group A. In group B, 88.9% of the children had IGF-I levels in the highest quintile (>0.84 SDS) and 27.8% had IGF-I levels above 2 SDS, compared to 43.8% (P=0.005) and 6.3% (NS) of children in group A, respectively. IGF-I levels were comparable for boys and girls.

IGFBP-3 levels increased significantly in both GH dosage groups and the increase was comparable between both groups. Compared to the population mean, IGFBP-3 levels remained significantly lower in group A (P=0.01) and were not statistically significant in group B. Levels were comparable for boys and girls.

During combined treatment, IGF-I levels only correlated with IGFBP-3 levels (r=0.50, P=0.003).

Growth response

After 1 year of combined treatment, height velocity SDS was significantly higher in group B (6.9 (4.0-7.4) SDS) compared to group A (4.3 (2.8-5.5) SDS, P=0.03) (Table 2). No differences were found between boys and girls. In group B, children with Tanner stage 2 at start of GnRHa treatment had a significantly greater height velocity SDS than children with Tanner stage 3 (7.1 (5.6-8.5) SDS vs. 4.0 (3.3-5.0) SDS, P=0.02).

Bone age was determined after 1 year of combined treatment. The median increase in bone age divided by the median increase in calendar age was similar in both GH dosage groups: 0.34 (0.00-1.0) years in group A and 0.08 (0.00-0.64) years in group B.

Multiple regression analysis

During multiple regression analysis, the following variables were associated with height velocity SDS during 1 year of combined treatment: fat mass SDS at start of GnRHa treatment (ß=0.83, P=0.008), GH dose (2 mg vs. 1 mg/m^2/day; ß=2.8, P<0.0001), Tanner stage 2 at start of GnRHa treatment (ß=2.0, P=0.01), target height SDS (ß=1.0, P=0.009), IGF-I SDS at start of GH treatment (ß=-0.79, P=0.009). This model explained 60% of the variation in first year growth response.

Discussion

Short SGA children treated with a GnRH analogue to suppress puberty and randomly receiving 1 mg or 2 mg GH/m^2/day show a GH dose-dependent increase in GH and IGF-I levels as well as in first year growth response.

During combined treatment, mean and maximum GH levels were lower in our study group, compared to levels in prepubertal SGA children treated with GH only [14]. In the latter study, GH levels were determined with the same assay in the same laboratory. We previously reported that GH levels significantly decreased after 3 months of GnRHa treatment, compared to levels before start of GnRHa treatment [27]. A reduction in GH levels during GnRHa treatment has also been reported in patients with precocious puberty [8, 28, 29]. Thus, the lower GH levels in our study group might well be a result of the simultaneous treatment with a GnRH analogue, next to GH. Nonetheless, short SGA children treated with GnRHa and 2 mg GH/m^2/day had mean serum GH levels of 53.4 mU/L and GH levels remained above 20 mU/L for almost 11 hours, demonstrating that these children have elevated GH levels for a great part of the day. There was a wide interindividual variation in mean GH levels in both GH dosage groups. This is in line with previous reports and was explained by variations in physiological mechanisms involved in degradation of GH at the site of injection and in the systemic circulation [14,30]. During combined treatment, GH levels increased significantly in both GH dosage groups, but to significantly higher levels in the 2 mg GH/m^2/day group. Dose-dependent rises in GH levels have been described in prepubertal short children born SGA [14], GH-deficient adolescents [11] and girls with Turner syndrome [12].

IGF-I levels significantly increased in both GH dosage groups and levels were significantly higher in the 2 mg GH/m^2/day group. Furthermore, a significantly greater percentage of children treated with GnRHa and 2 mg GH/m^2/day had IGF-I SD-scores in the highest quintile, compared to children treated with GnRHa and 1 mg GH/m^2/day. This is in line with results found in prepubertal SGA children, where a dose-dependent increase to comparable IGF-I levels was found [13,14]. Reassuringly, the percentage of children with IGF-I SD-scores above 2 SDS was not significantly different between both GH dosage groups.

The increase in IGFBP-3 levels was similar in the 1 mg and 2 mg GH/m^2/day group, which is in contrast to results found in prepubertal SGA children [13,14]. An explanation for the lack of a dose-dependent increase in IGFBP-3 levels could be the wide range in IGFBP-3 levels during GH treatment. Compared to the population mean, IGF-I levels

were significantly lower at start of GH treatment and significantly higher after 1 year of combined treatment. IGFBP-3 levels increased as well, but to a lesser extent than IGF-I levels. This will increase the levels of free, biologically active IGF-I which stimulates growth. These results are in line with those found in prepubertal short SGA children treated with GH [14,31].

Height velocity SDS was significantly higher in children treated with GnRHa and 2 mg GH/m^2/day, compared to children treated with GnRHa and 1 mg GH/m^2/day. Furthermore, multiple regression analysis demonstrated that GH dose was a predictor of the first year growth response during combined treatment. Importantly, the increase in bone age was similar in both GH dosage groups. Although GH dose is less important for long-term growth in cohorts who started GH treatment at a young age, a dose-dependent effect was found during the first 4-5 years of GH treatment, which is the expected duration of treatment of older SGA children [1,32]. Furthermore, several studies demonstrated that height velocity declines after the first year of GH treatment, especially when a GH dose of 1 mg/m^2/day is given [33,34]. Our results suggest that when GnRHa is combined with GH treatment, treatment with 2 mg GH/m^2/day might result in a better adult height than with 1 mg GH/m^2/day. However, only long-term data can provide a definitive answer whether adult height will be higher with the double GH dose. Next to adult height data, it is important to determine long-term IGF-I levels and metabolic outcome of combined treatment with GnRHa and either 1 or 2 mg GH/m^2/day.

Tanner stage at start of GnRHa treatment significantly influenced first year growth response: children with Tanner stage 2 had a greater height velocity SDS than children with Tanner stage 3. Pubertal stage, as a marker of prior estrogen exposure, was also inversely correlated with height velocity SDS during GnRHa treatment in girls with precocious puberty [35]. Our findings show that this is also true for short children born SGA, suggesting that combined treatment should best be started at an early pubertal stage.

Other predictors of height velocity SDS during 1 year of combined treatment were target height SDS, fat mass SDS and baseline IGF-I levels. Target height SDS is a consistent predictor of growth response in short prepubertal SGA children [1,33,36]. Fat mass was positively associated with the increase in IGF-1 during GH treatment in healthy, peripubertal children [37]. This might explain the positive association between fat mass SDS and height velocity SDS in our study. Baseline IGF-I levels were inversely correlated with first year growth response, suggesting that lower IGF-I levels at start

of GH treatment indicate a greater potential to respond to GH treatment [38,39]. Our findings demonstrate that the main predictors of GH-induced growth response in prepubertal SGA cohorts do also play a role in pubertal SGA children treated with a combination of GnRHa and GH.

In conclusion, short SGA children treated with GnRHa and either 1 mg or 2 mg GH/m^2/day show a dose-dependent increase in GH and IGF-I levels as well as first year growth response. However, long-term height and safety data need to be awaited before it can be concluded that combined treatment with GnRHa and 2 mg GH/m^2/day will result in a greater adult height than treatment with GnRHa and 1 mg GH/m^2/day, without adversely influencing metabolic outcome.

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Chapter

6

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Submitted

Randomized GH Trial Investigating the Effect of Combined Treatment with a GnRH Analogue and GH on Body Composition, Glucose Homeostasis and Lipid Metabolism in Short Children born Small for Gestational Age

Abstract

Context: It is unknown whether treatment with a gonadotropin releasing hormone analogue (GnRHa) for 2 years, next to growth hormone (GH), will improve adult height of short SGA children who start combined treatment at onset of puberty. No data concerning the safety of combined treatment in these children are available.

Objective: To determine body composition, blood pressure, insulin sensitivity (Si), ß-cell function (disposition index (DI)), and lipid profile during combined treatment with GnRHa and GH.

Subjects: Forty-one pubertal short SGA children, mean (± SD) age: 12.1 (±1.0) years.

Intervention: Children received leuprorelide acetate depot 3.75 mg subcutaneously every 4 weeks and were randomly assigned to receive 1 mg (group A) or 2 mg (group B) GH/m^2/day.

Outcome measures: Fat mass and lean body mass SDS corrected for height, percentage trunk and limb fat, blood pressure, Si and DI, lipids.

Results: During the study period, group A developed a higher fat mass SDS and percentage limb fat compared to group B. Percentage trunk fat increased in both GH dosage groups, but to a higher percentage in group A. Lean body mass SDS increased only in group B. Changes in blood pressure, Si, DI and lipids were similar in both GH dosage groups. Insulin sensitivity significantly decreased, but DI remained comparable to baseline. Lipids remained within normal reference ranges.

Conclusion: Except for a GH dose-dependent effect on body composition, in favor of treatment with 2 mg GH/m^2/day, next to GnRHa, no differences in metabolic profile between both GH dosage groups were found.

Introduction

Gonadotropin releasing hormone analogue (GnRHa) treatment has been used in children with central precocious puberty (CPP). Most studies have focused on adult height, bone mineral density and restoration of the reproductive system after long-term treatment with GnRH analogues [1-4]. Much less attention has been paid to changes in body composition and results were controversial. Some studies reported an increase in fat mass or BMI SDS during GnRHa treatment with a return to values comparable to those at baseline after discontinuation [3,5], whereas others reported no changes [6] or even a decreased BMI SDS during GnRHa treatment [7]. Lean mass SDS decreased significantly during GnRHa treatment [5].

In prepubertal short children born small for gestational age (SGA), growth hormone (GH) treatment effectively induces catch-up growth [8-10)]. Metabolic effects of GH treatment in prepubertal short SGA children include the development of a relative insulin resistance [11,12] with an improvement of blood pressure and lipid profile [13]. Fat mass SDS adjusted for gender and height declined significantly, whereas the increase in lean body mass SDS adjusted for gender and height reflected the normal increase as a result of the increase in height [14]. In a randomized clinical trial where prepubertal short SGA children were treated with 1 mg or 2 mg GH/m^2/day, no significant differences in BMI SDS, blood pressure, insulin levels and lipid profile were found between the 2 GH dosage groups [11,13].

Studies investigating combined treatment with GnRHa and GH in patients with idiopathic growth hormone deficiency or in children with idiopathic short stature have mainly focused on adult height data [15-18].

As of today, it is still unknown whether GH treatment in addition to postponement of puberty for 2 years will improve adult height of short SGA children who come under medical attention around onset of puberty. Furthermore, no data are available on the GH dose effect and the safety of combined treatment in these children. In the present randomized trial, we investigated the effect of combined treatment with GnRHa and 2 randomly assigned GH dosages (1 mg vs. 2 mg GH/m^2/day) on body composition, blood pressure, insulin sensitivity, ß-cell function and lipid profile in pubertal short children born SGA.

Materials and methods

Subjects

The study group consisted of 45 short children (29 girls) born SGA who were at the beginning of puberty. Children with the following inclusion criteria were included in the study: 1) birth length and/or birth weight SDS below -2 for gestational age [19], 2) chronological age of 8 years or older at start of the study, 3) current height SDS below -2.5 or a predicted adult height below -2.5 SDS (calculated as height at start of puberty plus 20 cm for girls and plus 30 cm for boys, according to Dutch references [20]), 4) Tanner breast stage 2 or 3 or a testicular volume of 4 ml or more [21], and a GnRH agonist test with a peak LH level of 10 IU/L or more, indicating central puberty [22]. Exclusion criteria were: 1) a complicated neonatal period, with signs of severe asphyxia (defined as Apgar score <3 after 5 minutes), 2) long-term complications of respiratory ventilation such as bronchopulmonary dysplasia, 3) endocrine or metabolic disorders, chromosomal defects, growth failure caused by other disorders (such as emotional deprivation, severe chronic illness and chondroplasia) or syndromes (except for Silver-Russell syndrome), 4) previous or present medication that could interfere with growth or GH treatment. The study was approved by the Medical Ethics Committee of the participating centers and written informed consent was obtained from parents or custodians and from children aged 12 years or older.

Four children (2 in each GH dosage group) were excluded during the 2-year observation period. One girl was classified as a non-responder. In another girl, GH dose was raised to 2 mg/m^2/day during the study period because of insufficient growth. A third girl and 1 boy were excluded because of non-compliance.

Study design

After 3 months of GnRHa treatment (leuprorelide acetate depots 3.75 mg subcutaneously every 4 weeks), children were randomized in two GH dosage groups, after stratification for gender, pubertal stage at start of GnRHa treatment (Tanner stage 2 or 3) and parental height SDS (one parent with height SDS below -2 or both parents with height SDS within the normal range). Children were assigned to either group A treated with 1 mg GH/m^2/day (~ 0.033 mg/kg/day) or group B treated with 2 mg GH/m^2/day (~ 0.067 mg/kg/day) Genotropin® (Somatropin) (Figure 1).

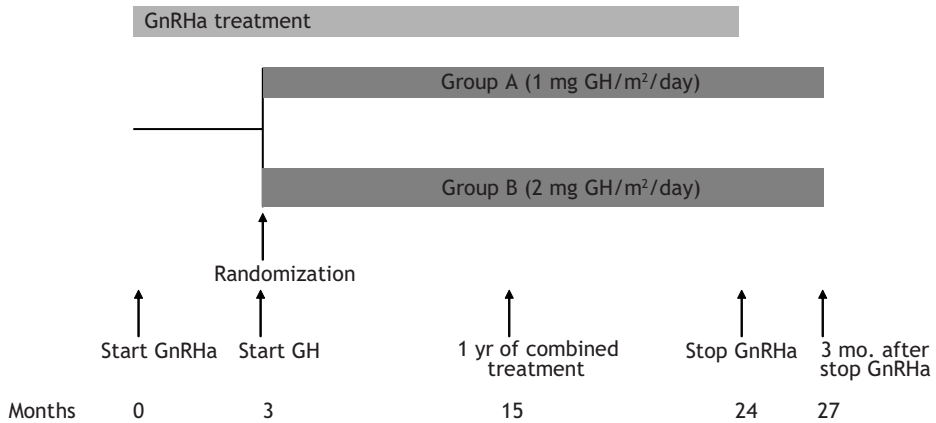

Figure 1. Schematic representation of the study design.

GH was administered subcutaneously once daily at bedtime. Three-monthly, the GH dose was adjusted to the calculated body surface area.

Pubertal stage according to Tanner was assessed by the same investigator (DvdK) during all visits [21]. Height was measured using a Harpenden stadiometer and expressed as SDS for calendar age and sex [20]. We previously demonstrated that all children had clinical arrest of puberty and prepubertal overnight LH and FSH profiles, indicating sufficient suppression of central puberty [23,24]. Systolic and diastolic blood pressures (BP) were measured with an automated device. Since height is an important determinant of blood pressure, BP was expressed as SDS to adjust for height and gender [25]. Fat mass and lean body mass were measured by Dual Energy X-ray Absorptiometry scans on one machine (DXA; type Lunar Prodigy, GE Healthcare, Chalfont St Giles, UK). Fat mass and lean body mass were expressed as SDS to adjust for gender and height (SDS_{height}) [26]. Percentage trunk fat was calculated as (trunk fat / total trunk mass) x 100. Percentage limb fat was calculated as (limb fat / total limb mass) x 100.

Glucose homeostasis

At baseline and during 1 year of combined treatment, a modified frequently sampled intravenous glucose tolerance test (FSIGT) with Tolbutamide was performed as previously described [27]. Insulin sensitivity (Si), glucose effectiveness (Sg), acute insulin response (AIR) and disposition index (DI) were calculated using Bergman's MINMOD MILLENNIUM software [28]. Si quantifies the capacity of insulin to stimulate glucose disposal and

Sg reflects the capacity of glucose to mediate its own disposal. The AIR, an estimate of insulin secretory capacity, was measured as the area under the curve from 0 to 10 minutes corrected for baseline insulin levels. DI is calculated as AIR*Si and is an estimate of ß-cell function.

Hormone assays

All blood samples were taken after an overnight fast. Insulin levels during FSIGTs were measured in one laboratory (IRMA; Medgenix, Biosource Europe, Nivelles, Belgium). The intra-assay coefficient of variance (CV) was 1.9% and the interassay CV was 6.3%. Fasting insulin levels after 3 months of GnRHa treatment and at 3 months after stop of GnRHa treatment were measured using a chemoluminescent assay on an Immulite 2000 analyzer (Diagnostic Products Corporation, Los Angeles, CA). Both methods were highly correlated (r^2=0.98), using the following formula: Y (Immulite) =0.6922X (IRMA) -0.1761. A conversion factor of 6.89 was used to transform data from mU/L (IRMA) to pmol/l (Immulite). HOMA insulin resistance index (HOMA-IR) was calculated using a computer model [29].

Total cholesterol (TC), LDL-cholesterol (LDL-c), HDL-cholesterol (HDL-c), non-esterified fatty acids (FFA) and triglycerides (TG) were determined as previously described [30]. Apolipoprotein A-1 (Apo-A1), Apolipoprotein B (Apo-B) and lipoprotein (a) (lp(a)) were determined by rate nephelometry on the Immage Immunochemistry system, according to manufacturers' instructions (Beckman Coulter, Mijdrecht, The Netherlands). Between-run CVs were 4.2%, 2.8% and 6.9% for the lipoproteins at levels of 0.94, 0.53 and 0.35 g/l respectively.

Statistics

Data at baseline are presented as mean (± SD). Percentage trunk fat, AIR, DI, insulin levels, HOMA-IR, triglycerides and lp(a) levels were log-transformed before analyses, in order to have a Gaussian distribution. SD-scores were compared with population means (zero SDS) using one-sample T-tests. To correct for multiple testing and some missing data, the changes over time and differences between groups A and B were analyzed using repeated measurements analysis with a categorical effect for time and an interaction term for time and GH dose. A P-value <0.05 was considered significant. Analyses at baseline were performed using the statistical package SPSS (version 11.0; SPSS Inc., Chicago, IL) for Windows. SAS 9.1 (SAS Institute Inc., Cary, / nC, USA) was used for repeated measurements analyses.

Results

Baseline clinical and laboratory characteristics

Clinical characteristics are shown in Table 1. Since body composition (Figure 2), blood pressure, insulin sensitivity, ß-cell function and lipid profile (Table 2) were not significantly different between the 2 groups, results are shown for groups A and B together, unless otherwise indicated.

Mean fat mass SDS_{height} and lean body mass SDS_{height} were significantly lower than the population mean ($P<0.0001$). Mean systolic BP was significantly higher than the population mean ($P<0.0001$). A mean systolic BP SDS above the normal range (>+2 SDS) was found in 27% of pubertal short SGA children.

Since body composition, insulin sensitivity and several lipid parameters were significantly different between boys and girls and between children with Tanner stage 2 and stage 3, all analyses were adjusted for gender and Tanner stage at baseline.

Table 1. Baseline clinical characteristics of both GH dosage groups.

	Group A 1 mg GH/m^2/day	Group B 2 mg GH/m^2/day
Number (female)	22 (13)	19 (13)
Gestational age (wks)	38.3 (± 2.4)	37.3 (± 3.5)
Birth weight SDS	-2.0 (± 1.1)	-1.9 (± 0.83)
Birth length SDS	-2.7 (± 1.1)	-2.4 (± 0.72)
Age (yrs)	12.1 (± 1.0)	12.1 (± 0.95)
Bone age (yrs)	11.4 (± 1.1)	11.1 (± 0.90)
Height SDS	-2.7 (± 0.68)	-2.8 (± 0.70)
Target height SDS	-0.65 (± 0.77)	-0.46 (± 0.75)
Tanner stage 2	18	15
Tanner stage 3	4	4

Data are expressed as mean (± SD)

Changes during 3 months of GnRHa treatment

During 3 months of GnRHa treatment, BMI SDS increased significantly (model estimate (95% CI): -0.98 (-1.28 to -0.68) to -0.90 (-1.22 to -0.57) SDS, P=0.045), but remained significantly lower than the population mean (P<0.0001). Blood pressure SDS, insulin levels and HOMA-IR remained comparable to baseline. HDL-c levels increased significantly and FFA levels decreased significantly compared to baseline (Table 2).

Changes during treatment with GnRHa and GH

Body composition

Changes in body composition are shown in Figure 2. During the study period, there was a significant GH dose effect on fat mass SDS_{height} (P=0.01), percentage trunk fat (P=0.03) and percentage limb fat (P=0.01). The GH dose effect on lean body mass SDS_{height} was almost significant (P=0.07).

During 1 year of combined treatment, fat mass SDS_{height} remained comparable to baseline in group A, but decreased significantly in group B (P<0.0001). At 3 months after stop of GnRHa treatment, fat mass SDS_{height} had significantly increased in both GH dosage groups (P=0.0001) and was significantly higher compared to baseline in group A (P<0.0001). In group B, fat mass SDS_{height} returned to values comparable to those at baseline. At 3 months after stop of GnRHa treatment, fat mass SDS_{height} in group A was similar to the population mean, but remained significantly lower in group B (P=0.03).

Percentage trunk fat increased significantly in group A (P=0.002) during 1 year of combined treatment, but remained comparable to baseline in group B. At 3 months after stop of GnRHa treatment, percentage trunk fat had significantly increased and was significantly higher compared to baseline in both GH dosage groups (P<0.001).

During 1 year of combined treatment, percentage limb fat remained comparable to baseline in group A, but decreased significantly in group B (P=0.007). At 3 months after stop of GnRHa treatment, percentage limb fat had significantly increased (P=0.002) and was significantly higher compared to baseline in group A (P=0.04). In group B, percentage limb fat returned to values comparable to those at baseline.

At 3 months after stop of GnRHa treatment, lean body mass SDS_{height} had significantly increased in group B (P=0.007), whereas it remained comparable to baseline in group A. In both GH dosage groups, lean body mass SDS_{height} remained significantly lower compared to the population mean.

