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METABOLIC RESPONSIVENESS TO GROWTH HORMONE IN CHILDREN

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**To Jens for all his support,
patience and love!**

**“Glück ist jetzt”
(Happiness is now)**

**Hope is definitely not the same thing as optimism.
It is not the conviction that something will turn out well,
but the certainty that something makes sense,
regardless of how it turns out.
(Václav Havel)**

ABSTRACT

Metabolic effects of growth hormone (GH) therapy in short children have not been clearly established owing to the previous lack of controlled trials studying the metabolic outcome in response to different GH doses despite the known effects of GH on insulin sensitivity, lipid profile, and body composition. It has previously been shown that individualized GH doses during catch-up growth significantly reduce the proportion of unexpectedly good and poor responders around a predefined individual growth target in short prepubertal children. In the research on which this thesis is based, 87 prepubertal children were treated with six different GH doses, individually chosen according to a prediction model of GH sensitivity regarding linear bone growth.

The first hypothesis was that the variance of the metabolic response during individualized GH treatment would be reduced. This was confirmed: individualized GH dosing during catch-up growth reduced the variance in insulin and HOMA by 34.2 % and 38.9 %, respectively (Paper I).

This led to the second hypothesis that metabolic responsiveness to GH treatment partly parallels and partly dissociates from the longitudinal growth response. This too was confirmed: a GH dose-dependent anabolic component was identified in contrast to a dose-independent lipolytic component (Papers I and II). This finding raised the question of whether different thresholds would achieve certain metabolic effects, such as anabolic effects, lipolytic effects, and glucose metabolism.

A pharmaco-proteomic approach was introduced in order to identify previous unknown biomarkers to predict metabolic responses to GH treatment as well as to investigate the physiology of GH (Papers III and IV). These results have been published recently, showing dissociations between GH-mediated longitudinal bone growth and bone mineralization and thereby confirming the second hypothesis named above (Paper III).

The third hypothesis was that patients with and without classical GH deficiency (GHD) had correspondingly different thresholds of responsiveness to GH treatment among different metabolic functions. The data confirmed the hypothesis and, moreover, increasing effective GH doses were needed to achieve specific metabolic effects (Paper V).

This research provides further evidence of the benefits of individualized GH dosing in order to maintain metabolic functions within age-adjusted reference values and to normalize body composition. In the long term, this might minimize metabolic and cardiovascular risk factors in children suffering from GHD or reduced GH responsiveness.

PUBLICATIONS AND MANUSCRIPTS

- I. **Ralph Decker**, Kerstin Albertsson-Wikland, Berit Kriström, Andreas F. M. Nierop, Jan Gustafsson, Ingvar Bosaeus, Hans Fors, Ze'ev Hochberg, Jovanna Dahlgren
"Metabolic outcome of GH treatment in prepubertal short children with and without classical GH deficiency"
Clinical Endocrinology (Oxf.) 2010;73:346–354.
- II. **Ralph Decker**, Kerstin Albertsson-Wikland, Berit Kriström, Maria Halldin, Jovanna Dahlgren
"Decreased GH dose after the catch-up growth period maintains metabolic outcome in short prepubertal children with and without classic GH deficiency"
(resubmitted to Clinical Endocrinology)
- III. Björn Andersson, **Ralph Decker**, Andreas F. M. Nierop, Ingvar Bosaeus, Kerstin Albertsson-Wikland, Gunnel Hellgren
"Protein profiling identified dissociations between growth hormone-mediated longitudinal growth and bone mineralization in short prepubertal children"
Journal of Proteomics 2011;74:89–100.
- IV. **Ralph Decker**, Björn Andersson, Andreas F. M. Nierop, Ingvar Bosaeus, Jovanna Dahlgren, Kerstin Albertsson-Wikland, Gunnel Hellgren
"Protein markers predict body composition during growth hormone (GH) treatment in short prepubertal children"
(manuscript)
- V. **Ralph Decker**, Anders Nygren, Kerstin Albertsson-Wikland, Berit Kriström, Andreas F. M. Nierop, Jan Gustafsson, and Jovanna Dahlgren
"Different thresholds of tissue-specific dose-responses to growth hormone in short prepubertal children"
(submitted to BMC Medical Informatics and Decision Making)

LIST OF ABBREVIATIONS

1D	One-dimensional
ACN	Acetonitrile
AITT	Arginine–insulin tolerance test
ALP	Alkaline phosphatase
AITT	Arginine–insulin tolerance test
ANOVA	Analysis of variance
Apo A-I	Apolipoprotein A-I
Apo A-II	Apolipoprotein A-II
Apo B	Apolipoprotein B
Apo C-I	Apolipoprotein C-I
App	Appendicular (arms and legs)
AUC	Area under the curve
BA _{HA}	Height-adjusted bone area
BMC	Bone mineral content
BMC _{HA}	Height-adjusted bone mineral content
BMC _{HAapp}	Height-adjusted appendicular bone mineral content
BMD	Bone mineral density
CHAPS	(3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
CM10	Weak cation exchange 10 protein chip array
CRP	C-reactive protein
CV	Coefficient of variation
Δ	Delta (changes between to time points)
DELFIA	Dissociation-enhanced lanthanide fluorescence immunoassay
DXA	Dual-energy X-ray absorptiometry
diffMPH _{SDS}	Difference from midparental height SDS
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
FIX	Fixed standard dose
FM	Fat mass
FFA	Free fatty acids
FFMI	Fat-free mass index
FSS	Familial short stature
GH	Growth hormone
GHD	Growth hormone deficiency
GH _{max}	Maximum GH secretion peak
GHRH	Growth hormone releasing hormone
GP-GRC	Göteborg Pediatric Growth Research Center
HBA	Haemoglobin A
HDL	High-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hGH	Human growth hormone
HOMA	Homeostatic model assessment
H50	Hydrophobic reverse phase surface protein chip array
ICP	Infancy–childhood–puberty (model of growth)
IGF-I	Insulin-like growth factor I

IGF-II	Insulin-like growth factor II
IGFBP-3	Insulin-like growth factor-binding protein 3
IL-1	Interleukin 1
IL-6	Interleukin 6
IMAC30	Immobilized metal affinity capture 30 protein chip array
IRP	International reference preparation
ISS	Idiopathic short stature
ITT	Intention to treat
JAK	Janus kinase
kDa	Kilodalton
LBM	Lean body mass
LDL	Low-density lipoprotein
LST	Lean soft tissue
LST _{HA}	Height-adjusted lean soft tissue
LST _{HAapp}	Height-adjusted appendicular lean soft tissue
MAPK	Mitogen-activated protein kinases
LVDd	Left ventricular diameter in diastole
mApo A-II	Monomeric apolipoprotein A-II
MES	3-N-morpholino ethane sulfonic acid
MPH	Midparental height
mU	Milliunits
m/z	Mass over charge
NMR	Nuclear magnetic resonance
OGP	n-Octyl β-D-glucopyranoside
PBS	Phosphate buffered saline
PCA	Principal component analysis
PP	Per protocol
RIA	Radioimmunoassay
RID	Reduced individualized dose
RNA	Ribonucleic acid
SAA4	Serum amyloid A4
SD _{res}	Standard deviation of the residuals
SDS	Standard deviation scores
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELDI-TOF MS	Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry
SGA	Small for gestational age
³⁵ SO ₄	Sulfate containing a radioactive isotope of sulphur
Sm	Somatomedin
SPA	Sinapinic acid
SS	Somatostatin
STAT	Signal transducer and activator of transcription
TFA	Trifluoroacetic acid
TNF-α	Tumour necrosis factor-alpha
Tris-HCL	Tris(hydroxymethyl) aminomethane hydrochloride
TTR	Transthyretin
UID	Unchanged individualized dose

CONTENTS

Abstract

Publications and manuscripts

List of Abbreviations

Contents

1	Introduction	10
2	Hypotheses	11
2.1	Aims	12
3	Background	13
3.1	GH physiology	13
3.1.1	GH secretion	13
3.1.2	GH sensitivity	16
3.1.3	GH effects	19
3.1.4	IGF-I	21
3.2	Growth and GH treatment	23
3.2.1	Growth	23
3.2.2	GH treatment	24
3.2.3	Prediction models to estimate GH responsiveness	26
3.2.4	Metabolic responsiveness to GH treatment	28
4	Patients and methods	32
4.1	Study design	32
4.2	Patients	34
4.3	Ethical consideration	35
4.4	Methods	36
4.4.1	Auxology measurements (Papers I-V)	36
4.4.2	Measurement of bone age (Papers I-II and V)	36
4.4.3	Measurement of body composition (Papers I-V)	36
4.4.4	Echocardiography (Paper V)	38
4.4.5	Spontaneous GH secretion (Papers I-V)	38
4.4.6	Laboratory analyses (Papers I, II, and V)	39
4.4.7	Proteomics (Papers III-IV)	40
4.4.8	Statistical methods	44
5	Results and comments	47
5.1	Catch-up growth part (Paper I)	47
5.1.1	Reduced variance of metabolic responses	47
5.1.2	Dissociation of anabolic and lipolytic GH effects	47
5.2	Maintenance growth part (Paper II)	50
5.2.1	Reduced variance of metabolic responses	50
5.2.2	Dissociation of anabolic and lipolytic GH effects	51
5.2.3	Bivariate regression	53

5.3	The proteomic approach (Papers III-IV)	55
5.3.1	Dissociation between bone growth and bone mineralization (Paper III)	55
5.3.2	Biomarkers associated with GH effects on body composition (Paper IV).....	55
5.3.3	Tissue-specific biomarkers associated with GH effects (Papers III and IV).....	56
5.4	Different Thresholds of GH effects (Paper V).....	58
5.4.1	Dose-dependency.....	58
5.4.2	Effective GH dose at 50% effect [ED 50%]	59
6	Discussion	61
6.1	Continuum of GH responsiveness.....	61
6.1.1	Continuum.....	61
6.1.2	Responsiveness.....	62
6.2	Metabolic responsiveness to GH treatment.....	63
6.2.1	Reduced variance of metabolic responses.....	63
6.2.2	Metabolic responses can dissociate from the growth response.....	65
6.3	Responsiveness of nutrition markers	66
6.4	Thresholds of metabolic responsiveness	67
7	Conclusions	69
8	Future perspectives	70
9	References	72
10	Acknowledgements	83
11	Outline of the papers	85
	Original manuscripts.....	86

1 INTRODUCTION

Metabolic effects of growth hormone (GH) therapy in short children have not been clearly established, owing to the previous lack of controlled trials studying the metabolic outcome in response to different GH doses despite the known effects of GH on insulin sensitivity, lipid profile, and body composition. It has been shown that individualized GH doses during catch-up growth significantly reduce the proportion of unexpectedly good and poor responders around a predefined individual growth target in short prepubertal children¹.

Using prediction models^{2, 3} to estimate individualized GH responsiveness makes it possible to avoid giving insufficient or excessive GH doses to children being treated for short stature¹. Growth in response to individualized GH treatment has been investigated in several studies in short prepubertal children^{1, 4, 5}; however, there have been few evaluations of metabolic outcomes following individualized GH treatment during different phases of growth⁶⁻⁸. GH effects beyond the promotion of linear growth in children comprise changes in body composition, insulin sensitivity, and protein and lipid metabolism^{9, 10}. The antagonistic effect of GH on insulin in particular has been considered to be an adverse effect, especially in children born small for gestational age (SGA)^{10, 11}, but high insulin levels without impaired insulin sensitivity may be needed for growth promotion, most obviously in fetal life¹².

Data show that the range of growth responses around a preset target, such as the mid-parental height standard deviation score (MPH_{SDS}), narrows after approximately two years of individualized treatment¹. This suggests that dosing optimized for each individual child in terms of the effects on height may be regarded as the same biological dose. Similarly, it was hypothesized that the metabolic response can also be individualized in the same way.

As healthy children approach the onset of puberty, there appears to be a decrease in GH secretion¹³ that does not compromise their growth, although height velocity decreases slightly¹⁴⁻¹⁶. A decreased GH dose during the years before puberty and after a previous catch-up period has never before been tested in a randomized study, but is presented in the current research. We showed recently that a 50% reduction in the individualized GH dose was sufficient to ensure channel-parallel growth after catch-up growth had been achieved¹⁷. Similarly, it was hypothesized that bisection of the individualized GH dose would result in a maintained metabolism and avoid hyperinsulinism.

2 HYPOTHESES

- A** Individualized GH dosing reduces the variance of metabolic outcomes
- B** Metabolic tissue responsiveness to GH treatment partly parallels and partly dissociates from longitudinal bone growth response
- C** Metabolic functions and body tissues exhibit different thresholds of GH responsiveness (**Figure 1**).

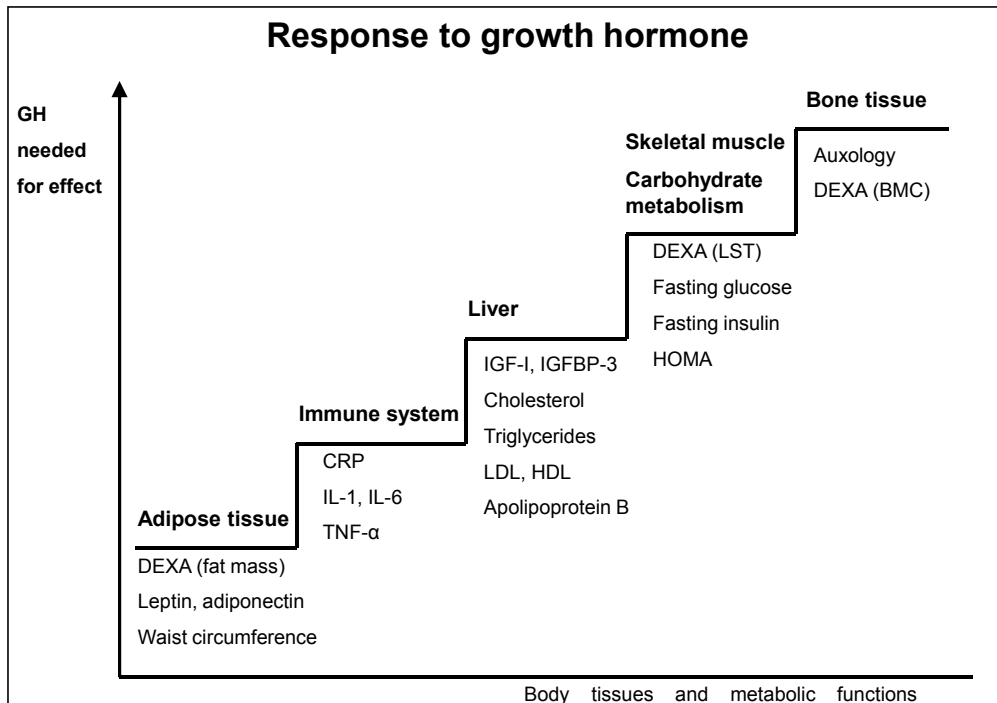


Figure 1 Different thresholds of GH response among tissues and metabolic functions are hypothesized. Surrogate markers are listed underneath the staircase: BMC (bone mineral content), CRP (C-reactive protein), DXA (dual-energy X-ray absorptiometry), HDL (high-density lipoprotein), HOMA (homeostatic model assessment), IGF-I (insulin-like growth factor I), IGFBP-3 (insulin-like growth factor binding protein 3), IL-1 (interleukin 1) IL-6 (interleukin 6), LDL (low-density lipoprotein), LST (lean soft tissue), TNF- α (tumour necrosis factor alpha).

2.1 AIMS

The overall aim was to study metabolic responsiveness by measuring metabolic responses with different approaches: Changes in group variability, contiguity between markers of the metabolic network, and dose-response relationships.

1. To evaluate whether the variance of metabolic response to GH treatment can be reduced by individualized GH treatment during catch-up growth (Paper I).
2. To evaluate whether the safety of continued individualized GH treatment is ensured by keeping metabolic variables within the reference range (Paper II).
3. To investigate whether a reduced GH dose during the maintenance growth period is beneficial for metabolic outcome (Paper II).
4. To get insight into metabolic factors associated with the anabolic effects of GH (Paper I and II).
5. To investigate whether a high-throughput proteomic approach can identify specific protein expression patterns associated with remodelling of body composition, longitudinal bone growth and bone mineralization during GH treatment (Paper III and IV).
6. To investigate whether there are tissue-specific dose-dependent thresholds for different GH effects (Paper V).

3 BACKGROUND

3.1 GH PHYSIOLOGY

3.1.1 GH secretion

3.1.1.1 Regulation

GH, a 22 kDa (kilodalton) large protein, is secreted in a pulsatile pattern from the somatotroph cells in the anterior pituitary with high peaks predominantly during night-time.

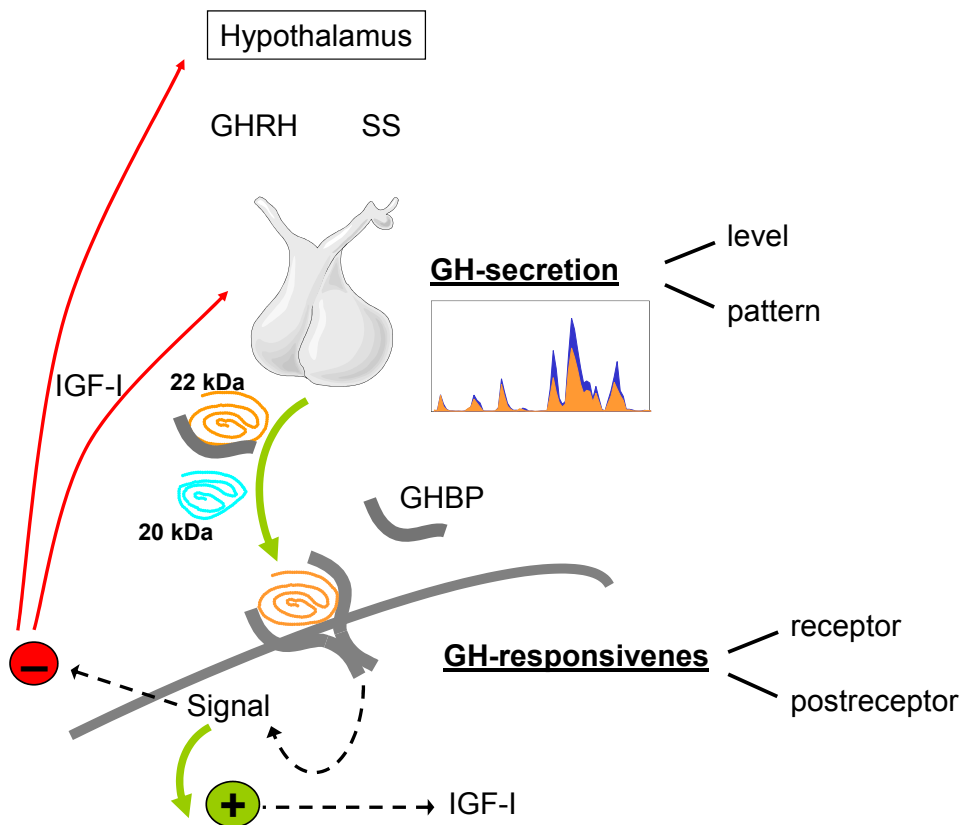


Figure 2 In blood, GH exists in a variety of isoforms. The major 22 kDa (kilodalton) isoform (or monomeric GH) constitutes approximately 50% of all GH in the blood and is believed to represent the biologically more active form of GH. A smaller proportion (~ 10%) of GH exists as a 20 kDa isoform, with reduced affinity to the GH receptor, while the remainder consists of a variety of GH fragments and GH aggregates. In addition, almost 50% of GH in blood is bound to the circulating high-affinity GH-binding protein (GHBP), which is structurally similar to the extracellular domain of the GH receptor. The extracellular signal resulting in negative feedback on hypothalamus and the pituitary is mediated by IGF-I. GHRH (growth hormone releasing hormone), SS (somatostatin), kDa isoforms of GH, (+) stimulation, (-) inhibition. Modified with permission of Chatarina Löfqvist, Growth hormone (GH) secretion in children, 2001 (ISBN 91-628-4503-9).

The secretion is regulated by two peptides from the hypothalamus, GH releasing hormone (GHRH) stimulating GH release and somatostatin (SS) inhibiting GH release. An intact interaction between these two is needed for the GH pulsatility. For optimal intracellular signalling and GH effects, a characteristic pulsatile pattern of diurnal GH secretion with sufficient peaks is required; in addition, GH levels should return to baseline between peaks, which is modulated by SS. GH has direct effects, such as lipolysis, and stimulates IGF-I secretion both locally and in the liver to mediate its indirect effects on metabolism, modulating body composition and promoting longitudinal bone growth. GH secretion is stimulated by stress, physical activity, hypoglycaemia, nutrition, amino acids, sleep, oestrogens, androgens, leptin, and thyroid hormones, but is inhibited by free fatty acids (FFA), insulin-like growth factor-I (IGF-I), and cortisol. The negative feedback loop inhibits its own secretion (**Figure 2**).

3.1.1.2 GH isoforms

Besides the most abundant 22 kDa GH isoform accounting for approximately 50% of the amount of GH in the blood, there are other immunoreactive molecular isoforms secreted from the pituitary. The second most common isoform is the 20 kDa isoform, which accounts for about 5-10%¹⁸. The 20-kDa isoforms have less binding capacity to the GH receptor than the 22-kDa isoforms. It has been suggested that the ratio of circulating non-22-kDa GH isoforms may have important implications for growth failure in some non-GH-deficient short children including SGA and girls with Turner syndrome¹⁹.

