Impact of the GH-IGF-I axis in adults – pharmacogenetic and genetic association studies

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Cover:
Word cloud of introduction section. http://www.wordle.net. Sizing represents the number of times the word appears in the text (more frequent = bigger size).

Till Lucas och Leia - de mest fantastiska barn på denna jord.

”Föräldrar till begåvade barn tror på ärftlighetsteorin”.
Joachim Fuchsberger

ABSTRACT

Growth hormone (GH) is a polypeptide hormone which is secreted from the anterior pituitary in a pulsatile pattern. GH is best known by its strong effects on longitudinal growth in children, but the importance of GH is maintained also in adulthood due to the powerful effects on cellular differentiation and fuel homeostasis. GH deficient (GHD) adults often present with abdominal obesity, insulin resistance and an almost doubled risk for cardiovascular mortality. These problems are to a varying extent reversed during GH replacement therapy (GHRT). Variability in treatment response is large and probably at least partly mediated by genetic variation. The effects of GH are mediated by the GH receptor (GHR), a transmembrane glycoprotein expressed on most human cell types. In humans, there are two isoforms of the GHR which differ in regards to retention (fl-GHR) or exclusion (d3-GHR) of exon 3. The two isoforms are simply two different alleles of a common *GHR* polymorphism (the *GHR* d3/fl polymorphism), which has been suggested to influence GH sensitivity.

This thesis is based on four studies, with the common, overall, aim to test the hypothesis that polymorphisms in genes within the GH-IGF-I axis influence body composition, metabolism and serum IGF-I concentrations.

In an initial study we found that the *GHR* d3/fl polymorphism was not associated with the 1 year changes in IGF-I concentrations and body composition in response to GHRT. During this study, which was fairly small, we realized the need for a different genotyping method for analyses of the *GHR* d3/fl polymorphism in larger cohorts. Therefore, in the subsequent study, we evaluated the use of tagSNP rs6873545 as a marker for the *GHR* d3/fl polymorphism, and showed that it does indeed perfectly tag the different *GHR* d3/fl alleles. In the next study, we investigated the impact of the *GHR* SNP rs6873545 and five other polymorphisms in genes within the GH-IGF-I axis on the early and the long-term IGF-I responses to GHRT in a large cohort of GHD adults. In this study we found that the *GHR* SNP rs6873545 and the *PIK3CB* SNP rs361072 were associated with the early, but not the long-term, IGF-I response. In the last study, we analysed the tagSNP rs6873545 in a study representative of the general Swedish population and found that homozygosity for the d3-GHR was associated with an adverse anthropometric and metabolic profile.

In conclusion, the results of this thesis suggests that 1) the *GHR* d3/fl polymorphism is indeed of functional importance, and influences both the response to GHRT in GHD adults as well as body composition and metabolism in the general Swedish population, and 2) other SNPs in genes within the GH-IGF-I axis may be of an equal importance in terms of the response to GHRT in GHD adults.

Key words: growth hormone, growth hormone receptor, genetic association study, pharmacogenetic, growth hormone deficiency, polymorphism, candidate gene approach, metabolism, anthropometry.

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LIST OF PAPERS

This thesis is based on the following papers and manuscripts, which are referred to in the text by their Roman numerals:

Paper I. **Influence of the exon 3-deleted/full-length growth hormone (GH) receptor polymorphism on the response to GH replacement therapy in adults with severe GH deficiency.**
Barbosa EJ, Palming J, Glad CA**,** Filipsson H, Koranyi J, Bengtsson BA, Carlsson LM, Boguszewski CL, Johannsson G.
J Clin Endocrinol Metab. 2009 Feb;94(2):639-44.

Paper II. **Rapid and high throughput genotyping of the growth hormone receptor exon 3 deleted/full-length polymorphism using a tagSNP.**
Glad CA**,** Johannsson G, Carlsson LM, Svensson PA.
Growth Horm IGF Res. 2010 Jun;20(3):270-3.

Paper III. **SNPs within the GH signalling pathway are associated with the early, but not the long-term, IGF-I response to GH replacement therapy in GHD adults.**
Glad CA**,** Barbosa EJL, Filipsson Nyström H, Carlsson LMS, Nilsson S, Nilsson AG, Svensson PA, Johannsson G.
Manuscript.

Paper IV. **The growth hormone receptor exon 3 deleted/full-length polymorphism is associated with body weight and body composition in the general population.**
Glad CA**,** Carlsson LMS, Sjöström L, Nilsson S, Larsson I, Svensson PA, Johannsson G.
Manuscript.

TABLE OF CONTENTS

ABBREVIATIONS 8

INTRODUCTION 9

GENETICS AND THE HUMAN GENOME 9

Genetic variation 10

Genetic linkage 11

Pharmacogenetics 11

THE GH-IGF-I AXIS 11

The GH signalling pathway 11

Adult GH deficiency (GHD) 12

PHARMACOGENETICS IN GH REPLACEMENT THERAPY 12

The *GHR* exon 3 deleted/full-length polymorphism 13

The *GHR* d3/fl polymorphism in GHRT 14

THE GH-IGF-I AXIS IN GENETIC ASSOCIATION STUDIES 14

AIMS 15

COHORTS and METHODS 16

STUDY POPULATIONS 16

The GH-2 study 16

The Swedish Obese Subjects (SOS) reference study 17

GENETIC ANALYSES 18

DNA extraction and quantification 18

Selection of candidate genes and SNPs 18

Genotyping methods 18

KEY RESULTS 22

GHRT RESPONSE PREDICTION 22

Paper I 22

Paper III 22

FACILITATED GENOTYPING 23

Paper II 23

THE GENERAL POPULATION 24

Paper IV 24

DISCUSSION 25

GHRT RESPONSE PREDICTION 25

The *GHR* d3/fl polymorphism 25

The GH signalling cascade 27

THE *GHR* d3/fl POLYMORPHISM IN THE GENERAL POPULATION 27

CONCLUDING REMARKS 29

SAMMANFATTNING PÅ SVENSKA 30

ACKNOWLEDGEMENTS 31

REFERENCES 33

PAPERS 43

ABBREVIATIONS

A	Adenine
BMD	Bone mineral density
BMI	Body mass index
bp	Base pair
C	Cytosine
CO	Childhood-onset
d3-GHR	Exon 3 deleted growth hormone receptor
DBP	Diastolic blood pressure
DEXA	Dual energy x-ray absorptiometry
DNA	Deoxyribonucleic acid
FFM	Fat free mass
fl-GHR	Full-length growth hormone receptor
G	Guanine
GH	Growth hormone
GHD	Growth hormone deficiency
GHR	Growth hormone receptor
GHRH	Growth hormone releasing hormone
GHRT	Growth hormone replacement therapy
GWAS	Genome-wide association study
HDL-C	High density lipoprotein cholesterol
HGP	Human genome project
HOMA-IR	Homeostatic model assessment insulin resistance
HWE	Hardy-Weinberg equilibrium
IGFBP-3	Insulin-like growth factor binding protein 3
IGF-I	Insulin-like growth factor I
ISS	Idiopathic short stature
ITT	Insulin tolerance test
JAK2	Janus kinase 2
LD	Linkage disequilibrium
LDL-C	Low density lipoprotein cholesterol
LTR	Long terminal repeat
MAF	Minor allele frequency
mRNA	Messenger ribonucleic acid
NFPA	Non-functioning pituitary adenoma
NFQ	Non-fluorescent quencher
NTC	No-template control
PCR	Polymerase chain reaction
PIK3CB	Phosphoinisitide 3 kinase, catalytic subunit beta
QoL	Quality of life
rhGH	Recombinant human growth hormone
RNA	Ribonucleic acid
SBP	Systolic blood pressure
SD	Standard deviation
SGA	Small for gestational age
SNP	Single nucleotide polymorphism
SOCS2	Suppressor of cytokine signalling 2
STAT5b	Signal transducer and activator of transcription 5b
T	Thymine
T2DM	Type 2 diabetes mellitus
tagSNP	Tagging single-nucleotide polymorphism
TBK	Total body potassium

INTRODUCTION

GENETICS AND THE HUMAN GENOME

Genetics is the science of genes, heredity and variation in living organisms. The modern science of genetics, which seeks to understand the process of inheritance, began in the mid-19th century with the work of the German-Czech Augustinian monk and scientist Gregor Johann Mendel (1822-1884). He studied garden peas, and discovered that organisms inherit traits via discrete units of inheritance, unknown at that time, which are now known as genes. In 1910, Thomas Hunt Morgan (1866-1945) postulated the theory that genes are positioned on chromosomes, and in 1953 the structure of the deoxyribonucleic acid (DNA) was determined by James D. Watson (1928-) and Francis Crick (1916-2004) [1], although the contribution by Rosalind Franklin should not be forgotten. Since then, the central dogma of molecular biology has been stated and also re-stated, and we now know that the flow of information in a cell goes from DNA, via ribonucleic acid (RNA), to protein [2].