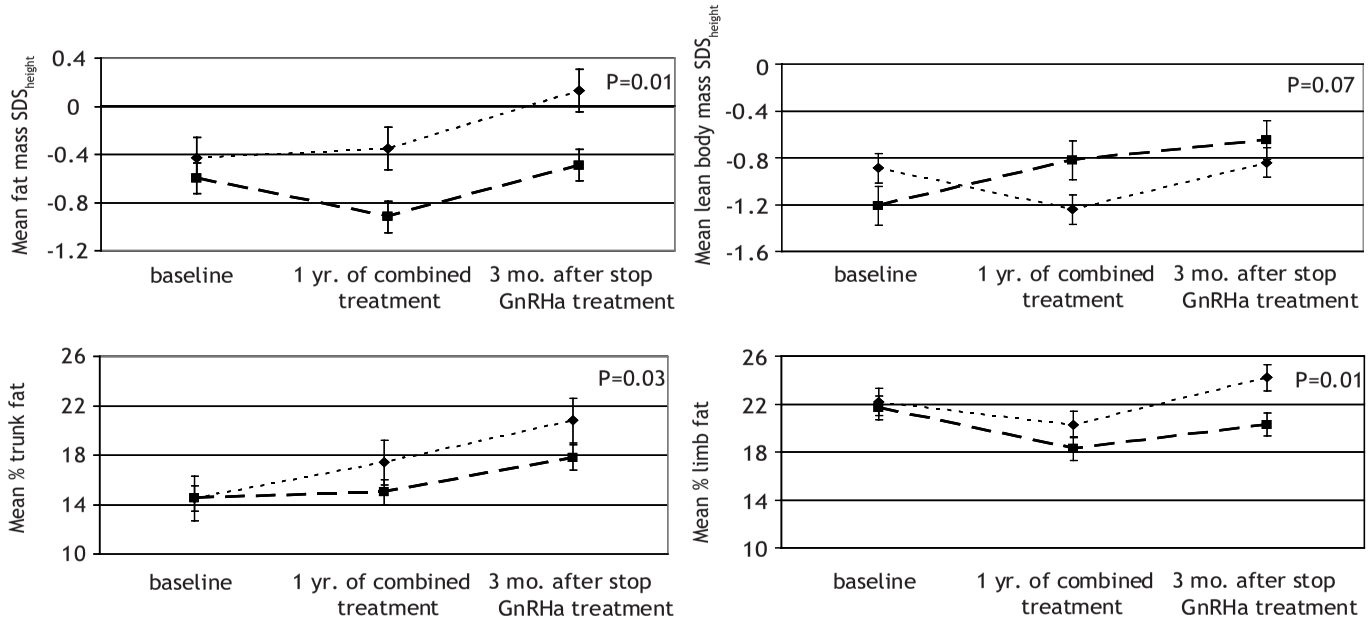

Figure 2. Changes in body composition and percentage trunk and limb fat during the study period. Data are expressed as mean (± SE). Diamonds represent values during treatment with GnRHa and 1 mg GH/m^2/day. Squares represent values during treatment with GnRHa and 2 mg GH/m^2/day. Mean % trunk fat = (trunk fat / total trunk mass) x 100; mean % limb fat = (limb fat / total limb mass) x 100.

Other measures

Blood pressure, insulin sensitivity, β-cell function and lipid profile were comparable between groups A and B. Changes in these parameters during the study period are shown for groups A and B together (Table 2).

Blood pressure

Systolic BP SDS did not change significantly during the study period (Table 2). Diastolic BP SDS increased significantly during 1 year of combined treatment and remained higher at 3 months after stop of GnRHa treatment, albeit still within the normal range.

Insulin sensitivity and β-cell function

Si decreased significantly and AIR increased significantly during 1 year of combined treatment. Sg and DI remained comparable to baseline (Table 2).

During 1 year of combined treatment, insulin levels and HOMA-IR increased significantly compared to baseline. At 3 months after stop of GnRHa treatment, insulin levels and HOMA-IR remained similar to those during 1 year of combined treatment.

Lipid profile

During 1 year of combined treatment, LDL-c, HDL-c, FFA, Apo-A1 and lp(a) levels increased significantly. At 3 months after stop of GnRHa treatment, LDL-c, FFA and Apo-A1 levels returned to levels comparable to baseline, whereas HDL-c and lp(a) levels remained significantly higher. TC and TG levels had increased significantly at 3 months after stop of GnRHa treatment, compared to baseline.

Mean lipid levels remained within the normal range during the study period. At baseline, lp(a) levels were above the normal range (>0.3 g/l) in 27% of children. During the study period, percentages were 27%, 34% and 24%, after 3 months of GnRHa treatment, after 1 year of combined treatment and at 3 months after stop of GnRHa treatment, respectively.

Table 2. Clinical and laboratory parameters during the study period.

	Baseline	3 mo. of GnRHa treatment	1 year of combined treatment	3 mo. after stop GnRHa treatment
Systolic BP SDS	1.59 (1.24-1.94)¶	1.45 (1.12-1.79)¶	1.26 (0.87-1.65)¶	1.39 (1.04-1.74)¶
Diastolic BP SDS	0.22 (0.00-0.45)	0.22 (0.02-0.41)	0.52 (0.27-0.78)[b]	0.57 (0.36-0.79)[c]
Si x 10^{-4}/min^{-1} (µU/ml)	7.38 (6.00-8.76)	ND	4.61 (3.71-5.50)[b]	ND
Sg x 10^{-2}/min^{-1}	3.47 (2.98-3.96)	ND	3.42 (2.94-3.89)	ND
AIR (mU/l)	421 (326-543)	ND	790 (643-971)[b]	ND
DI (AIR x Si)	2569 (2012-3279)	ND	3105 (2514-3838)	ND
Insulin (pmol/l)	48.1 (41.7-55.5)	42.4 (33.9-53.0)	75.0 (63.8-88.1)[b]	79.2 (66.7-94.0)[c]
HOMA-IR	0.91 (0.79-1.06)	0.85 (0.71-1.02)	1.39 (1.19-1.63)[b]	1.43 (1.20-1.70)[c]
TC (mmol/l) [3.0-5.5]	4.16 (3.99-4.33)	4.03 (3.86-4.21)	4.20 (4.00-4.40)	4.33 (4.13-4.53)[c]
LDL-c (mmol/l) [1.3-3.4]	2.28 (2.11-2.46)	2.33 (2.14-2.53)	2.44 (2.25-2.64)[b]	2.36 (2.19-2.52)
HDL-c (mmol/l) [0.9-1.9]	1.41 (1.31-1.52)	1.53 (1.41-1.66)[a]	1.63 (1.51-1.76)[b]	1.55 (1.44-1.66)[c,d]
TG (mmol/l) [0.4-1.6]	0.76 (0.66-0.86)	0.69 (0.61-0.80)	0.79 (0.67-0.94)	0.92 (0.75-1.13)[c]
FFA (mmol/l) [0.2-1.0]	0.52 (0.45-0.59)	0.33 (0.27-0.40)[a]	0.69 (0.59-0.79)[b]	0.51 (0.42-0.60)[d]
Apo-A1(g/l) [0.9-1.6]	1.39 (1.32-1.46)	1.35 (1.28-1.42)	1.56 (1.47-1.65)[b]	1.45 (1.38-1.53)[d]
Apo-B (g/l) [0.5-1.3]	0.71 (0.66-0.75)	0.70 (0.65-0.74)	0.73 (0.68-0.77)	0.72 (0.67-0.77)
Lp(a) (g/l) [≤ 0.3]	0.09 (0.06-0.13)	0.09 (0.06-0.14)	0.14 (0.09-0.21)[b]	0.14 (0.09-0.22)[c]

Data are expressed as model estimate (95% CI), after adjustment for gender and Tanner stage at baseline. AIRg, DI, insulin levels, HOMA-IR, triglycerides and lp(a) levels were log-transformed prior to analysis. The values between brackets represent reference ranges for healthy children; ND=not determined

¶ P<0.0001 compared to the population mean, [a] P<0.01: 3 mo. of GnRHa treatment, compared to baseline, [b] P<0.03: 1 year of combined treatment, compared to baseline, [c] P<0.03: 3 mo. after stop of GnRHa treatment, compared to baseline, [d] P<0.02: 3 mo. after stop of GnRHa treatment, compared to 1 year of combined treatment

Discussion

In this study, we investigated the effect of combined treatment with a GnRH analogue and either 1 mg or 2 mg GH/m^2/day on several metabolic parameters in pubertal short children born SGA. During 3 months of GnRHa treatment, BMI SDS increased significantly, whereas blood pressure, insulin sensitivity and lipid profile did not significantly change. During combined treatment, children treated with GnRHa and 1 mg GH/m^2/day developed a higher fat mass SDS_{height}, percentage trunk and limb fat. Lean body mass SDS_{height} increased only in children treated with GnRHa and 2 mg GH/m^2/day. Blood pressure, insulin sensitivity and lipid profile were similar in both GH dosage groups. As expected, insulin sensitivity significantly decreased, but the disposition index remained comparable to baseline in both GH dosage groups. Lipids remained within normal reference ranges.

At baseline, fat mass SDS_{height} and BMI SDS were significantly lower than the population mean in pubertal short children born SGA, which is in line with previous findings in prepubertal short SGA children [14]. During 3 months of GnRHa treatment, BMI SDS increased significantly, although values remained significantly lower than the population mean. In children with CPP, several studies reported an increase in fat mass or BMI SDS during GnRHa treatment [3,5,31], which might be related to a decrease in GH levels during GnRHa treatment [32]. In children treated with GnRHa and 1 mg GH/m^2/day, fat mass SDS_{height} increased to values significantly higher than those at baseline and comparable to the population mean. In children treated with GnRHa and GH 2 mg/m^2/day, fat mass SDS_{height} significantly decreased during 1 year of combined treatment and returned to baseline values at 3 months after stop of GnRHa treatment, remaining significantly lower than the population mean. GH has well-documented lipolytic effects [33] and treatment in prepubertal short SGA children resulted in a significant decrease in fat mass SDS_{height}, especially in the first treatment year [14]. The significant differences between the 2 GH dosage groups in our study can be explained by the fact that treatment with GH 2 mg/m^2/day counteracts the effect of simultaneous treatment with a GnRH analogue, whereas treatment with GH 1 mg/m^2/day is insufficient to prevent children from gaining fat mass during GnRHa treatment.

GH dose also had a dose-dependent effect on percentage trunk and limb fat. During the study period, percentage trunk fat increased in both GH dosage groups, but to higher values in children treated with GnRHa and 1 mg GH/m^2/day. Percentage

limb fat increased only in children treated with GnRHa and 1 mg GH/m^2/day. Changes in fat distribution during GnRHa treatment in children with CPP have not been investigated, but our findings are in line with the clinical experience that children with CPP gain especially more fat mass around the waist during GnRHa treatment. In prepubertal short SGA children treated with 2 mg GH/m^2/day, the percentage trunk fat remained comparable to untreated children [34]. Therefore, the increase in percentage trunk fat in our study group is most likely due to simultaneous treatment with a GnRH analogue. Epidemiological studies have shown that low birth weight followed by catch-up in weight during childhood and adolescence was associated with a higher risk of developing type 2 diabetes and cardiovascular disease, even within the normal weight range [35,36]. Follow-up until adult height is required to investigate whether changes in body composition in short SGA children treated with GnRHa and GH continue to exist. It is, however, important to make parents and adolescents aware of the risks of gaining fat mass.

At baseline, lean body mass SDS_{height} was significantly lower than the population mean. It was previously shown that older prepubertal short SGA children have a lower lean body mass SDS_{height}, compared to younger ones [14]. At 3 months after stop of GnRHa treatment, compared to baseline, lean body mass SDS_{height} had significantly increased in children treated with GnRHa and 2 mg GH/m^2/day, whereas values had not significantly changed in children treated with GnRHa and 1 mg GH/m^2/day. Prepubertal short SGA children treated with 1 mg GH/m^2/day showed no increase in lean body mass SDS_{height} beyond the normal increase as a result of gain in height [14]. In children with CPP, lean body mass SDS adjusted for gender and age decreased significantly during GnRHa treatment [5]. We therefore conclude that treatment with 2 mg GH/m^2/day, even in combination with a GnRH analogue, results in an increase in lean body mass SDS_{height} in older short SGA children.

At baseline, mean systolic BP SDS was significantly higher than the population mean and a systolic BP SDS above +2 SDS was found in 27% of short SGA children. High blood pressure in childhood has been associated with an increased risk of developing hypertension in adulthood [37]. Systolic BP SDS did not change significantly during the study period. This is compatible with previous findings, where a significant decrease in blood pressure SDS was found only after 3 years of GH treatment [13,38].

At baseline, insulin sensitivity was lower and insulin secretion was higher in pubertal short SGA children, compared to reported values in prepubertal short SGA children [30]. This was expected, since healthy pubertal children have a

physiologic insulin resistance with a compensatory increase in insulin secretion [39]. It was considered unethical to perform a second FSIGT test after 3 months of GnRHa treatment, since these children already underwent a baseline FSIGT test and overnight LH and FSH profiles at baseline and after 3 months of GnRHa treatment. We, therefore, compared fasting insulin levels and HOMA-IR after 3 months of GnRHa treatment with those at baseline. No significant changes were found, indicating that short-term GnRHa treatment does not affect insulin sensitivity, although these parameters are rather crude to assess insulin sensitivity.

During 1 year of combined treatment, Si and AIR were comparable between both GH dosage groups. This is in line with results found during oral glucose tolerance tests in prepubertal short SGA children [11]. As expected, during 1 year of combined treatment, Si significantly decreased and AIR significantly increased. Reassuringly, the disposition index remained comparable to baseline, reflecting that ß-cells were able to compensate for a reduction in insulin sensitivity by increasing their insulin secretion. At 3 months after stop of GnRHa treatment, fasting insulin levels and HOMA-IR remained comparable to those during 1 year of combined treatment.

At baseline, mean lipid levels were within the normal range, although lp(a) levels were above the normal range (>0.3 g/l) in 27% of pubertal short SGA children. High lp(a) levels have been associated with an increased risk of developing cardiovascular disease in several studies [40,41]. During the study period, the percentage of lp(a) levels above the normal range remained comparable to baseline. During the study period, lipid parameters were comparable between both GH dosage groups, which is in line with results found in prepubertal short SGA children [13]. Although some lipids showed a significant temporary increase (LDL-c, FFA, Apo-A1 levels), whereas others increased more steadily over time (TC and TG levels), the actual increase and subsequent decrease in levels was very small. The clinical significance of these changes in lipid parameters is therefore considered negligible.

In conclusion, our results indicate that combined treatment with a GnRH analogue and either 1 mg or 2 mg GH/m^2/day can be regarded as a potential treatment strategy for short SGA children who come under medical attention at onset of puberty. We found a GH dose-dependent effect on fat mass SDS_{height}, percentage trunk and limb fat and lean body mass SDS_{height} in favor of treatment with GnRHa and 2 mg GH/m^2/day. Blood pressure, insulin sensitivity and lipid profile were similar between the 2 GH dosage groups. Follow-up until adult height is necessary before a definitive conclusion can be drawn if this combined treatment strategy will result in an optimal adult height without adversely affecting metabolic outcome.

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Chapter 7

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Submitted

Insulin-like Growth Factor-Binding Protein-1: Serum Levels, Promoter Polymorphism and Associations with Components of the Metabolic Syndrome in Short Subjects Born Small for Gestational Age

Abstract

Context: IGFBP-1 is the only acute regulator of IGF-I bioavailability. Its production is suppressed by insulin, and low levels are associated with hyperinsulinemia and cardiovascular disease risk in adults. Data on IGFBP-1 levels in short SGA subjects are scarce and associations with *IGFBP1* promoter SNPs have not been established.

Objective: To determine IGFBP-1 levels in short SGA subjects compared with those in controls. To assess genotype frequency of the -575 G/A SNP and to determine its impact on IGFBP-1 levels.

Subjects: 272 short subjects born SGA and 330 subjects with normal stature (245 children, 85 adults).

Outcome Measures: Fasting levels of IGFBP-1, IGF-I, insulin and lipid parameters, body composition.

Results: IGFBP-1 SDS was comparable to controls in lean, short SGA children, but significantly lower in short SGA adults with normal fat mass ($P<0.001$). IGFBP-1 SDS correlated significantly with insulin levels, systolic BP SDS and various lipid parameters. Baseline IGFBP-1 SDS was lowest in SGA children with -575 GG genotype and significantly higher in SGA children with one or two copies of the A allele. In response to a given insulin level, children with the AA genotype had a significantly higher IGFBP-1 SDS compared to children with the GG genotype.

Conclusion: Normal IGFBP-1 levels in lean, short SGA children suggest a normal metabolic state, despite reported hyperinsulinemia. IGFBP-1 is modulated by polymorphic variability, and seems to be an additional player in the complex interaction between the IGF-IGFBP axis, glucose homeostasis and lipid metabolism.

Introduction

Epidemiological studies have shown that the development of type 2 diabetes mellitus and associated disorders such as hypertension, dyslipidemia and cardiovascular disease in adults are associated with low birth weight and postnatal catch-up in weight [1-3]. Although postnatal catch-up in height occurs in most children born small for gestational age (SGA), about 10% of children born SGA remain short through adult life.

Failure of catch-up growth is associated with low levels of insulin-like growth factor I (IGF-1) and insulin-like growth factor-binding protein 3 (IGFBP-3) [4,5]. IGF-I and its binding proteins play a role in glucose metabolism and homeostasis [6,7]. Insulin-like growth factor-binding protein 1 (IGFBP-1) is the only acute regulator of IGF-1 bioavailability. Its production in the liver is suppressed by insulin through binding to insulin-response elements in the IGFBP-1 gene promoter, thereby forming a link between glucose metabolism and the IGF axis [8,9]. Reduced serum IGFBP-1 levels are considered to reflect hyperinsulinemia and cardiovascular risk in adults [10,11], in women with polycystic ovary syndrome [12] and in prepubertal obese children [13]. Previous studies demonstrated that short subjects born SGA have increased insulin secretion [14-16]. However, limited studies with relatively small numbers of children determined IGFBP-1 levels in short SGA children, with conflicting results [17-20].

Thirty-six percent of the interindividual variability in circulating IGFBP-1 levels is genetically determined [21]. A relatively frequent polymorphism has been found within the *IGFBP-1* gene (*IGFBP1*) promoter (-575 G/A) and the minor allele of this single nucleotide polymorphism (SNP) was associated with a decreased prevalence of diabetic nephropathy in patients with type 2 diabetes mellitus [22]. No data exist on the impact of this SNP on circulating IGFBP-1 levels.

Given the increased risk of hyperinsulinemia in the SGA population, we hypothesized that their IGFBP-1 levels would be reduced and that this reduction may be modulated by genetic variability. The first aim of our study was therefore to determine serum IGFBP-1 levels in a large cohort of short subjects born SGA and a) to compare these levels to those found in age-matched controls with normal stature and b) to assess the relationship between IGFBP-1 levels and clinical and laboratory parameters. The second aim of our study was to assess genotype frequency of the *IGFBP1* SNP at the -575 locus and to determine whether variability in IGFBP-1 levels might be related to this SNP.

Materials and methods

Subjects

The group of short subjects born SGA comprised 193 prepubertal, 40 pubertal children and 39 young adults. None of the subjects were treated with growth hormone (GH). Inclusion and exclusion criteria for the cohorts of short SGA children have been previously described [5,14,23]. Briefly, subjects had a birth length and/or birth weight, and current height below -2 SDS and had no growth failure caused by other disorders. All young adults were recruited as part of the PROgramming factors for GRowth And Metabolism (PROGRAM) study [15]. Short adults included subjects born SGA (birth length <-2 SDS) with a short adult height (<-2 SDS). Healthy adult controls (n=85) who were born appropriate for gestational age (birth length >-1 SDS) with normal adult height (>-1 SDS) served as a control group. All study protocols were approved by the Medical Ethics Committee of all participating centers and written informed consent was obtained from parents or custodians and from children aged 12 years or older.

Normal reference values of fasting serum IGFBP-1 were established using blood samples from 245 healthy age-matched children and adolescents with normal stature who attended the outpatient clinic for minor elective surgery. Normal stature was defined as a height SDS between -2 and +2 according to Dutch references [24]. Children with chronic illness and syndromes were excluded. Collection and use of fasting blood samples from the control group was approved by the Medical Ethics Committee and written informed consent was obtained from all adolescents and parents or custodians of each child.

Clinical parameters

Height was measured using a Harpenden stadiometer and expressed as SDS to adjust for sex and chronological age using Dutch reference values [24]. Systolic and diastolic blood pressure (BP) was measured with an automated device. Since height is an important determinant of blood pressure, BP was expressed as SDS to adjust for height and sex [25]. Percentage fat mass was measured by Dual Energy X-ray Absorptiometry (DXA) and expressed as SDS to adjust for sex and height using Dutch reference values [26].

FSIGT test

A modified frequently sampled intravenous glucose tolerance test (FSIGT) with Tolbutamide was performed in 92 short SGA subjects, as previously described [27]. Insulin sensitivity (Si), glucose effectiveness (Sg), acute insulin response (AIR) and disposition index (DI) were calculated using Bergman's MINMOD MILLENNIUM software [28]. Insulin sensitivity quantifies the capacity of insulin to stimulate glucose disposal and glucose effectiveness reflects the capacity of glucose to mediate its own disposal. The AIR, an estimate of insulin secretory capacity, was measured as the area under the curve from 0 to 10 minutes corrected for baseline insulin levels. DI is calculated as AIR*Si and indicates the degree of glucose homeostasis.

Laboratory parameters

All blood samples were taken after an overnight fast, before start of GH treatment. Serum IGFBP-1 and IGF-I levels were measured in one laboratory. All IGFBP-1 levels were determined by a specific RIA which does not discriminate between differences in phosphorylation status of the protein, as described previously in detail [29]. The lower detection limit of the assay was 1 µg/l. The intra-assay coefficient of variation (CV) was 7.9% at 31.6 µg/l and 12% at 8.7µg/l. The interassay CV was 10% at 27.4 µg/l. Smoothed references for IGFBP-1 levels were constructed by the LMS method, using data of the 245 healthy age-matched children and adolescents and 85 healthy adult controls who were described above [30]. Serum levels of SGA subjects were expressed as SD-scores (SDS) to adjust for age and sex. All IGF-I was measured using an automated chemi-luminescence immunometric assay (Immulite-1000 systems, Siemens Healthcare Diagnostics, Tarrytown NY, USA). The lower detection limit was 12.0 ng/ml and the intra-assay CVs were 3.1, 3.6 and 3.7% at mean serum levels of 49, 114, and 418 ng/ml, respectively. Interassay CVs were 6.4, 6.9, and 6.3% at mean IGF-I serum levels of 51, 169, and 412 ng/l, respectively. IGF-I levels were expressed as SDS to adjust for age and sex, using reference data from a healthy Dutch population which was previously described [31].

Glucose levels were measured at the local hospital laboratories with automatic analyzers using a hexokinase-catalyzed glucose oxidase method. In a subgroup of 125 subjects (92 FSIGT and 33 fasting), insulin levels were measured in one laboratory using the same method (IRMA; Medgenix, Biosource Europe, Nivelles, Belgium). The intra-assay CV was 1.9% and the inter-assay CV was 6.3%.