Coded on two related genes (hGH-N and hGH-V), multiple alternative mRNA splicing mechanisms and posttranslational modifications occur. At the mRNA level, hGH-N undergoes alternative splicing into 20 kDa and 22 kDa isoforms. Post-translationally, the principal and most abundant GH form of the pituitary, 22K GH, undergoes modifications such as acetylation, deamidation and oligomerization²⁰.

3.1.1.3 Changes over life-span

GH secretion in rats increases in late fetal life to then decrease post-partum²¹ and changes from high-frequency oscillation during early infancy to pulsatility. In humans, in early childhood, the GH secretory pattern can be described with higher basal levels and lower peak amplitudes compared to later in childhood²². During childhood there is a relationship between GH secretion and growth^{23, 24}, as well as during puberty^{23, 25}. It has been shown that the GH maximal peak during a stimulation test was lower in the

years before puberty¹³. Thereafter, a marked (two- to four-fold) increase in GH secretion during puberty²⁵⁻³² (**Figure 3**) parallels the growth spurt, which occurs earlier in girls than in boys. This increase in GH secretion seems to be an effect of sex steroid hormones, not least oestrogens, and results from increased pulse amplitude during the day and at night without any change in pulse frequency^{26, 28-30}. Throughout adulthood, GH continues to be secreted in a pulsatile fashion but the secreted amount declines continuously. However, due to differences in oestrogen levels, postpubertal women secrete higher GH levels than postpubertal men for similar GH effects^{33, 34}.

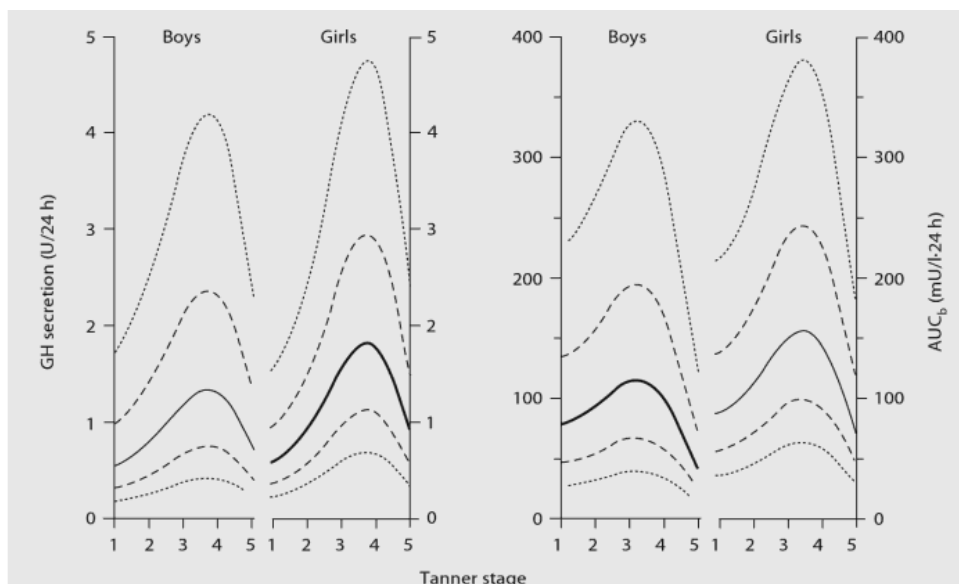


Figure 3 GH secretion rate during 24 hours (left) and area under the plasma concentration curve above baseline (AUC_b, right) for normal boys and girls at different stages of puberty. The figures show mean values (solid lines), ± 1 SD (dashed lines) and ± 2 SD (dotted lines). GH was assessed with a polyclonal assay with the IRP (international reference preparation) 66/217²⁵ With the permission of Kerstin Albertsson-Wikland (Albertsson-Wikland, K. & Rosberg, S. (2011) *Methods of Evaluating Spontaneous Growth Hormone Secretion*. In *Diagnostics of Endocrine Function in Children and Adolescents*. Karger AG, Basel, pp. 138-156)³⁵.

3.1.1.4 Impaired GH secretion

Mutations in the GH gene will lead to GH deficiency (GHD). The human GH/placenta lactogen gene cluster that gives rise to human GH is located on chromosome 17³⁶. Two genes located on chromosome 17 encode for GH: GH-1 (formerly named hGH-N, where N stands for normal), which is expressed in the pituitary, and hGH-V (V for variant), expressed in the placenta²⁰. Only the GH-1 gene is required for normal growth and health. Deletion or mutations in the GH-1 gene result in isolated GHD type IA^{37, 38} and is the most severe form of GHD. In these individuals, severe hypoglycaemia and

growth delay within six months after birth occur due to an absence of GH secretion³⁹, and an infancy–childhood transition is not observed in the growth charts of GHD patients until the onset of GH substitution⁴⁰. (See section 3.2. for more details).

It has been demonstrated that the congenital absence of the pituitary gland in humans does not result in decreased size at birth⁴¹. This is thought to be due to the direct effect of glucose and nutrition enabling normal placental function and growth factors such as IGF-I and IGF-II during fetal growth. On the other hand, mutations in the IGF-I and IGF-II genes, lead to major impairments in both prenatal and postnatal growth⁴².

3.1.2 GH sensitivity

The human GH receptor (GHR) was first cloned in 1987 by Leung et al.⁴³ and was further characterized by Godowski⁴⁴. The three-dimensional structure and interaction with human GH was elucidated by x-ray crystallography in 1992⁴⁵.

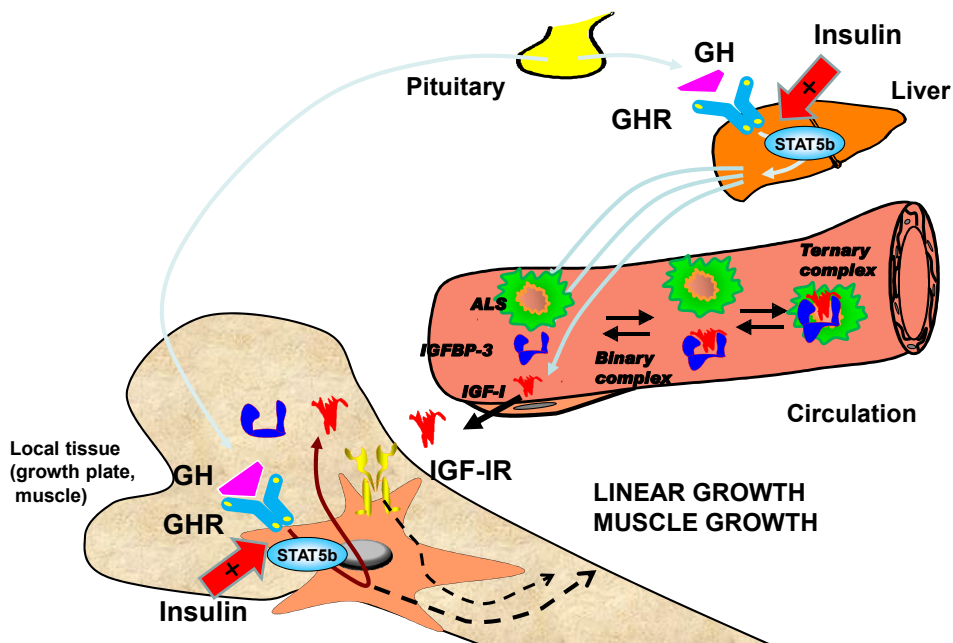


Figure 4 GH-IGF-I axis and its endocrine and autocrine/paracrine effects on growth. IGF-I has local (autocrine/paracrine) as well as endocrine effects⁴⁶. It has been proposed that free IGF-I may reflect the bioactivity of IGF-I in target tissues, thus relating a measurable parameter to biological responses⁴⁷. GH (growth hormone), GHR (GH receptor), IGF-IR (insulin-like growth factor I receptor), ALS (acid-labile subunit), STAT5b (signal transducer and activator of transcription 5B). Modified with permission of Peter Bang.

The receptor belongs to the class I cytokine receptor family also including the prolactin receptor and several cytokine receptors. This receptor family is characterized by the association with Janus kinases (JAKs) and signal transducer and activator of

transcription (STAT) 5b which couple ligand binding to tyrosine phosphorylation of signalling proteins recruited to the receptor complex⁴⁸.

The GHR is a transmembrane receptor. The coding part of the GHR gene consists of nine exons; exons 2 – 7 encode the extracellular domain, including the signal peptide, exon 8 encodes the transmembrane domain and exons 9 – 10 encode the cytoplasmatic domain of the receptor. The extracellular domain of the GHR is dimerized by a single hormone molecule, in turn activating the intracellular signal cascade that mediates the effects of GH ⁴⁹ (**Figure 4**). The soluble extracellular domain of the GH receptor acts as GH binding protein (GHBP)^{43, 50}.

In the brain of neonatal rabbits, the strongest immunoreactivity was evident in the cerebral cortex, in pyramidal cells of the hippocampus, and in neurones of the inferior and superior colliculi, brain stem reticular formation, dorsal thalamus and hypothalamus⁵¹. GH-mediated effects for specific GH receptors have been found in a variety of tissues⁵² (**Table 1**).

Table 1 Selected target tissues of direct GH and IGF-I effects

Selected target tissues	GH effect	IGF-I effect
Growth plate progenitor cells into chondrocytes	Differentiation ⁵³ IGF-I production	Clonal expansion/ hyperplasia ⁵⁴
Chondrocytes	Proliferation ⁵⁵	
Skeletal muscle cells	Hyperplasia ⁵⁶	
Preadipocytes into adipocytes	Low levels: Differentiation ⁵⁷ High levels: Clonal expansion ⁵⁸	Exponential growth of preadipocytes ⁵⁸
Liver, kidney, heart, adipose tissue	Proliferation ⁵⁹	
Hematopoietic progenitor cells	Proliferation ⁶⁰	
Smooth muscle	Proliferation ⁶¹	
Pancreatic B-cells	Proliferation ⁶²	
Fibroblasts	Proliferation ⁶³	

Mutations in the human GHR, such as 876-1 G>C⁶⁴ and heterozygous nonsense mutations (c.703C>T; p.Arg217X)⁶⁵ lead to high GH levels with very low IGF-I levels. The heterogeneity ranges from the most severe form, known as Laron syndrome, to less severe phenotypes such as partial GH insensitivity and idiopathic short stature (ISS)⁶⁵. It is not currently possible to evaluate in full detail the function of the intracellular cascades in the clinical setting, but with increasing knowledge we can expect diagnoses of ISS to diminish as new aetiologies are identified.

The regulation of IGF-I production by GH appears to be mediated entirely by signalling through the JAK2 pathway, via the phosphorylation of the STAT5b transcription factor⁶⁶. GH also signals through additional pathways, for example mitogen-activated protein kinases (MAPK), which are likely to be critical to the metabolic actions of GH⁶⁶ (Figure 5).

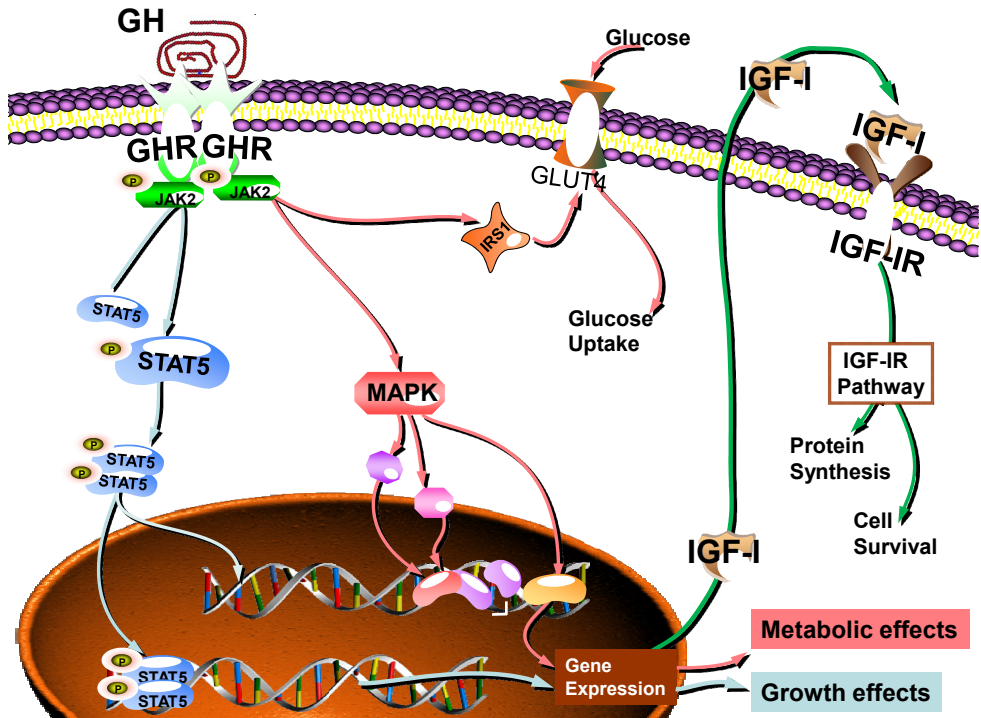


Figure 5 Growth hormone signalling. Multiple signalling pathways mediate the diverse effects of GH on growth and metabolism. Biologically active GH binds to two of its transmembrane receptors (GHR), causes dimerization of GHR, activation of the GHR-associated JAK2 (Janus kinase 2), and tyrosyl phosphorylation of both JAK2 and GHR⁶⁷. These events recruit and/or activate a variety of signalling molecules, including MAPKs (mitogen-activated protein kinases), STATs (signal transducers and activators of transcription), and IRS1 (insulin receptor substrate 1). These signalling molecules contribute to the GH-induced changes in enzymatic activity, transport function and gene expression that ultimately culminate in changes in growth and metabolism. Cross-talk among these signalling cascades in regulating specific genes suggests a GH-regulated signalling network. Activation of IRS1 by GH signalling results in increased glucose uptake by effecting the translocation of GLUT4 (glucose transporter protein 4) from an intracellular compartment to the plasma membrane⁶⁸. IGF-I expression is stimulated and acts via its own transmembrane receptor. Modified with permission of SABiosciences.

3.1.3 GH effects

3.1.3.1 General GH effects

GH is one of the major regulators of growth and metabolism. It is possible to survive without GH, but not without insulin or IGF-I⁶⁹. The effects of GH are multifaceted and include cellular growth, differentiation and intermediary metabolism. GH controls growth, fat accumulation, and sexual dimorphism of the male and female phenotype⁷⁰. GH is the only hormone that dose-dependently increases longitudinal bone growth⁷¹ by a direct effect on chondrocyte progenitor cells⁵³, which are then differentiated and produce IGF-I. Thus, GH stimulates longitudinal bone growth directly⁵³. The autocrine and paracrine action of IGF-I results in an increased number of cells by clonal expansion, thus hyperplasia⁵⁴. The growth-stimulating effect of GH is achieved partly through a direct action on cells and partly through stimulation of the liver to produce growth factors (IGFs).

Through knockout mice studies, knowledge has increased on the roles of circulating IGF-I versus autocrine effects of peripheral IGF-I⁷². In this mouse model, it was shown that liver-derived or endocrine IGF-I is not required for post-natal bone growth, but seems to be of vital importance for normal carbohydrate and lipid metabolism⁷³.

One of the alternative GH- enhancing pathways is stomach-derived ghrelin, which is a natural GH secretagogue in humans⁷⁴. Ghrelin is in turn diminished by hyperinsulinaemia, suggesting that insulin plays a role as physiological and dynamic modulator of plasma ghrelin and possibly mediates the effect of nutritional status on its concentration.⁷⁵ Ghrelin stimulates appetite and induces a positive energy balance leading to body weight gain; it also induces hyperglycaemia and reduces insulin secretion^{74, 76}.

3.1.3.2 GH effects on muscle tissue

In 1971, it was shown that muscle width and mass estimated by calf radiography increased during GH treatment in GH-deficient children⁷⁷. Using more accurate measurements, it was later shown that GH increases muscle mass and muscle strength⁷⁸. GH regulates the level of IGF-I mRNA in rat skeletal muscle in a gender-specific way⁷⁹. In humans, physiological GH bolus activates STAT5 signalling pathways in skeletal muscle, irrespective of ambient circulating glucose and insulin levels. Insulin resistance induced by GH occurs without a distinct suppression of insulin signalling proteins⁸⁰.

3.1.3.3 Metabolic effects of GH

GH stimulates hyperplasia and/or hypertrophy of metabolic active tissues. GH effects on carbohydrate, protein and lipid metabolism are classified as either acute insulin-like or chronic insulin-antagonistic (diabetogenic). GH and insulin act in an antagonistic manner with respect to carbohydrate metabolism. In healthy children, GH is one of the counter-regulatory hormones in hypoglycaemic states, together with adrenalin, glucagon and cortisol.

Current insights into the metabolic response of GH⁸¹ are given in **Table 2**.

Table 2 Insulin-like and insulin-antagonistic growth hormone effects.

Metabolic response	GH effect	Insulin-like	Insulin-antagonistic
Insulin resistance	+		●
Glucose uptake (m/a)	+ ⁽⁸²⁻⁸⁴⁾	●	
Glucose oxidation (m/a)	+ ^(83, 85)	●	
Glucose conversion to fatty acids (a)	+ ⁽⁸³⁾	●	
Glucose conversion to glycogen (m/a)	+ ^(82, 86)	●	
Glycogen stores (muscle and liver)	+ ⁽⁸⁷⁾	●	
Glycogenolysis (a)	- ⁽⁸⁴⁾	●	
Transport of amino acids (m)	+ ⁽⁸⁸⁻⁹⁰⁾	●	
Leucine oxidation (a)	+ ⁽⁹¹⁾	●	
Protein translational machinery	+ ⁽⁹²⁾	●	
Protein synthesis	+ ^(88-90, 92)	●	
Lipolysis (a)	+/- ⁽⁹³⁾	●	

(m) muscle; (a) adipose tissue; (+) stimulation; (-) inhibition

Transient insulin-like GH effects disappear after 3-4 hours incubation with GH. It is a moderate effect, characteristically half of the maximal response to insulin. Insulin-like GH effects are unaffected by inhibition of DNA-mediated RNA synthesis, hence indicating a signal transduction independent of gene activations⁹⁴. Neither IGF-I nor IGF-II accounts for these effects of GH⁹⁵.

The somatomedin hypothesis by Daughaday was based on the indirect action of GH through induction of an effector hormone (somatomedin = IGF-I)⁹⁶ (**Figure 6**).

Later it was shown that GH exerts direct effects on peripheral cells other than fat, muscle and liver cells, causing clonal expansion of haemopoietic stem cells⁶⁰. This gave rise to the dual effector theory, which states that GH stimulates the differentiation of progenitor cells that produce growth factors in peripheral tissues, e.g. IGF-I and their corresponding receptors for autocrine and paracrine action and clonal expression^{53, 54}.

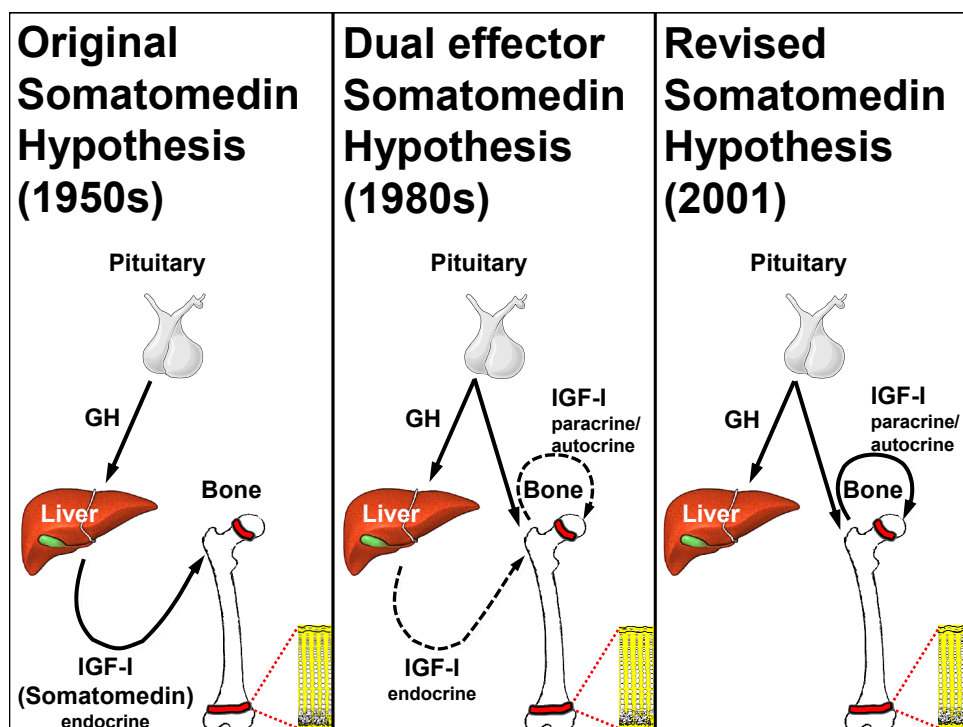


Figure 6 The original hypothesis proposed that the effect of GH on longitudinal body growth was mediated solely by liver-derived IGF-I. The revised hypothesis proposed that extra-hepatic tissues expressed IGF-I and are also involved in mediating local GH action on the growth plate. Data from tissue-specific gene-deletion experiments (IGF-I knockout models in mice) suggest that liver-derived IGF-I may not be essential for GH-stimulated postnatal growth and development⁶⁷.