Molecularly, DNA is made up of four bases, termed adenine (A), thymine (T), cytosine (C) and guanine (G). The bases are complementary, in the sense that A always binds to T, and C always binds to G, and so DNA is composed of two anti-parallel intertwining nucleotide chains of these bases, making up a double-helix polymer (Figure 1) with the bases bound to a backbone of alternating sugars and phosphate groups [3].

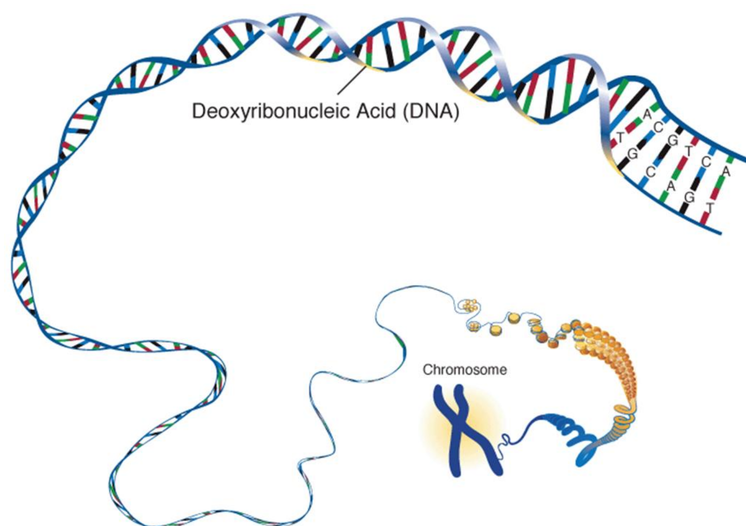

Figure 1. The level of DNA organization within the human cell. Complementary bases bound to a sugar- and phosphate backbone make up the DNA double-helix polymer. The DNA is further packaged and organized into a set of chromosomes, which reside in the cell nucleus. Courtesy: D. Leja / National Human Genome Research Institute, http://www.genome.gov/.

In eukaryotes (animals, plants, fungi and protists), the vast majority of genetic information is stored within the cell nucleus, and the DNA is tightly packaged into long structures called chromosomes. In 2003, the joint efforts of the Human Genome Project (HGP) and the private

effort by Celera Genomics, led to finalization of the sequencing of the human genome (the entirety of the human genetic information). The complete (99%) sequence of the human genome was subsequently published in 2004, and so to date we know that it contains 3.2 billion base pairs (bp) organized on 22 autosomal and two sex (X and Y) chromosomes and into 20.000-25.000 protein coding genes. Structurally, genes constitute approximately 1.2% of the genome and they are distributed on all chromosomes [4], with the remaining 98.8% being non-protein coding sequence. These non-coding sequences may appear unimportant, but in fact within these sequences lay regions important for regulation of gene expression etc., including promoters which are essential for initiation of RNA synthesis. Genes, in turn, have both coding regions (exons) and non-coding intermediary regions (introns) [5].

The genetic information is transferred from DNA to protein via transcription of the genetic code (sequence of bases) into messenger RNA (mRNA), which is then translated into protein. The mRNA sequence is read in triplets, starting with a start codon (AUG) and ending with a stop codon (UAA, UGA or UAG). The codons in between start and stop determine which amino acids are to be incorporated in the polypeptide chain. In total, there are 64 possible triplets that specify the 20 most common different amino acids found in proteins.

Genetic variation

In any two healthy unrelated individuals, only approximately 0.1% of the genome differs. As opposed to mutations, which are rare, polymorphisms are present in the human population with a frequency of >1%. There are different types of polymorphisms, including large, structural types such as deletions, insertions, duplications, translocations and inversions. However, in humans, the most common form of genetic variation (accounting for more than 90% of all genetic variation) is called a single-nucleotide polymorphism (SNP). On average, it is estimated that there is one SNP in every 300 bp, adding up to a total of 10 million of SNPs [6]. As the name implies, a SNP is merely a DNA sequence variation occurring when there are two alternative bases (alleles), or in some very rare circumstances more than two, present at a single nucleotide position (locus) in different individuals.

A SNP may or may not have functional consequences, depending on its location in the genome. A polymorphism located within the coding sequence of a gene may alter the amino acid sequence of the subsequent protein (a non-synonymous SNP) and lead to changes in structure and function of the protein. Also, a SNP may introduce a stop codon, leading to premature cessation of the translation process, which in most cases disrupts the function of that protein. Due to the degeneracy of the genetic code, a SNP can also be silent (a synonymous SNP), meaning that it doesn't change the amino acid sequence. The majorities of SNPs are located outside the coding parts of a gene and are referred to as being non-coding. However, these variants may still be functional if they are located in regions such as promoters, enhancers or silencers – which can be important for transcription factor binding etc. and thus lead to changes in gene expression.

In combination with random processes and environment, genetic variation is the cause of natural selection and it makes us all unique. Our genetic make-up influences everything from eye color to disease vulnerability and drug response. To study the correlation between a genetic variant and a phenotype, such as a disease, a disease-associated quantitative trait or a drug response, a genetic association study can be performed. Using a candidate gene approach, genetic variants to be tested for association are selected based on previous information, such as knowledge of the corresponding gene function, its location in a region previously linked to disease, or simply because it for some reason is an interesting candidate variant. Today it is possible to perform genome-wide association studies (GWAS), where hundreds of thousands of SNPs are tested simultaneously for association with disease or a trait.

Genetic linkage

When two genetic variants are located close to each other on a chromosome, they are often inherited together more frequently than expected by chance; a phenomenon termed linkage disequilibrium (LD). When two variants are so closely associated that they are always inherited together, they are said to be in complete LD. As such, a genetic variant which is in perfect LD with another variant can be used as a tagSNP (marker).

Pharmacogenetics

For most medications there is a large interpatient variability in efficacy and toxicity [7]. Factors such as age, sex, underlying disease status and other drugs administered to the patient influence the response to a medical treatment [7, 8]. It is estimated that genetic variability account for 20-95% of the variability in drug disposition (absorption, distribution, metabolism and excretion) and effects [9]. Pharmacogenomics is the research field aimed to investigate the genetic basis for differences in drug efficacy and toxicity, ultimately aiming to maximize efficacy and safety of a treatment [7, 8]. While pharmacogenomics uses genome-wide approaches to search for polymorphisms affecting the response to a medical treatment, its counterpart pharmacogenetics does the same but rather using a candidate-gene (small-scale) approach.

THE GH–IGF-I AXIS

In the 1920s, the presence of a growth-promoting substance from the anterior pituitary was demonstrated in experiments which showed the ability of pituitary extracts to restore and maintain growth in hypophysectomised animals [10]. In 1956, human growth hormone (GH) was isolated [11], and starting from the late 1950s GH deficient (GHD) children were treated with GH purified from cadaver pituitaries [12]. However, use of cadaver-purified GH was stopped, and the medication was withdrawn from the market in 1985 when it was shown to transmit Creutzfelt-Jacobs disease [13]. Since 1985, recombinant human GH (rhGH) has been commercially available.

Human pituitary GH protein is a four-helical bundle protein consisting of 191 amino acids. The protein is produced after transcription of the GH1 gene located on chromosome 17, position 17q24.2, and it is secreted from the pituitary in a pulsatile pattern. As the name implies, GH is an important stimulator of postnatal longitudinal growth and it has been shown to directly stimulate the cells in the growth plate to induce growth [14]. In addition, GH has major effects on fuel metabolism by influencing muscle (protein anabolic), fat (lipolytic) and regulation of blood glucose levels [15]. GH also has a profound effect on the brain and quality-of-life [16]. In addition, GH has some important indirect effects, predominantly mediated through GH-induced production of insulin-like growth factor I (IGF-I) from the liver. IGF-I shares both structural and functional homology with the insulin peptide, and also mimics some of its actions, such as stimulation of glucose and amino acid uptake and inhibition of gluconeogenesis [17-19]. Also, IGF-I is important for the stimulation of postnatal body growth. About 75% of the IGF-I found in the circulation originates from the liver, however through a liver-specific knock-out of IGF-I in mice it has been shown that body growth is rather determined by locally produced (autocrine/paracrine) IGF-I [20].

The GH signalling pathway

At the molecular level, GH effects are mediated by the GH receptor (GHR), encoded by the GHR gene located on chromosome 5, region p13.1-p12 [21, 22]. The GHR is a transmembrane glycoprotein belonging to the type I cytokine receptor superfamily [23, 24]. At the cell surface, GHR can exist in preformed dimers, and binding of a GH molecule to its dimerized GHR induces a series of rotations and conformational changes within the subunits [25], which enables

transphosphorylation of the Janus kinase 2 (JAK2) protein and subsequent activation of the Janus kinase 2/Signal transducer and activator of transcription 5 (JAK/STAT) pathway [26, 27]. The result, amongst other, is an increased generation of IGF-I. In addition, activation of the suppressor of cytokine signalling (SOCS) genes by GH mediates an inhibitory effect on the GH signalling [28], thereby producing an intracellular negative feed-back loop in the GHR signalling cascade. Also, some of the metabolic effects of GH are the results of activation of phosphoinisitide 3 (PI3) kinases [29], such as PIK3CB, which are independent of the GHR-induced IGF-I generation (Figure 2).