Total cholesterol (TC), LDL-cholesterol (LDL-c), HDL-cholesterol (HDL-c), and triglycerides were determined as previously described [14].

RFLP genotyping of the -575 G/A SNP

A 100-ng aliquot of genomic DNA was mixed with PCR buffer, supplemented by 1.0 µmol/L of each primers, 1 mmol/L $MgCl_2$, 0.1 mmol/L of each dNTP and 2 U of Taq DNA Polymerase (Life Technologies Inc., Burlington, Canada). Primer sense was 5'-GGC AGA GCC CTA GGA TGA AC-3' and antisense was 5'-GAT CTC CTG CAA AGC GTC TC-3'. The cycling parameters consisted of an initial incubation of 5 min at 94°C, followed by 34 cycles of 1 min at 96 C, 1 min at 63.8 C and 1 min at 72 C. The reaction was terminated after a final extension of 5 minutes at 72 C. Ten µl of PCR product was digested with 2.5 U of *Hpy*CH4 V (New England Biolabs, Pickering, Ontario, Canada) for 2-14 h at 37°C. Digestion products were visualized on 8% polyacrylamide gel (29:1) and stained with ethidium bromide. Due to the presence of three *Hpy*CH4 V sites in the PCR product, one of which is destroyed when there is a G in position -575, DNA fragment sizes of 160, 43 and 21 bp (G allele), or 110, 50, 43 and 21 bp (A allele) were observed.

Statistical analysis

Fasting IGFBP-1, insulin, Si, Sg, AIR, DI and triglyceride levels were log-transformed before analyses, to insure a Gaussian distribution. All data are presented as mean (± SD), except for the skewed parameters mentioned above, which are presented as median (interquartile range). ANOVA was used to determine differences between subgroups with regard to group characteristics. Bonferroni correction was used for pair wise group comparisons. SD-scores were compared to reference population means (zero SD-score) using one-sample t-tests. After log-transformation, Pearson's correlation coefficient was used for correlations. Genotype distributions for significant departure from the Hardy-Weinberg equilibrium and distributions among laboratory parameters were calculated using the x^2 test. Univariate analysis was used to estimate the percent variation in IGFBP-1 SDS that can be explained by genotype and the interaction between genotype and insulin levels. A P-value <0.05 was considered significant. Analyses were performed using the computer statistical package SPSS (version 11) for Windows.

Results

Clinical and laboratory data

Baseline clinical data are shown in Table 1. Compared to the population mean, percentage fat mass SDS was significantly lower in prepubertal and pubertal short SGA children. Percentage fat mass SDS was similar in short SGA adults compared to the reference population. Systolic BP SDS was significantly higher in all SGA groups and diastolic BP SDS was significantly higher in SGA adults, compared to the population means. A mean systolic BP SDS above the normal range (>+2 SDS) was found in 19% of prepubertal SGA children, 30% of pubertal SGA children and 23% of SGA adults.

Table 1. Baseline clinical characteristics of short prepubertal and pubertal children and short young adults born SGA.

	N	Prepubertal (n=193)	Pubertal (n=40)	Young adults (n=39)
Male/female	272	104/89	17/23	13/26
Gestational age (wks)	272	36.2 (± 3.90)[c]	37.4 (± 3.10)[d]	39.5 (± 1.5)[c,d]
Birth length SDS	210	-3.00 (± 1.47)	-2.89 (± 1.15)	-2.90 (± 0.79)
Birth weight SDS	272	-2.16 (± 1.10)	-2.20 (± 1.00)	-1.88 (± 0.96)
Current age (yrs)	272	7.49 (± 2.53)[b,c]	12.39 (± 1.40)[b,d]	20.8 (± 1.75)[c,d]
Height SDS	272	-2.99 (± 0.61)[c]	-2.84 (± 0.72)	-2.63 (± 0.55)[c]
Target height SDS	232	-0.46 (± 0.79)	-0.62 (± 0.88)	ND
% fat mass SDS	147	-0.86 (± 0.91)[a,b,c]	-0.38 (± 0.98)[a,b]	0.14 (± 0.84)[c]
Systolic BP SDS	268	1.10 (± 1.04) [a,b]	1.63 (± 1.00)[a,b]	1.18 (± 1.00)[a]
Diastolic BP SDS	268	0.45 (± 1.05)[c]	0.27 (± 0.65)[d]	0.95 (± 0.58)[a,c,d]

Results are expressed as mean (± SD)
[a] P<0.02 compared to age and sex-matched population means (0 SDS)
[b] P<0.03 prepubertal children vs pubertal children
[c] P<0.008 prepubertal children vs young adults
[d] P<0.04 pubertal children vs. young adults
ND = not determined

Baseline laboratory data are shown in Table 2. IGFBP-1 levels significantly decreased with age and were comparable to controls in prepubertal and pubertal SGA children. However, young SGA adults had significantly lower IGFBP-1 levels compared to controls (P<0.0001). In both short SGA subjects and controls, there was an inverse correlation between IGFBP-1 levels and age (r=-0.81, P<0.0001 and r=-0.74, P<0.0001, respectively).

Table 2. Baseline laboratory characteristics of short prepubertal and pubertal children and short young adults born SGA.

	N	Prepubertal (n=193)	Pubertal (n=40)	Young adults (n=39)
IGFBP-1 (µg/l)	272	172 (122-245)[b,c]	71.5 (53.0-106)[b,d]	24.0 (18.0-46.0)[c,d]
IGFBP-1 SDS	272	0.31 (± 0.97)[c]	0.26 (± 0.94)[d]	-0.87 (± 1.34)[a,c,d]
IGF-I SDS	271	-1.52 (± 1.19)[a,b,c]	-0.52 (± 1.15)[b]	-0.36 (± 0.86)[c]
Insulin (mU/L)	125	6.00 (4.67-8.00)[b,c]	11.0 (8.00-12.42)[b]	8.35 (7.00-10.50)[c]
Glucose (mmol/l)	260	4.77 (± 0.60)	4.70 (± 0.68)	4.61 (± 0.48)
Si x 10^{-4}/min^{-1} (µU/ml)	92	14.2 (10.9-19.8)[b,c]	6.4 (3.7-10.9)[b]	5.6 (3.1-9.6)[c]
Sg x 10^{-2}/min^{-1}	92	2.0 (1.6-3.0)[b]	3.5 (2.5-4.9)[b,d]	1.9 (1.5-2.2)[d]
AIR (mU/l)	92	283 (187-357)[b]	465 (297-776)[b]	409 (252-547)
DI (AIR x Si)	92	3715 (2693-5382)[c]	2811 (1854-4491)	2576 (1569-3839)[c]
Total cholesterol (mmol/l)	205	4.39 (± 0.82)[c] [3.0-5.5]	4.26 (± 0.60)[d] [3.0-5.5]	4.82 (± 0.91)[c,d] [≤ 5.0]
LDL-cholesterol (mmol/l)	203	2.43 (± 0.70)[c] [1.3-3.4]	2.32 (± 0.59)[d] [1.3-3.4)]	3.02 (± 0.78)[c,d] [< 3.2]
HDL-cholesterol (mmol/l)	204	1.45 (± 0.35) [0.9-1.9]	1.40 (± 0.34) [0.9-1.9]	1.32 (± 0.27) [m: >1.03; f: >1.29][*]
Triglycerides (mmol/l)	190	0.66 (0.54-0.88)[c] [0.4-1.6]	0.77 (0.60-1.03) [0.4-1.6]	0.83 (0.69-1.11)[c] [≤ 1.7][*]

Results are expressed as mean (± SD), except IGFBP-1, insulin, Si, Sg, AIR, DI and triglycerides, which are median (interquartile range)
The values between brackets represent reference ranges for healthy children and adults as determined by our hospital laboratory
m=males and f=females
[a] P 0.0001 compared to age and sex-matched population means (0 SDS)
[b] P<0.03 prepubertal children vs. pubertal children
[c] P<0.03 prepubertal children vs. young adults
[d] P<0.04 pubertal children vs. young adults
[*] According to NCEP-ATP III definitions of the metabolic syndrome

Compared to the population mean, IGF-I levels were significantly lower in prepubertal short SGA children. Mean fasting insulin, glucose and lipid levels were within the normal range. As expected, insulin sensitivity was significantly higher in prepubertal short SGA children, compared to pubertal SGA children and young SGA adults. In the latter groups, the lower insulin sensitivity was compensated by an increase in the acute insulin response.

Associations between IGFBP-1 SDS and clinical and laboratory parameters

Table 3 shows correlations between IGFBP-1 SDS and clinical and laboratory parameters in short SGA children (prepubertal and pubertal group combined). After adjustment for age, significant negative correlations were observed with insulin levels, systolic BP SDS, triglycerides and IGF-I SDS; significant positive correlations were observed with HDL-c. Correlations were stronger after adjustment for percentage fat mass SDS, in addition to age. Insulin levels were significantly associated with triglycerides ($r=0.39$, $P<0.0001$) and weakly with IGF-I SDS ($r=0.19$, $P=0.05$). IGFBP-1 SDS remained significantly correlated with insulin levels after additional corrections for triglycerides ($r=-0.31$, $P=0.03$) and IGF-I SDS ($r=-0.30$, $P=0.03$), next to age and percentage fat mass SDS.

In SGA adults, IGFBP-1 SDS was inversely correlated with the acute insulin response (AIR: $r=-0.43$, $P=0.04$), fat mass SDS ($r=-0.43$, $P=0.03$) and IGF-I SDS ($r=-0.51$, $P=0.001$) and positively correlated with insulin sensitivity (Si: $r=0.40$, $P=0.05$). Insulin levels and AIR were positively correlated with percentage fat mass SDS ($r=0.50$, $P=0.02$ and $r=0.54$, $P=0.009$, respectively).

Table 3. Correlations between IGFBP-1 SDS and various clinical and laboratory characteristics.

	Short SGA children (n=233)			
	IGFBP-1 SDS[a]		IGFBP-1 SDS[b]	
	R	P-value	R	P-value
% fat mass SDS	-0.05	0.59	-	-
Systolic BP SDS	-0.19	0.003	-0.26	0.004
Diastolic BP SDS	-0.05	0.50	0.03	0.77
IGF-I SDS	-0.20	0.002	-0.48	<0.0001
Insulin (mU/L)	-0.26	0.008	-0.40	0.003
Total cholesterol (mmol/l)	0.04	0.60	0.13	0.17
LDL-cholesterol (mmol/l)	0.05	0.52	0.04	0.65
HDL-cholesterol (mmol/l)	0.19	0.01	0.29	0.004
Triglycerides (mmol/l)	-0.31	<0.0001	-0.31	0.001

[a] Adjusted for age
[b] Adjusted for age and % fat mass SDS

IGFBP1 -575 G/A SNP

Genotyping of the -575 G/A SNP was performed in all SGA groups. Polymorphic variation was common, and the G allele (63.6%) was more frequent than the A allele (36.4%). Genotype distribution was in Hardy-Weinberg equilibrium (x^2=2.47; P>0.05) and was: GG=116 (42.6%), GA=114 (42.0%), AA=42 (15.4%).

In prepubertal SGA children, IGFBP-1 SDS was lowest in children carrying the GG genotype and significantly higher in children carrying 1 or 2 copies of the A allele (Figure 1). The variance in IGFBP-1 SDS that could be explained by genotype was 4.6% (P=0.001). We did not analyze the pubertal and young adult SGA groups because of the relatively small sample size.

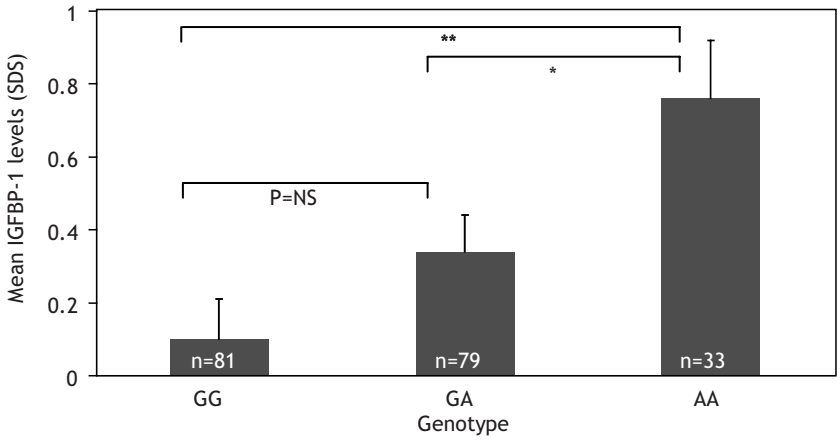

Figure 1. Mean IGFBP-1 SDS in prepubertal short SGA children according to genotype at locus -575. Data are expressed as mean (± SEM). ** P<0.01, * P<0.05

Associations between genotype, IGFBP-1 and insulin levels

In the prepubertal SGA group, the -575 G/A SNP significantly influenced the association between IGFBP-1 SDS and insulin levels, as well as between IGFBP-1 SDS and acute insulin response (interaction term genotype * insulin levels: R^2_{adj}=0.18, P=0.001; genotype * AIR: R^2_{adj}=0.19, P=0.006). This indicates that SGA children with comparable insulin secretion and carrying the GG genotype had a lower IGFBP-1 SDS, compared to SGA children carrying the AA genotype.

Discussion

In the present study, we compared fasting IGFBP-1 levels in a large cohort of non-GH treated short subjects born SGA with those of a control population with normal stature and comparable age. Furthermore, we evaluated if variability in IGFBP-1 levels is related to the -575 G/A *IGFBP1* SNP.

In our cohort of short SGA subjects, IGFBP-1 levels significantly decreased with age and were comparable to subjects with normal stature throughout childhood and adolescence. Only a few studies have determined IGFBP-1 levels in much smaller cohorts of short children born SGA. One study found lower and the other 3 studies found similar IGFBP-1 levels in short children born SGA, albeit compared to short children born appropriate for gestational age or children with catch-up growth after being born SGA, as opposed to a normal stature control group [17-20].

In our adult short young SGA cohort, IGFBP-1 levels were significantly lower compared to controls with normal stature. No data on IGFBP-1 levels in short SGA adults are available. One study performed in young SGA women who showed spontaneous catch-up growth also found reduced IGFBP-1 levels, compared to controls with normal stature [32].

Insulin is considered as the main regulator of IGFBP-1 production and we found a significant association between IGFBP-1 and insulin levels in our study group. FSIGT tests were performed in a subset of short SGA subjects and controls included in this study [14,15]. Prepubertal children and young adults born SGA secreted significantly higher amounts of insulin, compared to controls. Notably, the disposition index – a measurement that reflects how well beta cells are able to compensate for a reduction in insulin sensitivity by increasing their insulin secretion – was comparable between short SGA subjects and controls. Thus, the significantly lower IGFBP-1 levels found in SGA adults in this study, compared to adult controls, are likely a response to the higher insulin secretion.

The interrelationships between IGF-I, IGFBP-1, insulin and adipose tissue are complex. The IGF-IGFBP axis plays a role in glucose homeostasis; IGF-I infusion increases IGFBP-1 levels, increases insulin sensitivity, and improves lipid profile in patients with type 2 diabetes mellitus [33]; an inverse association between IGF-I and glucose levels in healthy adults was found to be independently modified by IGFBP-1 levels. In this latter study, only subjects with IGFBP-1 levels below the median demonstrated the inverse association between increasing tertiles of IGF-I and 2-h glucose levels [6]. Interestingly, in *IGFBP1* transgenic mice possessing supraphysiologic levels of IGFBP-1, insulin resistance and impaired glucose tolerance are observed, probably due to reduced bioavailability of IGF-I [34].

As mentioned above, IGFBP-1 levels were comparable between short SGA children and their age-matched controls. In our cohort, percentage fat mass SDS was low in prepubertal SGA children, but comparable to the population mean in SGA adults. IGFBP-1 SDS correlated significantly with percentage fat mass SDS in SGA adults. Previous studies showed that low birth weight followed by catch-up in weight during childhood and adolescence, even within the normal weight range, was associated with a higher risk of developing type 2 diabetes and cardiovascular disease [3,35]. We therefore hypothesize that the normal IGFBP-1 levels in SGA children in this study may be explained by their low fat mass, even in the presence of their reported higher insulin secretion, and may reflect a metabolically more favorable state.

Cardiovascular risk factors in adults include hypertension, high LDL-c and triglycerides and low HDL-c levels [36,37]. We found a high systolic blood pressure in a considerable proportion of short SGA subjects. High blood pressure in childhood has been associated with an increased risk of developing hypertension in adulthood [38]. Mean lipid parameters, on the other hand, were within the normal range. In addition to the correlations between IGFBP-1 SDS and insulin, IGFBP-1 SDS correlated significantly with various lipid parameters and systolic BP SDS, independent of percentage fat mass SDS. These data support the concept that IGF-I and its binding proteins, particularly IGFBP-1, are important for glucose homeostasis and lipid metabolism; they also support the previous finding that IGFBP-1 is an independent predictor of cardiovascular risk in adults [10,11].

In order to explore additional factors that may influence circulating IGFBP-1 levels, we examined the contribution of the -575 G/A SNP. This frequent promoter polymorphism has not been previously reported in relation to circulating IGFBP-1 levels. *IGFBP1* is highly conserved among species except in the promoter regions, where there is also considerable polymorphic variation between individuals. Nine polymorphic SNPs have been described, and 5 of these validated in SNPper (http://snpper.chip.org/). Only 2 of these SNPs show relatively high frequencies of the minor alleles (>10%). We chose to examine the highly polymorphic -575 G/A SNP based on its proximity to the transcription start site as well as to the many regulatory factor recognition motifs.

Frequencies of the G and A allele in the SGA population were comparable with frequencies previously found in Caucasian adults [22] (http://www.ncbi.nlm.nih.gov/SNP/). IGFBP-1 levels were lowest in SGA children carrying the GG genotype and significantly higher in children carrying the GA and AA genotype. The -575 G/A SNP explained 4.6% of the variability in IGFBP-1 levels in prepubertal SGA children, which is larger than the impact found in other studies looking at correlations between SNPs and serum analytes or phenotypes [39,40]. Future studies should be extended to haplotype analysis to examine the impact of the other previously validated promoter polymorphisms on IGFBP-1 levels. It is also important to determine the impact of the -575 G/A SNP on IGFBP-1 levels in a control group with normal stature, to investigate if our findings are unique to short SGA subjects.

The -575 G/A SNP significantly influenced the relation between IGFBP-1 and insulin secretion, such that SGA children with comparable insulin secretion and carrying the GG genotype had a lower IGFBP-1 SDS, compared to SGA children carrying the AA

genotype. This SNP is situated near at least 2 insulin response elements (nucleotides -282 to -265 and nucleotides -118 to -105) [8,9] and its role could thus be through modulation of insulin-dependent gene repression.

In conclusion, normal IGFBP-1 levels in short SGA children with a low fat mass may reflect a normal metabolic state, despite reported hyperinsulinemia in these children. However, short young SGA adults with normal fat mass had significantly lower IGFBP-1 levels, compared to controls, reflecting a higher insulin secretion. The -575 G/A SNP was significantly associated with IGFBP-1 levels and influenced the relation between insulin and IGFBP-1 levels. As the only acute regulator of IGF-I bioavailability, IGFBP-1 could therefore be an additional player in the complex interactions between the IGF-IGFBP axis, glucose homeostasis and lipid metabolism.

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Chapter 8

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Genetic and Epigenetic Variability in the Gene for IGFBP-3 (*IGFBP3*): Correlation with Serum IGFBP-3 Levels and Growth in Short Children Born Small for Gestational Age

Abstract

Context: IGF-I and IGFBP-3 play a central role in fetal and postnatal growth and levels are low in short SGA children. The -202 A/C and -185 C/T SNPs are located near elements involved in directing *IGFBP3* promoter activity and expression. Changes in promoter CpG methylation status affect transcription factor binding and transcriptional activation of *IGFBP3 in vitro*.

Objective: To assess the relationship between *IGFBP3* promoter SNPs, IGFBP-3 levels, spontaneous growth and growth response to GH treatment in short prepubertal SGA children. To assess promoter methylation status in a subgroup of short SGA subjects and controls.

Patients: 292 short prepubertal SGA children, 39 short young SGA adults and 85 young adults with normal stature.

Intervention: Short prepubertal SGA children received GH 1 mg/m^2/day.

Outcome measures: Fasting levels of IGF-I and IGFBP-3, baseline and delta height SDS.

Results: At baseline, IGFBP-3 levels were highest in SGA children with -202 AA genotype and lower in children with 1 or 2 copies of the C-allele (P<0.001). Children with C^{-202}/C^{-185} haplotype, compared to children with A^{-202}/C^{-185} haplotype, had lower IGFBP-3 levels (P=0.003) and were shorter (P=0.03). During GH treatment, children with C^{-202}/C^{-185} haplotype showed a significantly greater increase in IGFBP-3 SDS and in height SDS than children with A^{-202}/C^{-185} haplotype, resulting in similar IGFBP-3 levels and similar height SDS after 12 months of GH treatment. CpG methylation patterns showed a trend towards more methylation of CpGs involved in transcription factor binding in short young SGA adults compared to controls.

Conclusion: Polymorphic variation in the *IGFBP3* promoter region is correlated with IGFBP-3 levels, spontaneous growth and response to GH treatment in short SGA children.

Introduction

Insulin-like growth factor I (IGF-I), largely independently of GH, is one of the important growth factors during fetal life [1]. In postnatal life, the entire GH-IGF axis, including the insulin-like growth factor binding proteins (IGFBPs), assumes an increasingly important role in growth regulation. Children with persistent short stature who were born small for gestational age (SGA) have been shown to have significantly lower spontaneous GH secretion and IGF-I and IGFBP-3 levels compared to their healthy peers [2-4]. It has been hypothesized that changes in the GH/IGF/IGFBP axis might underlie the failure in catch-up growth. Several studies have demonstrated that growth hormone (GH) treatment results in significant catch-up growth in short children born SGA, although treatment response is variable [5,6].

IGFBP-3 binds the majority of IGF-I in the circulation and plays a role in modulating bioavailability of IGF-I in addition to having IGF-independent effects on growth [7-9]. IGFBP-3 levels are stimulated by GH and insulin, and show only minor diurnal variation [7,8,10]. Twin studies have shown that 60% of the interindividual variability in circulating IGFBP-3 levels is genetically determined [11]. Various polymorphic loci have been detected in the IGFBP3 gene (*IGFBP3*) and genotyping at one of these loci, the -202 A/C promoter single nucleotide polymorphism (SNP), has been correlated with circulating IGFBP-3 levels in several adult cohorts [12,13].