Recent studies of mice with liver-specific and inducible IGF-I gene knockout indicated that liver-derived IGF-I is not necessary for postnatal body growth⁶⁹.

3.1.4 IGF-I

GH has been shown to induce the production of a serum factor, which was originally called the “sulphation factor” because of its ability to induce sulfate incorporation (sulphation) by cartilage from hypophysectomized rats *in vitro*⁹⁷. The sulphation factor was renamed somatomedin, later identified as IGF-I and IGF-II⁹⁶. IGF-I was found to increase the incorporation of radiolabelled sulfate ($^{35}\text{SO}_4$) into costal cartilage of hypophysectomized rats and produce in adipose tissue an acute stimulation of glucose oxidation that was not suppressed by insulin anti-serum⁹⁸.

Unlike GH, whose insulin-like effects disappear within three to four hours, IGF-I-stimulated glucose oxidation in adipose tissue remains linear for the entire four-hour incubation period⁹⁸. Although acute stimulation of tissues with GH rendered them

refractory to renewed insulin-like stimulation by GH, no such refractoriness to the action of IGF-I was seen. Furthermore, acute stimulation with IGF-I failed to render tissues refractory either to IGF-I or to GH. Finally, IGF-I failed to reproduce the delayed lipolytic effects produced by GH in conjunction with glucocorticoids. These results make it highly unlikely that IGF-I alone accounts for the delayed metabolic effects of GH⁹⁸.

The stimulatory properties of IGF have been shown to differ from those of GH in that IGF increased glucose oxidation as much as twenty-fold more than human GH (hGH) did. Furthermore, all of the IGF preparations stimulated glucose oxidation after 48 hours under conditions in which hGH suppressed glucose metabolism. Thus, it was unlikely that *extracellular* IGFs mediate the effects of hGH on glucose metabolism in adipocytes⁹⁵.

Cord blood leptin and IGF-I levels during the last trimester and at birth have been found to correlate to fetal growth and birth size⁹⁹. During postnatal life, circulating IGF-I is predominantly liver-derived but is not essential for normal postnatal growth⁶⁷. Therefore, it is proposed that non-hepatic tissue-derived IGF-I may be sufficient for growth and development¹⁰⁰. The somatomedin hypothesis has been modified during the last decades⁶⁷ (**Figure 6**).

It has been reported that spontaneous GH_{max} during a 24-hour profile correlates with IGF-I standard deviation scores (SDS) in children¹⁰¹. It is likely that the low levels in serum IGF-I associated with GHD are modified by nutritional status, because the liver is the principal source of IGF-I in the circulation⁷³ and hepatic production of IGF-I is substantially influenced by nutritional factors¹⁰². There exists a wide variation of concentrations of these peptides among healthy children, illustrating that other factors may also stimulate production of variations of IGF-I and insulin-like growth factor binding protein 3 (IGFBP-3), independently of GH¹⁰³. To differentiate among pathologies of the GH–IGF-I axis associated with growth disorders, there is a need to measure both IGF-I and IGFBP-3 and consider their ratio¹⁰⁴. The relationship between age and log (IGFBP-3) is positive in prepuberty and early puberty¹⁰⁵.

3.2 GROWTH AND GH TREATMENT

3.2.1 Growth

Height is determined on a population level mostly by nutrition¹⁰⁶. In children, growth velocity (change in height over time), is an important marker of childhood health¹⁰⁶. Body energy storage triggers a predictive–adaptive response that modifies the transition into childhood¹⁰⁶. The growth process in children is often subdivided into the different phases of growth (infancy, childhood, and puberty). These growth phases are regulated differently. IGF-I from the placenta dominates in fetal life and thyroid hormone in infancy. This is followed by a GH dose-dependent phase during childhood, and finally the pubertal growth spurt – an effect of interplay between sex steroids and a twofold to fourfold increase in GH secretion (more in girls than in boys)^{101, 107, 108}. This growth model is called the infancy–childhood–puberty growth model¹⁰⁹, which has been extended by the juvenility growth phase between childhood and puberty¹⁰⁶ (**Figure 7**). However, through leptin, nutrition interacts with the hypothalamic stimulation of onset of puberty¹¹⁰. In the human growth plate, oestrogen and androgen receptors are expressed throughout pubertal development, with no difference between the sexes¹¹¹. The combination of estradiol and a functioning receptor is responsible for the fusion of the human epiphyseal growth plate^{112, 113}.

Growth should be evaluated according to age- and gender-adjusted growth charts on a national basis¹⁶. Heights between -2 SDS to +2 SDS are considered normal and short stature is defined as height below -2 SDS on a statistical (not physiological) basis. However, a growth pattern decelerating as well as deviating markedly from target height (MPH_{SDS}) is pathological.

Chronic diseases and psychosocial deprivation should be excluded as causes before GHD or GH insensitivity is examined¹⁶. Serum concentrations of IGF-I and IGFBP-3, reflect nutrition, inflammation, and even endogenous GH secretion but, despite this, are often used as a screening evaluation of GH status in short children¹¹⁴. If the child is healthy, a 12-hour night (for growth) or a 24-hour spontaneous profile with normal nutrition, activity and sleep during the investigation, together with IGF-I and IGFBP-3 measurements, are needed for an appropriate evaluation of the GH–IGF-I axis.

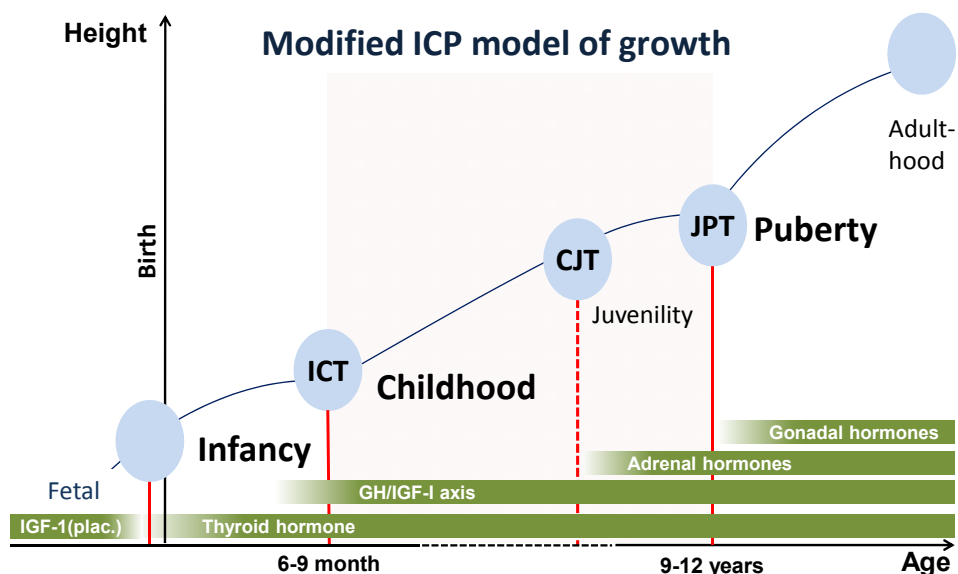


Figure 7 The childhood–juvenility growth phase studied in this thesis is marked with a grey area. The fetal growth phase is dominated by IGF-I from the placenta and thyroid hormone. During infancy, thyroid hormones plays a major role, alternating with the GH–IGF-I axis during childhood. The transition phases between the different growth phases are marked with circles, all indicating a hormonal axis to set in: At the infancy–childhood transition (ICT), the GH–IGF-I axis; at the childhood–juvenility transition (CJT), adrenal hormones; at the juvenility–puberty transition (JPT), the gonadal hormones. Modified with the permission of Kerstin Albertsson-Wikland from Hochberg, Albertsson-Wikland, *Pediatr Res* 64:2-7, 2008¹⁰⁶.

3.2.2 GH treatment

Children can be short as a consequence of low or absent GH secretion, i.e. GHD, or due to low or absent responsiveness to GH, i.e. insensitivity. In Sweden, the following considerations are taken into account in the decision to initiate GH treatment: firstly, the short child is growing channel-parallel or with pathologically low growth velocity and short stature, with height below -2.0 SDS and/or 1.5 SDS below MPH_{SDS} ; secondly, evidence of low GH secretion considering the relevant growth phase and nutritional status. If the GH_{max} is estimated by a provocation test, GH refractoriness due to a preceding spontaneous GH peak must also be considered. If the GHRH-GH axis is refractory to a new stimulus, an AITT will be falsely low¹¹⁵.

If the parents were healthy during childhood, the predefined goal is to reach a height near MPH_{SDS} . These parameters are taken into the prediction model of longitudinal bone growth² in order to estimate the predicted growth outcome, calculated on $33 \mu\text{g}/\text{kg}/\text{day}$, to decide whether or not GH treatment should be initiated.

After initiating GH treatment in short children, catch-up growth is usually observed¹¹⁶, with increasing height velocity and often normalizing height compared to MPH and/or age- and gender-related reference values. After this catch-up period of growth, height velocity for a channel-parallel growth is maintained during the following growth period until puberty, called the maintenance period of growth (**Figures 8 and 9**).

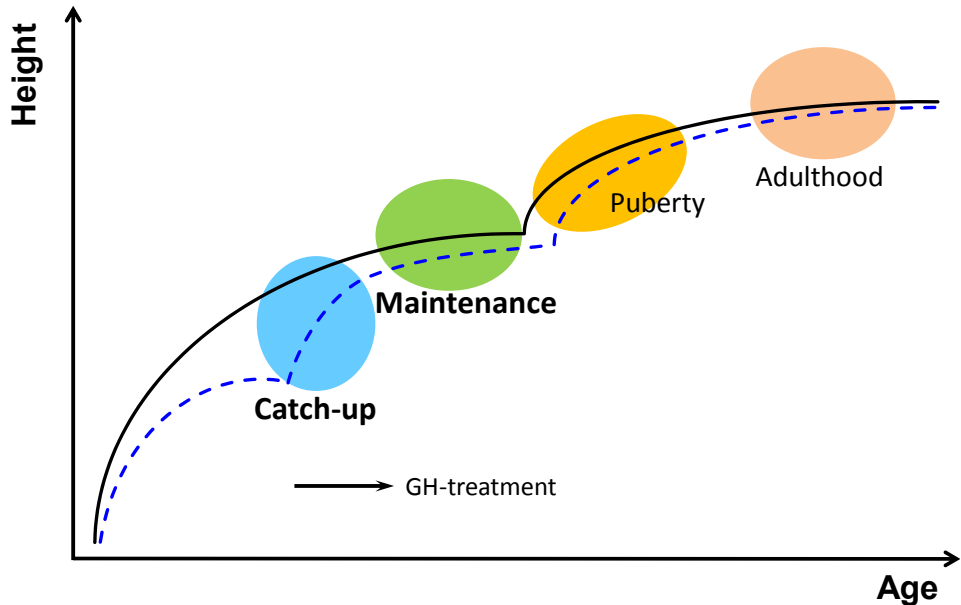


Figure 8 Catch-up and maintenance growth after initiated GH treatment. The black line is a sketch of the normative mean (0 SDS), while the blue dashed line marks the growth of an individual short child undergoing GH treatment. Modified with the permission of Kerstin Albertsson-Wikland.

In prepubertal short children with and without classical GHD, the increase in serum IGF-I levels in response to GH treatment is positively correlated to long-term growth¹¹⁷. However, in most countries, GH dosing in children is commonly standardized based on body weight or square meter body surface¹¹⁸ and not on GH responsiveness^{1, 119}, although it is observed that severe GH-deficient children need lower GH doses for an acceptable increase in height velocity. It is also observed that girls with Turner syndrome need higher GH doses for comparable growth responses⁴².

The response to GH treatment in children has up to now been measured as the first-year growth response in terms of height velocity, height_{SDS} or difference from MPH_{SDS} (diffMPH_{SDS})¹²⁰. The individual growth response to GH treatment varies widely among short children, depending on the child's responsiveness (which is influenced by the

underlying cause of short stature), as well as on age, developmental stage¹²⁰⁻¹²² and GH receptor polymorphisms¹²³.

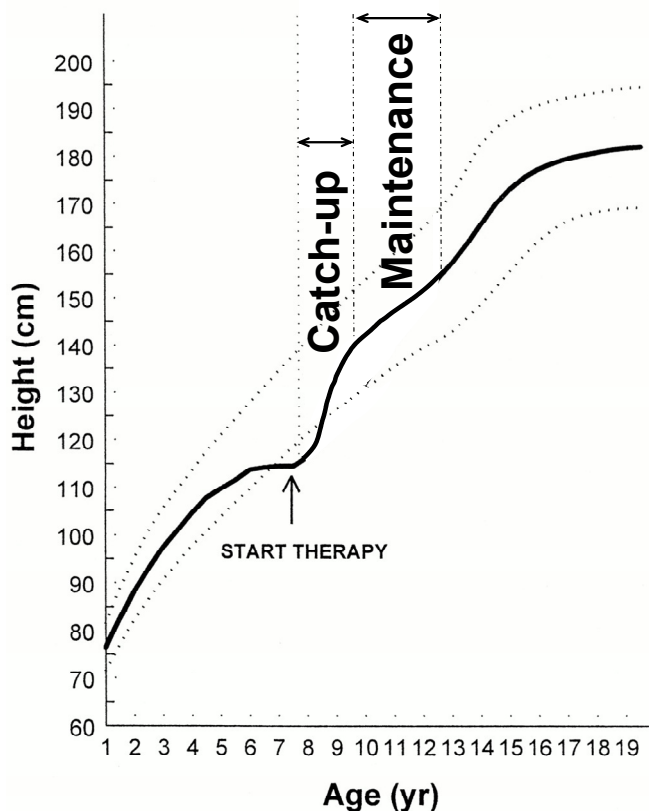


Figure 9 Catch-up growth and maintenance growth during GH treatment modified after Boersma B, Wit JM, Endocrine Reviews 1997;18:646-661 with permission of The Endocrine Society. Maintenance growth is the channel-parallel growth after reaching midparental height_{SDS} before puberty.

This is also the case in adult GHD populations³⁴. The individual responsiveness to GH treatment in GH-deficient adults is dependent on the level of GH-binding protein, body mass index, age and gender. Therefore, in the adult setting, IGF-I titration of GH treatment has been used for decades¹²⁴.

3.2.3 Prediction models to estimate GH responsiveness

Historically, treatment effects have been estimated retrospectively by measuring the response (change over time) of outcome variables such as height gain or metabolic response during GH treatment. Mostly, group means and group variation measurements (for example, SDS, range or variance) have been compared in relation

to a certain treatment. However, conclusions to be drawn from such retrospective effector–response evaluations provide information on the group level but not on an individual level.

Bivariate correlations give an estimate about the variation in one variable in relation to the variation in another. Correlations provide, similarly, an estimate of the connection between two variables on a group level. As the variability is often wide and, in addition, effects in medicine are often related to many variables, bivariate correlations are not always feasible for a prospective judgement (prediction) about the outcome in an individual patient. Principal component analysis (PCA) goes a step further. PCA is applied as a multivariate tool to reduce the amount of data in a network of variables and to get an overview of the main correlations between multitudes of variables.

Regression analysis has been widely used in medicine, as it can express an outcome as a function of one (bivariate) or more variables (multivariate).

Nevertheless, a predictive model can be constructed from a regression analysis. The predicted outcome is calculated from a mathematical function using regression coefficients from each effector variable, called predictors, by taking estimates from each individual into account. This calculation gives a prognostic value, with a statistical error expressed by the value of the residuals.

How well a model fits the data can be evaluated at the group level; the value of R^2 , or explained variance, is an estimate of how the observed values are correlated with the fitted values. In clinical practice, the applicability of a model is determined by how well it predicts the outcome in each single patient. Therefore, it is crucial that the model is validated using data from patients who fulfil the inclusion criteria for the model, but who were not among the patients whose data were used to derive the model. The model is considered to be statistically valid if the standard deviation of the residuals (SD_{res}) for the validation group of patients is in the same range as that observed for the group of patients from whose data the model was derived; this is called model validation¹²⁵. This leads from observation and deduction to a generalization of validity according to Karl Popper's theorem of theoretical sciences¹²⁶.

To our knowledge, so far, three groups have published prediction models for the growth response to GH treatment in individuals with GHD^{2, 3, 127}. Later on, the KIGS (Kabi International Growth Study) group went on to develop separate prediction models for ISS¹²⁸ and short SGA children¹²⁹, whereas the Gothenburg group

developed the same prediction model to be used for children having isolated GHD, ISS or SGA due to similar patterns of the growth response curve. This model was robust even though different diagnoses with diverse responsiveness were included¹³⁰.

Efforts have been made to individualize GH treatment in children by titrating IGF-I levels towards two predefined levels (established from a study setup) and evaluating the growth responses¹³¹ and the doses needed. IGF-based GH dosing has been found clinically feasible in both GHD and ISS patients¹³². In the first publication from the GH dose study¹, a multivariate non-linear prediction model was used to estimate individual growth response in GHD and ISS children on a standard GH dose. Indirectly, the responsiveness was estimated at the same time, guiding the GH dose selection together with $\text{diffMPH}_{\text{SDS}}$ in the group randomized to treatment with an individualized dose. The growth response variability was reduced by individualized GH dosing¹. The $\text{diffMPH}_{\text{SDS}}$ range at 2 years of treatment was reduced by 32% in the individualized-dose group compared to the standard-dose group¹.

The growth response during GH replacement in relation to GH secretion capacity, as estimated by a stimulation test or 24-hour GH profile, is a continuum¹³³ with a very broad range¹³⁴; GH secretion capacity also varies with body composition and type of growth phase (see 3.1.1.3, Changes over life-span). Because of this variability, it is at present unclear where to set the GH-secretion cut-off – if there should be one at all – to distinguish between GHD and idiopathic short stature (ISS)¹³⁴.

3.2.4 Metabolic responsiveness to GH treatment

Urinary calcium excretion was one of the first metabolic GH effects studied in the early 1940s^{135, 136}. The following metabolic actions of GH were elucidated in the early 1950s: (a) stimulation of somatomedin (IGF-I) production; (b) stimulation of thymidine incorporation into costal cartilage of hypophysectomised rats; (c) cause of glucosuria in pancreatectomized and dexamethasone-treated rats; (d) stimulation of protein synthesis; (e) amino acid and glucose transport into isolated diaphragms from hypophysectomized rats and (f) glucose utilization by isolated adipose tissue of hypophysectomized rats^{92, 137}.

Further GH mechanisms were studied in animals in the early 1960s. These studies looked at: plasma concentrations of free fatty acids (FFA), which increased; α -amino nitrogen and glucose tolerance, with positive balances in nitrogen, phosphate, sodium, potassium; and increased body weight¹³⁸.

In the 1970s it was found that short-term metabolic responsiveness to exogenous human GH increased with the degree of GHD in children¹³⁹. Severely GH-deficient children also have extremely low fasting insulin levels that are normalized when GH therapy is initiated⁴.

3.2.4.1 GH – Insulin interactions

It is well known that GH induces reversibly higher fasting and glucose-stimulated insulin levels during long-term GH therapy, indicating an underlying problem of insulin resistance^{10, 140}. Insulin resistance is characterized by a diminution of the ability of insulin to metabolize glucose and is manifested as follows: glucose intolerance with hyperglycaemia; a compensatory increase in plasma concentrations of insulin; dyslipidaemia with increased concentrations of triglycerides with diminished high density lipoprotein (HDL) cholesterol; elevation of blood pressure; abdominal obesity¹⁴¹. GH-induced insulin resistance is rapidly reversible, as observed in an experimental study in GH-deficient adults¹⁴².

3.2.4.2 Adipose tissue

One important peripheral site of GH action is adipose tissue. GH accounts for half of the adipogenic activity and the other adipogenic hormone is cortisol (cortone)^{143, 144}. Clinically, it has been observed that GH reduces fat mass in response to supplementation treatment. In vitro studies show that GH causes diabetic effects, such as increased lipolysis in adipocytes from healthy animals. High concentrations of GH result in exponential growth of preadipocytes⁵⁸; however, GH evokes insulin-like effects such as uptake and conversion of glucose and lipids, i.e. increased lipogenesis, and counteraction of lipolytic agents such as noradrenalin in cells prepared from GH-deficient subjects, i.e. hypophysectomized rats.

3.2.4.3 Adipokines

GH is involved in the regulation of body composition through both its lipolytic and anabolic effects¹⁴⁵. In healthy children, a strong positive correlation between leptin levels and body fat and a significant negative correlation between leptin levels and GH secretion has been found¹⁴⁶. Leptin concentrations at start of GH treatment correlate positively with growth response to GH treatment; high leptin levels can be used as a positive predictor for the growth response to GH treatment and have therefore been included in prediction models¹³⁰. It has also been reported that GH replacement therapy lowers plasma leptin and decreases fat mass in both children and adults with GHD^{6, 147, 148}.

In population studies, high plasma adiponectin levels were associated with reduced risk of myocardial infarction¹⁴⁹. Adiponectin expression and serum concentrations are reduced in obese and insulin-resistant states¹⁵⁰ and in patients with diabetes mellitus type 2¹⁵¹. It has also been shown that SGA children with catch-up growth have significantly lower adiponectin levels than those without catch-up growth¹⁵². Furthermore, GH treatment in SGA children leads to lower adiponectin levels due to a more insulin-resistant state¹⁵².