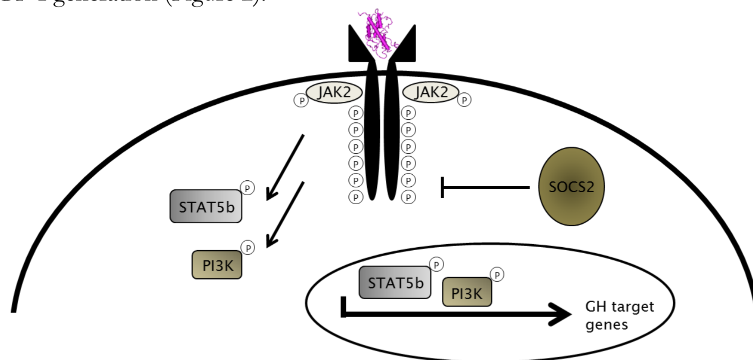

Figure 2. Schematic and simplified view of the GHR signalling cascade. Binding of GH to the GHR induces a series of conformational changes leading to phosphorylation of the associated JAK2 protein. A series of transphosphorylation steps then leads to activation of signalling molecules such as STAT5b and PI3K, and to translocation of these proteins into the cell nucleus. The result is a subsequent transcription of GH-inductive genes such as *SOCS2*, proteins of which then produces an intracellular negative feed-back loop on the GHR signalling. *Note that some proteins such as the MAPK/ERK and IRS1 have been left out from the illustration due to space limitation and for clarity of the overall picture.*

Adult GH deficiency (GHD)

Patients with adult hypopituitarism and GHD, most frequently caused by pituitary tumours and the treatment thereof [30], suffer from a phenotype which resembles the metabolic syndrome. The phenotypic picture include increased amount of body fat (particularly abdominal) [31-33] and decreased lean body mass [31, 33-35], reduced bone mineral density (BMD) [36-38] and increased fracture risk [39], dyslipidaemia [40-44], hypertension [44] and insulin resistance [45]. They also have decreased well-being and quality of life (QoL) [16, 46-49]. Most importantly, the mortality rate of adults with hypopituitarism not replaced with GH is twice that expected in the normal population and is commonly due to cardiovascular and cerebrovascular diseases [50-55]. For these patients, GH replacement therapy (GHRT) improves many of the symptoms and clinical signs associated with the deficiency [56]. The dosing of GH is individualized and guided by clinical and biochemical responses. The recommended GH starting dose is 0.1 to 0.3 mg/day depending on sex and age. To mimic the rhythmical pattern of endogenous GH secretion, GH is administered in the evening (before bedtime) through subcutaneous injections [57].

PHARMACOGENETICS IN GH REPLACEMENT THERAPY

Upon start of GH replacement, the individual responsiveness to GH is not known. Therefore, the treatment is initiated with a low dose of GH, independent of body weight, which is titrated upwards based on serum IGF-I levels and clinical response [58, 59]. Determination of the response to GHRT in adults includes clinical signs and symptoms of fluid retention, improvement in well-being, changes in body composition and biochemical serum markers of GH action, where the most sensitive is IGF-I. Despite stable serum IGF-I levels, metabolic endpoints

such as lipid profile and indices of glucose homeostasis have been shown to change over time [60], which suggests that there is a poor association between serum IGF-I and efficacy of GHRT. Sex, age, body mass index (BMI), GH dose and the route of oestrogen replacement in women have been linked to the response to GHRT [61-63], but the variability in GH-mediated effects on body composition, lipid- and glucose metabolism is less well known [64]. Most likely, the variability could be due to genetic factors. For instance, it has been suggested that 50% of the interindividual variation in baseline serum IGF-I levels in healthy subjects is genetically determined [65].

The *GHR* exon 3 deleted/full-length polymorphism

To date, candidate gene studies are the most common in the literature on pharmacogenomics in GHRT, and for obvious reasons the *GHR* gene has been an attractive candidate gene. The first report of GHR cDNA clones with differences within the coding region came in 1989, from Godowski *et al* [22]. In one of these clones the entire *GHR* exon 3 sequence was missing, giving rise to a GHR protein lacking 22 amino acids in the extracellular, GH binding, domain. Also, the resulting protein contains an aspartic acid instead of an alanine residue at the exon 2-4 junction [66].

The initial studies investigating the exon 3 deletion (d3) generated a hypothesis that the d3-GHR was caused by an alternative splicing event, and the expression of the two isoforms were subsequently studied. It was originally reported on a tissue-specific expression pattern of the two isoforms, with the d3-GHR primarily expressed in the placental villi and amnion [66, 67] and the full-length (fl)-GHR found exclusively in chorion and decidua [67]. Later, Mercado *et al* found that both isoforms were expressed in 19 tested tissues, but that the relative amount of the isoforms differed between the tissues [68]. However, following studies suggested that the expression pattern was rather subject-specific [69, 70] and/or developmentally regulated [70], than tissue-specific. In 1996, Stallings-Mann *et al* showed that the skipping of exon 3 was not tissue-specific, nor developmentally regulated, but actually subject-specific and due to a polymorphism in the *GHR* gene that follows a simple Mendelian mode of inheritance [71].

In 2000, Pantel *et al* shed some light on the origin of the polymorphism when they reported on the existence of two 99% identical retroelements flanking exon 3 [72]. The retroelements are 251 bp long, located 577 bp upstream and 1821 bp downstream of exon 3 and underlies the creation of the d3-GHR through an early homologous recombination event which has been passed on through generations. The resulting deletion involves besides the exon 3 also parts of introns 2 and 3. Several studies have investigated the actual effects of the loss of amino acid 7-28 in the GHR protein, but to date the importance of this region is still partly unknown. Even though the region appears to be conserved among GHR proteins in mammals, there is no homolog in the closely related prolactin receptor [73]. The GH binding capacities of the two GHR isoforms have been studied, but without demonstration of a significant difference [66, 74, 75]. Also, when modelled by crystallography, the amino acids encoded by exon 3 does not appear to interact in a significant matter with GH [76].

In 2004, Dos Santos *et al* transfected 293 HEK fibroblasts with vectors expressing both GHR isoforms, either alone or simultaneously, and showed that transduction of GH signalling was approximately 30% greater through d3-GHR homo- and heterodimers than through fl-GHR homodimers [77]. Efficacy of signal transduction was quantified by induction of the firefly luciferase gene coupled to the GH responsive *STAT5* promoter. This, however, is to date the only study showing a functional difference between the two GHR isoforms.

The *GHR* d3/fl polymorphism in GHRT

The *GHR* d3/fl polymorphism has been thoroughly investigated in several paediatric and adult populations receiving GH therapy; with subjects being either GHD or non-GHD. Non-GHD populations include children with idiopathic short stature (ISS), Turner syndrome and children who are born small for gestational age (SGA). Although some studies have shown no effects of the d3/fl polymorphism on response to GH therapy in children [78-82], other studies have indicated that carriers of the d3-allele respond better in terms of growth velocity [77, 83-86], gain in height [85, 87] and final height [84], than non-carriers, suggesting that the d3-GHR confers a more GH-sensitive receptor. In accordance, in adult GHD populations receiving GHRT there are data suggesting an increased sensitivity to GH in patients carrying the d3-GHR. For instance, it has been shown that d3-GHR carriers experience a higher increase in serum IGF-I levels, more marked decrease in total- and LDL-cholesterol levels and a higher increase in serum HDL-cholesterol levels during short-term GHRT [88].

THE GH-IGF-I AXIS IN GENETIC ASSOCIATION STUDIES

Surprisingly, there have been no reports on any influence of genetic variants within the *GHR* locus on adult height in genome wide association studies [89]. However, several SNPs in genes within or directly downstream of the GH signalling pathway have been identified, and previously shown to be of functional importance. First, two SNPs in the *JAK2* gene have been associated with central adiposity (rs7849191) and blood lipid levels (rs3780378) [90]. *STAT5b* SNP rs6503691 has been associated with breast cancer [91], and *SOCS2* SNP rs11107116 has been associated with adult height [92]. Lastly, *PIK3CB* SNP rs361072 has been associated with HOMA-IR [93], serum IGF-I levels and longevity [94].

AIMS

The overall aim of this thesis was to test the hypothesis that polymorphisms in genes within the GH-IGF-I axis impact on body composition, metabolism and serum IGF-I concentrations.

The specific aims of the different studies were:

Paper I.	to investigate the influence of the GHR d3/fl polymorphism on the response to GHRT in GHD adults.
Paper II.	to facilitate the genotyping of the GHR d3/fl polymorphism.
Paper III.	to investigate the influence of genetic variants within the GH signalling pathway on the IGF-I response to GHRT in GHD adults.
Paper IV.	to investigate the impact of the GHR d3/fl polymorphism on body composition and metabolism in the general population.