Developmental programming involving epigenetic modifications of growth regulating genes has received attention as a possible explanation for adverse health outcomes later in life [14,15]. Methylation is one of the predominant epigenetic modifications of DNA in mammalian genomes, leading to alterations in the binding affinity of transcription factors to DNA binding sites and subsequent reduced gene expression. We and others have shown that changes in the promoter CpG dinucleotide methylation status affects transcription factor binding and subsequent transcriptional activation of *IGFBP3* [16,17].

We hypothesize that genetic and/or epigenetic variability in *IGFBP3* may, in part, explain spontaneous and GH-stimulated growth in short children born SGA. Therefore, the first aim of the present study was to assess the relationship between the *IGFBP3* promoter SNPs, serum IGFBP-3 levels and growth. In addition, we analyzed if *IGFBP3* promoter SNPs are associated with GH treatment-related rises in IGFBP-3 levels and may be useful for predicting the growth response to GH treatment. Our third aim was to investigate *IGFBP3* promoter methylation status in a subgroup of short SGA

subjects and to compare results with the methylation status in healthy controls with normal stature.

Materials and methods

Subjects

The total study group consisted of 292 Caucasian short prepubertal children born SGA. Inclusion and exclusion criteria have been previously described [18-20]. Briefly, subjects had a birth length and/or birth weight, and current height below -2 SDS and had no growth failure caused by other disorders.

All children have been, or still are, treated with biosynthetic GH at a dose of 1 mg/m^2/day. GH was administered subcutaneously once daily at bedtime. GH dose was adjusted to the calculated body surface area every 3 months. After stratification, 35 of 292 children were randomized into a higher GH treatment group (2 mg GH/m^2/day). Another 10 children reached puberty during the 2 year follow-up period. These 45 children were excluded in the analyses concerning the associations between *IGFBP3* SNPs and GH treatment-related rises of IGFBP-3 levels and growth response.

Young adults were recruited as part of The PROgramming factors for GRowth And Metabolism (PROGRAM) study [21]. Short young adults (n=39) were subjects born SGA (<-2 SDS) with a short adult height (<-2 SDS). Healthy young adult controls (n=85) were born appropriate for gestational age (birth length >-1 SDS) and had a normal adult height (>-1 SDS). All study protocols were approved by the Medical Ethics Committee of all participating centers and written informed consent was obtained from the parents or custodians and from the subjects aged 12 years or older.

Clinical parameters

Height was measured using a Harpenden stadiometer and expressed as SDS for sex and chronological age using Dutch references [22]. Genetic target height was adapted from Dutch reference data with the addition of 4.5 cm for secular trend, taking into account the 6.5 cm adult height difference, on average, between men and women as follows: 1/2 x (height$_{father}$ + height$_{mother}$ + 13) + 4.5 for boys and 1/2 x (height$_{father}$ + height$_{mother}$ - 13) + 4.5 for girls. Body mass index (BMI) was calculated according to the formula weight/(height)^2 and expressed as SDS for age and gender using Dutch reference values for children [23].

Laboratory parameters

All blood samples were taken after an overnight fast and measured in one laboratory. IGF-I was measured using an automated chemi-luminescence immunometric assay (Immulite-1000 systems, Siemens Healthcare Diagnostics, Tarrytown NY, USA). The lower detection limit was 12.0 ng/ml and the intra-assay CVs were 3.1, 3.6 and 3.7% at mean serum levels of 49, 114, and 418 ng/ml, respectively. Interassay CVs were 6.4, 6.9, and 6.3% at mean IGF-I serum levels of 51, 169, and 412 ng/l, respectively. IGFBP-3 was measured by a specific RIA. The lower detection limit was 0.002 mg/l (absolute concentration) and interassay CVs were 9.3, 6.9 and 10.2% at mean serum IGFBP-3 levels of 0.97, 2.0 and 3.0 mg/l, respectively. Serum levels of total IGF-I and IGFBP-3 were expressed as SDS to adjust for age and sex, using reference data from a healthy Dutch population [24].

RFLP genotyping of the -202 and -185 SNP

The RFLP genotyping was as described [12]. The cycling parameters consisted of an initial incubation of 5 min at 94°C, followed by 35 cycles of 1 min at 96°C, 1 min at 64°C and 1 min at 72°C. The reaction was terminated after a final extension of 5 minutes at 72°C. Ten microliters of PCR product was digested with either 5 U of *Alw*21I (MBI Fermentas, Flamborough, Canada) for 2-14h at 37°C or *Sma*I (Life Technologies, Burlington, Canada) for 2-14 h at 30°C for the -202 and the -185 SNP, respectively. For the -202 SNP, alleles contained either A or C; for the -185 SNP, alleles contained either C or T. Digestion products were visualized on 2% agarose gel or 8% polyacrylamide gel (29:1) and stained with ethidium bromide, for the -202 and -185 SNP, respectively.

Methylation study

Methylation status was determined in 10 short prepubertal children born SGA, 5 homozygous for the A^{-202}/C^{-185} haplotype and 5 homozygous for the C^{-202}/C^{-185} haplotype; in 12 short young adult subjects born SGA, 8 homozygous for the A^{-202}/C^{-185} haplotype and 4 carrying the C^{-202}/C^{-185} haplotype (2 with CC/CC genotype and 2 with CC/CT genotype); and 14 young adult controls, 9 homozygous for the A^{-202}/C^{-185} haplotype and 5 carrying the C^{-202}/C^{-185} haplotype (all with CC/CT genotype).

Genomic DNA (250 to 500 ng), was modified using the EZ DNA Methylation Kit™ (Zymo Research, Orange, CA) according to manufacturer's instructions. The modification reaction was done in a thermal cycler with the following parameters:

4 cycles of 98°C for 10 min and 50°C for 4 h. A 225bp region (nucleotide -253 to -28; containing 23 CpGs if -202 position is A and 24 CpGs if -202 position is C) within the minimal promoter and localized within a CpG island, was amplified using primers and conditions as described [17]. The PCR product was cloned into a pCR® 4-TOPO® vector using the TOPO TA Cloning® Kit (Invitrogen, Burlington, Ontario, CA) according to manufacturer's instructions. Plasmid DNA was purified by the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, Oakville, Ontario, Canada) and sequenced using the T3 primer (5'- ATT AAC CCT CAC TAA AGG GA -3') and the ABI (Applied Biosystem) 3730 DNA Analyzer. To determine the methylation status of *IGFBP3*, sequencing results (10 clones per subject) were analyzed using the BiQ Analyzer Program [25]. The percentage methylation was calculated from the number of clones with a methylated CpG at the site indicated, divided by the total number of clones analyzed at that site (n=10 clones per subject).

In addition, we investigated the methylation status of a second region within the same CpG island, somewhat further downstream (nucleotide +40 to +190; containing 15 CpGs, the translation start site and part of the first exon) in 4 young women; 2 carrying the A^{-202}/C^{-185} haplotype and 2 carrying the C^{-202}/C^{-185} haplotype. Bisulfite modification of genomic DNA was done with 1 cycle of 98 C for 10 min and 64 C for 2.5 h. A fragment of 150 bp was amplified by nested PCR with 2 forward primers F-948 5'GTGTTTTGGGTTATTTYGGTTTTT and F-1039 5'TTTTTTGTTTGGATTTTATAGTTT with one common reverse primer R-1189 5'AAACAACACCAACAAAATCAA. The PCR conditions were comparable to those described [17]. The final product was purified using the QIAquick PCR purification kit according to manufacturer's instructions (QIAGEN Inc., Mississauga, ON, Canada) and directly sequenced with primers F-1039 and R-1189.

Statistical analysis

Genotype distributions for significant departure from the Hardy-Weinberg equilibrium were calculated using the X^2 test. ANOVA or T-tests were used for differences between groups. Data were expressed as mean (±SD). SD-scores were compared with their respective population means (zero SDS) using one-sample t-tests. Crosstabs were used to calculate distributions of genotypes or haplotypes (A^{-202}/C^{-185} and C^{-202}/C^{-185}) among various parameters. A general linear model was employed to estimate the percent variation in IGFBP-3 SDS that can be explained by genotype or haplotype. Pearson's correlation coefficient was used for correlations. Multiple linear regression

analysis was used to test multivariate relationships. Only results of the best fitting models (expressed as R-squared) are shown. Fisher's exact test was used to calculate differences in methylation pattern among various groups of SGA subjects and controls. A P-value <0.05 was considered significant. Analysis was performed using the computer statistical package SPSS (version 11) for Windows.

Results

Baseline

Clinical and laboratory characteristics

Table 1 shows the baseline clinical and laboratory characteristics of all short prepubertal SGA children. BMI SDS, IGF-I SDS and IGFBP-3 SDS were significantly lower than that of the reference population means (0 SDS).

Table 1. Baseline clinical and laboratory characteristics (mean ±SD).

	Prepubertal SGA children (n=292)
Male/female	155/137
Gestational age (weeks)	36.3 (±3.87)
Birth length SDS	-3.06 (±1.43)
Birth weight SDS	-2.28 (±1.14)
Current age (years)	6.95 (±2.42)
Baseline height SDS	-3.00 (±0.61)
Target height SDS	-0.47 (±0.79)
BMI SDS	-1.36 (±0.98)*
IGF-I SDS	-1.17 (±1.27)*
IGFBP-3 SDS¶	-1.32 (±1.07)*

* P<0.0001 compared to age- and sex-matched reference population
¶ IGFBP-3 levels were available for 277 prepubertal SGA children

-202 A/C SNP

Polymorphic variation at the -202 A/C locus was common. The genotype distribution was in Hardy-Weinberg equilibrium (X^2=0.75; P>0.05) and was: AA=62 (21%), AC=138 (48%), CC=89 (31%).

As depicted in Figure 1, IGFBP-3 levels were highest in short SGA children carrying the AA genotype and levels decreased significantly in a stepwise manner in children carrying one or two copies of the C allele. Using a general linear model, the percent variation in IGFBP-3 SDS that could be explained by the -202 A/C SNP was 4.0% (P=0.001). No significant differences in IGF-I levels were found between the various genotype groups.

Height SDS did not significantly differ between the various genotype groups, nor did birth length SDS, birth weight SDS, target height SDS and BMI SDS.

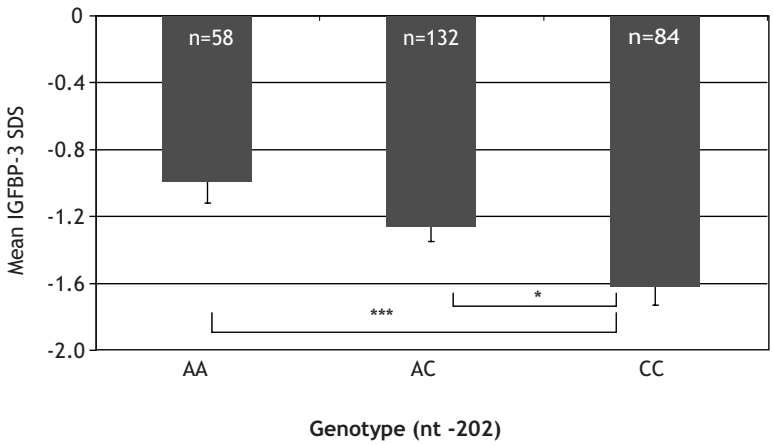


Figure 1. Mean IGFBP-3 SDS from 274 short prepubertal SGA children subdivided by the different genotype groups at the -202 locus (*P=0.01 and ***P<0.0001).

-185 C/T SNP

Genotype frequencies at the -185 C/T locus did not conform to the Hardy-Weinberg equilibrium test (CC=150 (52%), CT=108 (37%), TT=32 (11%); X^2=4.55; 0.025<P<0.05), although the -185 C/T SNP is in strong linkage disequilibrium with the -202 A/C SNP. No significant differences in IGFBP-3 SDS, IGF-I SDS, birth length SDS, birth weight SDS, height SDS, target height SDS and BMI SDS were found between the various genotype groups.

IGFBP3 promoter haplotype

In Table 2 the haplotype distribution of the 2 SNPs are depicted. As shown, the combination of an A allele at the -202 position with a T allele at the -185 position was not seen in our population.

Table 2. Distribution of the various haplotypes in short SGA subjects*.

	-185 C/T genotype		
-202 A/C genotype	**CC**	**CT**	**TT**
AA	21.5%	0.0%	0.0%
AC	22.5%	25.3%	0.0%
CC	7.6%	12.1%	11.1%

* 3 subjects of the entire cohort were unable to be genotyped

Short SGA children homozygous for C at the -202 and -185 position (C^{-202}/C^{-185} haplotype) had significantly lower IGFBP-3 levels compared to children homozygous for A at the -202 position and C at the -185 position (A^{-202}/C^{-185} haplotype) (-1.92 ±1.03 SDS vs. -0.95 ±1.21 SDS, P=0.003). In addition, children with C^{-202}/C^{-185} haplotype were significantly shorter compared to children with A^{-202}/C^{-185} haplotype (-3.31 ±0.58 SDS vs. -2.94 ±0.60 SDS, P=0.03). Using a general linear model, the percent variation in IGFBP-3 SDS that can be explained by haplotype was 12.2% (P=0.003). No significant differences in IGF-I SDS, birth length SDS, birth weight SDS, target height SDS and BMI SDS were found between the haplotype groups.

During GH treatment

When short SGA children were subdivided in 3 baseline IGFBP-3 SDS tertile groups, the catch-up in height SDS after 12 months of GH treatment was significantly greater in the children with IGFBP-3 levels in the lowest tertile group (IGFBP-3 SDS: -2.44 ±0.73 SDS, delta height SDS: 0.82 ±0.39 SDS), compared to children with IGFBP-3 levels in the highest tertile group (IGFBP-3 SDS: -0.17 ±0.53 SDS, delta height SDS: 0.66 ±0.32 SDS, P=0.006).

-202 A/C SNP

IGFBP-3 SDS significantly increased in all genotype groups during GH treatment. Levels showed a parallel increase in short SGA children carrying the AA and CC genotype, thus levels remained significantly lower in children carrying the CC genotype compared to children carrying the AA genotype. The increase in IGFBP-3 SDS, IGF-I SDS and height SDS was similar in all genotype groups.

-185 C/T SNP

The increase in IGFBP-3 SDS, IGF-I SDS and height SDS during GH treatment was similar in all genotype groups.

IGFBP3 promoter haplotype

IGFBP-3 SDS showed a greater increase in short SGA children carrying the C^{-202}/C^{-185} haplotype, compared to children carrying the A^{-202}/C^{-185} haplotype, resulting in comparable IGFBP-3 levels after 12 months of GH treatment. As depicted in Figure 2, children carrying the C^{-202}/C^{-185} haplotype showed a significantly greater catch-up in growth after 12 and 24 months of GH treatment, compared to children carrying the A^{-202}/C^{-185} haplotype, resulting in a comparable height SDS between both haplotype groups. The increase in IGF-I SDS was similar in both haplotype groups.

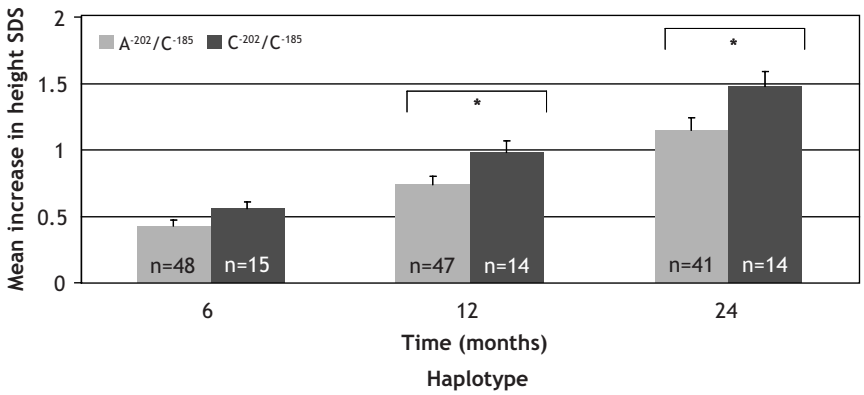


Figure 2. Mean height increase during GH treatment subdivided by the two haplotypes. (* P<0.05).

Multiple regression analyses

Potential variables explaining the mean increase in height SDS after 12 months of GH treatment were analyzed as independent factors in multiple regression analysis. These included baseline height SDS, target height SDS, gender, age, baseline IGFBP-3 SDS and increase after 12 months of GH treatment, baseline IGF-I SDS and increase after 12 months of GH treatment, and *IGFBP3* haplotype (n=61).

The mean increase in height SDS after 12 months of GH treatment was associated with the increase in IGFBP-3 SDS (β=0.11, P<0.0001), target height SDS (β=0.14,

P=0.02), the increase in IGF-I SDS (ß=0.06, P=0.03), age (ß=-0.04, P=0.06) and C^{-202}/C^{-185} haplotype (ß=0.15, P=0.09). This model explained 52% of the total variation in the first year growth response; haplotype explained 9% of the variation. After two years of GH treatment, the mean increase in height SDS was only explained by the 1st year growth response (ß=0.31, P<0.0001), and accounted for 38% of the variation in the second year growth response.

Methylation status

Clinical and laboratory characteristics of short SGA subjects included in the methylation study are shown in Table 3.

Analysis of 10 cloned PCR products per subject showed that overall, the percentage of methylation in the region flanking the SNPs was low, and a wide interindividual variability in methylation patterns was observed (Figures 3 and 4). Methylation status was comparable between short SGA subjects with C^{-202}/C^{-185} haplotype and A^{-202}/C^{-185} haplotype and between controls with C^{-202}/C^{-185} haplotype and A^{-202}/C^{-185} haplotype. In both haplotype groups, methylation status was also comparable between short children and short young adults born SGA. CpG 2-4, involved in Sp1 and p53 binding (Figure 3), showed a trend towards more methylation in short SGA adults with A^{-202}/C^{-185} haplotype, compared to adult controls with A^{-202}/C^{-185} haplotype (13.6% vs. 6.8%, P=0.07). When haplotype groups for short SGA adults and adult controls were combined this trend was still apparent (CpG 2-4: 13.4% vs. 7.9%, P=0.09).

Because of the relatively low levels of methylation found, we also explored an alternative, downstream site (but still within the same CpG island) in other leukocyte DNA samples from young normal stature adult women. Methylation status through direct sequencing showed comparable methylation percentages, suggesting that in leukocytes, the *IGFBP3* promoter generally does not exhibit high levels of CpG methylation (data not shown).

Table 3. Clinical and laboratory characteristics of short prepubertal and short young adult subjects born SGA and young adult controls.

	A^{-202}/C^{-185} haplotype (n=22)			C^{-202}/C^{-185} haplotype (n=14)		
	Short prepubertal SGA children (n=5)	Short young adult SGA subjects (n=8)	Young adult controls (n=9)	Short prepubertal SGA children (n=5)	Short young adult SGA subjects (n=4)	Young adult controls (n=5)
Birth length SDS	-2.69 (±1.07)	-2.67 (±0.46)	-0.12 (±0.77)	-3.53 (±0.64)	-3.09 (±1.18)	-0.11 (±0.97)
Birth weight SDS	-2.69 (±1.00)	-1.92 (±0.62)	-0.61 (±1.22)	-2.05 (±1.54)	-1.59 (±0.79)	0.01 (±1.00)
Height SDS	-2.68 (±0.34)	-2.61 (±0.52)	0.34 (±1.05)	-3.52 (±0.53)*	-2.81 (±0.24)	-0.10 (±1.15)
IGF-I SDS	-0.92 (±0.91)	-0.67 (±1.17)	-0.08 (±0.87)	-0.45 (±1.68)	0.25 (±0.60)	-0.23 (±1.45)
IGFBP-3 SDS	-0.26 (±0.69)	-1.20 (±0.95)	-0.50 (±0.34)	-3.40 (±0.74)***	-0.80 (±0.60)	-1.44 (±0.82)**

*P<0.05, C^{-202}/C^{-185} haplotype vs. A^{-202}/C^{-185} haplotype
**P<0.01, C^{-202}/C^{-185} haplotype vs. A^{-202}/C^{-185} haplotype
***P<0.001, C^{-202}/C^{-185} haplotype vs. A^{-202}/C^{-185} haplotype

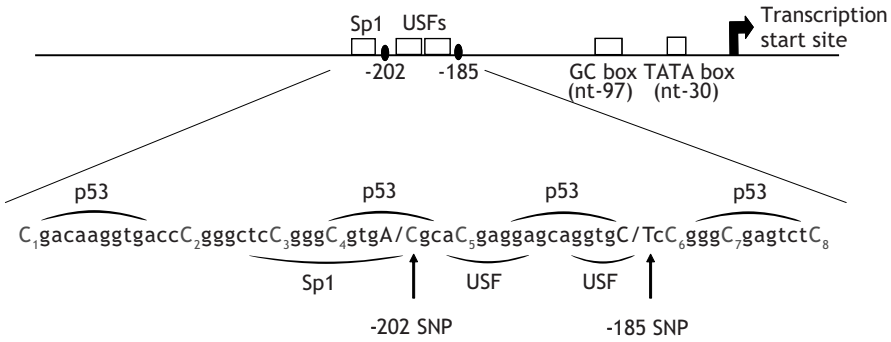
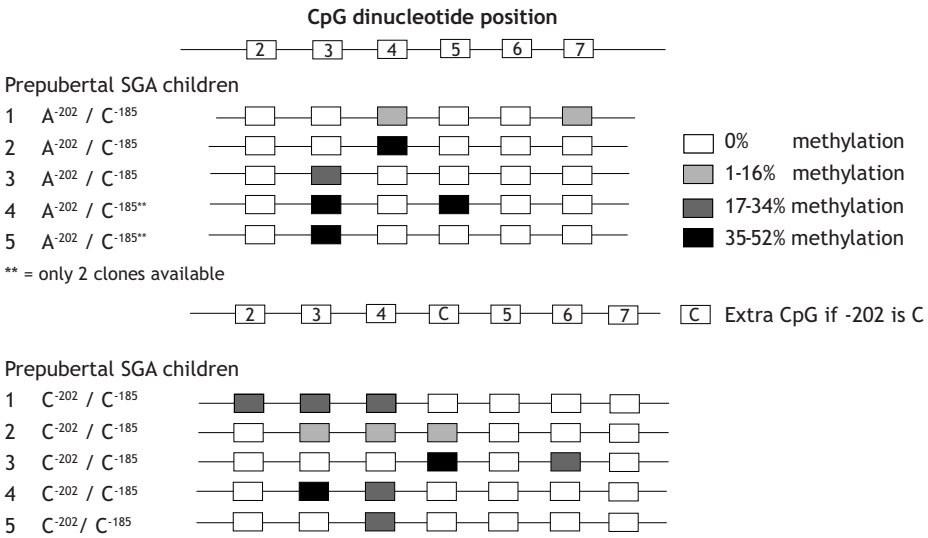


Figure 3. Schematic representation of part of the *IGFBP3* promoter region. This schematic details the CpG dinucleotides flanking the polymorphisms of interest which were studied for their methylation profiles.