Serum adiponectin levels, like leptin levels, decrease significantly after the onset of GH treatment in both GHD and SGA children, remaining low during the first year of GH treatment¹⁵³. This was considered to be an effect of increased insulin levels during insulin resistance¹⁵⁴.

Both leptin and adiponectin inhibit bone formation in vitro and it has been demonstrated that the inhibitory effect of adiponectin on bone formation is negated by insulin¹⁵⁵.

3.2.4.4 Lipids and pro-inflammatory cytokines

Non-GH-treated adult patients with GHD have been shown to have an increased cardiovascular risk, as manifested by elevated fasting and postprandial lipids and by increased body fat that was reversed by GH treatment¹⁵⁶. GH replacement reduces total and low-density lipoprotein (LDL) cholesterol and waist circumference¹⁵⁷. During GH treatment, total body fat decreases¹⁵⁸, while lean body mass and bone mineral density increase^{159, 160}. The pronounced elevation of inflammatory factors seen in non-GH treated GHD patients seems to be associated with the presence of increased levels of fasting and postprandial triglycerides, which may result in an increased susceptibility for premature atherosclerosis¹⁶¹. Pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF- α), can be affected by GH treatment in GHD children, suggesting a direct effect of GH on immune function¹⁶². Moreover, GH and IGF-I have been shown to play a role in T and B cell development in mice¹⁶³.

It has been demonstrated that, in fetal rat metatarsal bones, IL-1 and TNF- α act locally in synergy to suppress longitudinal growth; this is an effect that can be partially reversed by IGF-I¹⁶⁴. GH therapy given to patients with adult-onset GHD has been found to result in significant reductions in C-reactive protein (CRP) and apolipoprotein B (Apo-B), indicating a positive effect of GH on cardiovascular risk¹⁶⁵ in this population. IL-6, CRP and fibrinogen correlate with visceral obesity¹⁶⁶; IL-6 and permanently

elevated CRP levels were strongly associated with – and likely play a dominant role in – the development of this inflammatory process, which leads to insulin resistance, non-insulin-dependent diabetes mellitus type II, and metabolic syndrome¹⁶⁷. In children, this has not been studied before, as growth response has previously been the outcome variable most often observed. However, in the present study of prepubertal children, metabolic markers were the main focus.

4 PATIENTS AND METHODS

4.1 STUDY DESIGN

The study was performed as a prospective, multicentre, randomized (to either individualized or standard/fixed GH dose), interventional trial, for a two-year catch-up growth period (study number TRN 98-0198-003) and a following prepubertal maintenance growth period (study number NRA 6280003). The study consisted of two parts, each of two years' treatment duration: the catch-up growth period and the maintenance growth period. The pre-set growth goal was to reach MPH_{SDS} in the first two years of treatment and to achieve continuous channel-parallel growth during the maintenance growth period.

The hypothesis in the first study part, the catch-up period, was that, despite many individuals needing higher GH doses (due to estimated lower GH responsiveness), in the individualized treatment group they would all grow well without metabolic impairment and with smaller variance of responses compared to the group of children randomized to standard GH treatment.

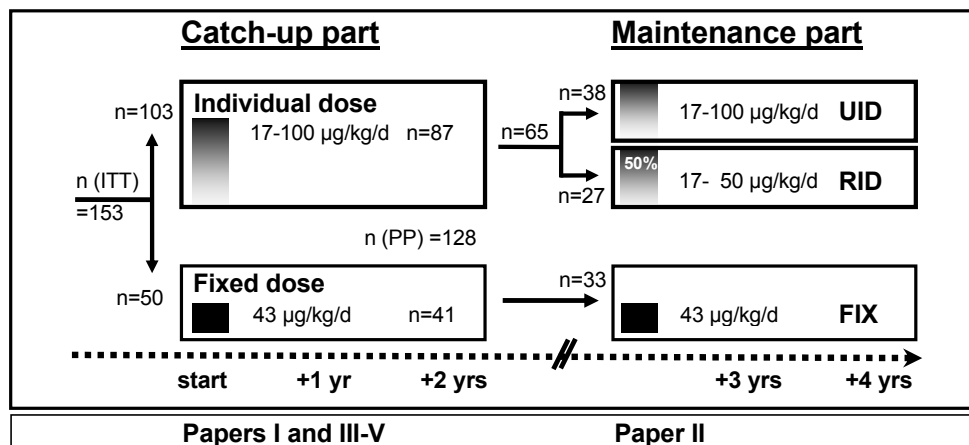


Figure 10 Study design of the GH dose study. The ITT (intention-to-treat) population consisted of 153 prepubertal short children. Out of the 128 per protocol children enrolled in the catch-up growth study part, 87 received an individualized GH dose within the range 17–100 µg/kg/day during two years and 41 were randomized to receive a fixed dose (FIX) of 43 µg/kg/day, thereby constituting the control group. In the second study part, the maintenance period, among the 87 children previously receiving individualized GH treatment, 38 were randomized to continue with an unchanged individualized dose (UID) and 27 to a 50% reduced individualized dose (RID); the 33 children in the control group continued with the unchanged fixed standard dose (FIX). However, the lowest dose of 17 µg/kg/day was not further reduced in the RID group. Papers I – V are depicted according to the appropriate study period.

This means, that the variance of the metabolic response during individualized GH treatment would be reduced.

In the second study part, the maintenance period, it was hypothesized that the GH dose could be reduced in patients treated individually without compromising growth or metabolic outcome compared to those receiving standard dose or unchanged individualized dose (**Figure 10**).

The selected individual GH dose was based on the child’s deviation from MPH_{SDS} and the individual GH responsiveness estimated from a validated multivariate prediction model² based on growth data from the first two years of life, parental heights, auxological data from the year before start of treatment, and GH secretion estimated during a 24-hour GH profile (**Table 3**)¹.

Predicted Δ height _{SDS} after 2 yr of GH treatment with 33 μ g/kg · d	Predicted difference in MPH _{SDS} after 2 yr of GH treatment with 33 μ g/kg · d				
	< -1.2	-1.2 to -0.8	-0.8 to -0.5	-0.5 to +0.5	> +0.5
>1.8	50	33	33	17	17
1.2 to 1.8	66	50	40	33	17
<1.2	100	66	50	33	17

Table 3 Treatment schedule for selection of GH doses in the group with individualized GH treatment with use of the two variables predicted diffMPH_{SDS} and predicted growth response after 2 years of treatment.

In the maintenance growth phase, the GH dose was randomly halved in 50% of the children receiving the individualized GH dose. However, no GH dose less than 17 μ g/kg/day was given.

The randomization variables in the catch-up study part were applied after a minimization procedure to age, gender, and the following auxological measurements: weight_{SDS} at birth; height_{SDS} one year before GH start; height_{SDS} at start; channel-parallel growth the year before start (yes/no); diffMPH_{SDS}; the predicted change in height_{SDS} during the first year of GH treatment at 33 μ g/kg/d; the GH_{max} AITT; and GH_{max} 24-hour profile.

The randomization variables in the maintenance study part were: (1) GH_{max} from AITT; (2) GH_{max} from the 24-hour profile; (3) age at start in the maintenance study and (4) gender.

4.2 PATIENTS

Of the 153 prepubertal children enrolled and participating in the intention-to-treat (ITT) population, two thirds were randomized to individualized GH dose and one third to fixed standard dose (FIX). Among the 128 children forming the per-protocol (PP) population in the first study part (catch-up period), with 41 receiving the fixed GH dose and 87 the individualized GH doses. Ninety children were diagnosed as having GHD and 38 had ISS, based on the classical definition of the maximum GH secretion peak (GH_{max}) with a cut-off of $\text{GH}_{\text{max}} < 24$ mU/L (monoclonal DELFIA) on AITT. However, 39 children were diagnosed with GHD and 89 with ISS based on the GH_{max} with a cut-off of $\text{GH}_{\text{max}} < 24$ mU/L on either AITT or a 24-hour GH secretion profile.

Thus, both provocation test (AITT) and a 24-hour spontaneous GH profile were performed in all children and the diagnosis (GHD or ISS) in this study was based on both investigations, resulting in 39 children receiving a diagnosis of GHD and 89 having ISS upon reclassification.

Inclusion in the study and analyses of the primary endpoints (evaluation of height gain), however, were based exclusively on the AITT test, as this is the gold standard for diagnosis of GHD in most parts of the world. The definition using a combined AITT or a 24-hour spontaneous GH profile was used in the metabolic analyses when categorizing the children as having GHD or ISS (**Table 4**).

GH_{max} during	Catch-up study part			Maintenance study part		
	GHD (n)	ISS (n)	(n)	GHD (n)	ISS (n)	(n)
AITT or 24 hour profile	39	89	128	33	65	98
24 hour profile only	49	79	128	39	59	98
AITT only	90	38	128	75	23	98

Table 4 Maximum GH secretion peak (GH_{max}) with a cut-off of $\text{GH}_{\text{max}} < 24$ mU/L (monoclonal DELFIA) defining growth hormone deficiency (GHD) according to a combined arginine–insulin tolerance test (AITT) or 24-hour spontaneous GH profile, a 24-hour spontaneous GH profile only, or AITT only. This resulted in different numbers of GHD and idiopathic short stature (ISS). The GH_{max} cut-off from the combined AITT and 24-hour spontaneous GH profile was used to define GHD or ISS (marked grey).

The protocol allowed children who were considered 'non-severe SGA' (birth weight or birth length between -2 and -2.5 SDS) to be included. In the catch-up part, eleven children were non-severe SGA, with nine of these having a GH_{max} in either AITT or 24 h profile ≥ 32 mU/L, and two between 25.3 and 31 mU/L. Eight SGA children were included in the maintenance part; Two had a GH_{max} below 32 mU/L and six ≥ 32 mU/L.

At the end of the first study part (after two to three years of catch-up growth), 98 patients were still prepubertal and continued into the second study part (maintenance growth period), with 27 randomized to receive reduced individualized dose (RID), 38 randomized to unchanged individualized dose (UID), and 33 constituting the control group with continued fixed standard dose (FIX). The detailed study population during the maintenance growth phase consisted of 98 patients according to **Figure 11**.

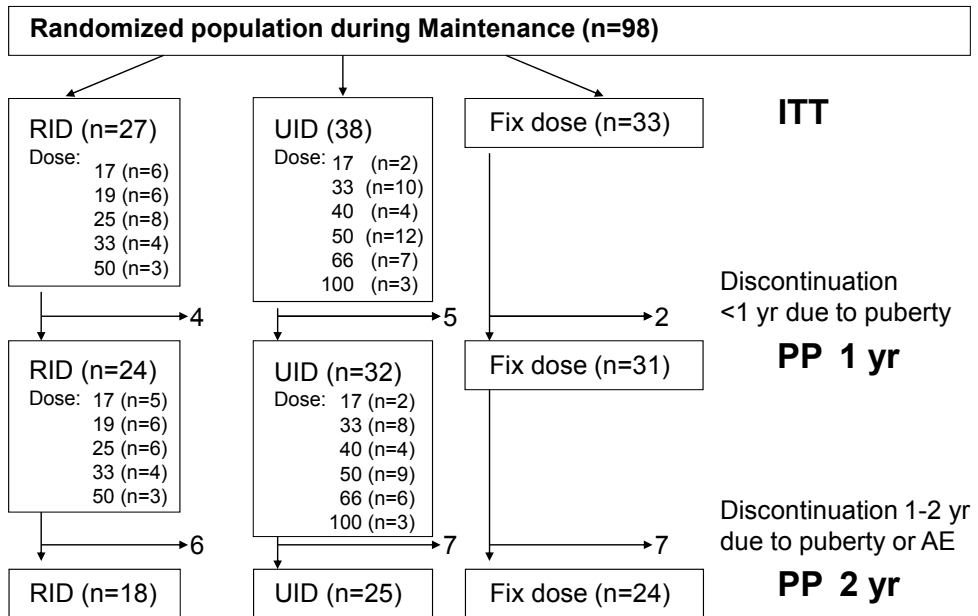


Figure 11 Randomized prepubertal study population during the maintenance growth phase. GH dose is given in $\mu\text{g}/\text{kg}/\text{day}$. n (number of patients), AE (adverse event), ITT (intention to treat), PP (per protocol), RID (reduced individualized dose), UID (unchanged individualized dose), FIX (unchanged fixed standard dose). Modified with the permission of Jovanna Dahlgren.

4.3 ETHICAL CONSIDERATION

The study protocol was approved by the Ethics Committees of the Universities of Gothenburg (for Göteborg and Halmstad), Umeå, Uppsala and Malmö and also by the Medical Product Agency of Sweden. Written informed consent was obtained from all parents and from children where possible. The study was performed in accordance with the Declaration of Helsinki and Good Clinical Practice.

4.4 METHODS

4.4.1 Auxology measurements (Papers I-V)

4.4.1.1 Anthropometry (Paper I-II and V)

Height was measured with a Harpenden stadiometer, taking the mean value of three independent measurements. Weight was measured using weighing scales. Accuracy was ± 0.1 kg.

Waist circumference was measured with a flexible tape, at the level of the umbilicus, and hip circumference at the level of the mons pubis to the nearest millimeter. Skin fold measurements of the subcutaneous adipose tissue were performed with a caliper according to a 3-folds method on the ventral (biceps) and dorsal (triceps) forearm and subscapular.

4.4.1.2 Growth evaluation (Papers I-V)

The measured height and weight values were transformed to SDS in relation to the Swedish reference values¹⁶; as well as the calculated BMI values¹⁶⁸.

The childhood component (applies only in prepubertal children)¹⁰⁹ of the Swedish population-based growth reference values¹⁶ was used for the height-related inclusion criteria and to express the SDS for prepubertal height for age and sex¹⁶, in order to assess changes in height, weight, and body mass index (BMI)¹⁶⁸ of the patients. Reference norms for newborns were used for SDS at birth¹⁶⁹. MPH was calculated based on the following: A simple linear function of MPH (x) was used to estimate target height (y): $y = 45.99 + 0.78x$ (boys), $y = 37.85 + 0.75x$ (girls), with a 95% predicted interval of about ± 10 cm^{16, 170}.

4.4.2 Measurement of bone age (Papers I-II and V)

Bone age from carpal radiographs was assessed throughout the studies by one skilled person at study start and thereafter yearly according to Tanner-Whitehouse II¹⁷¹.

4.4.3 Measurement of body composition (Papers I-V)

Body composition was measured by dual energy X-ray absorptiometry (DXA). It was used at each study centre using either a Lunar DPX-L scanner (GE Medical, Madison, WI) or a Lunar Prodigy (GE Medical). The DXA measure results in a three-compartment model consisting of fat mass (FM), lean soft tissue (LST) mass and bone mineral content (BMC)¹⁷². The alternative term lean body mass (LBM) was used instead of LST in Paper I when referring to the same compartment.

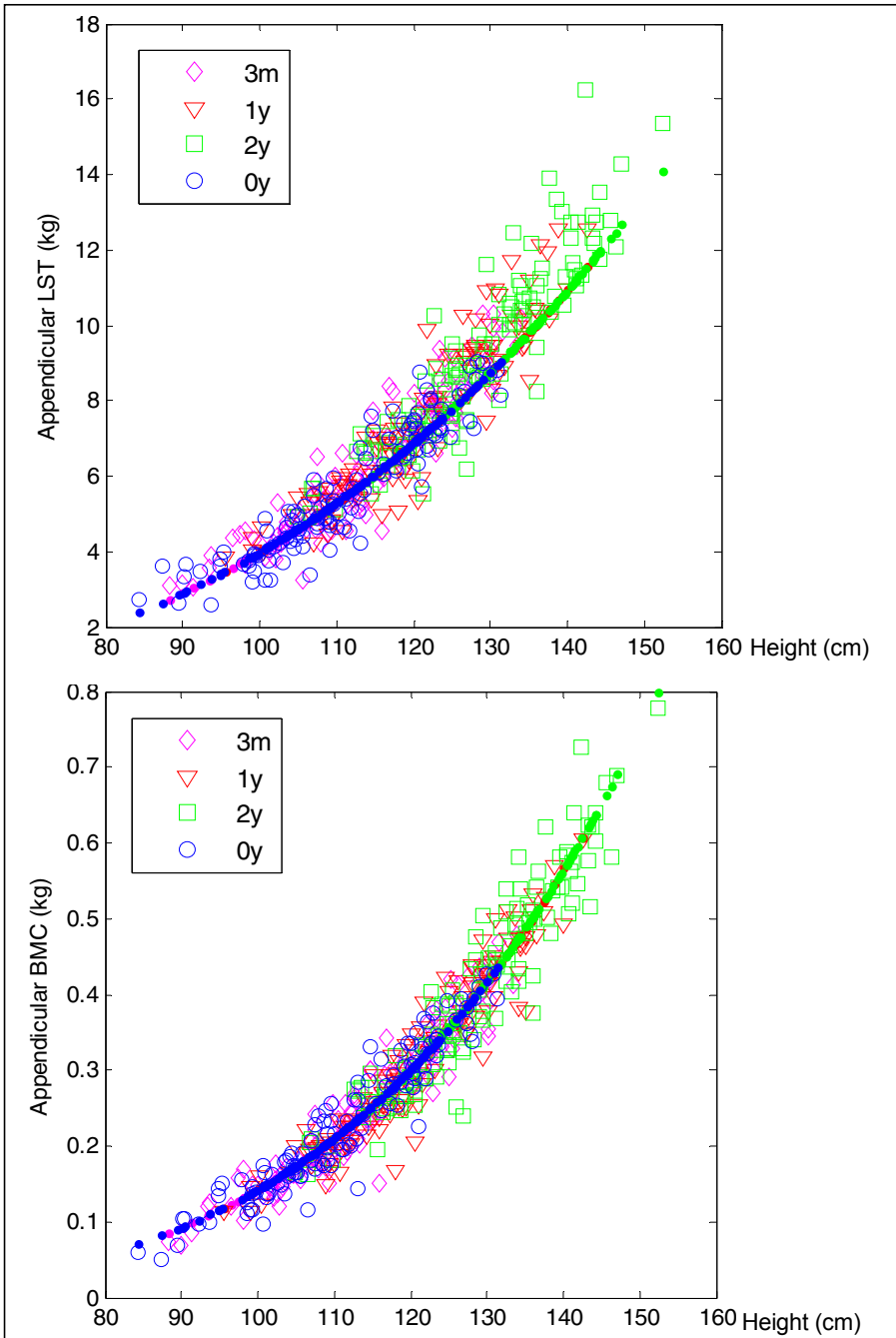


Figure 12 Appendicular (arms and legs) lean soft tissue mass, LST, (left panel) and appendicular bone mineral content, BMC, (right panel) at study start (0y), 3 months (3m), one year (1y) and two years (2y) of GH treatment were highly correlated with height. Explained variance (R^2) is 0.87 for LST and 0.91 for BMC for the measurements at study start (0y) used for fitting the power function that was thereafter applied to adjust for the 3-month, first and second year data. The filled symbols represent the values calculated using the equations and the unfilled symbols represent the observed values.

Regional body compartments were measured using LBM, LST, and BMC of arms and legs (called appendicular LBM, LST and BMC, respectively).

The results were comparable across DXA systems, and only small differences were detected between Lunar Prodigy and DPX systems¹⁷³. Each child was longitudinally measured with the same setting. Quality assurance tests were made on a daily basis, and a phantom spine was examined once weekly, as recommended by the manufacturer. DXA scan reproducibility expressed as coefficient of variation (CV) has been reported to be 0.18–1.97% for total body measurement and 0.96–6.91% for regional measurements¹⁷². SDS for total body BMC, bone mineral density (BMD), LBM, and fat were calculated according to Dutch normative data using the same method¹⁵⁹.

Regional measurements of body compartments were made to adjust for height because normative data are missing in children. Appendicular LST and BMC were strongly correlated with height (**Figure 12**) and therefore the measurements were height adjusted (_{HA}), resulting in the new variables LST_{HAapp} and BMC_{HAapp}. The optimal non-linear equations for adjusting LST_{HAapp} and BMC_{HAapp} at start of GH treatment were the following power functions: $LST_{HA} = 3.934 \cdot 10^{-3} \cdot \text{height}(\text{cm})^{3.003}$ and $BMC_{HA} = 9.451 \cdot 10^{-7} \cdot \text{height}(\text{cm})^{4.090}$, respectively. The equations were constructed by robust fitting on DXA measurements at start of treatment only with explained variance (r^2) = 0.87 for LST and r^2 = 0.91 for BMC and were then also applied to three months, the first and second year of treatment in order to adjust for height.

4.4.4 Echocardiography (Paper V)

Echocardiography was performed by four paediatric cardiologists and one experienced sonographer per study site. Each child was examined by the same sonographer (a cardiologist). Interventricular septum thickness (IVSd), left ventricular diameter (LVDD) and left ventricular posterior wall (LVPWd) thickness were measured in diastole using M-mode. Left ventricular mass (LVM) was calculated using Devereux's anatomically corrected formula¹⁷⁴.

4.4.5 Spontaneous GH secretion (Papers I-V)

Spontaneous GH secretion was measured in terms of both secretory rate and pulsatile pattern including GH_{max} during a 24-hour profile with integrated 20-min sampling interval²³. AITT was performed by administering 0.5 g arginine-HCl/kg bodyweight i.v. (max 30 g) of a 10% arginine-HCl solution in 0.9% NaCl at a constant rate over 30 min followed sequentially by insulin 0.1 IU/kg bodyweight i.v. as a bolus¹⁷⁵.