COHORTS AND METHODS

STUDY POPULATIONS

The studies included in this thesis were approved by the regional ethical review board at the University of Gothenburg, and written informed consent was obtained from all participants prior to study inclusion.

The GH-2 study

This is an on-going prospective longitudinal study of adults with hypopituitarism and severe GHD who are treated at the outpatient Centre for Endocrinology and Metabolism (CEM) at the Sahlgrenska University Hospital, Gothenburg, Sweden. The first patient was included as early as in 1993, and as of today there are around 450 patients receiving GH treatment within this study making it the largest single-centre study of adult GHD. Patients from this study were included in Paper I (n=124), II (n=183) and III (n=313). General characteristics of the subjects included in Papers I and III are shown in Table 1. The most frequent GH stimulation test for confirmation of GHD was an insulin tolerance test (ITT; 77%), but other tests such as the GHRH-arginine, GHRH-pyridostigmine or glucagon were also used. In some patients, diagnosis was made based on a low serum IGF-I together with ≥ 3 additional pituitary deficiencies [57]. The most common cause of GHD was a non-functional pituitary adenoma (NFPA). All childhood-onset (CO) GHD subjects had been without GH treatment before retesting of GHD in transition from childhood to adult GH treatment. Patients with Cushing's syndrome or acromegaly were in remission when entering the study protocol.

All patients received rhGH with an average starting dose of 0.23 mg/day. The dose was titrated individually during GHRT based on age- and sex-adjusted serum IGF-I concentrations, after 1 and 4 weeks and every 3 months thereafter, to maintain IGF-I levels between the median and the upper limit of the normal reference range. When necessary, patients received adequate replacement therapy with glucocorticoids, thyroid hormone, desmopressin and/or sex hormones.

Methodological considerations: This is a long-term study where patients are enrolled consecutively, which generates some considerations to mention. Firstly, ever from the time when this study was started there have been several changes in treatment preferences, diagnostic criteria, biochemical methods for serum measurements and GH dosing strategies that may be of importance. During the time of inclusion into the study, different diagnostic tests have been used. Today, an ITT is the golden standard and indeed that test has been used for the majority of patients. One could speculate that different diagnostic tests and cut-offs could have led to some patients being included into the study with a somewhat different degree of severity of GHD. However, it is known that GH is commonly the first hormone that disappears during the development of hypopituitarism [95], and so most patients with pituitary disease and multiple pituitary hormonal deficiencies would be suspected to also have GHD. Serum IGF-I is the major response variable in Paper I and III, and during the years the biochemical methods for serum concentration measurements have changed. Although the methods have been shown to be correlated, there is still a concern in regards to some uncertainness in exact serum IGF-I levels and the comparisons thereof. Lastly, and most importantly, the first patients enrolled in the study (n≈50) were treated using an mg/kg body weight strategy adapted from the experience of peadiatricians. This treatment strategy was shown to generate GH doses in the unphysiological range, resulting in side-effects mostly related to fluid retention [33, 34, 96]. The strategy was later changed into a fix starting dose with individual dose titration, which reduced the problem with side-effects but showed similar good treatment results [58, 59].

Table 1. Characteristics of the subjects in the GH-2 study.

	Paper I	Paper III
n	**124**	**313**
Male gender (%)	79 (63.7)	182 (58.1)
Age (yrs; range)	50.1 (18-76)	49.7 (17-77)
Adult onset GHD (%)	87.1	89.8
Isolated GHD (%)	9.7	11.3
Aetiology		
NFPA (%)	56 (45.2)	128 (40.9)
Idiopathic (%)	12 (9.7)	29 (9.3)
Prolactinoma (%)	13 10.5)	27 (8.6)
Craniopharyngioma (%)	11 (8.9)	24 (7.7)
Other* (%)	32 (25.8)	105 (33.5)
Pre-study treatment		
Surgery (%)	73 (58.9)	163 (54.7)
Radiotherapy (%)	6 (4.8)	16 (5.4)
Both (%)	23 (18.5)	48 (16.1)

*other aetiologies include empty sella, previous cushing or acromegaly, meningioma, apoplexy, Sheehan, trauma, sarcoidosis, cystic lesion, histocytosis, congenital hypopituitarism, dysgerminoma, granular cells tomour, hamartoma, medulloblastoma, rhabdomyosarcoma, septo-opto dysplasia, TSH adenoma and Wegener granulomatosis. NFPA = nonfunctioning pituitary adenoma.

The Swedish Obese Subjects (SOS) reference study

The SOS reference study includes subjects from the Swedish cities Mölndal and Örebro. The subjects were randomly selected from a population registry to constitute a reference group to the SOS intervention study, presented elsewhere [97]. The SOS reference study includes 1135 subjects (46.2% men), with an average age of 49.5 yrs (range 35.6 - 61.8) and average BMI of 25.3 kg/m^2 (range 17.6 - 45.4). Extensive information about the subjects was collected at each subject's physical examination by means of questionnaires. Age, sex, menopause status, self-reported diabetes (yes/no) and hypertension (yes/no) was reported for all subjects. Also, the subjects reported on a four-grade scale of leisure-time physical activity during the last 12 months period, with 4 being the most active. Body composition was measured by whole-body counting of the 40K isotope to determine total body potassium (TBK) content and estimate fat free mass (FFM) [98]. Systolic (SBP) and diastolic (DBP) blood pressure were measured on-site at examination day. Lab variables measured in serum included triglycerides, total cholesterol, high density lipoprotein (HDL) cholesterol and low density lipoprotein (LDL) cholesterol, insulin, IGF-I and IGF-binding protein 3 (IGFBP-3). Glucose was measured in whole blood. General characteristics of the subjects are shown in Table 2.

Methodological considerations: This study population includes subjects from two Swedish cities, which were at the time for inclusion regarded as representative of the Swedish population. However, the subjects were all investigated at the same time period, which makes the study vulnerable to societal effects. One should therefore be careful if comparing to more recent populations, whose subjects may have a somewhat different environmental background. Also, for a general population, 1135 subjects is a fairly small study. However, one of the strengths of this study is that the subjects were thoroughly phenotyped, using pre-determined standardized procedures.

Table 2. General characteristics of the subjects in the SOS reference study.

	n	Mean	Range (min – max)
Male gender (%)	1135	524 (46.2)	
Postmenopausal (%)	611	23.1	
Age (yrs)	1135	49.5 (7.0)	35.6 – 61.8
Anthropometry			
Height (cm)	1135	172.1 (9.2)	150.0 – 201.0
Weight (kg)	1135	75.1 (14.2)	43.7 – 138.1
BMI (kg/m^2)	1135	25.3 (3.8)	17.6 – 45.4
Waist-to-Hip ratio	1134	0.88 (0.08)	0.59 – 1.49
Calf circumference (cm)	1135	34.3 (3.3)	24.5 – 53.0
FFM* (kg)	919	55.7 (11.5)	34.5 – 94.6
Biochemistry			
S-IGF-I (μg/L)	1118	207.0 (64.9)	48 – 659
S-insulin (mU/L)	1087	8.6 (5.1)	2.6 – 55.7

*estimated from TBK measurements. Numbers within brackets represent SD.

GENETIC ANALYSES

DNA extraction and quantification

Genomic DNA was isolated from frozen venous whole blood. In the GH-2 study, DNA isolation was performed using a standard protocol for the FlexiGene DNA kit (Qiagen, Hilden, Germany). DNA yield was quantified using the ND-1000 spectrophometer (NanoDrop Products, Thermo Fischer Scientific, Wilmington, DE, USA). In the SOS reference study, DNA was isolated using the Mag Maxi Plus kit (AGOWA GmbH, Berlin, Germany) and a semi-automated magnetic bead technique on a Microlab Star instrument (Hamilton Robotics, Reno, NV, USA). DNA yield and quality was determined using PicoGreen® on an ABI Prism 7900HT Sequence Detection System instrument from Applied Biosystems (Foster City, CA, USA).

Selection of candidate genes and SNPs

The selection of candidate genes in Paper III was based on their well-established function within the GH-IGF-I axis according to previous publications. Further selection of SNPs within these genes was based on reports on functionality and/or associations with aspects of GH function/effects. Due to the limited size of the GH-2 cohort, we chose to only include SNPs with a minor allele frequency (MAF) > 10% in the HapMap CEU Panel.

Methodological considerations: The candidate-gene approach used in Paper III has some obvious strengths but also some drawbacks. First, there is an increased chance of the selected SNPs to be of functional importance due to the previous associations. However, as compared to performing a GWAS, the chances of finding something unexpected are severely decreased. In addition, there is always a possibility that some SNPs of importance are missed in the selection process, since they are simply not previously examined in a relevant aspect. The same is true because of the MAF cut off limit; other SNPs of lower MAF may be of equal or larger importance, but missed during the stringent selection procedure (due to the limited sample size).

Genotyping methods

The basic first step in all methods used for genotyping is a polymerase chain reaction (PCR), a method allowing for rapid amplification of DNA that was developed in 1983 by Kary Mullis and

colleagues. The different primer and probe sequences, as well as cycling conditions etc. can be found in the respective Papers.