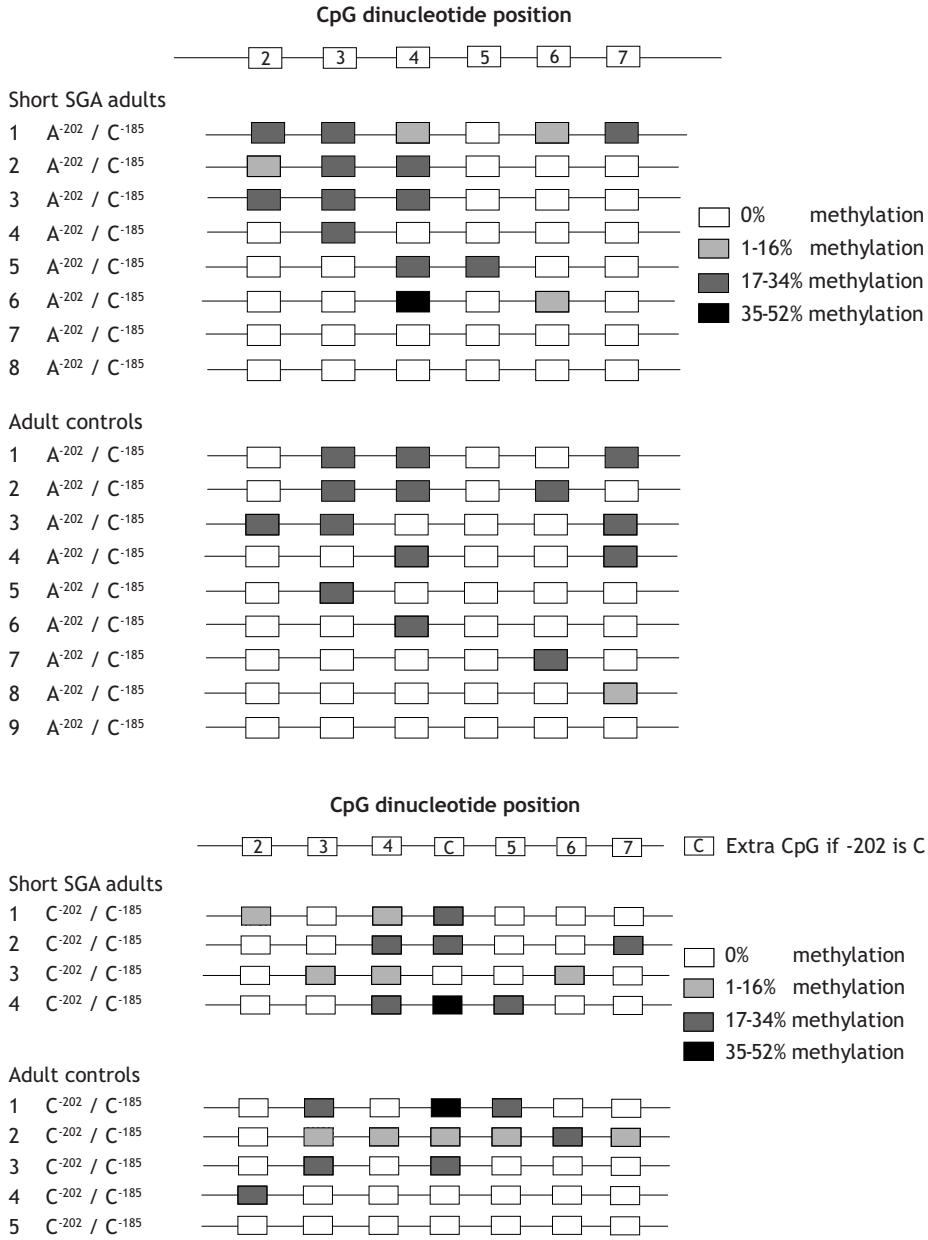


Figure 4. Methylation patterns of CpGs 2-7 per subject, in short SGA children with A^{-202}/C^{-185} haplotype and C^{-202}/C^{-185} haplotype (Figure 4a), short SGA adults and adult controls with A^{-202}/C^{-185} haplotype (Figure 4b), and short SGA adults and adult controls with C^{-202}/C^{-185} haplotype (Figure 4c). The color-legends for the various percentages of methylation are depicted in the figures. The percentage methylation was calculated from the number of clones with a methylated CpG at the site indicated, divided by the total number of clones analyzed at that site (n=10 clones per subject).

Discussion

Since our original publications showing the importance of the -202 A/C *IGFBP3* promoter SNP in regulating IGFBP-3 levels in adult men and women [12,13], our findings have been replicated in various independent adult cohorts [26-29]. In this Caucasian cohort of short SGA children, frequencies of the A and C allele for the -202 SNP were comparable with frequencies found in Caucasian adults [12,13], suggesting that allelic variants of this SNP are not associated with the SGA phenotype. IGFBP-3 levels were highest in short SGA children carrying the AA genotype and significantly lower in children carrying the AC and CC genotype, which is in line with previous studies performed in adults [12,13].

The -202 A/C SNP explained 4.0% of the variability in IGFBP-3 levels. This percentage is lower than percentages found in adults, which varied between 5.6% and 9.7% [12,13,26,29]. The impact of this SNP is nonetheless significantly larger than that found in other studies looking at correlations between SNPs and serum analytes or phenotypes [30,32]. Clearly, SNPs with even small effects on phenotype have been shown to provide important contributions to understanding complex diseases at the population level, such as cardiovascular disease [33] and cancer [34], as well as to predicting response to therapies [35].

We have previously shown that the -202 A/C SNP is functional *in vitro*; in reporter gene constructs the C-allele had approximately 50% of the promoter activity of the A-allele [12]. This is no doubt due to its localization within the minimal promoter, near elements involved in directing promoter activity, and its implication in the binding of methyl-sensitive transcription factors [16,36,37].

Genotype frequencies for the -185 C/T SNP were not in Hardy-Weinberg equilibrium, which we have seen in some, but not all of the cohorts we have genotyped (unpublished). The reason for this is not yet clear but may be that the -185 C/T SNP is a relatively more recent polymorphism. It is probably not due to negative selection pressure with regards to its impact on *IGFBP3* transcriptional activity since even the A^{-202}/T^{-185} haplotype, not found in any cohorts sequenced to date, did not show any anomalous behavior when we tested it in reporter gene transcription assays [16].

It is well known that the interaction of multiple SNPs within a genetic locus (haplotype) could theoretically influence the phenotype to a larger extent. Indeed, we found a greater impact of A^{-202}/C^{-185} and C^{-202}/C^{-185} haplotypes on IGFBP-3 levels and baseline height SDS, than the single -202 A/C SNP. Short SGA children carrying

the C^{-202}/C^{-185} haplotype had significantly lower IGFBP-3 levels and were shorter compared to children carrying the A^{-202}/C^{-185} haplotype. Haplotype explained 12.2% of the variability in baseline IGFBP-3 levels. Given the fact that 60% of the variability in circulating IGFBP-3 levels can be explained by genetic factors [11], this haplotype seems to play an important role in the regulation of *IGFBP3* expression and therefore growth in this population. However, it must be noted that in 3 recent genome-wide association studies in cohorts within the normal height range, none of the members of the GH-IGF axis appeared within the list of genes having the most influence on normal adult stature [38].

As expected, IGFBP-3 levels significantly rose during GH treatment and catch-up in height SDS was greatest in children with the lowest baseline IGFBP-3 levels. This is a robust phenomenon seen in daily practice as well as in various growth prediction models, and is thought (along with IGF-I levels), to provide information about endogenous GH levels [39-41].

After 12 months of GH treatment, the increase in IGFBP-3 levels and height SDS was significantly greater in children carrying C^{-202}/C^{-185} haplotype, resulting in similar IGFBP-3 levels and height SDS, compared to children carrying A^{-202}/C^{-185} haplotype. The increase in IGFBP-3 levels predicted the variability in catch-up growth after 12 months of GH treatment, a time point which is a strong predictor for subsequent growth and adult height, as shown by us and others [42,43]. A prediction model for first year growth response based on the largest SGA cohort did not include IGFBP-3 levels [43]. Our results show that determination of IGFBP-3 levels could be helpful in providing a more reliable prediction of an individual's response to GH treatment.

One of the greatest challenges in the treatment of short SGA children is dealing with the large interindividual variability in growth response during GH treatment. Age, target height SDS and duration of treatment are important variables, but after accounting for these factors there remains a wide variation which is difficult to explain. Short children born SGA are a heterogeneous group of patients and genetic variability probably accounts for part of the wide variability in growth response, as shown in this study.

Environmental influences have been hypothesized to result in changes in the epigenetic regulation of genes during fetal and post-natal development [44]; these changes are believed to contribute to adult diseases [15]. Epigenetic contributions to adverse outcome in SGA are mainly based on hypotheses and animal models and

no studies determining methylation status in short SGA subjets have been performed. We were interested in the methylation status of the *IGFBP3* promoter region, because we previously found that *IGFBP3* haplotype was associated with different methylation profiles. Furthermore, we showed that changes in the methylation status surrounding both SNPs affected transcription factor binding and subsequent transcriptional activity [16]. In this previous study, methylation status was determined by direct sequencing on fewer individuals; we used a more rigorous, more quantitative methodology, involving cloned PCR products, in the present study.

We found very low global methylation percentages in both investigated regions of the *IGFBP3* promoter. This was perhaps to be expected since our population is young, and methylation of CpG islands increases with increasing age [45]. Another explanation could be the fact that *IGFBP3* is ubiquitously expressed, reflecting the importance of maintaining this CpG island in an unmethylated and thus active state. Finally, influences on epigenetic regulation may be tissue-specific [15]. There was, however, a wide interindividual variability in methylation patterns in both short SGA subjects and controls; this could be a mechanism for explaining variation in disease risk [15]. Methylation status was comparable between subjects carrying A^{-202}/C^{-185} and C^{-202}/C^{-185} haplotypes, as well as controls carrying A^{-202}/C^{-185} and C^{-202}/C^{-185} haplotypes.

We did detect a trend towards higher methylation patterns of specific CpGs involved in binding of 2 transcription factors in short young SGA adults, compared to age-matched controls with normal stature. Sp1 is an important factor in the activation of genes in response to insulin [36] and insulin is one of the regulators of *IGFBP3* expression [10,16]. Methylation of the Sp1 binding site leads to decreased binding of Sp1, resulting in reduced transcriptional activity [46]. The tumor suppressor p53 is a transcription factor known to upregulate *IGFBP3* expression. *In vitro* studies showed that hypermethylation of p53 consensus sequences in the *IGFBP3* promoter results in reduced *IGFBP3* expression in HepG2 cells [37]. Further studies focusing on these specific CpGs and perhaps using additional tissues besides leukocytes are warranted.

Since IGFBP-3 is involved in cell growth and apoptosis, most research involving circulating IGFBP-3 levels and genetic variability in the *IGFBP3* promoter region were conducted in epidemiological cancer studies. Although higher circulating IGFBP-3 levels and the -202 CC genotype have been inversely associated with the risk of developing certain types of cancer [27,29], some authors found that IGFBP-3 levels

and the -202 CC genotype were positively associated with cancer risk [28,47,48], while others found no association [26,49]. It also remains to be determined whether circulating levels of IGFBP-3 reflect the level and action of IGFBP-3 and/or the IGFs in various tissues. Thus using blood IGFBP-3 levels to predict cancer risk does not seem to be promising.

In conclusion, polymorphic variation in the *IGFBP3* promoter region is associated with IGFBP-3 levels, spontaneous growth and response to GH treatment in short SGA children. Although we found a trend towards higher methylation percentages in CpGs involved in binding of 2 transcription factors in SGA adults versus controls, the overall methylation of the *IGFBP3* promoter CpG island under study was low. Our results suggest that variability in *IGFBP3* genotype and epigenetics should continue to receive attention in our search for factors explaining persistent short stature in SGA patients.

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Chapter 9

General Discussion

The present thesis describes various endocrine outcome variables in pubertal short children born SGA before start of treatment, during GnRHa treatment and during combined treatment with a GnRH analogue and GH. The following variables were studied: (i) spontaneous LH and FSH secretion and the effect of GnRHa treatment on LH and FSH secretion, (ii) the effect of GnRHa treatment, as well as the effect of combined treatment with GnRHa and either 1 or 2 mg GH/m^2/day on the GH-IGF-IGFBP axis and first year growth response, and (iii) the effect of combined treatment with GnRHa and 1 or 2 mg GH/m^2/day on body composition, insulin sensitivity and ß-cell function, and lipid profile. Furthermore, genetic and epigenetic variability in 2 IGFBP gene promoters in relation to circulating levels, growth and metabolic outcome were evaluated in a large cohort of short SGA subjects. The following variables were studied: (iv) serum IGFBP-1 levels, associations with clinical and laboratory parameters, and the impact of an *IGFBP1* promoter SNP on circulating IGFBP-1 levels and (v) genetic and epigenetic variability in the *IGFBP3* promoter in relation to IGFBP-3 levels, spontaneous growth and growth response to GH treatment.

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

Part 1: Clinical aspects

Spontaneous LH and FSH secretion

Overnight LH and FSH levels before and during GnRHa treatment

In 2 studies performed in girls and boys, we evaluated serum LH and FSH levels during an overnight profile in pubertal short SGA children, at onset of puberty and after 3 months of GnRHa treatment (leuprorelide acetate depot 3.75 mg subcutaneously every 4 weeks). In both studies, children with Tanner stage 2 had a low-frequency and low-amplitude pulsatile pattern during the night and early morning. In girls with breast stage 3, both the amplitude and frequency of LH and FSH pulsatility were higher and pulses were found from the evening until the early morning. This is in line with findings in previous studies [1,2]. After 3 months of GnRHa treatment, all girls and boys showed clinical arrest or regression of puberty. During the overnight profiles, AUC_0, mean and maximum LH and FSH levels had significantly decreased

to very low levels, indicating that LH and FSH secretion patterns in GnRHa-treated short SGA children are similar to reported profiles in healthy prepubertal children [1-3]. We conclude that GnRHa treatment with leuprorelide acetate depot 3.75 mg subcutaneously every 4 weeks results in adequate pubertal suppression.

In Europe and Asia, a leuprorelide acetate depot of 3.75 mg sc every 4 weeks is the first treatment choice for postponement of puberty in children with central precocious puberty (CPP) or early puberty, where the recommended dose in the USA is 7.5 mg sc every 4 weeks. Although, peak LH (3.75 mg group: 1.73 ±0.99 IU/L, 7.5 mg group: 1.30 ±0.74 IU/L) and FSH levels (3.75 mg group: 3.9 1±1.98 IU/L, 7.5 mg group: 2.86 ±1.91 IU/L) were slightly higher with the lower GnRHa dose [4], no differences in sex steroid levels and in long-term height results between European and USA patients were reported [4-6]. Our present studies demonstrate that the lower GnRHa dose is effective, as it leads to similar overnight LH and FSH secretion patterns as those found in prepubertal children.

The GnRH agonist test after 3 months of GnRHa treatment

In the Netherlands, consensus was reached that a peak LH below 3 IU/L (95^{th} percentile of prepubertal peak LH response) during a GnRH agonist test (peak LH_{GnRH}), in combination with prepubertal oestradiol levels below 50 pmol/l and prepubertal testosterone levels below 1 nmol/l, should be used as cut-off levels to indicate sufficient pubertal suppression [7-9]. According to the peak LH cut-off level, 33% of short SGA girls and 43% of short SGA boys had an insufficiently suppressed puberty after 3 months of GnRHa treatment. LH and FSH profiles were comparable between children with a peak LH_{GnRH} above or below 3 IU/L. Furthermore, median (interquartile range) oestradiol (19 (13.0-25.0) pmol/l) and testosterone (0.25 (0.10-0.53) nmol/l) levels were very low. Our findings are in line with the only comparable study, performed in 6 children (2 boys) with CPP [10]. In this study, children with clinically well suppressed puberty had overnight LH levels similar to prepubertal children, whereas children who showed clinical progression of pubertal development had increased overnight LH levels, similar to pubertal children. The peak LH during the GnRH stimulation test was, however, comparable between clinically suppressed and non-suppressed children. These results demonstrate that clinical signs of pubertal arrest correlate better with spontaneous overnight LH secretion, compared to peak LH levels during stimulation tests.

Various alternatives for the classical GnRH stimulation test, such as a single LH level 40 minutes after subcutaneous LHRH administration [11], one single LH level 2 hours or 12 hours after depot GnRHa administration [12,13] and spontaneous 24-h urinary gonadotropin excretion during GnRHa treatment [14] have been proposed and discussed in literature. Nowadays, the GnRH agonist test (leuprorelide acetate stimulation) is frequently used as an alternative [15,16]. However, no study has investigated the peak LH cut-off level indicating sufficient pubertal suppression, thus one may consider the cut-off level of 3 IU/L as rather arbitrary [4,11,12,17]. In our studies, we could demonstrate that a peak LH cut-off level of 3 IU/L might be too low for the GnRH agonist test.

Conclusions and clinical implications

In our studies in short SGA children, we have demonstrated that treatment with leuprorelide acetate depot 3.75 mg sc every 4 weeks results in an effective inhibition of central puberty, as shown by clinical signs of pubertal arrest and prepubertal overnight LH and FSH secretion patterns after 3 months of treatment.

The results of the GnRH agonist tests were known before LH levels during the overnight profiles were available. According to the Dutch consensus guideline, the schedule of leuprorelide acetate depot injections was changed to every 3 weeks instead of every 4 weeks in children who had a peak LH_{GnRH} above 3 IU/L. However, this adjustment to a higher frequency of injections proved to be unnecessary once we received the results of the overnight LH profiles.

Admitting children to a hospital in order to perform overnight LH and FSH profiles is not feasible in daily clinical care. It is, therefore, important to have a reliable peak LH_{GnRH} indicating sufficient pubertal suppression. Since 100% of the children had prepubertal LH profiles after 3 months of GnRHa treatment, we were unable to determine which peak LH_{GnRH} cut-off level indicates sufficient pubertal suppression, based on the LH profiles. Future research is warranted in order to determine a new peak LH cut-off level during a GnRH agonist test.

The GH-IGF-IGFBP axis before and during GnRHa treatment

Overnight GH levels before and during GnRHa treatment

We performed overnight GH profiles in pubertal short SGA children to determine GH levels at baseline and after 3 months of GnRHa treatment. In pubertal short SGA

girls, we also compared baseline GH levels to levels found in prepubertal short SGA girls [18]. In the latter study, GH levels were determined in the same laboratory with the same assay as in our present study.

At baseline, pubertal short SGA girls with Tanner stage 2 or stage 3 had mean GH levels comparable to prepubertal short SGA girls. In girls with normal stature, GH levels increase during puberty with the highest levels found at Tanner stage 3 and stage 4 [19]. Our data suggest that the lack of a rise in GH levels in early pubertal short SGA girls might explain the less intense pubertal growth spurt found in short SGA children who do not receive GH treatment [20,21].

During 3 months of GnRHa treatment, AUC_0, mean and maximum GH levels decreased significantly and mean GH levels became significantly lower compared to prepubertal short SGA girls. Our results are in line with those found in the few GH profile studies performed in children with CPP [22-24]. Baseline mean and maximum GH levels correlated positively with those after 3 months of GnRHa treatment, indicating that girls who have lower GH levels at baseline, also have lower GH levels during GnRHa treatment. There was a wide interindividual variability in GH secretion in response to GnRHa treatment: 8 girls had a more than 40% decrease in mean GH levels, whereas 3 girls actually had an increase in mean GH levels. These 3 girls had prepubertal LH and FSH levels during the overnight profiles and peak LH_{GnRH} was below 3 IU/L, indicating that puberty was sufficiently suppressed after 3 months of GnRHa treatment. We found no correlations between mean GH levels and oestradiol levels or between mean GH levels and mean and maximum LH levels during the overnight LH profile. Thus, girls with the same degree of pubertal suppression had different GH levels during GnRHa treatment. Furthermore, no differences in bone age, fat mass SDS, Tanner stage, oestradiol and LH levels, IGF-I and IGFBP-3 SDS were found between girls who showed a more than 40% decrease, a decrease between 0% and 40% or an increase in mean GH levels.

GH levels in pubertal short SGA boys were compared to levels in boys with normal stature and a similar pubertal stage [25]. Based on standard samples, our assay was comparable with the assay used by Rose et al [25].

At baseline, pubertal short SGA boys had GH levels comparable to those found in boys with normal stature and a similar pubertal stage. In prepubertal short SGA children, some authors reported significantly lower GH levels compared to controls [26,27], whereas others found similar levels [28]. Since endogenous GH secretion is similar in prepubertal girls and boys, no distinction in gender was made in the articles

investigating GH levels in prepubertal children. The wide interindividual variability in GH levels found in SGA children is indicative of the heterogeneity of SGA cohorts and probably reflects a continuum in GH secretion, ranging from GH deficiency to normal GH secretion.

After 3 months of GnRHa treatment, overnight GH profile characteristics had not significantly changed compared to baseline. Rose et al previously demonstrated that mean GH levels in healthy boys with testicular volumes of 5 to 10 ml remained near prepubertal levels, and a significant increase in spontaneous GH and IGF-I levels was found when Tanner stage 3 and a testicular volume of 10-15 ml was reached [25]. In our study group, all boys had testicular volumes between 4 and 8 ml. Thus, our study confirms that GH levels are comparable for early pubertal and prepubertal boys.

IGF-I and IGFBP-3 levels before and during GnRHa treatment

Baseline IGF-I and IGFBP-3 SDS in pubertal short SGA girls and boys were significantly lower than the respective population means, which is in line with previous studies describing low IGF-I and IGFBP-3 levels throughout childhood in subjects born SGA [15, 29-32].