4.4.6 Laboratory analyses (Papers I, II, and V)

GH, IGF-I, IGFBP-3, leptin and adiponectin assays were performed at the Göteborg Pediatric Growth Research Center (GP-GRC) laboratory (accredited number 1899).

Spontaneous **GH** secretion was measured in terms of both secretory rate and pulsatile pattern including GH_{max} ; in addition, 24-hour GH profiles with integrated 20-min samples were taken²³. GH concentrations were measured using a time-resolved immunofluorometric assay (Wallac, Finland), with the WHO International Reference Preparation (IRP) 80/505 as the standard¹⁷⁶. The intra-assay CVs were 2%, 2%, and 1% at 3 mU/L, 30 mU/L, and 70 mU/L, and the interassay CVs were 3%, 4%, and 3%, respectively. GH concentrations were transformed to comparable levels using transformation factors derived in the laboratory¹⁷⁷ to a polyclonal dissociation-enhanced lanthanide fluorescence immunoassay (DELFI A), Pharmacia Diagnostics, Uppsala, Sweden). The GH_{max} cut-off value for the conventional diagnosis of classical GHD was <24 mU/L, corresponding to <32 mU/L (polyclonal DELFI A), and corresponding to a cut-off value <10 μ g/L using IRP 66/127^{35, 177}.

For **IGF-I**, an IGFBP-blocked radioimmunoassay (RIA) was used without extraction, and in the presence of IGF-II levels 250-fold in excess of normal (Mediagnost, Tübingen, Germany). The intra-assay CVs were 18.5%, 11.4%, and 7.1% at 9 μ g/L, 33 μ g/L, and 179 μ g/L, respectively. The interassay CVs were 29%, 11.3%, and 6.3% at 9 μ g/L, 34 μ g/L, and 190 μ g/L, respectively.

IGFBP-3 was measured by RIA as previously described¹⁷⁸. The intra-assay CVs were 7.6%, 9.5%, 6.1%, and 7.8% at 716 μ g/L, 1075 μ g/L, 3929 μ g/L, and 7061 μ g/L, respectively. The interassay CVs were 20.8%, 11.6%, 10.8%, and 10% at 725 μ g/L, 1859 μ g/L, 3919 μ g/L, and 6078 μ g/L, respectively. IGF-I and IGFBP-3 levels were converted into SDS¹⁰⁸. The ratio of IGF-I/IGFBP-3_{SDS} was calculated for each sample^{105, 108}.

Serum **leptin** concentrations were measured by RIA (Linco Research, St. Charles, USA) with a range of 0.3–100.0 μ g/L and intra-assay CVs of 7.0% at 2.4 μ g/L, 5.2% at 6.6 μ g/L and 4.9% at 14.0 μ g/L. The interassay CVs were 12.5% at 1.4 μ g/L, 7.6% at 6.7 μ g/L and 5.6% at 15.1 μ g/L¹⁷⁹.

Adiponectin concentrations were measured in duplicate by an enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, Minnesota, USA). The intra-assay CVs were 5%, 7%, and 10% at 7 mg/L, 11 mg/L, and 30 mg/L,

respectively¹⁸⁰. The interassay CVs were 12%, 9%, and 16% at 2 mg/L, 10 mg/L, and 30 mg/L.

Lipoproteins, triglycerides, ALP, insulin and glucose were measured the Sahlgrenska University Hospital in the Department of Clinical Chemistry, Institute of Laboratory Medicine (accredited number 1240).

Total high-density lipoprotein (**HDL**), low-density lipoprotein (**LDL**) cholesterol and **triglycerides** were analysed using an enzyme-based method (Modular P, Roche Diagnostics, Mannheim, Germany). CVs were 1.57% at 0.26 mmol/L, and 2% at 0.26 mmol/L for HDL, and 2.4% at 2.5 mmol/L, and 1.4% at 2.4 mmol/L for LDL, respectively for inter- and intra-assay CVs.

ALP was analysed using a colorimetric method in the presence of magnesium ions and zinc ions. P-nitrophenylphosphate is cleaved by alkaline phosphatase; phosphate and p-nitrophenol are formed and the latter measured by colorimetry. The intra-assay CVs are 0.9% at 1,76 μ kat/L and 0.89% at 5,75 μ kat/L. The interassay CVs are 4.9% at 1,76 μ kat/L and 0.9% at 5,75 μ kat/L.

Serum **insulin** concentrations were measured by RIA (Pharmacia and Upjohn, Uppsala, Sweden) expressed with an intra-assay CV of 4.5–8.3% at 1.3 mU/L and interassay CV of 6–7% at 1.3 mU/L.

Plasma **glucose** was measured using the hexokinase method (Roche Diagnostics, Mannheim, Germany) with a 4% CV for sample concentrations between 5 and 15 mmol/L (intra- and interassay). Insulin resistance was estimated by homeostasis model assessment (HOMA), calculated as $[(\text{fasting serum insulin} \times \text{fasting plasma glucose})/22.5]$. Analyses were performed at accredited university hospital laboratories.

4.4.7 Proteomics (Papers III-IV)

4.4.7.1 Serum denaturation and fractionation

Frozen serum samples were thawed on ice and spun at 10 000 rpm for 10 min at 4°C. Each serum sample (10 μ L) was denaturated by the addition of 20 μ L of U9 buffer (9 M urea, 2% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate, a zwitterionic detergent), 50 mM Tris-HCL (tris(hydroxymethyl) aminomethane hydrochloride) and 1% DTT (dithiothreitol, a small-molecule redox reagent), pH 9.0), then oscillated in a vortex mixer at 4°C for 30 min.

Sample fractionation was performed on a Q HyperD F resin plate (180 μ L resin, Bio-Rad Laboratories, Hercules, CA, USA). The plate was prewashed and equilibrated with U1 solution (1 M urea, 0.2% CHAPS, 50 mM Tris-HCL, pH 9.0) prior to the addition of samples to the 96-well fractionation plate. The anion exchange fractionation included the following elution steps:

(1) 50 μ M Tris-HC, 0.1% OGP (a non-ionic detergent, n-Octyl β -D-glucopyranoside), pH 9; (2) 50 μ M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, a zwitterionic organic chemical buffering agent), 0,1% OGP, pH 7; (3) 100 μ M Na Acetate, 0,1% OGP, pH 5; (4) 100 mM Na Acetate, 0,1% OGP, pH 4; (5) 50 μ M Na Citrate, 0.1% OGP, pH 3; and (6) 33.3% isopropanol, 16.7% acetonitrile, 0.1% TFA (trifluoroacetic acid).

4.4.7.2 SELDI-TOF MS serum protein profiling

To identify protein biomarkers of interest, the surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) technique was used (**Figure 13**). SELDI-TOF MS is a high-throughput technique that is used to identify protein biomarkers in many different sample types and for many diseases¹⁸¹⁻¹⁸³. This method is based on mass spectrometry and retention chromatography performed on protein chip arrays with varying chromatographic properties.

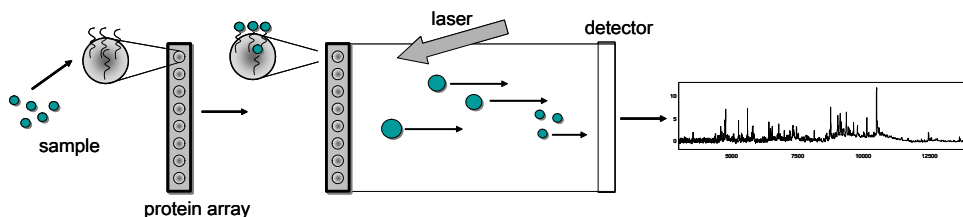


Figure 13 Schematic illustration of the SELDI-TOF MS technique. Serum sample are applied on the spots of a ProteinChip[®] array. A laser is fired on the spots. Proteins are accelerated in an electric field in vacuum towards the detector and a mass spectrum (m/z) is created. Printed the permission of Björn Andersson.

Each array contains a chemically pre-activated surface. Serum fractions 5 and 6 (pH 3 and organic solvent) were analysed together using weak anion-exchange (CM10) arrays, fraction 1 (flow through) was analysed using a metal-binding array (IMAC30) and fraction 4 (pH 4) was analysed using a hydrophobic reversed-phase (H50) array. Serum samples were applied on protein chip arrays which were loaded into the SELDI instrument together with a 50% solution of sinapinic acid (SPA) (Bio-Rad Laboratories) in 0.5 % trifluoroacetic acid (TFA) used as the matrix (**Figure 14**).

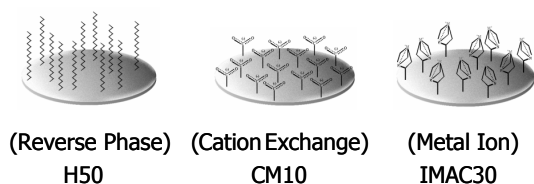


Figure 14: Various ProteinChip[®] array surfaces. Chromatographic surfaces are composed of reversed-phase (H50), ion exchange (CM10) or immobilized metal-affinity capture (IMAC30) chemistries. The figure was provided by Bio-Rad.

Time-of-flight spectra were generated using a PBS IIc ProteinChip[®] reader (Bio-Rad Laboratories). Proteins were eluted by laser desorption and ionization and accelerated in an electric field in vacuum. The time of flight (TOF) of the ionized proteins through the electric field-free flight tube was measured by the mass spectrometry detector. The time to reach the detector is a function of their mass and charge (m/z). The lighter ions will reach the detector first because of their higher velocity.

The intensities of the peaks in the spectra are proportional to the amount of the specific protein, reflecting the protein expression in serum. All samples were analysed in duplicate.

To minimize experimental variation, all samples were randomized and analysed concurrently within 1 week by the same operator; one reference serum sample was randomly applied on each protein chip array and the same calibration equation was used for all samples.

4.4.7.3 1D SDS-PAGE analysis

Fractionated and purified serum was loaded on to the gel to separate the proteins. The 1-dimensional (1D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run under denaturation conditions using NuPAGE[®] Bis-Tris gels 4-12% and NuPAGE[®] MES (3-N-morpholino ethane sulfonic acid) SDS Running buffer, both from Novex (San Diego, CA, USA). 1x NuPAGE[®] MES Sample Buffer, 0.05M DTT and protein samples were mixed and denatured for 3 min at 95°C prior to loading. NuPAGE[®] Antioxidant was added to the running buffer in the upper buffer chamber of the Xcell SureLock[™] Mini-Cell (Novex) to keep the proteins in a reduced state. Gels were run at 200 V for 35 min using a POWER PAC 1000 (Bio-Rad Laboratories) power supply. Staining was performed using Colloidal Blue Staining Kit (Novex). Precision Plus Protein[™] unstained standards were used as the molecular weight standard and the gels were scanned using a Fluor-S Multi Imager and Quantify One Software, all purchased from Bio-Rad Laboratories.

4.4.7.4 Passive elution

To be able to use mass spectrometry analysis for protein identification, the proteins in the low molecular weight range were passively eluted from the 1D SDS-PAGE gel by excising the band after Coomassie staining. The excised band was washed with 50% acetonitrile (ACN)/50 mM Ambic (3 x 15 min or until the gel slice was destained). The gel pieces were dehydrated in 100% ACN, heated to 50°C for 5 min and placed in a Speed vac until completely drained. Thereafter, 45% formic acid, 30% ACN and 10% isopropanol were added to the cover bands and the samples were sonicated for 30 min in a water bath at room temperature and left at room temperature for 3-4 hours before subsequent analysis.

A small amount of the passively eluted proteins was analysed on a normal phase (NP20) array to confirm that the same m/z identity and peak pattern could be seen as in the original SELDI-TOF MS spectra profile.

4.4.7.5 Protein identification

To confirm the identity of the biomarkers, immunodepletion was performed using antibodies and dynabeads (Invitrogen, Carlsbad, CA, USA) coated with anti-rabbit immunoglobulin G (IgG). Dynabeads were washed twice in 1 mL PBS (Phosphate Buffered Saline, PAA Laboratories GmbH, Pasching, Austria) and resuspended in 0.5 mL PBS. 4 µg of antibody was added per 50 µL dynabeads and incubated overnight at 4°C on a rotation mixer (REAX 2, Heidolph, Schwabach, Germany). Unbound antibodies were removed by washing twice in 1 mL PBS .

The beads were resuspended in 50 µL PBS. Twenty µL of fractionated serum were then added followed by incubation for 1 hour at 4°C on a rotation mixer and two washes in 1 mL PBS. The depleted serum was analysed on the same protein chip arrays as those on which the biomarkers were found.

4.4.8 Statistical methods

For all analyses, the assumptions of normality were assessed by analysis of skewness, kurtosis and frequency histograms. A p-value of <0.05 was considered to be statistically significant.

4.4.8.1 Basic statistics

Evaluations of the null hypothesis and differences between groups were made using a two-sample independent t-test for independent variables and a Mann-Whitney U-test as the corresponding non-parametric alternative (Paper III). Paired-sample t-tests were performed to calculate differences in each variable during the two years of GH treatment and Wilcoxon matched-pairs were used as the non-parametric alternative (Paper III). One-way ANOVA with SGA as a covariate was used to test differences in means in the entire PP population and for subgroups when comparing ISS with GHD or when comparing the two GH treatment regimens (standard with individualized GH treatment). A non-parametric comparison of group means (robust test of equality of means – Welch test or Brown–Forsythe test) was conducted when variances of dependent variables were not equal across groups.

Linear and quadratic regression analysis was performed to demonstrate dependency of some metabolic variables to growth response and GH dose (Paper I). A linear regression line was plotted based on the GH dose effect on the dependent metabolic variables for each of the six different GH dose groups. Delta values (Δ -values) of the metabolic variables were calculated quantifying the increase at 2 year of treatment compared to baseline. S-shaped piecewise linear regression models were fitted with GH dose as the predictor variable and the delta of the metabolic variables as response variables. The GH dose effect was given by the maximum range of the fitted piecewise function. Half of the GH dose effect on the respective variables was calculated halfway the lowest and the highest level of the piecewise function [50% Δ effect] with corresponding 90% confidence bands. A S-shaped piecewise linear regression line was plotted based on the GH dose effect on the dependent metabolic variables. The effective GH dose [ED 50%] required to achieve [50% Δ effect] was calculated based on the fitted piecewise linear regression equation with corresponding 90% confidence band. One-way analysis of variance (ANOVA) with GH dose as bounded continuous predictor was performed to test the S-shaped piecewise linear GH effect. A non-parametric comparison of group means (robust test of equality of means – Welch test

and Brown–Forsythe test) was conducted when variances of dependent variables were not equal across groups (Paper V).

Spearman correlations (2-tailed) were performed to evaluate relationships between non-parametric variables. Levene's test for equality of variance was performed to evaluate differences in standard deviation (SD) between the treatment groups.

4.4.8.2 Data reduction technique (Papers I-II)

PCA has been applied for the analysis of metabolic data. PCA is a powerful descriptive and data-reduction technique when used to assess variables related to a complex system¹⁸⁴ such as the metabolic network. Its properties, as well as the interpretation of the components, have been investigated extensively. Rotation by the well-established 'Varimax' method¹⁸⁵ was performed to focus on the most important interrelationships between metabolic variables. In the component figures of the PCA analysis, correlations of the variables with the rotated components are displayed graphically. Longer vectors have stronger correlations with one or two components. The cosine of the angle between two long vectors approximates their mutual correlation. Therefore, clusters with high mutual positive correlations among the vectors are formed by vectors close together with small angles in between (0° , cosine = 1). Negative correlations between two variables are visible if they point at opposite directions (180° , cosine = -1). Variables with an angle of 90° (cosine = 0) have almost no correlation. All statistical methods were performed using SPSS version 17.0 (SPSS Inc., Chicago, Illinois, USA).

4.4.8.3 Proteomics (Papers III-IV)

Data handling was performed using ProteinChip Data Manager (Bio-Rad Laboratories). All spectra were baseline-subtracted and normalized according to total ion current and then visually inspected. Patients were excluded from further data analysis if profiles clearly differed between the duplicate samples or if the overall quality was low in one or both of the spectra (i.e. high noise, overall low peak intensity or an abnormal normalization factor in combination with visually deviating spectra). This process resulted in the identification of 147 valid peaks for CM10 (67 peaks), IMAC30 (46 peaks) and H50 (34 peaks) in the m/z area between 2.3 and 30.0 kDa. The average CVs for the peaks detected in all of the reference samples were 30.1% for CM10, 34.1% for IMAC30 and 32.8% for H50.

Multivariate data analysis was used to identify potential biomarkers of interest. Samples from children with GHD and ISS and from both groups combined (total population) were analysed at baseline and after one year of GH treatment. Intensities

of peaks were analysed at start and at one year, and the changes in profiles over one year of treatment were analysed.

Every sample was analysed in duplicates using Matlab[®] software (version 7.7.0 R2008b, The Mathworks). The peak data were analysed using multidimensional scaling (MDS) to explore the relationships between the peaks and to identify outlying patients.

Stepwise regression analysis was computed to find subsets of peaks that correlated with variables of body composition. To study the robustness of the data analyses, random permutation tests were performed on the complete stepwise regression procedure. For each number of selected peaks, it was tested for 999 permutations if the permuted r^2 was equal to or above 90% of the calculated true r^2 . Random permutation tests resulting in a p-value <0.05 were considered significant.

5 RESULTS AND COMMENTS

5.1 CATCH-UP GROWTH PART (PAPER I)

5.1.1 Reduced variance of metabolic responses

In contrast to the means of metabolic GH effects, where no differences were found when comparing individualized and standard GH treatment, a reduced variance by 34.2% (SD 5.7 vs. 8.7 in the individualized and the standard dose group, respectively) was found for insulin and for HOMA by 38.9% (SD 1.5 vs. 2.4), $p < 0.05$ (**Figure 15**)^{Paper I}.

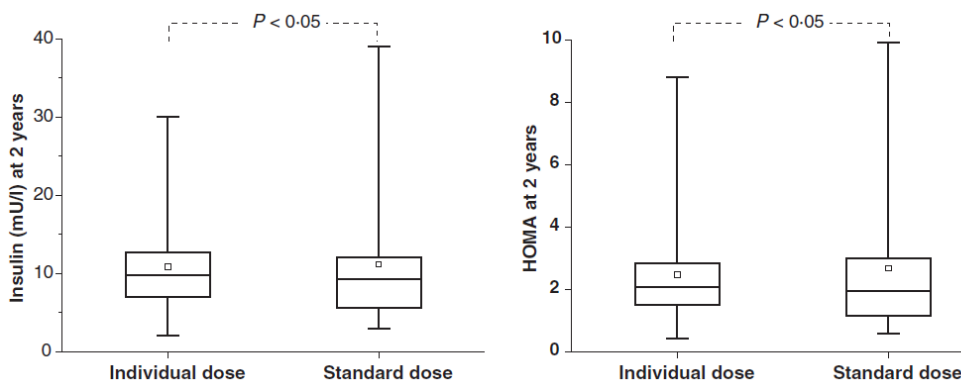


Figure 15 Distribution of insulin and homoeostasis model assessment (HOMA) in the standard and the individualized GH dose groups (The distributions of values at two years for insulin and HOMA according to randomization group are shown. The standard deviation around the mean is significantly narrower for insulin (34.2 %) and for HOMA (38.9%) in the group receiving an individualized GH dose compared with the group receiving a standard dose. The boxes represent 50% (3rd and 1st quartile) of measured values, with the line indicating the median and the small box indicating the mean; the whiskers indicate the maximum and minimum. Levene's test for equality of variances gives p-values at a 95% confidence interval). (**Paper I**)

Auxological and metabolic variables at start and after two years of individualized GH treatment were comparable between the ISS and GHD groups, except for GH secretion, weight, BMI, and fat mass at start.

5.1.2 Dissociation of anabolic and lipolytic GH effects

Clusters with high inter-correlations of anabolic GH effects (LBM_{SDS} , $height_{SDS}$, BMC_{SDS} , $weight_{SDS}$, $IGF-I_{SDS}$, and $IGFBP-3_{SDS}$) and lipolytic GH effects ($fat\ mass_{SDS}$ and leptin) were found in the ISS group by performing principal component analysis (PCA). Both clusters were separated from each other, showing no inter-correlations between their variables. Furthermore, solely the anabolic cluster showed a dose dependency (**Figure 16**)^{Paper I}.

Similar patterns were observed when PCA was re-run separately for the GHD group. In both groups, fasting insulin and HOMA constituted a single vector cluster between the anabolic and lipolytic vector clusters.