In Papers I and II, genotyping of the *GHR* d3/fl polymorphism was performed using a conventional multiplex PCR method with fragment detection by gel electrophoresis, which was developed by Pantel *et al.* Briefly, a multiplex PCR was performed using three primers G1, G2, and G3 (GenBank accession no. AF155912) as previously described [72], which allows for amplification of the polymorphic area (Figure 3A). The amplified PCR products were separated, allowing for genotype calling, by electrophoresis on a 1.2% ethidium bromide-stained agarose gel, see Figure 3B.

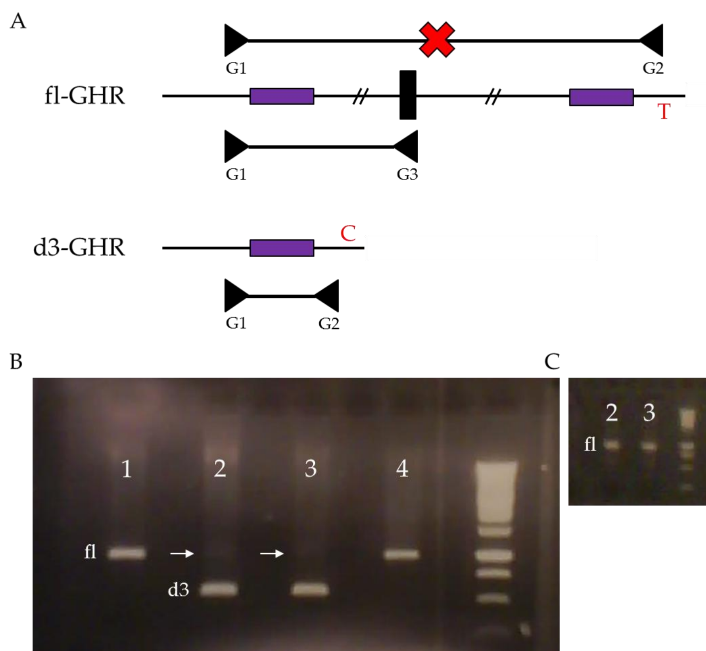

Figure 3. The *GHR* d3/fl polymorphism. A. Schematic view of the *GHR* exon 3 locus. Black box represents exon 3, purple boxes represents the LTR sequences flanking exon 3. Black arrows represents primers G1, G2 and G3 used in the conventional PCR genotyping assay. Red cross signifies that the fl-GHR fragment is not produced using primers G1 and G2, due to the length of the fragment and competition over PCR reagents. C/T in red represents the respective rs6873545 alleles tagging the *GHR* d3/fl polymorphism. **B.** EtBr-stained gel electrophoresis. Samples in lanes 1 and 4 are fl-homozygotes (one strong band of 935-bp amplicon). White arrows in lanes 2 and 3 highlight the difficulty in determining the presence of the 935-bp amplicon in some d3/fl subjects. **C.** The same samples 2 and 3 from panel B in a second round of PCR using only primers G1 and G3, with subsequent amplification of the fl-allele (935-bp amplicon).

In part of Paper II and in Papers III and IV, the genotyping of the *GHR* d3/fl polymorphism was performed using TaqMan SNP genotyping of tagSNP rs6873545. In brief, an rs6873545-specific TaqMan genotyping assay (C__28966089_10) was ordered from Applied Biosystems. The assay contains forward and reverse primers for amplification of the polymorphic site and two allele-specific probes labelled with a 3’ non-fluorescent quencher (NFQ) and a 5’ fluorescent dye (either VIC or FAM). In the PCR reaction, the probes anneal to its complementary sequence.

The DNA polymerase then extends the template-bound primers and cleaves the probes that are hybridized to target DNA. Any non-complementary probe will be un-bound to DNA, and hence will not be cleaved in the reaction. Cleavage of target-bound probes generates allele-specific fluorescence, due to separation of the NFQ from reporter dye, which is then measured in an ABI Prism 7900HT Sequence Detection System instrument (Applied Biosystems; Figure 4).

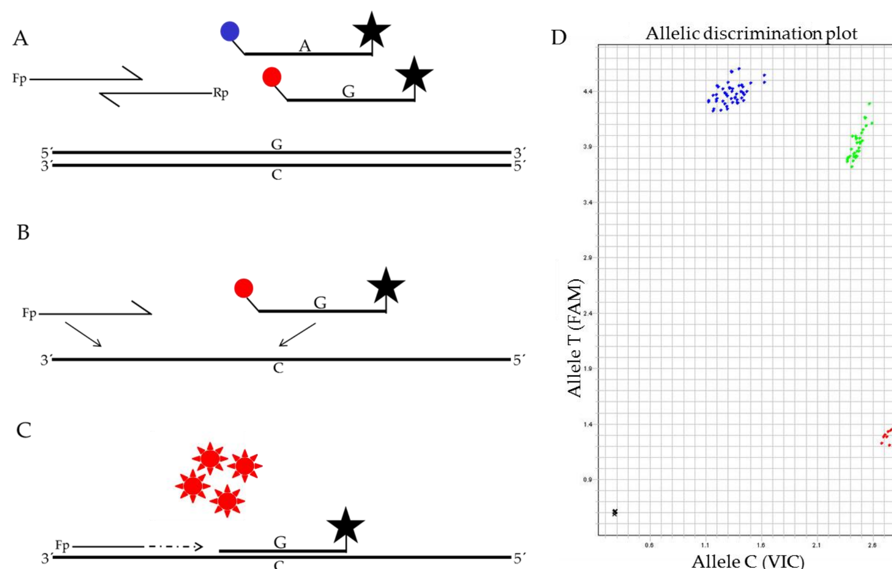

Figure 4. The principles and components of the TaqMan SNP genotyping assay. For simplicity, panels A-C represent a homozygote individual. **A.** Forward and reverse primers and two rs6873545-specific probes with bound fluorescent markers and non-fluorescent quencher (NFQ) are added in the PCR reaction. **B.** During the PCR, the target DNA is denatured, allowing for complementary binding of primers and probes. Due to the proximity of the fluorescent dye to the NFQ, no signal is generated. **C.** In the exonuclease reaction, elongation of the annealed primer results in cleavage of bound probe and release of the fluorescent dye from the proximity of the NFQ thus generating a fluorescent signal. **D.** The relative fluorescence is measured, and signals are visualized, in an allelic discrimination scatter plot.

In Paper III, most of the genotyping was performed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry on the iPLEX MassArray Sequenom platform. Genotyping was performed at the Mutation Analysis Facility (MAF), Karolinska Hospital, Huddinge, Sweden. The iPLEX assay is composed of several steps. First, two sets of primers per SNP are designed using the SpectroDesigner software (Sequenom Inc., San Diego, CA, USA); two amplification primers and two allele-specific extension primers. The amplification primers are designed to generate amplicons of 80-120bp in length, containing the polymorphic site, whereas the extension primers are designed in order to attach with the 3' end immediately adjacent to its complimentary SNP allele. Experimentally, the procedure starts with a standard PCR, generating amplicons containing the SNPs of interest, followed by another round of PCR (iPLEX gold reaction) where the extension primers are used. The PCR products are then analysed on a MassARRAY compact mass spectrometer, where the amplicons are separated based on mass. Finally, the genotypes are called using the SpectroTYPER RT 4.0.5 software (Sequenom Inc.; Figure 5).

Methodological considerations: There are some differences between the different genotyping methods that could be mentioned. Genotyping using multiplex PCR with gel electrophoresis is a simple method using standard lab-utensils available at most labs around the world, suitable for genotyping of a small number of samples. However, it requires a large amount of DNA for the analysis which may not always be available. In addition, a second round of PCR is required in order to confirm the absence of fl-fragments whenever a perfect d3/d3 or d3/d3 with weak 935-bp amplicon (suspected d3/fl genotype) is detected, which highlight the shakiness of the multiplex efficiency, due to the complexity of the polymorphic area, and calls for another genotyping method for analysis in larger cohorts. Also, it raises the question of whether the d3-allele has been over reported in the literature, which may have skewed the conclusions drawn from these earlier reports. TaqMan SNP genotyping, on the other hand, requires much less template DNA and enables rapid and high throughput genotyping either in a 96-well or 384-well format. In addition, introduction of human errors is eliminated because allelic discrimination is performed automatically instead of by visual inspection. It does, however, require access to specific genotyping equipment, such as the ABI Prism 7900HT Sequence Detection System instrument. For genotyping of a large number of SNPs in large cohorts, the Sequenom platform is very advantageous. It allows for simultaneous analysis of up to 20 SNPs in a single pool, and it is rapid and much less expensive than TaqMan SNP genotyping of the same number of SNPs. However, the equipment is almost exclusively available at consortiums and core facilities, due to the vast cost. Also, the primer-pool design can be tricky, wherefore some SNPs may fail design and have to be analysed separately using TaqMan SNP genotyping.