After 3 months of GnRHa treatment, IGF-I and IGFBP-3 SDS had not significantly changed. This is in line with some studies performed in children with CPP [33,34], although others found a reduction in free IGF-I levels during GnRHa treatment [35]. However, GnRHa-treated short SGA girls with a more than 40% reduction in mean GH levels had a significantly greater decrease in IGF-I and IGFBP-3 levels, compared to girls who showed a reduction in mean GH levels between 0% and 40%.

In short SGA girls, the decrease in mean GH levels during 3 months of GnRHa treatment correlated significantly with the decrease in IGF-I and IGFBP-3 levels. In short SGA boys, IGF-I SDS correlated significantly with mean GH levels, both at baseline and after 3 months of GnRHa treatment. IGFBP-3 SDS correlated significantly with mean GH levels after 3 months of GnRHa treatment. Thus, IGF-I and IGFBP-3 levels do not only reflect spontaneous GH secretion in healthy children [36], but also in short SGA children treated with a GnRH analogue.

Conclusions and clinical implications

We demonstrated that pubertal short SGA girls lack the normal increase in GH secretion seen in pubertal girls with normal stature. Both girls and boys had IGF-I and IGFBP-3 levels significantly lower than the population mean. Our results indicate that

the relatively low GH, IGF-I and IGFBP-3 levels during puberty might be responsible for poor pubertal growth, at least in short SGA girls.

During 3 months of GnRHa treatment, mean serum GH levels decreased significantly in short SGA girls and IGF-I and IGFBP-3 levels decreased to a greater extend in GnRHa-treated girls with a more than 40% reduction in mean GH levels, compared to girls with a reduction in mean GH levels between 0% and 40%. GnRHa treatment has been associated with a significant decrease in height velocity [22,37,38], which might in part be explained by a reduction in serum GH levels [22-24]. Since all children in our study started with GH treatment after 3 months of GnRHa treatment, it was not possible to reliably determine height velocity. Our results nonetheless indicate that reduced GH levels might result in reduced growth during GnRHa treatment, at least in short pubertal SGA girls.

The GH-IGF-IGFBP axis during combined treatment with GnRHa and GH

GH, IGF-I and IGFBP-3 levels during combined treatment

We performed a randomized trial in order to evaluate the effect of treatment with a GnRH analogue in combination with 1 mg or 2 mg GH/m^2/day on GH, IGF-I and IGFBP-3 levels in pubertal short SGA children. During 1 year of combined treatment, GH levels increased significantly and levels were significantly higher in GnRHa-treated children who received 2 mg GH/m^2/day, compared to GnRHa-treated children who received 1 mg GH/m^2/day. Dose-dependent rises in GH levels have been reported in prepubertal short SGA children [18], in GH-deficient patients [39] and in girls with Turner syndrome [40]. GH levels in short SGA children who were treated with a GnRH analogue and 2 mg GH/m^2/day remained above 20 mU/L for almost 11 hours, demonstrating that these children have elevated GH levels for a great part of the day. Nevertheless, mean and maximum GH levels were lower in our study groups, compared to levels in prepubertal short SGA children treated with 1 mg or 2 mg GH/m^2/day. In both studies, GH levels were determined with the same assay in the same laboratory. As described above, GH levels decreased during 3 months of GnRHa treatment. In the present study, we demonstrated that GH treatment is unable to induce an increase in GH levels to similar levels as those found in GH-treated prepubertal short SGA children.

IGF-I SDS increased significantly in both GH dosage groups and levels were significantly higher in the 2 mg GH/m^2/day group, compared to the 1 mg GH/m^2/day

group. Furthermore, a significantly greater percentage of children treated with GnRHa and 2 mg GH/m^2/day had IGF-I SD-scores in the highest quintile (>0.84 SDS), compared to children treated with GnRHa and 1 mg GH/m^2/day (88.9% vs 43.8%). This is in line with results found in prepubertal short SGA children [15,18]. Reassuringly, the percentage of children with IGF-I SDS above +2 SDS was not significantly different between the 2 GH dosage groups. Compared to findings in prepubertal short SGA children treated with 1 mg or 2 mg GH/m^2/day, the percentage of children with IGF-I levels above +2 SDS was similar in children treated with GnRHa and 1 mg or 2 mg GH/m^2/day (6% vs 6%, 28% vs 37%) [18].

The increase in IGFBP-3 levels was similar in both GH dosage groups. The lack of a dose-dependent increase might be explained by the wide range in IGFBP-3 levels during GH treatment, possibly masking a significant difference between both GH dosage groups.

First year growth response during combined treatment

Height velocity SDS was significantly greater in GnRHa-treated children receiving 2 mg GH/m^2/day, compared to GnRHa-treated children receiving 1 mg GH/m^2/day (6.9 (4.0-7.4) SDS vs 4.3 (2.8-5.5) SDS). Multiple regression analysis demonstrated that the GH dose was associated with the first year growth response during combined treatment. Importantly, the increase in bone age was similar in both GH dosage groups. Although the GH dose is less important for long-term growth in children who start GH treatment at a young age, several studies found a GH dose-dependent effect during the first 4-5 years of GH treatment [41,42], which is the expected treatment period of older short SGA children. In the present study, growth response was restricted to the first year. Follow-up is necessary before definitive conclusions can be made about the effects of combined treatment with GnRHa and either 1 mg or 2 mg GH/m^2/day on adult height.

The first year growth response was comparable for girls and boys. Tanner stage at start of GnRHa treatment, on the other hand, significantly influenced the first year growth response: children with Tanner stage 2 at start of GnRHa treatment had a significantly greater height velocity SDS than children with stage 3. Pubertal stage was also inversely correlated with height velocity SDS during GnRHa treatment in girls with CPP [43]. Thus, combined treatment should best be started at an early pubertal stage.

Conclusions and clinical implications

In our randomized GH trial, short SGA children treated with GnRHa in combination with either 1 mg or 2 mg GH/m^2/day show a dose-dependent increase in GH and IGF-I levels, as well as in first year growth response. In short SGA children who come under medical attention at the onset of puberty, the optimal GH dose and whether postponement of puberty will improve adult height is still unknown. Although adult height data need to be awaited, our results suggest that treatment with 2 mg GH/m^2/day in combination with a GnRH analogue might be more effective.

Metabolic profile during treatment with GnRHa and GH

Body composition during combined treatment

No data are available on the metabolic effects of combined treatment with a GnRH analogue and GH in pubertal short SGA children. We, therefore, investigated the GH dose effect of combined treatment with a GnRH analogue and 2 randomly assigned GH dosages (1 mg vs 2 mg GH/m^2/day) on body composition, blood pressure, insulin sensitivity and ß-cell function, and lipid profile.

At baseline, fat mass SDS and lean body mass SDS adjusted for gender and height (SDS_{height}) were significantly lower than the population mean, which is in line with findings in prepubertal short SGA children [44]. At 3 months after stop of GnRHa treatment, fat mass SDS_{height} and percentage limb fat ((limb fat / total limb mass) x 100) had increased to values significantly higher than those at baseline in children treated with GnRHa and 1 mg GH/m^2/day. In children treated with GnRHa and 2 mg GH/m^2/day, fat mass SDS_{height} and percentage limb fat decreased significantly during 1 year of combined treatment and returned to baseline values at 3 months after stop of GnRHa treatment. In children with CPP, GnRHa treatment resulted in an increase in fat mass or BMI SDS [45,46]. GH treatment in prepubertal short SGA children, on the other hand, resulted in a decrease in fat mass SDS_{height} [44]. Based on our data, we conclude that simultaneous treatment with 2 mg GH/m^2/day counteracts the fat-accumulating effect of GnRHa treatment, whereas treatment with 1 mg GH/m^2/day is insufficient to prevent children from gaining fat mass. During the 2-year study period, percentage trunk fat ((trunk fat / total trunk mass) x 100) increased in both GH dosage groups, but to a significantly higher percentage in children treated with GnRHa and 1 mg GH/m^2/day, compared to children treated with GnRHa and 2 mg GH/m^2/day. This indicates that short SGA children develop

relatively more fat mass around the waist, but that the increase is less when GnRHa treatment is combined with 2 mg GH/m^2/day. In prepubertal short SGA children treated with 2 mg GH/m^2/day, the percentage trunk fat remained comparable to untreated children [47]. Therefore, the increase in percentage trunk fat in our study group is most likely due to the treatment with a GnRH analogue.

During the study period, lean body mass SDS_{height} had only increased in children treated with GnRHa and 2 mg GH/m^2/day. Thus, treatment with 2 mg GH/m^2/day – even in combination with GnRHa which is known to decrease lean body mass SDS adjusted for gender and age in children with CPP [45] – results in an increase in lean body mass SDS_{height} in short SGA children.

Blood pressure, glucose homeostasis and lipid profile during combined treatment

Blood pressure (BP), insulin sensitivity and ß-cell function, and lipid parameters were comparable between children treated with GnRHa and 1 mg GH/m^2/day and children treated with GnRHa and 2 mg GH/m^2/day.

At baseline, mean systolic BP SDS was significantly higher than the population mean and 27% of pubertal short SGA children had a systolic BP above +2 SDS. High blood pressure in childhood has been associated with an increased risk of developing hypertension in adulthood [48]. During the study period, systolic BP SDS did not significantly change, which is in line with previous studies in prepubertal short SGA children where a decrease was found only after 3 years of GH treatment [44,49].

FSIGT tests were performed at baseline and during 1 year of combined treatment. In order to compare insulin sensitivity during 3 months of GnRHa treatment and at 3 months after stop of GnRHa treatment, we also determined fasting insulin levels and calculated HOMA-IR [50]. During 3 months of GnRHa treatment, insulin levels and HOMA-IR remained comparable to baseline. Thus, short-term GnRHa treatment does not affect insulin sensitivity. As expected, during 1 year of combined treatment, Si decreased significantly and AIR increased significantly. Reassuringly, the disposition index (DI) remained comparable to baseline, reflecting that beta cells were able to compensate for a reduction in insulin sensitivity by increasing their insulin secretion. At 3 months after stop of GnRHa treatment, fasting insulin levels and HOMA-IR remained similar to those during 1 year of combined treatment.

Although some lipids showed a significant temporary increase (LDL-cholesterol, free fatty acids, apolipoprotein A-1 levels), whereas others increased more steadily over time (total cholesterol and triglyceride levels), the actual increase and

subsequent decrease in levels was very small and within the normal range during the 2-year study period. The clinical significance of these changes in lipid levels is therefore negligible.

Conclusions and clinical implications

We found a GH dose-dependent effect on fat mass SDS_{height}, percentage trunk and limb fat and lean body mass SDS_{height} in favor of treatment with GnRHa and 2 mg GH/m^2/day. Percentage trunk fat increased in both GH dosage groups, but to higher values in children treated with GnRHa and 1 mg GH/m^2/day, compared to children treated with GnRHa and 1 mg GH/m^2/day. Epidemiological studies have demonstrated that low birth weight followed by catch-up in weight during childhood and adolescence, even within the normal weight range, was associated with a higher risk of developing type 2 diabetes and cardiovascular disease [51,52]. It is therefore important to make parents and adolescents aware of the risks of gaining fat mass.

Blood pressure, insulin sensitivity and lipid profile were similar between both GH dosage groups. Reassuringly, systolic blood pressure SDS remained comparable to baseline and diastolic blood pressure SDS and lipid parameters remained within the normal range. Furthermore, children were able to compensate for the GH-dependent decrease in insulin sensitivity.

Follow-up until adult height with continuous monitoring of all above-mentioned metabolic parameters, next to IGF-I and IGFBP-3 levels, is necessary before definitive conclusions can be drawn concerning the safety of combined treatment. Our results nevertheless suggest that treatment with either 1 mg or 2 mg GH/m^2/day, in addition to a GnRH analogue, can be regarded as a potential treatment strategy for short SGA children who come under medical attention at onset of puberty.

Part 2: Genetic aspects

IGFBP-1 levels and associations with clinical and laboratory parameters

Reduced serum IGFBP-1 levels are considered to reflect hyperinsulinemia and cardiovascular risk in adults [53-55] and in prepubertal obese children [56]. Previous studies showed that short subjects born SGA have increased insulin secretion. Reassuringly, the disposition index was comparable between short SGA subjects and controls [57-59]. We hypothesized that IGFBP-1 levels would be lower compared to

levels in age-matched controls with normal stature. To answer this question, we compared fasting IGFBP-1 levels in a large cohort of 272 short SGA subjects with those in a large control group of 330 subjects with normal stature and comparable age.

Short young SGA adults had significantly lower IGFBP-1 levels compared to controls with normal stature. No data on IGFBP-1 levels in short SGA adults are available. One study performed in young SGA women with normal stature also found reduced IGFBP-1 levels, compared to controls [60]. Insulin is considered the main regulator of IGFBP-1 production through binding to insulin-response elements in the IGFBP-1 gene promoter [61,62]. Thus, the significantly lower IGFBP-1 levels found in short SGA adults in our study, compared to adult controls, are likely a response to a higher insulin secretion.

IGFBP-1 levels in short SGA children throughout childhood and adolescence were comparable to those in children with normal stature. Four studies have determined IGFBP-1 levels in much smaller cohorts of short SGA children. One study found lower [63] and the other 3 studies found similar IGFBP-1 levels in short children born SGA [64-66]. None of these studies used children with normal stature as a control group. Short SGA children are lean: fat mass SDS adjusted for gender and height was significantly lower in short SGA children than in the population mean. Short young SGA adults, on the other hand, have a fat mass SDS similar to the population mean. Epidemiological studies found an association between low birth weight followed by catch-up in weight during childhood and adolescence and a higher risk of developing type 2 diabetes mellitus and cardiovascular disease [51,67,68]. IGFBP-1 SDS correlated significantly with fat mass SDS in short SGA adults. We therefore postulate that the normal IGFBP-1 levels in short SGA children with a low fat mass reflect a normal metabolic state, despite reported hyperinsulinemia in these children.

IGFBP-1 SDS correlated inversely with systolic BP SDS, insulin levels, triglycerides and IGF-I SDS, and positively with HDL-cholesterol levels, independent of age and fat mass SDS. These data support the concept that IGF-I and its binding proteins, particularly IGFBP-1, are important for glucose homeostasis and lipid metabolism [61, 69].

Genetic variability in the *IGFBP1* promoter

In order to determine if serum IGFBP-1 levels are influenced by genetic variability in the IGFBP1 gene promoter (*IGFBP1*), we assessed the contribution of the -575 G/A

IGFBP1 single nucleotide polymorphism (SNP). This SNP lays in the proximity of the transcription start site and many regulatory factor recognition motifs. The minor (A) allele was associated with a decreased prevalence of diabetic nephropathy in patients with type 2 diabetes mellitus [70]. Frequencies of the G (63.6%) and A (36.4%) allele in our Caucasian short SGA population were comparable with frequencies found in Caucasian adults [70]. IGFBP-1 SDS was highest in short SGA children carrying the rarer AA genotype and significantly lower in children carrying the GA and GG genotype. Thus, serum IGFBP-1 levels were significantly related to the -575 G/A SNP. In prepubertal short SGA children, 4.6% of the variability in IGFBP-1 levels was explained by this SNP.

The -575 G/A SNP significantly influenced the relation between serum IGFBP-1 and insulin levels. Prepubertal short SGA children with comparable insulin secretion and carrying the GG genotype had significantly lower IGFBP-1 levels, compared to children carrying the GG genotype. The -575 G/A SNP is situated near several insulin response elements (IREs). Transcription factors interact with these elements, thereby regulating the expression of *IGFBP1* in response to insulin. This promoter SNP, possibly in combination with other as yet unidentified SNPs, could play a role through altering the binding of transcription factors to these IREs, thereby modulating insulin-dependent gene repression [62,71].

Conclusions and clinical implications

From our study we conclude that IGFBP-1 levels reflect the metabolic state in short SGA subjects: levels were comparable to controls in lean, short SGA children, whereas levels were significantly lower in short young SGA adults with a normal fat mass. IGFBP-1 levels correlated with established clinical and metabolic cardiovascular risk factors. As the only acute regulator of IGF-I bioavailability, IGFBP-1 might be an additional player in the complex interactions between the IGF-IGFBP axis, glucose homeostasis and lipid metabolism.

The -575 G/A *IGFBP1* promoter SNP was significantly associated with IGFBP-1 levels and with the relation between insulin and IGFBP-1 levels. Common polymorphisms with small effects on phenotype can provide important contributions to understanding complex diseases. Future studies should be extended to haplotype analysis to examine the impact of other promoter polymorphisms on IGFBP-1 levels.

Genetic variability in the *IGFBP3* promoter: correlations with serum levels and growth

Twin studies demonstrated that 60% of the interindividual variability in circulating IGFBP-3 levels is genetically determined [72]. Various polymorphic loci have been detected in the IGFBP3 gene (*IGFBP3*) and the -202 A/C promoter SNP has been correlated with circulating IGFBP-3 levels in several adult cohorts [73,74]. We hypothesized that genetic variability in *IGFBP3* may, in part, explain circulating IGFBP-3 levels, spontaneous and GH-stimulated growth in short children born SGA. We, therefore, assessed the relationship between 2 *IGFBP3* promoter SNPs, IGFBP-3 levels and growth, both at baseline and during GH treatment in 292 Caucasian, prepubertal short SGA children.

IGFBP-3 levels were highest in short SGA children carrying the AA genotype and significantly lower in children carrying the AC and CC genotype. The -202 A/C SNP is functional *in vitro*; reporter gene constructs demonstrated that the C-allele had approximately 50% of the promoter activity of the A-allele [73]. This is most likely due to its localization near elements involved in directing promoter activity, and its implication in the binding of transcription factors [75]. The interaction of multiple SNPs within a genetic locus (haplotype) could theoretically influence the phenotype to a larger extent. We therefore investigated another SNP (the -185 C/T SNP) adjacent to the -202 A/C SNP. Indeed, we found a greater association between A^{-202}/C^{-185} and C^{-202}/C^{-185} haplotypes and IGFBP-3 levels as well as baseline height SDS, than between the single -202 A/C SNP and IGFBP-3 levels. Short SGA children carrying the C^{-202}/C^{-185} haplotype had significantly lower IGFBP-3 levels and were significantly shorter compared to children carrying the A^{-202}/C^{-185} haplotype. The haplotype explained 12.2% of the variability in baseline IGFBP-3 levels, whereas the -202 A/C SNP explained only 4.0% of the variability.

During 1 year of GH treatment, catch-up in height SDS was greatest in children with the lowest baseline IGFBP-3 levels. This is a robust phenomenon seen in daily practice as well as in various growth prediction models [76,77]. Multiple regression analyses showed that the increase in IGFBP-3 levels predicted the variability in catch-up growth during 1 year of GH treatment, a period which strongly predicts subsequent growth and adult height [78]. Furthermore, de Ridder et al found that IGFBP-3 levels at start of GH treatment influence the effect of treatment: a higher GH dose is more effective if IGFBP-3 SDS is (very) low [77]. Thus, determination of IGFBP-3 levels is recommended to provide a more reliable prediction of an individual's response to GH treatment.

One of the greatest challenges in the treatment of short SGA children is dealing with the large interindividual variability in growth response during GH treatment. A great part of this variability remains after accounting for important variables such as age at start of treatment, target height SDS and duration of treatment. We demonstrated that after 1 year of GH treatment, the increase in IGFBP-3 and height SDS was significantly greater in children carrying the C^{-202}/C^{-185} haplotype compared to children carrying the A^{-202}/C^{-185} haplotype. This resulted in similar IGFBP-3 SDS and height SDS during GH treatment.

Epigenetic variability in the *IGFBP3* promoter

Methylation is one of the epigenetic modifications of DNA in mammalian genomes, leading to alterations in the binding affinity of transcription factors to DNA binding sites and subsequent reduced gene expression. Environmental influences during fetal and postnatal development could hypothetically result in changes in the epigenetic regulation of genes [79] and these changes might contribute to adult disease [80]. Until now, no studies have investigated the methylation status in short SGA subjects.

Global methylation percentage in the investigated region of the *IGFBP3* promoter was very low. This could be explained by the fact that our study group was young (mean (± SD) age in adults: 20.8 (± 1.68) years) and methylation of CpG islands increases with increasing age [81]. Furthermore, *IGFBP3* is ubiquitously expressed, reflecting the importance of maintaining this CpG island in an unmethylated and thus active state. Finally, gene expression is tissue- and cell-type specific and epigenetic mechanisms maintain this specificity [80]. Methylation status was comparable between subjects carrying the A^{-202}/C^{-185} and C^{-202}/C^{-185} haplotypes, as well as between controls carrying the A^{-202}/C^{-185} and C^{-202}/C^{-185} haplotypes. We found a wide interindividual variability in methylation patterns in both short SGA subjects and controls; this variability could be a mechanism for explaining variation in disease risk [80]. On the other hand, the wide variability makes it difficult to detect significant differences in a small study group.

We did detect a trend towards higher methylation patterns of specific CpGs involved in binding of 2 transcription factors in short SGA adults, compared to age-matched controls with normal stature (13.6% vs 6.8%). The first transcription factor, Sp1, is an important factor in the activation of genes in response to insulin [82,83] and insulin is one of the regulators of *IGFBP3* expression [61,75,84]. Methylation of the Sp1 binding site leads to decreased binding of Sp1, resulting in reduced

transcriptional activity [85] and hypothetically to lower IGFBP-3 levels. The second transcription factor, p53, is known to upregulate *IGFBP3* expression. *In vitro* studies showed that hypermethylation of p53 consensus sequences in the *IGFBP3* promoter results in reduced *IGFBP3* expression in HepG2 cells [86] and thus lower IGFBP-3 levels. Although short SGA adults had somewhat higher methylation percentages of the CpGs involved in the binding of these 2 transcription factors, a methylation percentage of 13.6% is relatively low. It is, therefore, questionable that epigenetic variability in this part of the *IGFBP3* promoter influences *IGFBP3* expression and thus circulating IGFBP-3 levels. Functional studies are required to answer this question.

Conclusions and clinical implications

Polymorphic variation in the *IGFBP3* promoter region is associated with IGFBP-3 levels, spontaneous growth and response to GH treatment in short SGA children. Haplotype explained 12.2% of the variability in baseline IGFBP-3 levels. Since 60% of the variability in circulating IGFBP-3 levels can be explained by genetic factors, this haplotype seems to contribute to the regulation of *IGFBP3* expression and therefore growth in this population. Haplotype was significantly associated with first year growth response to GH treatment. Thus, as shown in this study, genetic variability of growth-related genes probably accounts for part of the wide range in growth response found in short children born SGA. In addition to haplotype, the first year growth response was associated with an increase in IGFBP-3 levels. We, therefore, suggest that determination of IGFBP-3 levels at start of GH treatment can further aid the prediction of an individual's response to GH treatment.