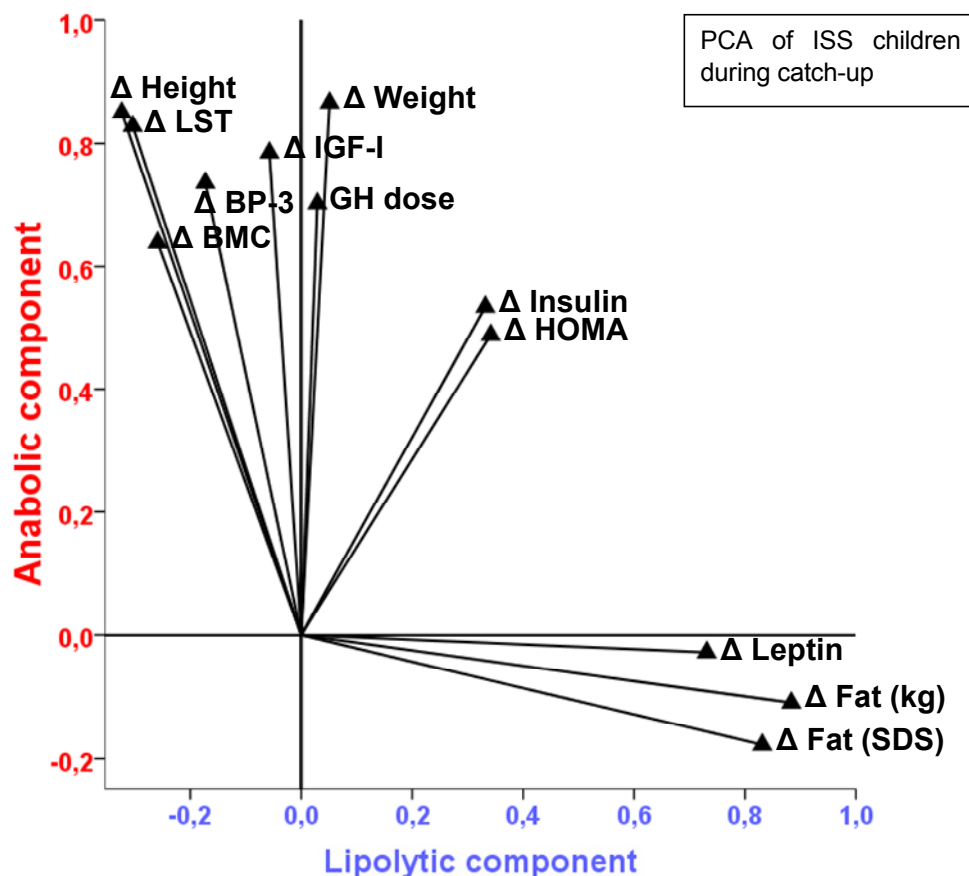


Figure 16 Two-dimensional principal component analyses (PCA) of response variables from the group of children with ISS during catch-up growth. The vector for each variable consists of a correlation with an anabolic component (y-axis) and a lipolytic component (x-axis). This PCA explains 61% of the total variance observed in all variables. Vectors with close mutual angles form a vector bundle. Changes (Δ) between start and two years of catch-up treatment are included in the analysis. Height ($\text{height}_{\text{SDS}}$); weight ($\text{weight}_{\text{SDS}}$); IGF-I (insulin-like growth factor I SDS); BP-3 (IGF-binding protein 3 SDS); LST (lean soft tissue mass SDS); BMC (bone mineral content SDS); Fat, kg (fat mass in kg) and Fat, SDS (fat mass in SDS); leptin (ng/ml); insulin (mU/L); HOMA (homoeostasis model assessment, mmol/mU). (**Paper I**)

Comment: Dissociation between the anabolic and lipolytic effects of GH was found. However, a substantial lipolytic effect was seen but no dose effect could be found. This suggests that the threshold for lipolysis is below the dose range administered. During

the catch-up growth part, insulin was partly related to the anabolic GH effects, indicating insulin to be a contributing growth factor.

By individualizing the GH dose based on estimated GH responsiveness using auxological data and spontaneous GH secretion in the Gothenburg growth prediction model², the anabolic response was predicted with reduced variance despite higher than standard GH doses given to many children randomized to individual dosing. The growth target was achieved¹ and at the same time the variance in fasting insulin levels (reflecting insulin sensitivity) was reduced, which means that both under- and over-treatment was avoided.

In the individualized group, those children who were estimated to have high GH responsiveness received a GH dose lower (40, 33 or 17 ug/kg/d) than the control group receiving the standard weight-based 43 ug/kg/d. This contributed also to the reduced variance.

Thus, the main results in these study parts were that the GH responsiveness reflected by the estimated growth response was also found valid to estimate responsiveness in carbohydrate metabolism. Lipolytic effects were observed but dose responses could not be demonstrated by the GH dose range used in our study and insulin was found to be a growth-contributing factor.

5.2 MAINTENANCE GROWTH PART (PAPER II)

5.2.1 Reduced variance of metabolic responses

The variation (SD around the mean) of fasting insulin (by 50%, $p < 0.05$) and HOMA (by 55.1%, $p < 0.05$) was markedly reduced in the RID group compared to the UID group, for insulin (3.7mU/L vs. 7.5 mU/L) and for HOMA (0.8 mU/L vs. 1.8 mU/L), respectively after two years of treatment in the maintenance study. The variations in LST (by 27.8%, 4.1 vs. 5.7 kg, $p < 0.05$) and BMC (by 31.3%, 0.22 vs. 0.32 kg, $p < 0.05$) were reduced in the RID group compared to UID at one year of treatment, while there was no longer a difference in variation after two years (**Figure 17**). No differences were found between RID and FIX or UID and FIX^{Paper II}.

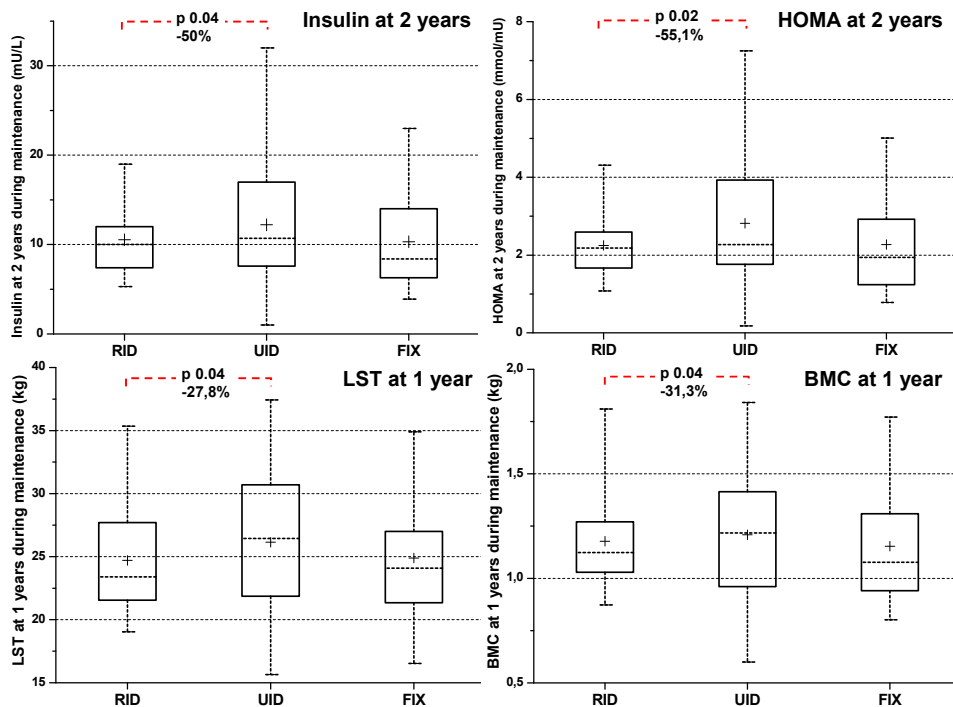


Figure 17 Variables during the maintenance growth period in the reduced individualized dose (RID) group compared with the unchanged individualized dose (UID) group and unchanged fixed standard dose (FIX) group. Distribution of fasting insulin levels, HOMA (homeostasis model assessment), lean soft tissue mass (LST), and bone mineral content (BMC) is shown. The standard deviation around the mean is significantly narrower for insulin (-50%), $p = 0.04$ RID compared with UID. The boxes represent the 1st and 3rd quartiles, with the line indicating the median (the cross indicating the mean); the whiskers indicate the maximum and minimum. Levene's test for equality of variances gives p-values at a 95% confidence interval. (**Paper II**)

5.2.2 Dissociation of anabolic and lipolytic GH effects

PCA performed on all patients in the PP population at two years (RID and UID together, $n = 43$) showed a cluster of anabolic variables with high inter-correlations of anabolic GH effects, Δ LST, Δ heights_{SDS}, and Δ BMC correlated with the GH dose (Figure 18). The 'adipose tissue/ insulin cluster' consisted of Δ leptin, Δ weight, Δ IGF-1_{SDS}, Δ insulin, and Δ HOMA, with Δ fat mass apart from this cluster. The two clusters were separate from each other, showing low inter-correlations with each other^{Paper II}.

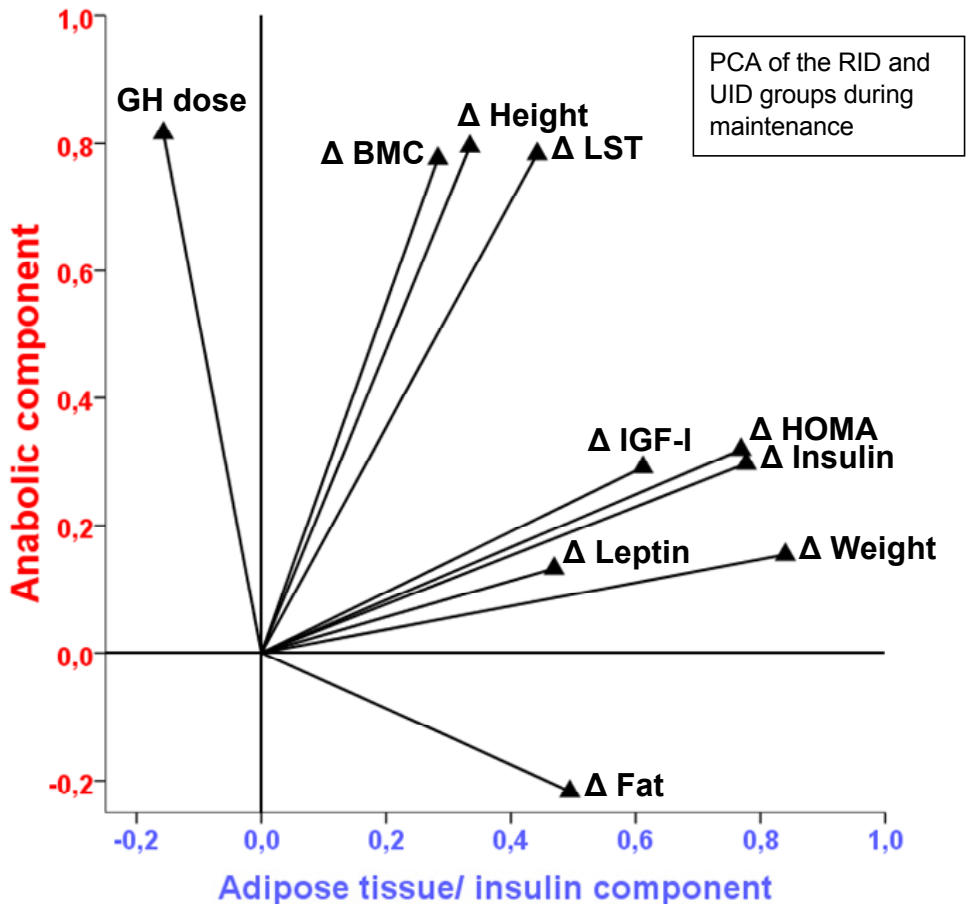


Figure 18 Two-dimensional principal component analysis (PCA) from the group of 43 individualized treated children comprising of the reduced individualized dose (RID), $n = 18$ and the unchanged individualized dose (UID), $n = 25$, during maintenance growth. The vector for each variable consists of a correlation with an anabolic component (y-axis) and a lipolytic component (x-axis). This PCA analysis explains 60.3% of the total variance observed in all variables depicted. Vectors with close mutual correlations form vector bundles with angles pointing in the same direction. Changes (Δ) between start and two years of maintenance treatment are included in the analysis. IGF-I (insulin-like growth factor I, SDS), LST (lean soft tissue mass, kg), BMC (bone mineral content, kg), Fat (fat mass, kg), HOMA (homoeostasis model assessment, mmol/mU). (Paper II)

Patients treated with the fixed standard dose showed less clear vector bundles of the responses (**Figure 19**). The correlations have to be interpreted carefully because of the limited number of patients ($n = 24$) and the fact that the dose has no variation ($43 \mu\text{g}/\text{kg}/\text{day}$). Nevertheless, the former anabolic vector bundle is much broader with IGF- I_{SDS} partly negatively correlated with height gain. Insulin and HOMA responses in the fixed GH dose group form a vector bundle together with weight response (**Figure 19**), precisely as in the individually treated children (**Figure 18**).

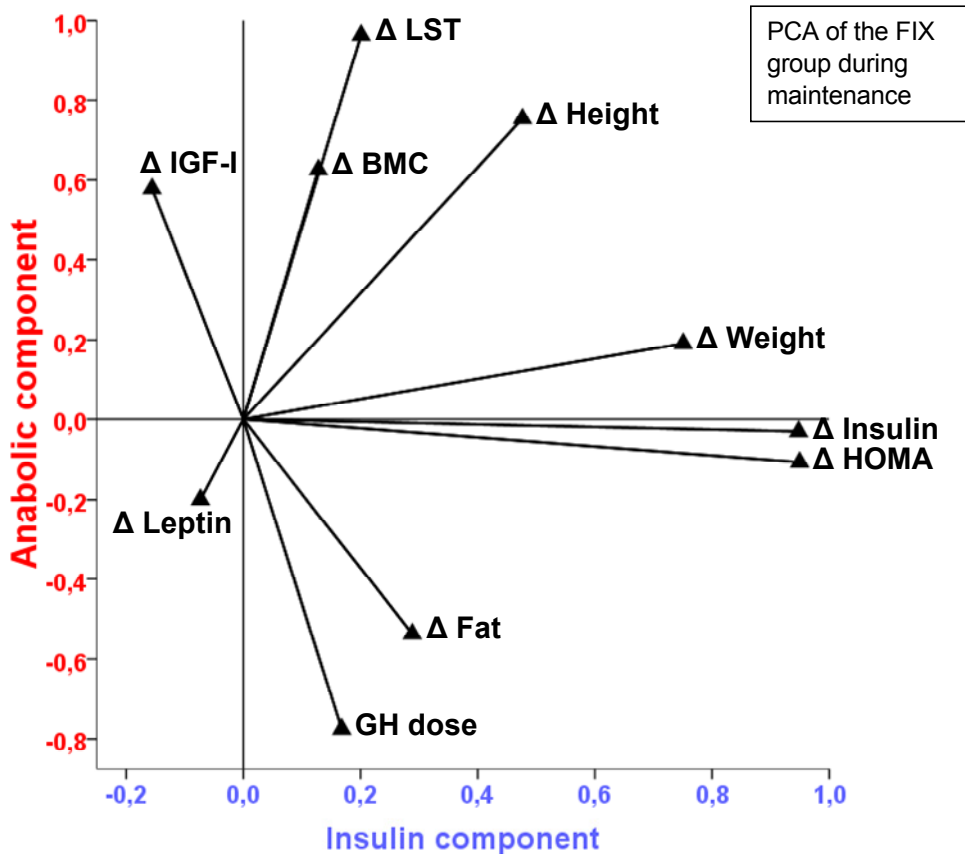


Figure 19 Two-dimensional principal component analysis (PCA) from the group of 24 children treated with the fixed standard dose (**FIX**) during maintenance growth, $n = 24$. The vector for each variable consists of a correlation with an anabolic component (y-axis) and a lipolytic component (x-axis). This PCA analysis explains 59.9% of the total variance observed in all variables depicted. Vectors with close mutual correlations form vector bundles with angles pointing in the same direction. Changes (Δ) between start and two years of maintenance treatment are included in the analysis. IGF-I (insulin-like growth factor I, SDS), LST (lean soft tissue mass, kg), BMC (bone mineral content, kg), Fat (fat mass, kg), HOMA (homoeostasis model assessment, mmol/mU).

When a subgroup of 18 children with channel-parallel growth within ± 0.3 heights SDS after two years (Δ Heights SDS 0-2 years), which was the primary endpoint of

maintenance treatment, was analysed, it was observed that the “anabolic” cluster (Δ lean soft tissue, Δ height_{SDS}, and Δ bone mineral content) was less dose-dependent compared to **Figure 16** during catch-up growth. Δ Fat mass correlated with Δ insulin and Δ HOMA and formed a weak ‘lipolytic’ cluster (shown by short vectors), indicating that there was only a minor impact on the lipolytic component in this subgroup. Δ leptin was found to be highly inter-correlated with the ‘anabolic’ cluster (**Figure 20**).

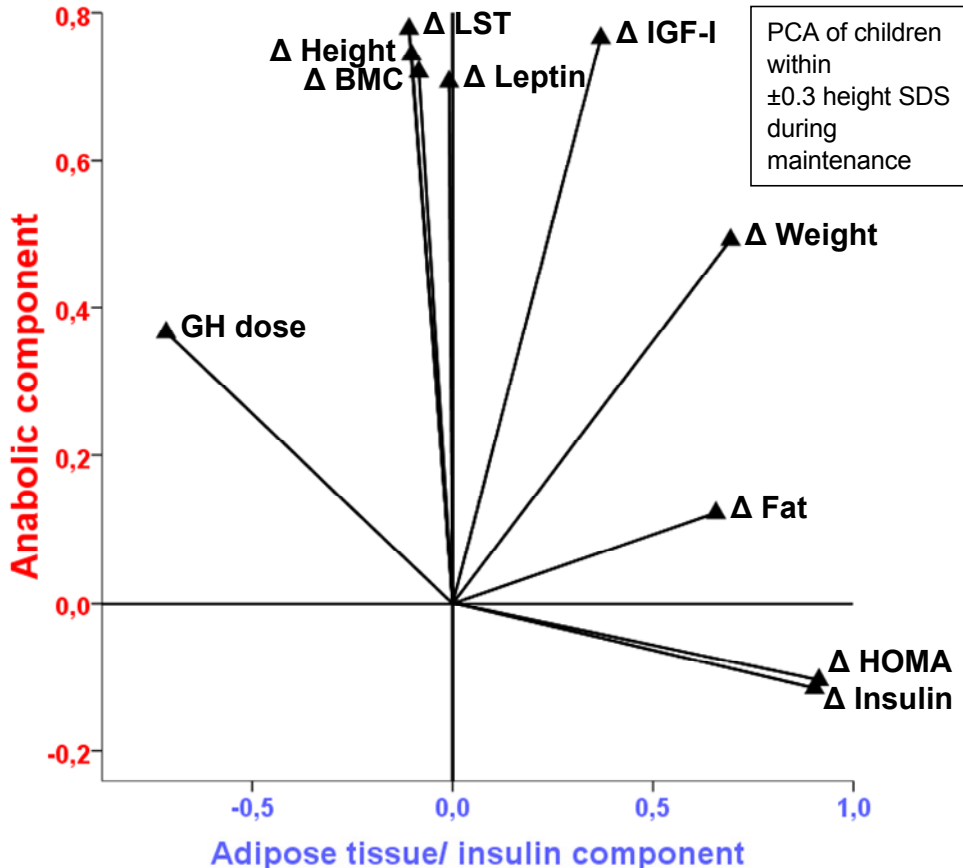


Figure 20 Two-dimensional principal component analysis (PCA) from the group of 18 children growing channel-parallel within ± 0.3 height_{SDS} after two years of maintenance growth. They included 10 children receiving the reduced individualized dose (RID), 4 receiving the unchanged individualized dose (UID), and 4 in the fixed dose group (FIX). Vectors with close mutual correlations form vector bundles with angles pointing in the same direction. Changes (Δ) between start and two years of maintenance treatment are included in the analysis. IGF-I (insulin-like growth factor I SDS), LST (lean soft tissue mass, kg), BMC (bone mineral content, kg), Fat (fat mass, kg), HOMA (homoeostasis model assessment, mmol/mU). (**Paper II**)

5.2.3 Bivariate regression

When comparing two growth factors, a parallel relationship between increases in insulin and increases in IGF-I was observed although absolute IGF-I levels did not

have any impact on insulin levels at any time; this suggests the involvement of separated responsiveness, which in this thesis is defined as responses of posttranscriptional mechanisms. (Figure 21).

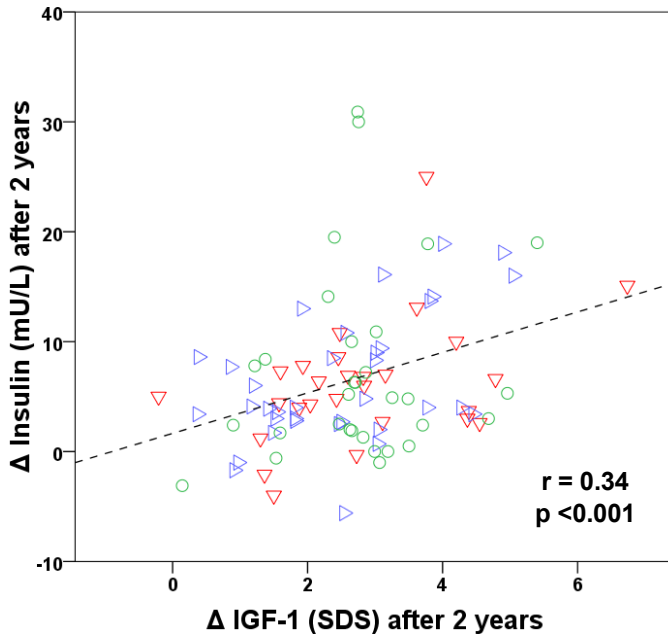


Figure 21 Linear regression of delta Δ insulin and Δ IGF-I after two years of GH treatment during the maintenance growth period. Δ is calculated between start and two years of maintenance treatment for the total per-protocol (pp) population. Regression coefficient r and p -value are given. Line of fit at total pp is shown. RID (reduced individualized dose), UID (unchanged individualized dose), FIX (fixed standard dose).