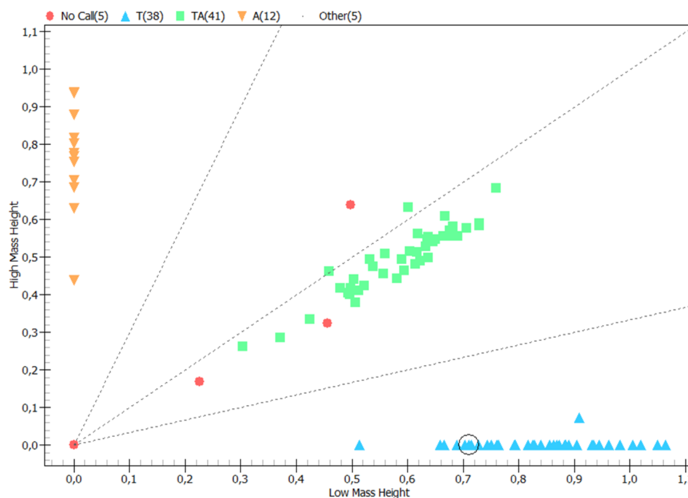

Figure 5. SNP genotyping using the Sequenom platform. Scatter plot showing the separation of alleles. Blue triangles represent T7T samples, orange triangles represent A/A samples and green boxes represent heterozygotes (A/T).

KEY RESULTS

GHRT RESPONSE PREDICTION

Paper I

In Paper I, we analysed the *GHR* d3/fl polymorphism in a cohort of 124 well-characterized GHD adults, who were studied after 12 months of GHRT. Using the conventional multiplex PCR developed by Pantel *et al*, a second PCR round was conducted whenever a perfect d3/d3 or d3/d3 with weak 935bp amplicon was suspected, which resulted in a reassignment of 57% of the originally assigned d3/d3 into d3/fl.

We found that the frequency of the d3-GHR was 26% (10% d3/d3, 32% d3/fl and 58% fl/fl). Due to the small sample size and the fact that only 10% were homozygous for the d3-GHR, we divided the subjects into two genotype groups. Group 1 included only fl/fl subjects (n=72), and group 2 included combined d3/fl and d3/d3 subjects (n=52). Men and women were studied separately. At baseline, there were no significant differences in clinical characteristics of the subjects in group 1 and 2. At 12 months of GHRT, the men in group 1 (fl/fl) had a lower median daily GH dose than the men in group 2 (p=0.03). However, although there was a similar trend, the cumulative GH dose did not differ significantly (Table 3). There were no significant differences in the change in IGF-I levels and BF between the two groups.

Table 3. 12 months characteristics of the 124 GHD adult subjects on GHRT, according to sex and genotype group.

		fl/fl		d3/fl + d3/d3	
	All patients	Men	Women	Men	Women
GH dose (mg/day)	0.4 (0.1, 0.9)	0.3 (0.1, 0.7)	0.5 (0.3, 0.9)	0.4 (0.3, 0.8)	0.4 (0.3, 0.8)
Cum GH dose (mg)	124 (69, 405)	118 (69, 297)	128 (76, 256)	129 (74.9, 405)	128 (70.5, 220)
IGF-I (µg/L)	234 (73, 634)	258 (97, 609)	188 (92, 506)	304 (75, 634)	209 (73, 455)
Δ IGF-I (µg/L)	145.5 (-32, 481)	154.0 (-32, 368)	115.5 (27, 481)	187 (42, 438)	124 (-32, 231)
BF (kg)	19.3 (2.4, 41)	18.2 (3, 41)	25 (11, 41)	15.0 (2.4, 38.2)	22.1 (14.2, 38.0)
Δ BF (kg)	-2.9 (-15, 10)	-3.5 (-15, 10)	-1.4 (-14.5, 3.8)	-2.4 (-11, 7.8)	-3.4 (-7.6, 8.7)

Data presented as median (range). Δ values represent value at 12 months – value at baseline.

Paper III

In Paper III, we analysed six SNPs in the *GHR* and the GH signalling pathway (*JAK2*, *STAT5b*, *SOCS2* and *PIK3CB*) genes in a cohort of 313 well-characterized consecutive GHD adults, who underwent GH replacement for 12 months. The subjects were studied before and after 1 week, 6 months and 1 year of GH replacement. The rapid response was defined as the percentage of change in IGF-I levels from baseline to one week of GH replacement. The long-term response was investigated as 1) the percentage of change in serum IGF-I levels from baseline to 6 and 12 months, and 2) the percentage of change in serum IGF-I levels from 1 week to 6 and 12 months. *GHR* exon 3 deleted/full-length (d3/fl) polymorphism was analysed using tagSNP rs6873545.

We found that the *GHR* d3/fl tagSNP rs6873545 and the *PIK3CB* SNP rs361072 were significantly associated with the rapid response to GH replacement (p=0.016 and p=0.025, respectively). Homozygotes of the major allele of SNP rs6873545 had on average a 45.3% larger increase in serum IGF-I levels, than individuals carrying the minor allele. Conversely, variant carriers of SNP rs361072 showed a 37.5% larger increase in serum IGF-I levels than homozygotes of the major allele. As compared to baseline, SNP rs6873545 (*GHR*) was associated

with the percentage of change in IGF-I levels at 6 months (*p*=0.041) and at 1 year (*p*=0.041). SNP rs361072 (*PIK3CB*) was significantly associated with the percentage of change in IGF-I levels at 6 months (*p*=0.047) of GH therapy. When comparing the serum IGF-I response between 1 week and 6 and 12 months, no SNP was significantly associated with the long-term responses to GH replacement therapy (Table 4).

Table 4. Summary of SNP info, effect size in percentage of change in serum IGF-I and *p*-values from regression analyses in 313 adult GHD patients during 1 year of GHRT.

dbSNP ID	Gene	1 week (n=253)	6 months (n=251)	1 year (n=256)
rs6873545	*GHR*	-45.3 *(0.016)*	-19.7 (0.457)	-24.5 (0.382)
rs361072	*PIK3CB*	37.5 *(0.025)*	-3.3 (0.894)	-9.6 (0.700)
rs7849191	*JAK2*	8.8 (0.610)	1.7 (0.945)	-0.8 (0.947)
rs6503691	*STAT5b*	-9.1 (0.717)	-3.9 (0.910)	-4.0 (0.913)
rs11107116	*SOCS2*	29.9 (0.119)	-19.2 (0.477)	-16.4 (0.564)

Table shows results from linear regression analyses adjusted for sex, age and GH dose. The long-term (6 months and 1 year) percentage of change in IGF-I levels was calculated using the 1 week IGF-I levels as reference value, to denote that the long-term response corresponds to the change in IGF-I levels taking place after the early response has occurred. Effect sizes refer to the adjusted difference between the heterozygote group and homozygotes of the major allele for each SNP, negative values correspond to a lower increase in IGF-I levels during GHRT for the heterozygotes versus the homozygotes of the major allele. Significant *p*-values are shown in Italics.

FACILITATED GENOTYPING

Paper II

In Paper II, we analysed the *GHR* d3/fl polymorphism in a cohort of 183 GHD adults, an extension to the cohort reported in Paper I. All subjects were genotyped using the conventional multiplex PCR, and also with TaqMan SNP genotyping of *GHR* SNP rs6873545 which has been reported as a tagSNP for the *GHR* d3/fl polymorphism by Lettre *et al* [99].

We found that the frequency of the d3-GHR was 24.0% (7.7% d3/d3, 32.2% d3/fl and 60.1% fl/fl). Genotyping success rate was 100% using tagSNP rs6873545, meaning that all samples were successfully called. In addition, the assigned genotypes were in total accordance with the genotypes achieved using the conventional genotyping method. Also, three samples which had previously not been assigned a genotype using the conventional method, due to technical difficulties, were successfully typed using the tagSNP. Figure 6 shows allelic discrimination plot from the TaqMan SNP genotyping of the *GHR* d3/fl polymorphism using tagSNP rs6873545.

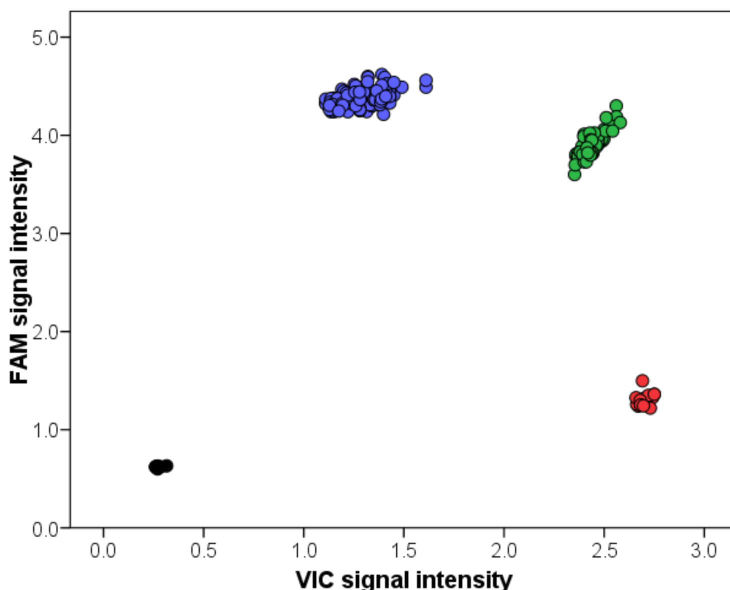

Figure 6. Allelic discrimination of the *GHR* d3/fl polymorphism as determined by TaqMan SNP genotyping. The plot shows signal intensity values of fluorescent reporter dyes FAM™ and VIC® for each sample and clear clustering of fl/fl (T/T; blue), fl/d3 (T/C; green) and d3/d3 (C/C; red) genotypes. Black dots represent no-template control (NTC).