Although we found a trend towards higher methylation percentages in CpGs involved in binding of 2 transcription factors in short SGA adults versus adult controls with normal stature, the overall methylation of the investigated *IGFBP3* promoter CpGs was low. Future studies focusing on these specific CpGs, perhaps using additional tissues besides leukocytes, are warranted.

General conclusions, practical implications and directions for future research

Since GH treatment was approved by the US Food and Drug Administration (FDA) in 2001 and by the European Agency for Evaluation of Medicinal Products (EMEA) in 2003, short children born SGA comprise a large group of GH-treated children. Various studies demonstrated that GH treatment in prepubertal short SGA children effectively and safely induces catch-up growth [32,42,44,87,88]. Better growth responses and greater adult height were achieved when children started growth hormone treatment at an early age. Some short SGA children, however, come under medical attention at onset of puberty. In a group of pubertal short SGA children, we investigated safety and efficacy parameters of treatment with 1 mg or 2 mg GH/m^2/day, next to treatment with a GnRH analogue for 2 years.

We demonstrated that GnRHa treatment with leuprorelide acetate 3.75 mg subcutaneously every 4 weeks results in an effective inhibition of central puberty, as shown by clinical signs of pubertal arrest and prepubertal overnight LH and FSH secretion patterns. In the Netherlands, a consensus-based LH cut-off level of 3 IU/L is used to indicate sufficient pubertal suppression. According to this cut-off level, 33% of short SGA girls and 43% of short SGA boys in our study had an insufficiently suppressed puberty after 3 months of GnRHa treatment. However, no study has actually investigated the peak LH cut-off level indicating sufficient pubertal suppression, thus one may consider a cut-off level of 3 IU/L as rather arbitrary. In our studies, 100% of the children had prepubertal LH profiles after 3 months of GnRHa treatment. We were, therefore, unable to determine which peak LH_{GnRH} cut-off level indicates sufficient pubertal suppression, based on the LH profiles. We strongly recommend future research in a larger study group, including boys and girls with different pubertal stages and with sufficient and insufficient pubertal suppression according to their LH profiles, in order to determine a new peak LH cut-off level during a GnRH agonist test.

Pubertal short SGA girls lack the usual increase in GH levels found in pubertal girls with normal stature. GnRHa treatment resulted in a significant decrease in GH levels to levels lower than those found in prepubertal short SGA girls. This decrease was accompanied by a decrease in IGF-I and IGFBP-3 levels in GnRHa-treated girls with a more than 40% reduction in mean GH levels. During 1 year of combined treatment with GnRHa and either 1 mg or 2 mg GH/m^2/day, GH and IGF-I levels and height velocity

SDS showed a significant dose-dependent increase. Reassuringly, the percentage of children with IGF-I SDS above the normal range (+2 SDS) was not significantly different between the 2 GH dosage groups. Mean and maximum GH levels were lower in our study groups, compared to levels in prepubertal short SGA children, demonstrating that treatment with either 1 mg or 2 mg GH/m^2/day is unable to induce an increase in GH levels to similar levels as those in prepubertal children. Although we found a GH-dose dependent effect on growth response during the first treatment year, it is necessary to follow these children until adult height in order to investigate whether combined treatment with GnRHa and 2 mg GH/m^2/day will result in a better adult height, compared to treatment with GnRHa and 1 mg GH/m^2/day.

During a 2-year study period, we showed that GH dose has an effect on fat mass SDS_{height}, percentage trunk and limb fat and lean body mass SDS_{height} in favor of treatment with 2 mg GH/m^2/day, in addition to GnRHa. Systolic and diastolic blood pressure, insulin sensitivity and lipid profile were similar between both GH dosage groups and combined treatment had no adverse effects on these metabolic parameters. Our results indicate that combined treatment with a GnRH analogue and either 1 mg or 2 mg GH/m^2/day can be considered as a safe treatment strategy in the short run for short SGA children who come under medical attention at onset of puberty. However, follow-up until adult height with monitoring of body composition, glucose homeostasis and lipid profile, next to IGF-I and IGFBP-3 levels, is required before definitive conclusions can be drawn concerning the long-term safety of combined treatment.

IGFBPs regulate the bioavailability of IGFs, but also have IGF-independent effects on growth and metabolism. IGFBP-1 is the only acute regulator of IGF-1 bioavailability. IGFBP-3 binds the majority of circulating IGF-I, serving as the major carrier protein. Short SGA children with a low fat mass had IGFBP-1 levels similar to controls, whereas short SGA adults with a normal fat mass had significantly lower levels. IGFBP-1 SDS correlated significantly with several metabolic parameters. Although a wide range in IGFBP-1 levels exists in children and adolescents, both in short SGA subjects and in controls, the range is much smaller in young adults. IGFBP-1 levels measured at baseline were closely related to levels after 1 year follow-up in patients with type 2 diabetes mellitus [89]. IGFBP-1 might, thus, serve as a marker to characterize subjects with an adverse metabolic outcome. This will require determination of IGFBP-1 levels in prospective cohorts and clinical intervention studies.

The -575 G/A *IGFBP1* promoter SNP was significantly associated with IGFBP-1 levels, explaining 4.6% of the variability in IGFBP-1 levels. Polymorphic variation in the *IGFBP3* promoter region was associated with IGFBP-3 levels, spontaneous growth and growth response to GH treatment in prepubertal short SGA children. The haplotype explained 12.2% of the variability in baseline IGFBP-3 levels. We demonstrated that genetic variability in 2 *IGFBP* promoter regions was associated with various clinical and laboratory parameters in short SGA subjects. Future studies focusing on genetic, as well as epigenetic, variability in genes important in growth and metabolism might aid in our search for factors explaining persistent short stature and metabolic profile in SGA subjects.

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Chapter

10

Summary

Chapter 1

This chapter gives an overview of definitions, prevalence and possible causes of small size at birth. In addition, the GH-IGF-IGFBP axis and genetic regulation of genes involved in this axis are described. Clinical and endocrinological aspects associated with SGA are discussed. Previously reported effects of GH treatment on growth, body composition, insulin sensitivity and lipid profile are summarized. Treatment options in short SGA children who present at the beginning of puberty are discussed. Reported effects of GH treatment, GnRHa treatment or a combination on growth and metabolic outcome in specific groups of children are discussed. Finally, the aims and outline of this thesis, as well as the study designs of the SGA study, IUGR-1 study, IUGR-2 study, IUGR-3 study and PROGRAM study are presented.

Chapter 2

Very limited data exist on spontaneous overnight LH and FSH profile patterns during GnRHa treatment. We, therefore, performed overnight LH and FSH profiles in 21 short SGA girls. Since performing overnight profiles in order to determine sufficient pubertal suppression during GnRHa treatment is not feasible in routine clinical care, a GnRH agonist test was performed in all girls. In the Netherlands, several years ago consensus was reached that a peak LH below 3 IU/L (peak LH_{GnRH}) with oestradiol levels below 50 pmol/l indicates sufficient pubertal suppression. These cut-off levels are, however, rather arbitrary.

After 3 months of GnRHa treatment, none of the girls had clinical progression of puberty and 8 girls (5 girls with B2 and 3 girls with B3) had clinical regression of puberty. AUC_0, mean and maximum LH and FSH levels during the overnight profile had significantly decreased to very low, prepubertal levels. Peak LH_{GnRH}, on the other hand, was between 3.1 and 7.3 IU/L in 7 out of 21 girls (33%). Oestradiol levels were below 50 pmol/l in 6 out of 7 girls and was 58 pmol/l in one girl. No significant differences in LH and FSH profiles were found between girls with a peak LH_{GnRH} above or below 3 IU/L.

In conclusion, our results demonstrate that GnRHa treatment with leuprorelide acetate 3.75 mg subcutaneously every 4 weeks results in an effective inhibition of central puberty in short SGA girls, as shown by clinical signs of pubertal arrest and prepubertal overnight LH and FSH secretion patterns. A peak LH cut-off level of 3

IU/L falsely indicated insufficient suppression of puberty in 33% of the girls, thus this cut-off level might be too low for the GnRH agonist test. Since 100% of the girls had prepubertal LH profiles after 3 months of GnRHa treatment, we could not determine a new peak LH cut-off level during the GnRH agonist test, based on the LH profiles. We recommend future research in a larger and more heterogeneous group in order to determine a new peak LH_{GnRH} cut-off level.

Chapter 3

We evaluated LH and FSH secretion patterns in 10 short SGA boys, before and after 3 months of GnRHa treatment. We also determined the number of short SGA boys with sufficient pubertal suppression according to the GnRH agonist test, using a consensus-based peak LH cut-off level of 3 IU/L (peak LH_{GnRH}) with testosterone levels below 1 nmol/l. After 3 months of GnRHa treatment, none of the 14 boys showed clinical progression of puberty, 2 boys showed clinical regression of puberty. AUC_0, mean and maximum LH and FSH levels had significantly decreased to very low, prepubertal levels. Testosterone levels had significantly decreased from 6.5 (1.3-9.9) nmol/l to 0.25 (0.10-0.53) nmol/l. Peak LH_{GnRH} varied between 3.3 and 5.2 IU/L in 6 boys (43%), whereas testosterone levels were below 1 nmol/l in all 6 boys. No significant differences in LH and FSH profiles were found between boys with a peak LH_{GnRH} above or below 3 IU/L.

No data on GH, IGF-I and IGFBP-3 levels were available in short SGA boys, either before or during GnRHa treatment. Overnight GH profiles in 10 pubertal short SGA boys showed that mean GH levels in boys with genital stage 2 were comparable with mean GH levels found in boys with normal stature and similar genital stage. IGF-I and IGFBP-3 levels were significantly lower than the population mean. After 3 months of GnRHa treatment, GH profile characteristics and IGF-I and IGFBP-3 levels remained comparable to baseline. Mean GH levels also remained comparable to controls. It was previously demonstrated that GH and IGF-I levels increase significantly when Tanner stage 3 and a testicular volume of > 10-15 ml was reached. Since all boys in our study group had testicular volumes between 4 and 8 ml, this could explain why we did not find significant changes in GH and IGF-I levels after 3 months of GnRHa treatment.

In conclusion, treatment with leuprorelide acetate depots of 3.75 mg every 4 weeks results in an effective inhibition of central puberty in short SGA boys, as shown by clinical signs of pubertal arrest and prepubertal overnight LH and FSH secretion

patterns. In contrast, the GnRH agonist test falsely indicated insufficient pubertal suppression in almost half of the boys. Low IGF-I and IGFBP-3 levels were found at the start of puberty, although mean GH levels were normal for pubertal stage. GH, IGF-I and IGFBP-3 levels did not significantly change during 3 months of GnRHa treatment, consistent with reports showing that prepubertal and early pubertal boys have similar GH levels.

Chapter 4

Postponement of puberty with a GnRH analogue is one treatment option in pubertal short SGA children. It was, however, unknown if GnRHa treatment has an effect on GH, IGF-I and IGFBP-3 levels in these children. We performed overnight GH profiles and measured IGF-I and IGFBP-3 levels in 24 short SGA girls (17 girls with Tanner stage 2 and 7 girls with Tanner stage 3), before and after 3 months of GnRHa treatment. We compared GH profile characteristics with those in prepubertal short SGA girls.

At baseline, mean GH levels in pubertal short SGA girls were comparable to levels in prepubertal short SGA girls. IGF-I and IGFBP-3 SDS were significantly below the population mean. After 3 months of GnRHa treatment, mean GH levels had significantly decreased and levels were significantly lower than those found in prepubertal short SGA girls. Eight girls who had a more than 40% decrease in mean GH levels also had a significantly greater decrease in IGF-I and IGFBP-3 levels, compared to girls with a decrease in mean GH levels between 0% and 40%.

In conclusion, pubertal short SGA girls lack the usual increase in GH levels found in pubertal girls with normal stature during Tanner stages 2 and 3. Mean GH levels significantly decreased during 3 months of GnRHa treatment to levels even lower than those found in prepubertal short SGA girls. Combining GnRHa treatment with GH treatment might therefore improve adult height in short SGA girls.

Chapter 5

Some short SGA children only come under medical attention at onset of puberty. Whether postponement of puberty is beneficial for adult height improvement in these short SGA children and which GH dose is most effective, was unknown. Also, no data were available on the effect of combined treatment with a GnRH analogue and GH 1 mg (group A) vs 2 mg/m^2/day (group B) on GH, IGF-I and IGFBP-3 levels and short-term

growth. We showed that after 1 year of combined treatment, mean GH levels had significantly increased in both GH dosage groups and levels were significantly higher in group B compared to group A. Following the sc. GH injection at 20.00h, GH levels remained significantly longer above 40 mU/L and 20 mU/L in group B, compared to group A. In both GH dosage groups, GH levels were lower in our study group, compared to reported levels in prepubertal SGA children. GH levels decreased during 3 months of GnRHa treatment. In chapter 4, we demonstrated that GH treatment is unable to induce an increase in GH levels to similar levels as those found in GH-treated prepubertal short SGA children.

IGF-I levels were significantly higher in group B compared to group A. In group B, 88.9% of the children had IGF-I levels in the highest quintile (>0.84 SDS), compared to 43.8% of children in group A. Reassuringly, the percentage of children with IGF-I SDS above the normal range (>+2 SDS) was not significantly different between the 2 GH dosage groups (27.8% vs. 6.3%). IGFBP-3 levels increased significantly in both GH dosage groups and the increase was comparable between both groups.

During 1 year of combined treatment, height velocity SDS – based on prepubertal height velocity references adapted for a wide age range – was significantly higher in group B (6.9 (4.0-7.4) SDS) compared to group A (4.3 (2.8-5.5) SDS). In group B, children with Tanner stage 2 at start of GnRHa treatment had a significantly greater height velocity SDS than children with Tanner stage 3 (7.1 (5.6-8.5) SDS vs 4.0 (3.3-5.0) SDS). Combined treatment should, therefore, best be started at an early pubertal stage.

In conclusion, short SGA children treated with GnRHa and either 1 mg or 2 mg GH/m^2/day show a dose-dependent increase in GH and IGF-I levels, and first year growth response. Our results suggest that combined treatment with GnRHa and 2 mg GH/m^2/day might be more effective than 1 mg GH/m^2/day, but adult height data and effects on metabolic parameters need to be awaited before definitive conclusions can be drawn.

Chapter 6

Before our study, no data were available on the effects of combined treatment with GnRHa and GH (1 mg vs 2 mg GH/m^2/day) on body composition, blood pressure, insulin sensitivity and ß-cell function, and lipid profile in pubertal short SGA children. In a randomized trial, we investigated the effect of combined treatment on these outcome measures in 45 short SGA children (29 girls).

BMI SDS increased significantly during 3 months of GnRHa treatment, but remained significantly lower than the population mean (-0.98 (-1.28 to -0.68) to -0.90 (-1.22 to -0.57) SDS). Blood pressure, insulin sensitivity and lipid profile did not significantly change compared to baseline.

During the 2-year follow-up period, children treated with GnRHa and 1 mg GH/m^2/day developed a higher fat mass adjusted for gender and height (SDS_{height}), and percentage trunk and limb fat, compared to children treated with GnRHa and 2 mg GH/m^2/day. Compared to baseline values, percentage trunk fat increased significantly in both GH dosage groups. Fat mass SDS_{height} and percentage limb fat only increased significantly in children treated with GnRHa and 1 mg GH/m^2/day, whereas lean body mass SDS_{height} only increased in children treated with GnRHa and 2 mg GH/m^2/day. Blood pressure, insulin sensitivity and ß-cell function, and lipid profile were similar in both GH dosage groups. As expected, insulin sensitivity decreased significantly, but the disposition index remained comparable to baseline in both GH dosage groups, indicating that ß-cells were able to compensate for a reduction in insulin sensitivity by increasing their insulin secretion. Lipids remained within normal reference ranges.

In conclusion, GH dose has an effect on fat mass SDS_{height}, percentage trunk and limb fat and lean body mass SDS_{height} in favor of treatment with 2 mg GH/m^2/day, when used in addition to GnRHa treatment. Blood pressure, insulin sensitivity and lipid profile were similar for both GH dosage groups and combined treatment had no unexpected adverse effects on these metabolic parameters. Our results indicate that combined treatment with a GnRH analogue and either 1 mg or 2 mg GH/m^2/day can be regarded as a potential treatment strategy for short SGA children who come under medical attention at onset of puberty. Follow-up until adult height is, however, necessary before we can conclude which GH dose in combination with GnRHa will result in an optimal adult height without adversely affecting metabolic outcome.

Chapter 7

Reduced serum IGFBP-1 levels are considered to reflect hyperinsulinemia and cardiovascular risk in adults. Previous studies demonstrated that short SGA subjects have increased insulin secretion. Data on IGFBP-1 levels in short SGA subjects were scarce and associations with the -575 G/A *IGFBP1* promoter single nucleotide polymorphism (SNP) had not been established. We determined serum IGFBP-1 levels in 272 short

SGA subjects and compared levels to those found in 330 age-matched controls with normal stature. We also determined whether variability in IGFBP-1 levels might be related to the -575 G/A SNP.

IGFBP-1 levels were comparable to controls in prepubertal and pubertal short SGA children who generally have a low percentage fat mass SDS. Short young SGA adults, however, had a normal percentage fat mass SDS and significantly lower IGFBP-1 levels compared to controls. After adjustment for age and percentage fat mass SDS, IGFBP-1 SDS was inversely correlated with insulin levels (r=-0.40), systolic BP SDS (r=-0.26), triglycerides (r=-0.31) and IGF-I SDS (r=-0.48), and was positively correlated with HDL-cholesterol (r=0.29) in short SGA children. In short SGA adults, IGFBP-1 SDS was inversely correlated with the acute insulin response (r=-0.43), percentage fat mass SDS (r = -0.43) and IGF-I SDS (r=-0.51).

In prepubertal short SGA children, IGFBP-1 SDS was lowest in children carrying the GG genotype and significantly higher in children carrying 1 or 2 copies of the A allele. The variance in IGFBP-1 SDS that could be explained by genotype was 4.6%. The -575 G/A SNP significantly influenced the association between IGFBP-1 SDS and acute insulin response: short SGA children with comparable insulin secretion and carrying the GG genotype had a lower IGFBP-1 SDS, compared to children carrying the AA genotype.

In conclusion, IGFBP-1 levels reflect the metabolic state in short SGA subjects: levels were comparable to controls in lean, short SGA children, whereas levels were significantly lower in short young SGA adults who have a normal fat mass. IGFBP-1 levels correlated with several cardiovascular risk factors. The -575 G/A *IGFBP1* promoter SNP was significantly associated with IGFBP-1 levels and with the relation between insulin secretion and IGFBP-1 levels. As IGFBP-1 is the only acute regulator of IGF-I bioavailability, IGFBP-1 might be an additional player in the complex interactions between the IGF-IGFBP axis, glucose homeostasis and lipid metabolism.

Chapter 8

We assessed the relationship between 2 *IGFBP3* promoter SNPs (-202 A/C SNP and -185 C/T SNP), serum IGFBP-3 levels, spontaneous growth and the growth response to GH treatment in 292 prepubertal short SGA children. Furthermore, we compared the *IGFBP3* promoter methylation status between a subgroup of short SGA subjects (n=22) and healthy controls with a normal stature (n=14).

IGFBP-3 SDS was highest in short SGA children carrying the -202 AA genotype and significantly lower in children carrying one or two copies of the C allele. The variance in IGFBP-3 SDS that could be explained by the -202 A/C SNP was 4.0%. Short SGA children carrying the C^{-202}/C^{-185} haplotype had significantly lower IGFBP-3 levels (-1.92 ±1.03 SDS) and were significantly shorter (-3.31 ±0.58 SDS) compared to children carrying the A^{-202}/C^{-185} haplotype (IGFBP-3 SDS: -0.9 5 ±1.21 SDS, height SDS: -2.94 ±0.60 SDS). The variance in IGFBP-3 SDS that could be explained by haplotype was 12.2%. After 12 months of GH treatment, short SGA children carrying the C^{-202}/C^{-185} haplotype had a significantly greater catch-up in IGFBP-3 SDS and height SDS, compared to children carrying the A^{-202}/C^{-185} haplotype, resulting in comparable IGFBP-3 and height SD-scores after 12 months of GH treatment.

The methylation percentage in the region flanking both SNPs was low. Methylation status was comparable between short SGA subjects with the C^{-202}/C^{-185} haplotype and the A^{-202}/C^{-185} haplotype and between controls with the C^{-202}/C^{-185} haplotype and the A^{-202}/C^{-185} haplotype. Three CpGs, involved in binding of transcription factors Sp1 and p53, showed a trend towards more methylation in short SGA adults with the A^{-202}/C^{-185} haplotype, compared to adult controls with the A^{-202}/C^{-185} haplotype (13.6% vs. 6.8%).

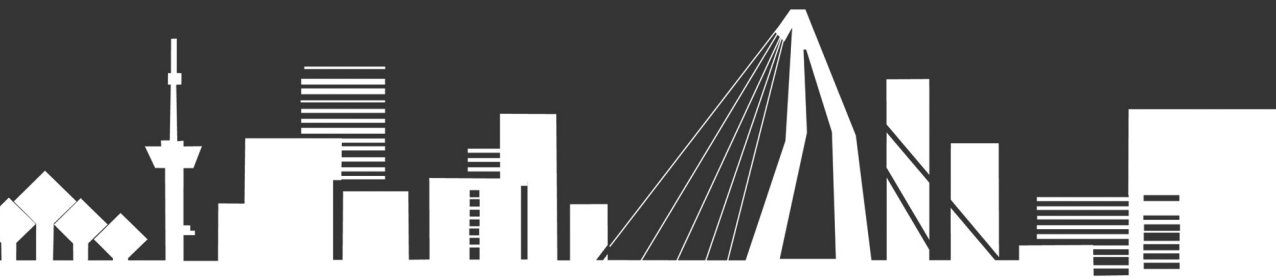
In conclusion, polymorphic variation in the *IGFBP3* promoter region is associated with IGFBP-3 levels, spontaneous growth and growth response to GH treatment in short SGA children. Although we found a trend towards higher methylation percentages in CpGs involved in binding of 2 transcription factors in SGA adults versus controls, the overall methylation of the *IGFBP3* promoter region under study was low.