Comment: The GH dose can be reduced once $height_{SDS}$ has reached the target, i.e. close to MPH_{SDS}^{17} , in order to avoid over-treatment in terms of metabolic outcome. During the maintenance growth period, IGF-I seems no longer to be the major growth factor compared to the catch-up growth phase. Instead, in the children growing closest to SDS channel-parallel, leptin gains importance as a growth factor, promoting both longitudinal bone growth, muscle growth and bone mass growth.

This is illustrated in the PCA of the maintenance growth part (Figure 18), as the vector of the $IGF-I_{SDS}$ response is further apart from the anabolic response (LST, height, BMC) with larger angles, compared to the catch-up growth part (Figure 16). The leptin response is very close to the anabolic response, with minor angles in the children growing SDS channel-parallel despite few patients in this group.

5.3 THE PROTEOMIC APPROACH (PAPERS III-IV)

5.3.1 Dissociation between bone growth and bone mineralization (Paper III)

The hypothesis that GH-mediated effects on height-adjusted (HA) bone mineralization are regulated partly by the same mechanisms that are involved in longitudinal growth and partly by different mechanisms was tested in the GHD group of patients (n=39) (Paper III). All regulated peaks identified at 0–1 years that were selected in any significant regression model correlated with Δ BMD, Δ BMC_{HA}, Δ BA_{HA} or Δ height 0–1, 1–2 and 0–2 years on GH treatment were grouped. Out of a total of 37 selected peaks, 3 were present in models correlated both with Δ BMD or Δ BMC_{HA} (mineralization) and Δ height, 27 were uniquely present in models correlated with Δ BMD or Δ BMC_{HA}, and 7 peaks were uniquely present in models correlated with Δ height (Figure 22)^{Paper III}.

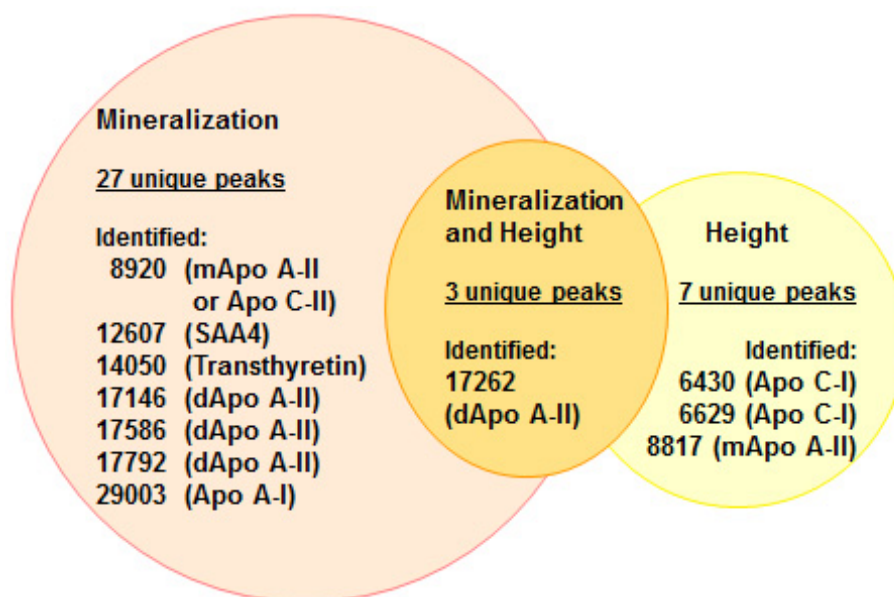


Figure 22 Protein peaks that were selected in any significant regression model for changes in bone mineralization and/or longitudinal bone growth during GH treatment in the GH-deficient group. Molecular weight is given in daltons. Apo A-I (Apolipoprotein A-I), mApo A-II (monomeric apolipoprotein A-II), dApo-AII (dimeric Apo A-II), Apo C-I (apolipoprotein C-I), Apo C-II (apolipoprotein C-II), SAA4 (Serum amyloid A4). (Paper III)

5.3.2 Biomarkers associated with GH effects on body composition (Paper IV)

In Paper IV, the appendicular body compartment was studied further. That is the sum of LST mass of arms and legs. Appendicular LST correlates best with muscle mass. A regression model consisting of three peaks at start was found in the GHD group explaining the first year changes of LST_{HAapp} ($r^2=0.50$). No other regression models were found for LST_{HAapp} in the other groups or for other time periods.

At start, a regression model of three peaks including a monomeric isoform of Apolipoprotein A-II (mApo A-II) was found to explain the variance of the second year changes in BMC_{HAapp} ($r^2=0.49$) in the GHD group. No other models correlating with BMC_{HAapp} were found in other groups or for other time periods.

FM at two years on GH treatment during the catch-up growth period correlated with peak patterns in the total group ($r^2=0.33$) and in the ISS group ($r^2=0.25$) using regression models of six or three peaks, respectively. The model explaining the variance of FM at two years of treatment in the total group consisted of mApo A-II and dimeric Apolipoprotein A-II (dApo A-II) isoforms, Serum amyloid A4 (SAA 4), and three so far unidentified protein peaks. The model explained the variance of FM at two years in the ISS group consisted of a dApo A-II isoform and two so far unidentified peaks (**Table 5**)^{Paper IV}.

Group	DXA	Peaks (n)	R ²	p<	Predictor peaks (m/z)	
Total	Δ 0-1y	LST _{HAapp}	-	-	n.s	-
	Δ 1-2y	BMC _{HAapp}	-	-	n.s	-
	at 2y	FM (total)	6	0.33	0.01	3042, 3219, 4713, 8920 (mApo A-II) , 12607 (SAA 4) , 17390 (dApo A-II)
ISS	Δ 0-1y	LST _{HAapp}	-	-	n.s	-
	Δ 1-2y	BMC _{HAapp}	-	-	n.s	-
	at 2y	FM (total)	3	0.25	0.05	9642, 12453, 17390 (dApo A-II)
GHD	Δ 0-1y	LST _{HAapp}	3	0.50	0.05	9827, 12872, 15363
	Δ 1-2y	BMC _{HAapp}	3	0.49	0.05	3821, 4156, 8875 (mApo A-II)
	at 2y	FM (total)	-	-	n.s	-

Table 5 Biomarkers at start related to body composition during GH treatment. DXA (dual energy X-ray absorptiometry), SELDI-TOF MS (surface-enhanced laser desorption/ionisation time-of-flight mass spectroscopy), m/z (mass over charge), ISS (idiopathic short stature), GHD (growth hormone deficiency), Total (combined ISS and GHD children), LST_{HAapp} (height-adjusted appendicular lean soft tissue), BMC_{HAapp} (height-adjusted appendicular bone mineral content), FM (fat mass of total body, kg), mApo A-II (monomeric apolipoprotein A-II), dApo A-II (dimeric Apo A-II), SAA4 (serum amyloid 4). (**Paper IV**)

5.3.3 Tissue-specific biomarkers associated with GH effects (Papers III and IV)

Tissue-specific biomarkers were found to be associated with GH effects when comparing findings from Paper III, where the focus was on total body DXA data, and Paper IV, where appendicular DXA data was the focus. A summary of tissue-specific biomarkers, both identified proteins and unidentified peaks associated with GH effects

on longitudinal bone growth or bone mineralization, is presented in **Table 6**. Of the peak changes that explained the variance of Δ LST_{HAapp}, one was unique for Δ LST_{HAapp}.

Five peak changes were specifically associated with GH effects on bone mineralization. Three peak changes, i.e. a transthyretin isoform, were associated with effects on FM^{Paper IV}.

Peak m/z and protein identity	
Δ LST _{HAapp}	7939
Δ BMC _{HAapp}	3374, 3821, 4156, 4212, 7440
FM (total)	4713, 13890 (TTR) , 15873

Table 6 Summary of Δ peaks uniquely selected in regression models correlating with specific body composition. m/z (mass over charge), LST_{HAapp} (height-adjusted appendicular lean soft tissue), BMC_{HAapp} (height-adjusted appendicular bone mineral content), FM (fat mass of total body, kg), TTR (transthyretin). (**Paper IV**)

Comment: The data suggest specific serum proteins, for example the nutritionally regulated transthyretin, being involved in changes of distinct body compartments. The mass/charge differences of the same isoforms are postulated to be the result of different post-translational modifications. Taken together, the result suggests that post-translational modifications could be of importance for tissue-specific effects of GH.

5.4 DIFFERENT THRESHOLDS OF GH EFFECTS (PAPER V)

5.4.1 Dose-dependency

Variables were analyzed as Δ values between start and at 2 years of GH treatment. For each of the six dose groups (17, 33, 40, 50, 67, 100 $\mu\text{g}/\text{kg}/\text{d}$), the mean Δ was calculated (Δ dose-group mean), for the 87 children receiving individualized GH treatment. The dose-response relationship of the metabolic outcome variables is fitted with S-shaped piecewise linear regression (Figure 23). The GH dose effect is given by the maximum range of the fitted piecewise function. Half of the GH dose effect on respective variables was calculated halfway the lowest and the highest level of the piecewise function [50% Δ effect].

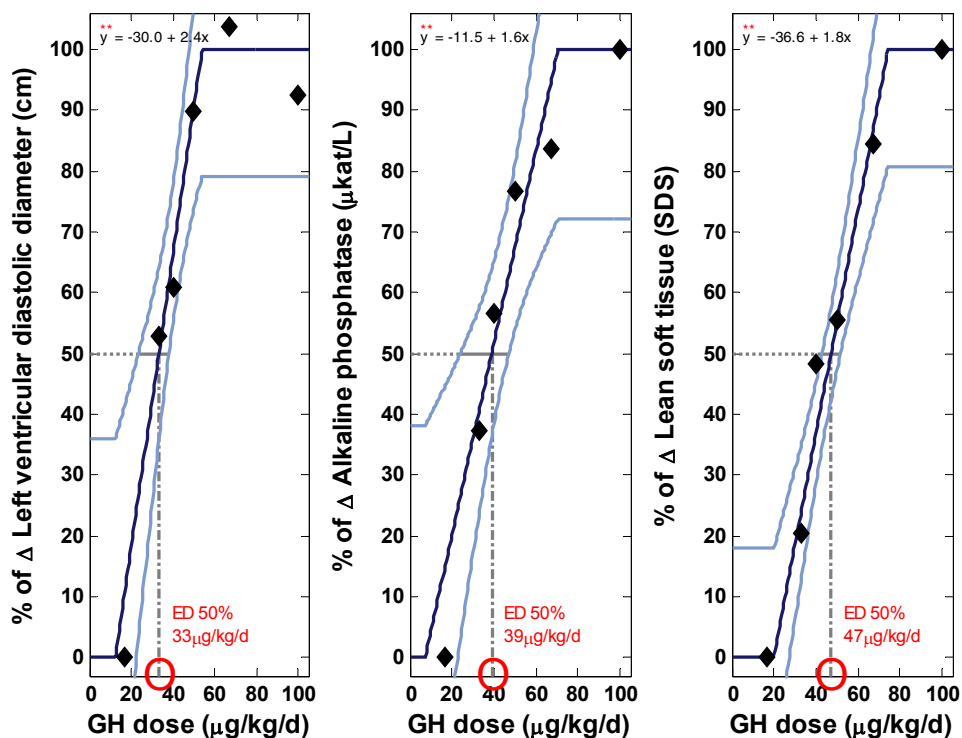


Figure 23 Dose-response relationship of metabolic outcome variables fitted with S-shaped piecewise linear regression lines with corresponding 90% confidence bounds. The y-axis is scaled as percent (%) of the maximum range of the fitted piecewise function. The lowest level of the piecewise function is set to 0% and the highest to 100%. The diamonds indicate the percentages Δ dose-group means (between start and 2 years) of the metabolic variables on the y-axis vs GH dose on the x-axis. LVDd: Left ventricular diameter in diastole, ALP: alkaline phosphatase, LST: lean soft tissue. The effective GH dose [ED 50%] required to achieve half of the dose effect is calculated according to the equation of the S-shaped piecewise linear GH dose effect. * $p < 0.05$, ** $p < 0.01$. (Paper V)

When performing ANOVA and linear regression, substantial lipolytic effects demonstrated by the variables fat mass and leptin were seen in all groups but no dose-response differences were observed between the GH dose groups.

5.4.2 Effective GH dose at 50% effect [ED 50%]

Dose-dependent increases of the dose-group means of Δ LVDd, Δ ALP, Δ LST_{SDS}, Δ insulin, Δ height_{SDS}, and Δ IGF-I_{SDS} at 2 years of individualized GH treatment are plotted in **Figures 23 and 24**. All values in Figure 23 are given as percentages of the maximum range of the fitted piecewise function, where the lowest function value is set to 0% and the highest to 100%. Figure 24 show the same data, but now with the original scale of the Δ metabolic variables and with the individual sample values added.

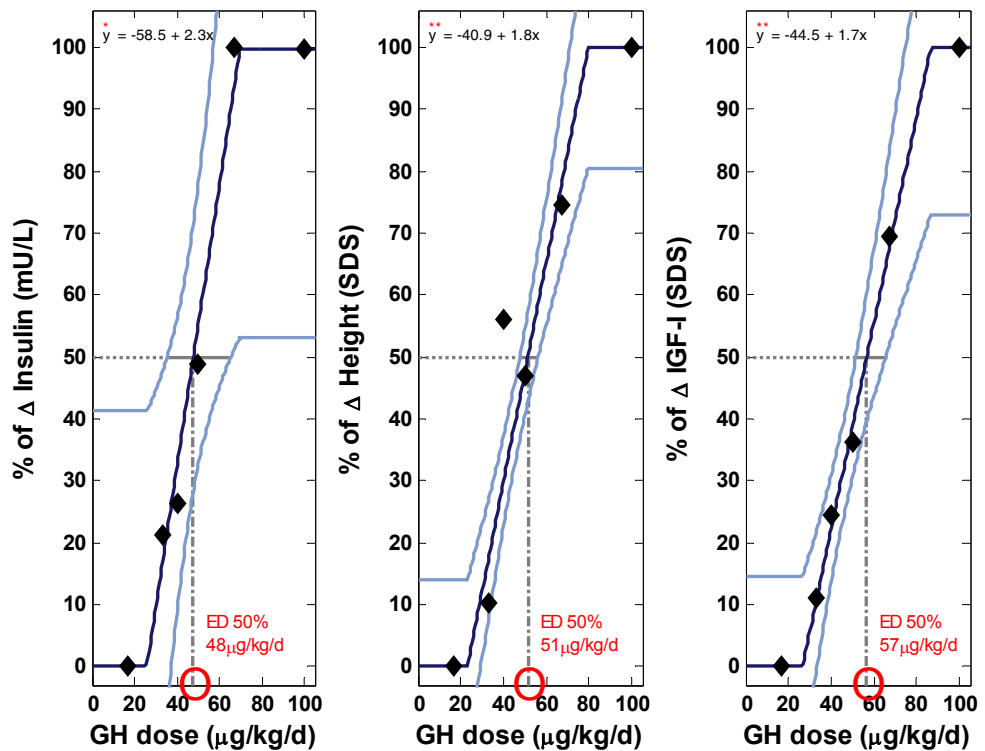


Figure 24 Dose-response relationship of metabolic outcome variables fitted with S-shaped piecewise linear regression lines with corresponding 90% confidence bounds. The y-axis is scaled as percent (%) of the maximum range of the fitted piecewise function. The lowest level of the piecewise function is set to 0% and the highest to 100%. The diamonds indicate the percentages Δ dose-group means (between start and 2 years) of the metabolic variables on the y-axis vs GH dose on the x-axis. IGF-I: insulin-like growth factor I. The effective GH dose [ED 50%] required to achieve half of the dose effect is calculated according to the equation of the S-shaped piecewise linear GH dose effect. * $p < 0.05$, ** $p < 0.01$. (**Paper V**)

The effective GH dose [ED 50%] required to achieve [50% Δ effect] was calculated based on the fitted piecewise linear regression equation. The higher the ED 50%, the lower is the responsiveness of a given variable. The ED 50% was lowest for Δ LVDD; i.e. the most responsive variable and highest for Δ IGF-I_{SDS}; i.e. the least responsive variable. Δ Alkaline phosphatase (ALP), Δ LST_{SDS}, Δ insulin, and Δ height_{SDS} lay in the interval between in ascending order. Differences between the Δ dose-group means for the metabolic variables and the piecewise linear GH dose effect were tested by one-way ANOVA (**Figures 23 and 24**)^{Paper V}.

*Comment: There are GH dose-dependent thresholds for different metabolic effects, with cardiac tissue being more sensitive to GH treatment than height gain. Increases in insulin were more sensitive than IGF-I_{SDS}, confirming the finding in **Paper I** that insulin may be required as a growth factor during the catch-up growth period in prepubertal short children.*

6 DISCUSSION

6.1 CONTINUUM OF GH RESPONSIVENESS

6.1.1 Continuum

There is an on-going debate about ISS, provoking dissent on its definition, pathophysiology, treatment, impact on quality of life and metabolism¹⁸⁶. There is nonetheless wide agreement about the current definition of ISS, ‘...a condition in which the height of the individual is more than 2 SDS below the corresponding mean height for a given age, sex and population group, without evidence of systemic, endocrine, nutritional or chromosomal abnormalities’. Specifically, children with ISS have normal birth size and are GH sufficient^{187, 188}.

The study reported in this thesis followed the above definition with two additions: All children were also at least 1 SDS below their MPH_{SDS} and, to be evaluated as GH sufficient, *both* GH_{max} at provocation test and the GH_{max} during a spontaneous 24-hour profile were taken into account. GH responsiveness was addressed individually by estimating the growth response as if the child were on a GH dose of 33 µg/kg/d by using the growth prediction model². Furthermore, higher GH doses were given to children with a lower predicted gain in height_{SDS} and at the same time greater estimated difference of height_{SDS} and MPH_{SDS} at two years of treatment¹.

It is well known that children who enter treatment with a greater deviation from target height (diffMPH_{SDS}) grow better during the first years of GH treatment⁵, thus showing a higher response to GH, an indirect measure of the degree of GH responsiveness¹.

In previous studies it has been shown in groups of GHD, ISS and SGA prepubertal children that a greater distance to target height (diffMPH_{SDS}) is one of the important variables that is positively related to the 1st year growth response^{2, 3, 131, 134}.

Similar metabolic changes were indeed seen in GHD and ISS, supporting the hypothesis that any chosen cut-off of endogenous GH secretion to distinguish between GHD and ISS is arbitrary. The metabolic data support the hypothesis of a continuous transition from GHD to ISS. More important is that the growth periods during GH treatment are taken into account, in that the dose can be successfully reduced without causing metabolic impairment.

6.1.2 Responsiveness

The definition of sensitivity has here been separated from responsiveness. Thus, GH sensitivity is here used to determine the ability of the GH receptor to act on a stimulus from its binding hormone – i.e. the GH molecule, whereas GH responsiveness is used to determine the entire intracellular signalling towards a certain GH effect.

In this research project, the metabolic response was measured as the extent of metabolic responsiveness. Responses were measurable effects mainly of GH treatment and, to a lesser degree, GH secretion, while responsiveness was an attribute used to conceptualize the ability of an organism to respond to a given stimulus, GH. The concept of responsiveness has its origin in the early 20th century, when actions of the first known hormones were studied¹⁸⁹. Thereafter it has been continuously used in the field of endocrinology. There are different means of adapting treatment to compensate for different responsiveness to GH. Responses to GH can be similar, even if there are differences in responsiveness, if other determinants of responsiveness are adapted, for example, adjusting the GH dose^{1, 17, Paper I} or selecting patients for GH secretion capacity, age at onset of treatment, pubertal stage, gender, acute illness or underlying diseases, genetic characteristics¹²³ and nutrition^{3, 120, 139, 190, 191}.

Responsiveness to GH has been shown in this project not to be constant over time and to be influenced by age and different growth phases in the the infancy–childhood–puberty (ICP) growth model¹⁰⁹. Younger GH-treated short children with varying diagnoses, have repeatedly been shown^{2, 130, 134, 192-194} to have higher GH response to a given dose than older children¹⁹⁵. The younger the short child at onset of GH treatment, the better the growth response¹³⁴. The response may be influenced by selection bias, as those starting GH treatment before the age of five years may have more severe short stature¹⁹⁵. In this project this bias was addressed by the randomization procedure when selecting children to the treatment groups.

The impact of a multitude of genes on GH responsiveness has been discussed¹⁹⁶. Monogenetic defects of the GH–IGF pathway should not be regarded as ISS. Cumulative effects of yet unknown gene polymorphisms associated with adult height may result in ISS as illustrated by the 6-cm difference between individuals with more than 30 ‘tall’ alleles and those with less than 15¹⁹⁶⁻¹⁹⁸.

To estimate the growth response to GH treatment in GHD children, prediction models have been developed by different research groups^{2, 3, 127}. Thereafter, the KIGS group

developed separate models for short children with ISS¹⁹⁹ and SGA¹²⁹ whereas the Gothenburg group developed one model to be used for short children with isolated GHD, ISS or SGA¹³⁰.