THE GENERAL POPULATION

Paper IV

In Paper IV, we analysed the *GHR* d3/fl polymorphism using tagSNP rs6873545 in the SOS reference study, which comprises 1135 subjects that were randomly selected from a population registry.

We found that the frequency of the d3-GHR was 24.0% (5.6% d3/d3, 36.8% d3/fl and 57.6% fl/fl). Genotyping success rate was 99.1%. Subjects homozygous for the d3-GHR weighed approximately four kilos more (p=0.014), had larger waist-to-hip ratio (WHR; p=0.045), waist- (p=0.020) and calf circumference (p=0.0002) and more fat free mass (FFM) estimated from total body potassium (TBK; p=0.021) than grouped fl/d3 and fl/fl subjects (d3-recessive genetic model). Also, d3-GHR carriers had reduced levels of IGF-binding protein 3 (IGFBP-3), compared to fl/fl subjects (p=0.008), and borderline higher serum insulin levels (p=0.09). Genotype was not associated with serum IGF-I levels (p=0.4).

DISCUSSION

Half a decade has passed since Raben in 1962 first described the effects of GH administration to a 35-year-old GH deficient woman. After only two months of treatment she noted "increased vigour, ambition and sense of well-being" [100], which we now acknowledge the classical and profound effects of GH and IGF-I on the brain and QoL [101, 102]. The reports from Raben, and the one-year-later description of the physiological consequences of hypopituitarism in adults by Falkheden [103], spurred the interest in GH replacement to hypopituitary adults. After the introduction of recombinant human GH, the first treatment trials with GH to adults with hypopituitarism were conducted in the mid-1980s [33, 96], demonstrating profound metabolic effects, which further established the important role of GH in adults. Today, adults with hypopituitarism and GHD are regularly being considered for GH replacement due to the well-known consequences of adult GHD [56, 57, 104]. Long-term replacement therapy has also become more common, as discontinuation of treatment has been shown to negatively affect QoL, fat mass and distribution, lipid profile and markers of systemic inflammation [105], highlighting the need for these patients to be treated. However, in contrast to GH treatment in children, where the primary endpoints are growth velocity and final adult height, the evaluation of treatment response in adults is more complex and includes factors such as changes in body composition, lipid- and metabolic profile, bone health and QoL.

GHRT RESPONSE PREDICTION

The variability in individual responsiveness to GH treatment is large, and dependent on multiple factors both in children and in adults. In children with GHD, Turner syndrome and children who are born SGA, mathematical models to predict growth response using patient characteristics and treatment modalities have been developed [106-111]. The generated algorithms have been shown to explain a high degree of observed variability of the response [107]. In GHD adults, the response to GH in body composition has been only poorly correlated with the GH dose, whereas factors such as age, sex, BMI and GHBP levels have been shown to weakly predict response [59, 63, 112, 113]. The IGF-I response has been correlated with GH dose [114], sex [115] and the route of oestrogen replacement [62, 116-120]. However, even after adjustments for these clinical factors the response variability remains largely unexplained, suggesting that the individual genetic background plays an important role in the responsiveness to GH.

The *GHR* d3/fl polymorphism

The *GHR* d3/fl polymorphism has been considered an interesting candidate polymorphism for pharmacogenetic studies in GH replacement therapy, mainly because of its location within the *GHR* gene and also because it is highly frequent in most populations. The polymorphism has been investigated in both paediatric and adult populations of patients undergoing GH replacement therapy. In Paper I, we found that there was no association between the *GHR* d3/fl polymorphism and 12 months GH treatment response in terms of change in serum IGF-I levels and/or body composition. However, we did observe that fl/fl men required a lower mean daily GH dose to normalize serum IGF-I levels during treatment, than did grouped d3/fl and d3/d3 men. In Paper III, we found that the *GHR* d3/fl polymorphism, analysed using tagSNP rs6873545, was associated with the early, 1 week, response to GH replacement in terms of the percentage increase in serum IGF-I levels from baseline. When adjusted for sex, age and the baseline GH dose, fl/fl subjects had a larger percentage increase in serum IGF-I levels at 1 week than the two other genotype groups (d3/fl and d3/d3, analysed separately). The polymorphism was also associated with the long-term response to GH replacement when using the baseline

serum IGF-I as reference, however, this association was lost when using the 1 week serum IGF-I as reference value.

The findings in Paper I and III are contrasting to previously published data, in that the fl/fl subjects required a lower GH dose (Paper I, men only) and were responding better to GH replacement (Paper III, men and women combined). In terms of serum IGF-I response to GHRT in GHD adults, there have been some other reports of the GHR d3/fl polymorphism with conflicting data. In a Dutch cohort of 99 adult GHD patients, subjects bearing at least one d3-allele had a larger increase in serum IGF-I concentrations after 12 months of GHRT [88]. Similarly, in a UK study of 194 GHD adults, d3-GHR homozygosity was associated with a greater serum IGF-I response to 12 months of GHRT [121]. Two other studies have, however, shown no effect of the GHR d3/fl polymorphism on the IGF-I response to GHRT in adults [122, 123]. In regards to an effect on the GH dose required during GHRT, in a study of 133 German GHD adults on 1 year of GHRT, d3-GHR carriers require approximately 25% less GH than fl/fl subjects [124], which is in contrast to the findings in our Paper I. In agreement with the findings in Paper I, both Giavoli *et al* [123] and Adetunji *et al* [122] have shown that there were no effects of the d3-GHR on BF response during 1 year of GHRT.

The discrepancies between our study in Paper III and other studies may be reflective of several differences between the studies in regards to different cohort compositions of age, sex, aetiologies of GHD, other hormone deficiencies and their replacement, treatment regimens etc. In addition, major differences exist in regards to the size of cohorts, end-point studied, length of replacement, difficulties with the commonly used genotyping method and different genetic models used in analyses. In fact, recently, a co-dominant model of inheritance has been proposed in a Bayesian meta-analysis [125].

Most importantly, in addition to our cohort being larger than cohorts in previous reports, one of the other major strengths of the study in Paper III is that we chose to separate the response into an early (1 week), and long-term (6 and 12 months) response. The reason is that the GH treatment regime used, the individualized GH dose titration [59], unfortunately most likely masks the effect of genetic predictors of response since the response variable (IGF-I concentrations) is used as a marker to govern the GH dose adjustments consecutively during the treatment period. At 1 week of GH treatment, however, neither GH dose titration nor other confounders such as diet and life-style choices are expected to have impacted on the treatment response. This problem is further highlighted by the fact that the polymorphism was associated with the long-term IGF-I response when the baseline IGF-I was used as reference, but not when using the 1 week IGF-I as reference. This indicates that the observed association to long-term response was in fact driven by the short-term response. To our knowledge, this is the first study to investigate the association between the *GHR* d3/fl polymorphism and the very early treatment response to GH. Another strength of our study is that we used the percentage of change in IGF-I levels as the end-point, which we consider to be a more suitable variable for response prediction whereas previous studies have focused on crude serum IGF-I concentrations or delta values thereof. A delta value only tells the difference from baseline, but says nothing about the magnitude of change, as does the percentage of change. For instance, a patient starting GH therapy with a serum IGF-I of 50 μg/L which increases to 100 μg/L after 1 year of GHRT, will have a delta IGF-I of 50 μg/L, but a 100% increase in IGF-I levels indicating a good response. Conversely, a patient starting GH replacement with a serum IGF-I of 100 μg/L, which increases to 150 μg/L after 1 year of GH therapy, will have the same delta IGF-I as in the previous example (50 μg/L), but a lower percentage of change in serum IGF-I levels (20%). Therefore, the first patient probably has a more sensitive GH-IGF-I axis.

The GH signalling cascade

In Paper III, we found that *PIK3CB* SNP rs361072 was associated with the 1 week IGF-I response to GHRT. When using the baseline IGF-I as reference, the SNP was also associated with the 6 month IGF-I response.

PIK3CB encodes an isoform of the catalytic subunit of phosphoinositide 3-kinase (PI3K), a key effector of insulin signalling. PI3K is involved in mediating part of the metabolic responses to GH. Interestingly, the G-allele of *PIK3CB* SNP rs361072 has been shown to create a GATA binding site capable of increasing transcription of *PIK3CB* [93]. In addition, this SNP has also been shown to be associated with serum IGF-I levels and longevity [94]. In accordance with previous findings, which have shown that homozygotes of the G-allele of this SNP had higher free serum IGF-I levels [94], in our study the G-allele was associated with a higher percentage of increase in IGF-I levels during GHRT.