Chapter 9

In the general discussion, we discuss our findings in relation to the current literature. We conclude this chapter with general considerations and directions for future research.

Chapter

11



Hoofdstuk 1

Dit hoofdstuk beschrijft de definities, prevalentie en mogelijke oorzaken van een kleine lengte en/of een laag gewicht bij de geboorte. De GH-IGF-IGFBP as en regulatie van genen die binnen deze as een rol spelen, worden beschreven. Vervolgens worden klinische en endocrinologische aspecten die samenhangen met SGA besproken. Eerder beschreven effecten van groeihormoonbehandeling op de groei, lichaamssamenstelling, insulinegevoeligheid en lipiden worden samengevat. De behandelingsopties en mogelijke effecten op de groei en metabole parameters bij pubertaire SGA geboren kinderen met een kleine lengte worden besproken. Tenslotte wordt een overzicht gegeven van de doelstellingen van dit proefschrift en wordt de opzet van de SGA-studie, IUGR-1 studie, IUGR-2 studie, IUGR-3 studie en PROGRAM studie gepresenteerd.

Hoofdstuk 2

Wij onderzochten de nachtelijke LH en FSH spiegels bij 21 kleine SGA geboren meisjes, aan het begin van de puberteit en na 3 maanden GnRHa behandeling. Het maken van 12-uurs profielen om de mate van onderdrukking van de puberteit te bepalen, is in de dagelijkse praktijk niet mogelijk. Daarom ondergingen alle meisjes ook een GnRH agonist test. In Nederland wordt aangehouden dat een maximale LH spiegel van 3 IU/L met een maximale oestradiolspiegel van 50 pmol/l een goede onderdrukking van de puberteit weerspiegelt. Deze afkapwaarden zijn desalniettemin arbitrair.

Na 3 maanden GnRHa behandeling vertoonden alle meisjes een klinische stilstand van de puberteit en hadden 8 meisjes (5 meisjes met Tanner stadium 2 en 3 meisjes met stadium 3) een lagere puberteitsscore. Alle LH en FSH waarden (AUC_0, gemiddelde en maximum) tijdens het profiel waren signficant gedaald naar zeer lage, prepubertaire waarden. De maximale LH-spiegel tijdens de GnRH agonist test was daarentegen tussen de 3.1 en 7.3 IU/L bij 7 van de 21 meisjes (33%). Oestradiolspiegels waren onder de 50 pmol/l in 6 van de 7 meisjes en was 58 pmol/l in 1 meisje. We vonden geen significante verschillen in LH en FSH profielwaarden tussen de meisjes met een maximale LH-spiegel boven of onder de 3 IU/L tijdens de GnRH agonist test.

Onze resultaten laten zien dat behandeling met leuprorelide acetaat 3.75 mg subcutaan elke 4 weken leidt tot een effectieve onderdrukking van de centrale puberteit, aangetoond door de klinische stilstand van de puberteit alsmede de prepubertaire LH en FSH secretiepatronen. Een LH-afkapwaarde van maximaal 3 IU/L tijdens de GnRH agonist gaf bij 33% van de meisjes ten onrechte een onvoldoende onderdrukking van de puberteit aan. Deze afkapwaarde is dus mogelijk te laag voor de GnRH agonist test. We konden geen nieuwe LH-afkapwaarde tijdens de GnRH agonist test bepalen omdat alle meisjes prepubertaire LH profielen hadden. Toekomstig onderzoek in een grotere en meer heterogene groep kinderen is van belang om een nieuwe LH-afkapwaarde vast te stellen.

Hoofdstuk 3

Wij onderzochten de LH en FSH afgiftepatronen bij 10 kleine SGA geboren jongens, zowel voor de start als 3 maanden na de start van de GnRHa behandeling. Tevens onderzochten wij bij hoeveel jongens uit een groep van 14 kleine SGA jongens (12 jongens met Tanner stadium 2 en 2 jongens met stadium 3) een goede onderdrukking van de puberteit door de GnRH agonist test werd aangegeven. Hierbij hielden wij de op een consensus gebaseerde maximale LH-waarde van 3 IU/L met een maximale testosteronspiegel van 1 nmol/l aan. Na 3 maanden GnRHa behandeling vertoonden alle jongens klinisch een stilstand van de puberteit en was bij 2 jongens het puberteitsstadium afgenomen. Alle LH en FSH waarden (AUC_0, gemiddelde en maximum) tijdens het nachtelijk 12-uurs profiel waren signficant gedaald naar zeer lage, prepubertaire waarden. Testosteronspiegels waren significant gedaald van 6.5 (1.3-9.9) nmol/l naar 0.25 (0.10-0.53) nmol/l. De maximale LH-spiegels tijdens de GnRH agonist varieerde tussen de 3.3 en 5.2 IU/L bij 6 jongens (43%). Testosteronspiegels waren lager dan 1 nmol/l in alle 6 de jongens. De LH en FSH profielwaarden waren vergelijkbaar tussen de jongens met een maximale LH-spiegel boven of onder 3 IU/L tijdens de GnRH agonist test.

Er waren geen gegevens bekend over GH, IGF-I en IGFBP-3 spiegels tijdens GnRHa behandeling bij kleine SGA jongens. Voor start van de GnRHa behandeling waren de gemiddelde GH-spiegels in de groep kleine SGA jongens vergelijkbaar met de spiegels in een groep jongens met een normale lengte en vergelijkbaar puberteitsstadium. De gemiddelde IGF-I en IGFBP-3 spiegels waren signficant lager dan de populatie gemiddelden. Na 3 maanden GnRHa behandeling bleven de GH-profielwaarden

(AUC_0, gemiddelde en maximum) en IGF-I en IGFBP-3 spiegels vergelijkbaar met de waarden voor start van de GnRHa behandeling. De gemiddelde GH spiegels bleven ook vergelijkbaar met de controlegroep. Eerder onderzoek bij jongens met een normale lengte heeft aangetoond dat GH en IGF-I spiegels pas stijgen wanneer een testikelvolume van >10-15 ml is bereikt. Omdat voor de start van de GnRHa behandeling alle jongens in onze studiegroep een testikelvolume van 4-8 ml hadden, kan dit verklaren waarom we na 3 maanden GnRHa behandeling geen significante veranderingen in GH en IGF-I spiegels vonden.

Samenvattend resulteert behandeling met leuprorelide acetaat 3.75 mg subcutaan elke 4 weken in een efficiënte remming van de centrale puberteit, aangetoond door de klinische stilstand van de puberteit alsmede de prepubertaire LH en FSH secretiepatronen. Een LH-afkapwaarde van maximaal 3 IU/L tijdens de GnRH agonist gaf ten onrechte een onvoldoende remming van de puberteit aan bij bijna de helft van de jongens. Voor start van de GnRHa behandeling waren IGF-I en IGFBP-3 spiegels laag, hoewel gemiddelde GH-spiegels normaal waren voor het puberteitsstadium. GH, IGF-I en IGFBP-3 spiegels veranderden niet tijdens de 3 maanden GnRHa behandeling, wat overeenkomt met het feit dat deze spiegels vergelijkbaar zijn tussen prepubertaire en vroeg-pubertaire jongens.

Hoofdstuk 4

Uitstel van de puberteit door middel van GnRHa behandeling is één van de behandelingsopties in pubertaire kleine SGA geboren kinderen. Het effect van GnRHa behandeling op GH, IGF-I en IGFBP-3 spiegels was echter niet bekend. Wij onderzochten veranderingen in nachtelijke GH-spiegels en bepaalden IGF-I en IGFBP-3 spiegels bij 24 kleine SGA geboren meisjes (17 meisjes met Tanner stadium 2 en 7 meisjes met stadium 3). Tevens vergeleken wij de GH-profielwaarden (AUC_0, gemiddelde en maximum) met de waarden zoals beschreven bij prepubertaire, kleine SGA meisjes.

Voor de start van de GnRHa behandeling waren de gemiddelde GH-spiegels in de groep pubertaire, kleine SGA meisjes vergelijkbaar met die in de groep prepubertaire, kleine SGA meisjes. IGF-I en IGFBP-3 spiegels waren significant lager dan de populatie gemiddelden. Na 3 maanden GnRHa behandeling waren de gemiddelde GH-spiegels significant gedaald en waren de spiegels in de GnRHa-behandelde groep meisjes significant lager dan die in de prepubertaire groep meisjes. Tevens vertoonden de 8

GnRHa-behandelde meisjes met een meer dan 40% daling van de GH-spiegels ook een significant grotere daling van IGF-I en IGFBP-3 spiegels, vergeleken met de GnRHa-behandelde meisjes met een daling van de GH-spiegels tussen de 0% en 40%.

Concluderend, pubertaire, kleine SGA geboren meisjes vertonen niet de stijging in GH-spiegels zoals wordt gevonden bij pubertaire meisjes met een normale lengte. De gemiddelde GH-spiegels daalden significant tijdens de 3 maanden GnRHa behandeling naar waarden significant lager dan die bij prepubertaire, kleine SGA meisjes. Het combineren van GnRHa behandeling met GH-behandeling zou daarom de volwassen lengte in deze groep kinderen kunnen bevorderen.

Hoofdstuk 5

Het is onbekend of uitstel van de puberteit middels GnRHa behandeling leidt tot een betere volwassen lengte en welke GH dosis daarbij het meest effectief is. Tevens waren er geen gegevens beschikbaar over het effect van gecombineerde behandeling met GnRHa en 1 mg GH/m^2/dag (groep A) of 2 mg GH/m^2/dag (groep B) op GH, IGF-I en IGFBP-3 spiegels en 1^e-jaars groei.

Na 1 jaar gecombineerde behandeling waren de nachtelijke GH-spiegels significant gestegen in beide groepen, en alle waarden (AUC_0, gemiddelde en maximum) waren significant hoger in groep B. Na de subcutane GH injectie om 20.00u bleven de GH-spiegels in groep B significant langer boven de 40 mU/L en 20 mU/L, vergeleken met groep A. In beide groepen waren de gemiddelde en maximum GH-spiegels echter lager in onze studiegroep ten opzichte van prepubertaire, kleine SGA kinderen. Zoals beschreven in hoofdstuk 4, daalden de gemiddelde GH-spiegels significant na 3 maanden GnRHa behandeling. In dit hoofdstuk toonden wij aan dat GH-behandeling niet resulteert in een stijging van de GH-spiegels tot vergelijkbare waarden zoals gevonden bij GH-behandelde, prepubertaire SGA kinderen.

IGF-I spiegels waren significant hoger in groep B, vergeleken met groep A. In groep B had 88.9% van de kinderen een IGF-I spiegel in de hoogste quintiel (>0.84 SDS) ten opzichte van 43.8% in groep A. Het percentage kinderen met IGF-I spiegels boven de normale range (>+2 SDS) was vergelijkbaar tussen beide groepen. De IGFBP-3 spiegels stegen significant in beide groepen en de stijging was vergelijkbaar tussen beide groepen.

Na 1 jaar gecombineerde behandeling was de groeisnelheid significant hoger in groep B (6.9 (4.0-7.4) SDS) ten opzichte van groep A (4.3 (2.8-5.5) SDS). In groep B

hadden kinderen met Tanner stadium 2 voor de start van de GnRHa behandeling een significant hogere groeisnelheid dan kinderen met stadium 3 (7.1 (5.6-8.5) SDS vs. 4.0 (3.3-5.0) SDS). Gecombineerde behandeling moet dus het liefst worden gestart aan het begin van de puberteit.

Samenvattend toonden kleine SGA kinderen die worden behandeld met GnRHa en 1 mg of 2 mg GH/m^2/dag een dosis-afhankelijke stijging van GH en IGF-I spiegels, alsmede van de 1^e-jaars groeirespons. Onze resultaten suggereren dat behandeling met een GnRH analoog in combinatie met 2 mg GH/m^2/dag mogelijk effectiever is dan met 1 mg GH/m^2/dag. Het vervolgen van deze groep kinderen tot de volwassen lengte, alsmede onderzoek naar de metabole effecten van deze gecombineerde behandeling, is echter noodzakelijk voordat definitieve conclusies kunnen worden getrokken.

Hoofdstuk 6

Het effect van gecombineerde behandeling met een GnRH analoog en 1 mg of 2 mg GH/m^2/dag op de lichaamssamenstelling, bloeddruk, insulinegevoeligheid en het lipidenprofiel was bij pubertaire, kleine SGA geboren kinderen nog niet eerder onderzocht. Wij onderzochten tijdens een gerandomiseerde studie de effecten van gecombineerde behandeling op deze parameters in een groep van 45 (29 meisjes) kleine SGA geboren kinderen.

De BMI SDS steeg tijdens 3 maanden GnRHa behandeling significant, hoewel de SD-scores signficant lager bleven dan het populatie gemiddelde (van -0.98 (-1.28 tot -0.68) naar -0.90 (-1.22 tot -0.57) SDS). De bloeddruk, insulinegevoeligheid en het lipidenprofiel waren vergelijkbaar met de waarden voor start van de GnRHa behandeling.

Tijdens een studieperiode van 2 jaar ontwikkelden kinderen die werden behandeld met GnRHa en 1 mg GH/m^2/dag een hogere vetmassa SDS gecorrigeerd voor het geslacht en de lengte (vetmassa SDS_{lengte}), en een hoger centraal en perifeer vetpercentage, vergeleken met kinderen die werden behandeld met GnRHa en 2 mg GH/m^2/dag. Vergeleken met de waarden vóór start van de GnRHa behandeling steeg het centraal vetpercentage significant in beide GH groepen. De vetmassa SDS_{lengte} en het perifeer vetpercentage stegen alleen in de groep kinderen behandeld met GnRHa en 1 mg GH/m^2/dag. De vetvrije massa SDS_{lengte} (spiermassa) steeg alleen in de groep kinderen die werden behandeld met GnRHa en 2 mg GH/m^2/dag.

Tijdens de gecombineerde behandeling waren de bloeddruk, insulinegevoeligheid en het lipidenprofiel vergelijkbaar tussen de 2 GH dosisgroepen. Zoals verwacht daalde de insulinegevoeligheid. De dispositie index bleef echter in beide GH groepen vergelijkbaar met de waarden voor de start van de GnRHa behandeling. De lipidenspiegels bleven binnen de normale grenzen.

Onze bevindingen laten zien dat GH-behandeling met 2 mg/m^2/dag in combinatie met GnRHa een positieve invloed had op de vetmassa SDS_{lengte}, het centraal en perifeer vetpercentage en op de vetvrije massa SDS_{lengte}. De bloeddruk, insulinegevoeligheid en het lipidenprofiel waren vergelijkbaar tussen beide GH groepen en wij vonden op deze korte termijn geen nadelige effecten van de gecombineerde behandeling op deze parameters. Gecombineerde behandeling met 1 mg of 2 mg GH/m^2/dag en GnRHa kan dus worden beschouwd als een potientiële behandeling voor kleine SGA geboren kinderen. Het vervolgen van deze groep kinderen tot de volwassen lengte is echter noodzakelijk voordat kan worden geconcludeerd welke GH dosering, naast GnRHa, tot de meest optimale volwassen lengte leidt zonder nadelige metabole effecten.

Hoofdstuk 7

Verlaagde IGFBP-1 spiegels geven een hogere insulinesecretie weer en zijn geassocieerd met een groter risico op cardiovasculaire ziekten bij volwassenen. Voorgaande studies hebben laten zien dat kleine SGA geboren personen een verhoogde insulinesecretie hebben. Gegevens over IGFBP-1 spiegels bij kleine SGA personen zijn echter schaars en associaties met de -575 G/A *IGFBP1* promoter single nucleotide polymorphism (SNP) zijn niet eerder onderzocht. Wij hebben, in een groep van 272 kleine SGA geboren personen, IGFBP-1 spiegels bepaald en vergeleken met spiegels in een groep van 330 controlepersonen met vergelijkbare leeftijd en een normale lengte.

In de prepubertaire en pubertaire groep kleine SGA kinderen met een laag vetpercentage SDS waren IGFBP-1 spiegels vergelijkbaar met de controlegroep. Kleine SGA geboren jong-volwassenen met een normaal vetpercentage SDS hadden significant lagere IGFBP-1 spiegels ten opzichte van de controlegroep. Na correctie voor leeftijd en geslacht was IGFBP-1 SDS negatief gecorreleerd met insulinespiegels (r=-0.40), systolische bloeddruk SDS (r=-0.26), triglyceriden (r=-0.31) en IGF-I SDS (r=-0.48), en positief gecorreleerd met HDL-cholesterol spiegels (r = 0.29). In de

groep kleine SGA jong-volwassenen was IGFBP-1 SDS negatief gecorreleerd met de insulinesecretie (r=-0.43), het vetpercentage SDS (r=-0.43) en IGF-I SDS (r=-0.51).

In de prepubertaire groep kleine SGA kinderen waren IGFBP-1 spiegels het laagst in de groep met het GG genotype en significant hoger in de groep met het GA en AA genotype. Het genotype verklaarde 4.6% van de variatie in IGFBP-1 spiegels. De -575 G/A SNP beïnvloedde de associatie tussen IGFBP-1 SDS en insulinesecretie: kleine SGA kinderen met vergelijkbare insulinesecretie en drager van het GG genotype hadden lagere IGFBP-1 spiegels ten opzichte van kinderen met het AA genotype.

Onze studie laat zien dat IGFBP-1 spiegels een indruk geven van de metabole status van kleine SGA personen: spiegels waren vergelijkbaar met de controlegroep in de groep kleine SGA geboren kinderen met een lage vetmassa, terwijl de spiegels signficant lager waren in kleine SGA jong-volwassenen met een normale vetmassa. IGFBP-1 spiegels correleerden met verschillende cardiovasculaire parameters. De -575 G/A *IGFBP1* promoter SNP was geassocieerd met IGFBP-1 spiegels en de relatie tussen insulinesecretie en IGFBP-1 spiegels. IGFBP-1 speelt dus zeer waarschijnlijk een rol binnen de complexe interacties tussen de IGF-IGFBP as, de glucosehuishouding en het lipidenmetabolisme.

Hoofdstuk 8

Wij onderzochten de relatie tussen 2 *IGFBP3* promoter SNPs (-202 A/C SNP en de -185 C/T SNP), IGFBP-3 spiegels, spontane groei en de groeirespons tijdens GH-behandeling bij 292 prepubertaire, kleine SGA geboren kinderen. Tevens vergeleken wij de *IGFBP3* promoter methyleringsstatus bij 22 kleine SGA personen ten opzichte van 14 controlepersonen met een normale lengte.

IGFBP-3 spiegels waren het laagst in de groep kinderen met het -202 CC genotype en significant hoger in de groep kinderen met het -202 AC en -202 AA genotype. Het genotype verklaarde 4.0% van de variatie in IGFBP-3 spiegels. Kleine SGA kinderen met het C^{-202}/C^{-185} haplotype hadden significant lagere IGFBP-3 spiegels (-1.92 ±1.03 SDS) en waren significant kleiner (-3.31 ±0.58 SDS) ten opzichte van kinderen met het A^{-202}/C^{-185} haplotype (IGFBP-3 SDS: -0.95 ±1.21 SDS, lengte SDS: -2.94 ±0.60 SDS). Het haplotype verklaarde 12.2% van de variatie in IGFBP-3 spiegels. Na 12 maanden GH-behandeling hadden kleine SGA kinderen met het C^{-202}/C^{-185} haplotype een significant hogere stijging van IGFBP-3 spiegels en een significant betere inhaalgroei dan kinderen met het A^{-202}/C^{-185} haplotype. Dit resulteerde in vergelijkbare IGFBP-3 spiegels en lengte na 12 maanden GH-behandeling.

Het methyleringspercentage in de regio rondom beide SNPs was laag. De methyleringsstatus was vergelijkbaar tussen kleine SGA personen met het C^{-202}/C^{-185} en het A^{-202}/C^{-185} haplotype, alsmede tussen controlepersonen met het C^{-202}/C^{-185} en het A^{-202}/C^{-185} haplotype. Drie CpGs die betrokken zijn bij de binding van de transcriptiefactoren Sp1 en p53 toonden een net niet significant hoger methyleringspercentage in de groep kleine SGA personen met het A^{-202}/C^{-185} haplotype ten opzichte van controles met het A^{-202}/C^{-185} haplotype (13.6% ten opzichte van 6.8%).

Onze bevindingen laten zien dat genetische variatie in de *IGFBP3* promoterregio is geassocieerd met IGFBP-3 spiegels, spontane groei alsmede de groeirespons tijdens GH-behandeling in een groep kleine SGA geboren kinderen. Hoewel er een trend aanwezig was dat CpGs die zijn betrokken bij de binding van 2 transcriptiefactoren meer gemethyleerd zijn in SGA jong-volwassenen ten opzichte van controlepersonen, was het methyleringspercentage van de onderzochte regio laag.

Hoofdstuk 9

In de algemene discussie worden de resultaten van de verschillende studies in relatie tot de huidige literatuur besproken. Wij sluiten dit hoofdstuk af met algemene overwegingen en suggesties voor toekomstig onderzoek.

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Curriculum Vitae of Daniëlle van der Kaay

Daniëlle van der Kaay was born in Rozenburg on August 8th, 1978. In 1996, she graduated from high school (Christelijke Scholengemeenschap Westland-Zuid, Vlaardingen), and started her medical training at the Erasmus University of Rotterdam. During her medical training, she participated in several committees of the Medical Factorial Society of the Erasmus University. In 2000 she participated in a research project investigating novel approaches in the treatment and prevention of meningococcal infections, which resulted in 2 articles (dr. J.A. Hazelzet and dr. E.D. de Kleijn). In 2001, she traveled through Australia and New Zealand for 2 months. After obtaining her medical degree in March 2003, Daniëlle started working as a resident at the Department of Pediatrics in the Groene Hart Hospital in Gouda. In July 2003, she started as a research fellow at the Department of Pediatrics, Division of Endocrinology, Erasmus MC-Sophia Children's Hospital in Rotterdam (prof.dr. A.C.S. Hokken-Koelega). From January 2007 until July 2008, Daniëlle performed genetic research in a Dutch study cohort at the Department of Endocrinology of the Centre Hospitalier Universitaire Ste-Justine in Montreal, Canada (prof.dr. C.L. Deal). In October 2008, she will start as a pediatric resident in the Sophia Children's Hospital in Rotterdam. In January 2009 Daniëlle will start her pediatric trainingship in the Sophia Children's Hospital in Rotterdam.

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