The use of prediction models estimating the individual GH growth responsiveness of each child, facilitated individualized GH dosing, and the results from the present study show that the gap was bridged from different phenotypes to both metabolic as well as growth response outcomes without needing to know the detailed underlying mechanisms of the individual child's disorder. The presumption that there should be a diagnosis of ISS on the one hand or classical GHD on the other hand was challenged; ISS was characterized by low GH responsiveness, as one extreme of a continuum with GH secretion deficiency, denoted by high GH responsiveness, as the other extreme^{2, 133, 134, 200}.

6.2 METABOLIC RESPONSIVENESS TO GH TREATMENT

Insulin sensitivity and GH responsiveness have been shown to have mutual influences in the growing child¹³⁴. However, little is known about the effects of GH on insulin sensitivity in children, especially if the dose is individualized according to responsiveness estimated by a growth prediction model. In severely GH-deficient adults, low GH dose (0.1 mg/day) decreased fasting glucose levels and improved insulin sensitivity with little effect on body composition²⁰¹.

There are isolated studies of metabolic responsiveness in regard to serum levels of leptin^{6, 146, 202}, insulin²⁰¹, IGF-I and IGFBP-3^{200, 203, 204}, body composition and bone strength^{205, 206} and always with the same weight based GH dose to all, disregarding possible individual GH responsiveness. In this study, changes of metabolic markers were confirmed; lean body mass and bone mass increased and fat mass decreased, IGF-I increased, while fasting insulin increased during the catch-up period of GH treatment without any significant difference between the treatment groups^{Paper I}.

6.2.1 Reduced variance of metabolic responses

Analogous to the existing approach to GH treatment of adults²⁰⁷, efforts have been made to individualize GH treatment in children by titrating IGF-I levels⁴. However, this is achieved at the price of higher GH doses up to 346 µg/kg/d⁴. Contrary to this, the present study shows that the GH dose can be individualized and extreme doses avoided using a prediction model that estimates the individual responsiveness of

longitudinal bone growth, which leads to a reduced variability in growth response compared to fixed weight-based standard GH dosing¹.

A main finding of this research project was that the variation of the insulin response was also reduced in the same way as the variation of longitudinal bone growth by using the same prediction model. During the catch-up growth period, narrower variation of fasting insulin and HOMA was demonstrated after two years of individualized GH treatment in comparison with a fixed standard GH dose. In other words, the individual GH dose administered, although different for each child, could be regarded as the same biological dose in terms of both longitudinal bone growth and metabolism (**Figure 25**). This means that some children do need a dose that is numerically 'higher' but biologically similar^{1, 132}.

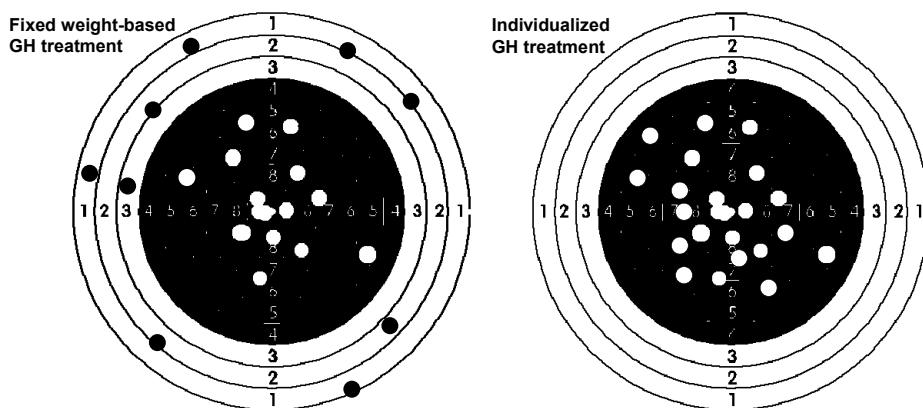


Figure 25 The dartboard illustrates the goal of optimizing treatment so that short children treated with GH will reach the preset height–metabolic goal by individualizing the GH dose. Response to individualized GH treatment is more stable around a predefined treatment goal compared to fixed weight-based GH dosing.

The results indicate that the observed reduction in variation observed for insulin and HOMA in children treated on an individual bases was reached by predicting (albeit in an unorthodox manner) the responses of insulin and HOMA, and thus parts of the metabolic GH responsiveness, at the same time as the longitudinal bone growth responsiveness.

It can be concluded that individualized GH treatment, based on the estimation of longitudinal growth responsiveness, does not lead to excessive values of insulin. However, a generalization of this conclusion is restricted to the population studied,

short prepubertal children with a wide range of GH secretion. Future studies are needed to confirm these findings.

The data from the maintenance period points out that the GH dose has to be adjusted by reduction to 50% after the catch-up growth is terminated. Nevertheless, the dose was arbitrarily halved, which was not based on a prediction model. In the children who did not receive a reduced dose during the maintenance period (the unchanged individualized dose group as well as in the fixed standard dose group), high insulin levels in can be expected in the long run.

The GH dose during the catch-up growth period was not solely chosen based on individual responsiveness but also corrected for the distance to MPH_{SDS}, leading to higher doses than would have been predicted by responsiveness itself.

6.2.2 Metabolic responses can dissociate from the growth response

Some of the metabolic functions and metabolic active tissues show changes parallel to the longitudinal growth response and some deviate from it, suggesting dissociation. As a corollary of the complexity of nature, different metabolic functions cannot be looked at in isolation. Metabolism is a complex network with mutual interactions.

In this thesis, inter-correlations of linear growth with bone mass growth and muscle mass growth, called the anabolic GH effects, are examined. They have been shown to be dose-dependent, which confirms previous findings of dose-dependent height gain in children²⁰⁸.

Lipolytic GH effects, namely decrease in fat mass and leptin, are not dependent on the GH dose being within the range 17– 100 µg/kg/day with a threshold supposedly being lower than 17 µg/kg/day. The lipolytic GH effects are not related to the anabolic effects as shown by PCA. This leads to the possibility that anabolic GH effects can be modulated by adipose tissue-derived hormones, as is suspected for leptin and suggested by the findings in the maintenance growth part of this study. This is an expected result, as leptin levels at GH start of treatment correlate with the growth response⁶. Moreover, in vitro studies have shown that leptin exerts direct effects on the growth plate²⁰⁹.

The data presented here suggests that insulin is a mediator of exogenous GH during the catch-up growth period, indirectly mediating the anabolic growth signal. The signal is mildly increasing insulin resistance. Thus, insulin per se is an important growth factor during the catch-up growth period in response to GH treatment.

Elevated insulin levels during GH treatment may not only be due to GH-induced hyperglycaemia and insulin resistance²¹⁰ but may also be a mechanism to compensate for a local insulin deficiency on the level of myocytes, expressed by the LBM and LST. Like the expression of IGF-I due to muscle loading, the enhanced expression of IGF-2 mRNA in muscle hypertrophy also appears to have a significant GH-independent component²¹¹, which may be due to anabolic effects of insulin. Insulin stimulates protein synthesis via its own cognate receptor, while IGF-I utilizes both IGF-I and IGF-I/insulin receptor hybrids²¹².

6.3 RESPONSIVENESS OF NUTRITION MARKERS

The pharmaco-proteomic approach in Paper III confirmed different tissue responses. The discovery of biomarkers that were uniquely associated with bone mineralization, and others uniquely with linear bone growth, suggests different mechanisms regulated during GH treatment or, alternatively, mechanisms that modulate bone mineralization and linear bone growth itself.

The biomarkers identified so far were different serum apolipoproteins, which are important nutrition markers. In 2009 similar apolipoproteins were identified including Apo A-I, Apo A-II, Apo C-I, Apo C-III, and SAA 4 as well as transthyretin as markers of longitudinal growth response suggesting that these proteins may play a role in tuning GH sensitivity²¹³. Interestingly, different isoforms of a protein could be associated with specific effects on different tissues, indicating that post-translational modifications might be of importance in the regulation of these processes. However, further validation will be needed to assess the clinical significance of the results.

Skeletal muscle is a large and important body composition compartment in children, a major site of energy expenditure, insulin resistance, and anabolism²¹⁴. However, current knowledge is based on the composition of the total body. Benefits can be gained by studying parts of the body in detail, which make it possible to focus more accurately on tissue functions and their interactions, for example the musculoskeletal unit during GH treatment with its marked changes in body composition and impact on metabolism. Based on the publications presented in this thesis, proteomic tools are now available to identify biomarkers not only related to longitudinal bone growth^{181, 213}, but also associated with bone mineralization and body composition in GH-treated short prepubertal children.

Lipoproteins were identified that correlated with linear growth response to GH treatment in short prepubertal children^{Paper III}, indicating that they may play a role in

promoting growth itself or in modulating anabolic GH effects. Alternatively, lipoproteins may merely be influenced by GH without any effect on growth. Moreover, transthyretin is a major nutrition marker and correlated with bone mineralization. Furthermore, serum protein profiles that correlated with changes in body composition, i.e. appendicular LST and BMC, in response to GH treatment were identified. The majority of the proteins identified, represent different apolipoproteins; Apolipoprotein A-I (Apo A-I), monomeric and dimeric isoforms of apolipoprotein A-II (Apo A-II), apolipoprotein C-I (Apo C-I), and serum amyloid A4 (SAA4). In Paper IV, transthyretin and hemoglobin A (HBA) were also found to correlate.

6.4 THRESHOLDS OF METABOLIC RESPONSIVENESS

Heart muscle, ALP, skeletal muscle, insulin, height and IGF-I increase in the catch-up period during two years of GH treatment in a dose-dependent manner. One-way analysis of variance (ANOVA) showed differences in response thresholds between the six different GH doses^{Paper V}. This is presumed to be due to the fact that the GH dosing was adapted to the individual responsiveness based mainly on longitudinal growth prediction, and not all metabolic responses follow that. However, some are close to height gain.

Supporting the findings of alternative GH-induced growth factors (i.e. insulin and leptin), the IGF-I_{SDS} response was found to need higher GH doses than the growth response (in other words, IGF-I has a higher threshold) suggesting that IGF-I is not the only growth factor mediating the growth signal towards the growth plate and skeletal muscle cells.

In the same way, the bone growth marker ALP, a reliable and early sign of increased bone metabolism after initiation of GH treatment, needs lower GH doses than longitudinal growth response; despite the fact that Δ BMC, representing the formation of mineralized bone mass, was highly correlated with anabolic GH response.

Gradual GH dose-responses were found with different thresholds, namely that effective GH doses at half of the GH effect show different thresholds of the metabolic functions and tissues. This has led to a modified “staircase” of GH responsiveness (**Figure 26**) which is different adding measured responsiveness to the previous hypothesis (**Figure 1**).

The main difference compared to the original staircase is the different order of skeletal muscle, insulin and longitudinal growth on one hand and IGF-I on the other being less

responsive. Myocardium has been added and has been shown being most responsive of all investigated variables. Markers of the immune system according to the original hypothesis have not been investigated. Adipose tissue did not show any dose-response, but is supposed to be most responsive according to lipolytic GH effects demonstrated in this study. However, the lipolytic effect may have been overridden within the dose range of 17–100 $\mu\text{g}/\text{kg}/\text{d}$ used in this study. It can be presumed that the threshold of the lipolytic GH effect reached the upper stationary part of an sigmoid-shaped dose-response curve. From the literature, substantial lipolytic GH effects in adults with GHD have been reported for doses below 10 $\mu\text{g}/\text{kg}/\text{day}$ ²¹⁵⁻²¹⁷. Skeletal muscle tissue was more responsive than cardiac tissue, and insulin levels were more responsive than IGF-I.

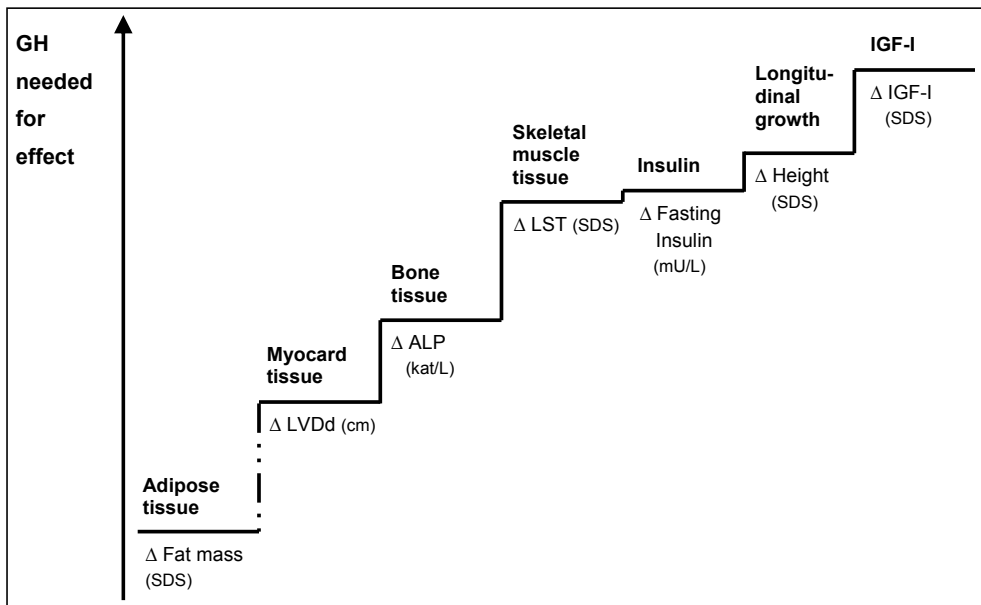


Figure 26 Different thresholds of GH response of tissues and metabolic functions are hypothesized from the results discussed in this thesis. Surrogate markers are listed underneath the staircase. Δ (delta), ALP (alkaline phosphatase), cm (centimetre), IGF-I (insulin-like growth factor I SDS), LST (lean soft tissue), LVdD (left ventricular diameter in diastole), SDS (standard deviation score).

7 CONCLUSIONS

This unique study design – using different GH doses – shows that the variability of metabolic makers is a practical means of investigating the responsiveness of each single child. Moreover, a multivariate approach makes the interactions within a metabolic network better understandable.

1. Individualized GH dosing during catch-up growth reduces the variance in insulin and HOMA and results in equal metabolic responses irrespective of the diagnosis of GHD or ISS (Paper I).
2. Continued individualized GH treatment with reduced dosing during the maintenance growth period reduced the number of children with hyperinsulinism (Paper II).
3. The GH dose can be decreased in children treated with an individualized dose, keeping metabolic variables within the reference range, once height_{SDS} has reached the midparental height target, in order to avoid under- and over-treatment in terms of metabolic outcome (Paper II).
4. When SDS channel-parallel growth is reached, IGF-I seems no longer to be the only growth factor. There are indications, that insulin during catch-up growth and leptin during maintenance growth gained importance as growth factors, promoting both longitudinal bone growth, muscle growth and bone mass growth (Paper II).
5. A proteomic approach could be used to identify specific protein expression patterns associated with longitudinal bone growth, bone mineralization, and body composition in response to GH treatment. Different nutrition markers, such as apolipoproteins and hemoglobin subunit alpha were associated with remodelling of body composition in response to GH treatment (Papers III, IV).
6. Dose-dependent thresholds were found for different GH effects with adipose tissue, myocardium and skeletal muscle tissue being more sensitive to GH treatment than height gain. Increases in insulin were more marked than IGF-I changes, which were found to be least influenced by GH (Paper V).

8 FUTURE PERSPECTIVES

Both under- and over-treatment can be avoided with individually adapted GH doses. It is suggested that the underlying individual GH dosing based on a prediction model and furthermore by reducing the GH dose during the maintenance growth period should be implemented in clinical practice in order to improve medical efficacy as well as cost-efficiency.

The combination of a prospective study design, well-characterized patients, randomization to the treatment groups and a multivariate data analysis approach establishes a valuable platform to increase our understanding of the GH effects with metabolic modulators.

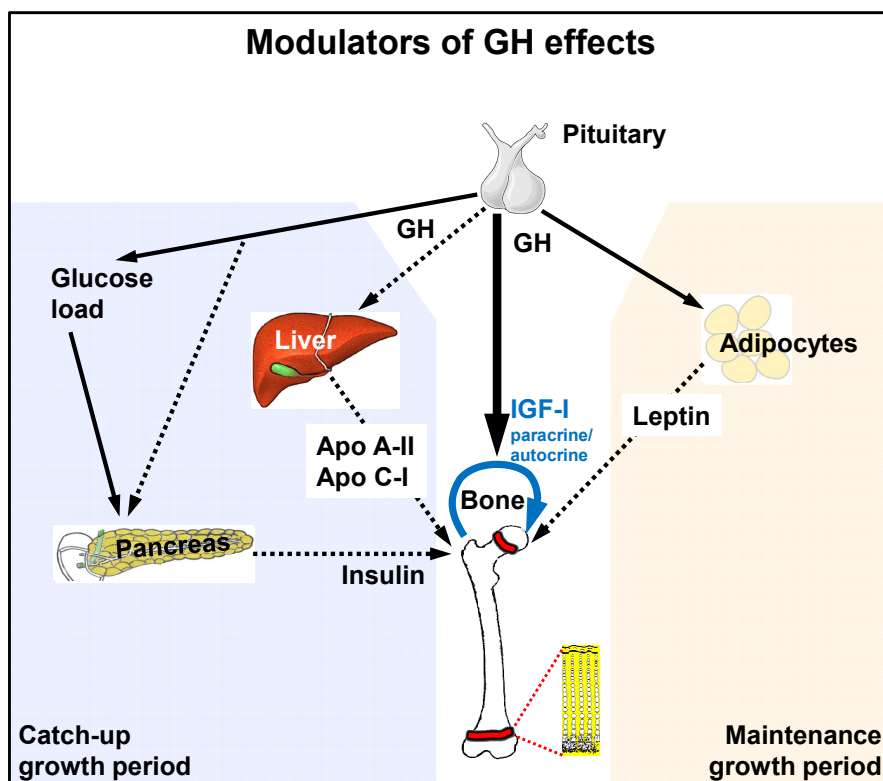


Figure 27 GH acts directly on the growth plate by stimulating autocrine and paracrine IGF-I secretion. Liver IGF-I is not essential for bone growth. In this thesis, during the catch-up growth period, GH effects were found to be associated with insulin, as supported by a mouse model²¹⁸, and apolipoprotein (Apo) A-II and Apo C-I were identified biomarkers associated with longitudinal bone growth. Insulin was dose-dependently increasing during GH treatment, which is possible through a GH-induced glucose load or directly by stimulating insulin secretion. During the maintenance growth period, predominantly in the children growing SDS channel-parallel, leptin was found to be associated with growth.

Based on the findings from this thesis, an extended somatomedin hypothesis with additional modulators of the response to GH needs to be confirmed in larger well randomized clinical studies and animal models (**Figure 27**).

Proteomic methods are laborious and therefore not suitable in clinical practice. They are used to generate hypotheses in clinical sciences. To implement the knowledge obtained from proteomic sciences into clinical practice, the generated hypotheses need to be tested and validated in blood or easy-accessible tissues by established methods.

In the future we may develop models for the prediction of metabolic responses per se, that is, insulin, body composition, bone metabolism, and quality of life parameters. Not only proteomic but also metabolomics and genetic approaches may lead to even more accurate prediction models. To validate these prediction models, validation studies will be necessary in order to generalize the predictions and to implement them into clinical practice.

Little is known about the cardiovascular risk of low GH responsiveness. Childhood-onset GHD leads to an increased cardiovascular risk in adult life as manifested by elevated fasting and postprandial lipids and by increased body fat that is reversed by GH treatment²¹⁹. Individualized GH dosing is to go for in the future in the light of current reports in children with isolated GHD, ISS or SGA, of a (potentially) higher incidence of deaths during GH treatment with a dose relationship^{220, 221}.

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11 OUTLINE OF THE PAPERS

A short description of each of the papers in this thesis is presented below.

Paper I describes how individualized GH dosing during catch-up growth reduces the variance in insulin and HOMA and results in equal metabolic responses irrespective of the diagnosis of GHD or ISS.

Paper II shows that the GH dose can be decreased once height_{SDS} has reached the target, i.e. approaching midparental height in order to avoid over-treatment in terms of metabolic outcome. Continued individualized GH treatment during the maintenance growth period was secured by reducing hyperinsulinism.

When SDS channel-parallel growth was reached during the maintenance growth period, IGF-I seems no longer to be the only major growth factor. Instead, leptin gained importance as a growth factor, promoting both longitudinal bone growth, muscle growth and bone mass growth.

Paper III concludes that a proteomic approach could be used to identify specific protein expression patterns associated with bone mineralization in response to GH treatment and that height-adjusted bone mineralization and longitudinal bone growth were regulated partly by the same and partly by different mechanisms. Protein isoforms with different post-translational modifications might be of importance in the regulation of these processes.

Paper IV shows a proteomic approach using SELDI-TOF MS being suitable to identify biomarkers that explain the variance in body composition during GH treatment. Different apolipoproteins and haemoglobin subunit alpha were associated with remodelling of body composition in response to GH treatment.

Paper V reveals dose-dependent thresholds for different GH effects with muscle tissue being more sensitive to exogenous GH than height gain; cardiac tissue was found to be most responsive. Insulin was more sensitive than IGF-I, which was found to be least sensitive, suggesting that insulin may be required as a growth factor during the catch-up growth period in prepubertal short children.