THE *GHR* d3/fl POLYMORPHISM IN THE GENERAL POPULATION

The role of GH in the regulation of human longitudinal growth and metabolism is undisputable and it is therefore not unlikely that a genetic factor influencing the individual sensitivity to GH would also have an impact. However, the influence of the *GHR* d3/fl polymorphism has been particularly evaluated in GHD populations, wherefore less is known about its effect in the general population with regards to anthropometry, body composition and metabolism.

In Paper IV, randomly selected adults from the general Swedish population who were homozygous for the d3-GHR were heavier and had larger waist circumference and WHR than fl-GHR carriers, indicative of a less favourable phenotype. In addition, they also had tended to have higher fasting insulin levels. This may be surprising bearing in mind the lipolytic effects of GH in the therapeutic situation.

Small birth size has been associated with late-onset diseases such as type 2 diabetes mellitus, hypertension and heart disease [126]. There are speculations on the reason for this observed association, and one possible explanation is that genetic variants that influence the disease risk also confers reductions in foetal growth [127]. In uterus, the foetus is exposed to high concentrations of placental GH, which is responsible for regulating its metabolism. However, the foetal longitudinal growth is mainly stimulated by insulin and IGF-I and not by GH. The d3-GHR has been found to be more prevalent among children born short for gestation age (SGA) with verified intrauterine growth restriction and also associated with a decreased third-trimester foetal growth velocity and an increased postnatal growth velocity [128]. In addition, associations between d3-GHR homozygosity and smaller birth size and earlier age at pubertal onset [129], and lower birth- and placental weight [130], have been shown. It is feasible to speculate that foetuses bearing the presumable more GH-sensitive d3-GHR become less insulin sensitive as a compensatory mechanism due to high activity of the GH-axis in these subjects, which could result in a poorer intrauterine growth. As such, the *GHR* d3/fl polymorphism could be a genetic factor influencing the association between insulin-mediated foetal growth (and therefore also birth weight), insulin resistance and other related metabolic aberrations which could last into adulthood [127]. The higher body weight, waist circumference and WHR in d3-GHR homozygotes observed in our randomly selected population could be related to an increased insulin exposure in these subjects and the reduced serum levels of IGFBP-3 in d3-GHR carriers might reflect a relative insulin resistance, which is known to be associated with an increased proteolysis of IGFBP-3 [131].

To our knowledge, our study was the first to investigate the putative influence of the *GHR* d3/fl polymorphism in a general, seemingly healthy, population of adult men and women. Previously, the polymorphism has been studied in subjects with normal glucose tolerance and impaired glucose tolerance including type 2 diabetes mellitus (T2DM). In that study, there was a significantly lower frequency of the d3-allele among the T2DM. However, amongst the patients with T2DM, subjects carrying the d3-GHR had significantly higher BMI [132], indicating that the d3-GHR confers a less favourable metabolic profile in this group. However, it is important to note that in this study, the genotype frequencies within the IGT and T2DM groups were not distributed in accordance with the HWE wherefore the results may be somewhat skewed.

CONCLUDING REMARKS

Most of the work presented in this thesis has focused on the *GHR* d3/fl polymorphism. Figure 7 shows a summary of the main results. It was a surprise to find that in our large study of adults with GHD, the fl/fl subjects were the ones that showed the best early response to GHRT, which opposes to the general opinion. However, previous studies were smaller and have focused on the long-term response, which may explain part of the discrepancies. In terms of long-term response to GH replacement, confounders such as GH dose titration, diet and life-style choices will probably influence and mask some of the effect of genetics on the response. For that reason, the early response is more suitable to study in pharmacogenetic studies. In the general population, the d3-GHR was associated with a less favourable anthropometric profile. What could possibly be the benefit of 24% of the population to carry the d3-GHR, when it in fact predisposes to metabolic disturbances?

The polymorphism has triggered the curiosity of many researchers for more than 20 years of time, and yet there are still no conclusive data on GH replacement predictive value etc. As for most medications, the response to GH replacement is probably multifactorial and I doubt that there will ever be one genetic variant that explains all variability. Most likely, there are still unknown genetic variants to be discovered that may be of an equal importance, and the GH response is most likely explained by a set of genetic variants each contributing with a fairly small, but still significant, effect. I would also like to suggest that while the early IGF-I response can be used to study the influence of genetic variants on GH responsiveness; it is the long-term outcome of GH treatment which is of the largest importance. Therefore, it would be of great importance to test variants such as the *GHR* d3/fl polymorphism for association with the long-term (10-15 yrs) incidence of heart disease, QoL etc, some of the most important parameters to be monitored during GH replacement.

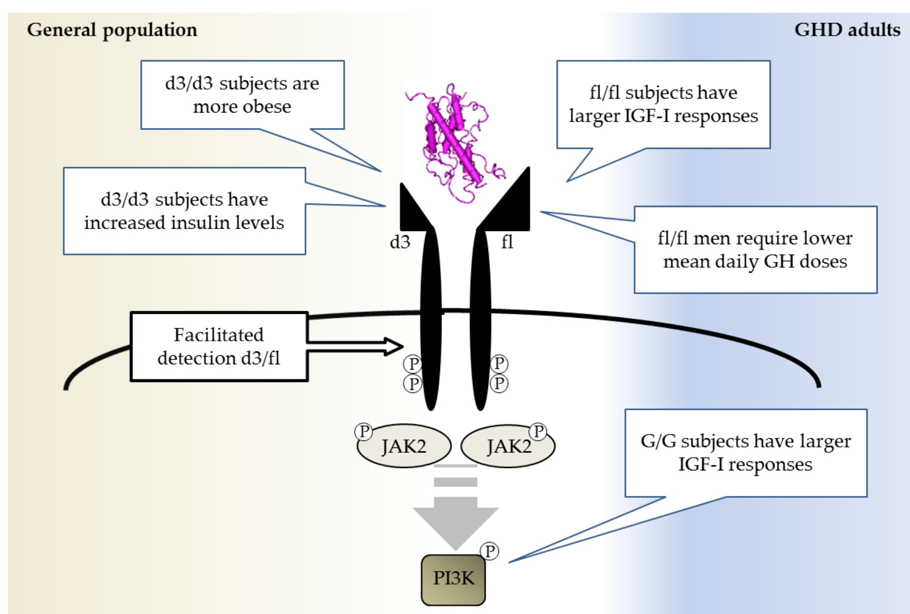

Figure 7. **Summary of main results.**

SAMMANFATTNING PÅ SVENSKA

Tillväxthormon är ett polypeptidhormon som produceras i hypofysen i hjärnan och som frisätts i ett pulsatilt mönster. Som namnet antyder är tillväxthormon väldigt viktigt för regleringen av längdtillväxt hos barn, men hormonet har viktiga funktioner även hos vuxna. Bland annat är tillväxthormon viktigt för regleringen av celldifferentiering och proliferation, och påverkar kroppssammansättning och metabolism.

Vuxna som har brist på tillväxthormon, oftast på grund av hypofystumörer eller dess behandling, drabbas i stor utsträckning av bukfetma, diabetes mellitus och högt blodtryck och löper dessutom en nästan dubblerad risk för död i hjärt- kärlsjukdom. Med anledning av detta erbjuds dessa patienter idag ofta en livslång ersättningsbehandling med syntetiskt tillväxthormon. Hittills har det visat sig vara svårt att optimera behandlingen; det är stor variation i svaret på behandling och det saknas prediktorer för detta. Därför letar man numera i stor utsträckning efter genetiska variationer som påverkar hur olika personer svarar på behandlingen.

I denna avhandling har jag undersökt genetiska variationer i tillväxthormonsystemet. Mycket fokus har legat på att studera en variant av receptorn för tillväxthormon, som tidigare visats påverka känsligheten för tillväxthormonbehandling. Sammantagningsvis fann jag att individer som bar på två kopior av den långa varianten av tillväxthormonreceptorn krävde lägre doser av tillväxthormon under 1 års behandling (bara män), samt att de svarade bättre på korttidsbehandling (män och kvinnor). Jag fann också att en genetisk variant av en signaleringspeptid nedströms om tillväxthormonreceptorn också var kopplad till ett bättre svar på korttidsbehandling med GH. Vidare visade jag att man kan använda en förenklad metod för analys av den kortare tillväxthormonreceptorn, som gör det lättare och mer tids- och materialeffektivt att analysera denna intressanta genetiska variant. Slutligen fann jag i en svensk normalpopulation med friska individer att personer som bar på två kopior av den kortare varianten av tillväxthormonreceptorn vägde mer och hade ett större midjeomfång, vilket tyder på en ogynnsam kroppssammansättning.

Sammanfattningsvis visar denna avhandling att genetiska variationer inom tillväxthormon-systemet kan påverka behandlingssvaret för tillväxthormonbehandling hos vuxna samt inverka på fetmarelaterade markörer hos friska individer ur den svenska normalbefolkningen.

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