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Identification of novel growth hormone-regulated factors

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To Magdalena

ABSTRACT

The studies described in this thesis aimed to identify novel factors involved in the regulation of longitudinal growth and bone mineralization in response to growth hormone (GH) treatment.

This was done by performing a single factor study (Paper I) where it was found that growth response was negatively correlated with adiponectin levels during the first year of GH treatment in short prepubertal children. Thereafter a genomic approach using microarray was used to identify GH and insulin-like growth factor I responsive genes in primary cultured human chondrocytes, from the growth plate, where GH has direct and indirect effects (Paper II). The *COMP* gene was found to be up-regulated by GH, which was confirmed using ELISA in short prepubertal children.

In Papers III–V a pharmacoproteomic approach was used to identify novel GH-regulated protein markers for longitudinal growth and bone mineralization. Serum protein expression profiles during the first year of GH treatment were analysed using SELDI-TOF in two different study groups. In Paper III changes in protein peak intensities allowed 82% of children to be correctly classified as good or poor responders. In Paper IV and V it was found that it was possible to predict the 2-year growth response and bone mineralization and by comparing the proteins in the regression models it was found that these are partly dissociated mechanisms. The proteins identified in Paper III-V were Apolipoprotein (Apo) A-I, Apo A-II, Apo C-I, Apo C-III, transthyretin, serum amyloid A4 and haemoglobin beta. All proteins except haemoglobin beta were related to the high-density lipoprotein. Robust statistical methods were used and developed to ensure valid proteomic data as well as reliable results.

In conclusion: different techniques from ELISAs to genomics and proteomics were used to identify novel GH-dependent factors. Our results suggest that nutritional factors may have a role in determining GH responsiveness. In future, this knowledge could be useful in the development of tools for the diagnosis and individualized treatment of short children, independently of low GH secretion or low GH sensitivity.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Målet med de studier som ingår i denna avhandling var att finna nya tillväxthormonssreglerade faktorer som är av betydelse för längdtillväxt och benmineralisering hos barn. Det långsiktiga målet är att genom en ökad kunskap om vilka mekanismer som styr tillväxthormonets effekter i kroppen, kunna förbättra behandlingen av kortvuxna barn oavsett låg tillväxthormoninsöndring eller låg känslighet för tillväxthormon. Längdtillväxt är en komplex process som till stor del är beroende av ärftliga faktorer, hormonell reglering och nutrition. Kunskapen om de exakta mekanismer som styr denna process är idag begränsad.

Inom ramen för projektet har olika tekniker använts för att studera biomarkörer i serumprover från kortvuxna barn. I första delarbetet studerades sambandet mellan adiponectin och tillväxt. Därefter studerades genuttrycket med hjälp av microarray teknik i primärodlade kondrocyter stimulerade med tillväxthormon och insulin-lik tillväxtfaktor. Resultaten verifierades i serumprover tagna vid start av tillväxthormon-behandling och under behandling. I delarbete III-V användes den masspektrometri baserade tekniken SELDI-TOF för att studera proteinuttryck i serum.
Vi fann att minskningen av adiponectin under första året av tillväxthormon-behandling korrelerar med tillväxtsvaret. Nya tillväxthormon reglerade faktorer identifierades med microarray teknik, däribland cartilage oligomeric matrix protein (COMP). Proteiner för att separera hög- och lågsvarare på tillväxthormonbehandling och för att prediktera tillväxtsvar och benmineralisering hittades med proteomiktekniken SELDI-TOF. Även proteiner, som indikerar en dissociering mellan längdtillväxt och benmineralisering, hittades. Alla proteiner som hittades var relaterade till high-density lipoprotein (HDL), det ”goda” kolesterolet.

Genom att kombinera traditionella analysmetoder med moderna avancerade genomik och proteomik tekniker har vi ökat kunskapen om tillväxthormonreglerade faktorer. Det kan på sikt göra det möjligt att förbättra såväl diagnostiken och kriterier för behandling och att anpassa behandlingen för den enskilde individen.

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, published or in manuscript, which will be referred to by their Roman numerals:

I. **Andersson B**, Carlsson LMS, Carlsson B, Albertsson-Wikland K, Bjarnason R. The decrease in adiponectin correlates to growth response in growth hormone treated children. *Hormone Research. 2009;71(4):213-8*.

II. Bjarnason R, **Andersson B**, Kim HS, Olsson B, Swolin-Eide D, Wickelgren R, Kristrom B, Carlsson B, Albertsson-Wikland K, Carlsson LM. Cartilage oligomeric matrix protein increases in serum after the start of growth hormone treatment in prepubertal children. *J Clin Endocrinol Metab. 2004 Oct;89(10):5156-60.*

III. Hellgren G, **Andersson B**, Nierop AF, Dahlgren J, Hochberg Z, Albertsson-Wikland K. A proteomic approach identified growth hormone-dependent nutrition markers in children with idiopathic short stature. *Proteome Science. 2008;6;35*.

IV. **Andersson B**, Hellgren G, Nierop AF, Hochberg Z, Albertsson-Wikland K. A proteomic approach identified apolipoprotein protein expression pattern to be correlated with growth hormone treatment response in short prepubertal children. *Proteome Science 2009, 7:40.*

V. **Andersson B**, Decker R, Nierop AF, Bosaeus I, Albertsson-Wikland K, Hellgren G. Protein profiling identified dissociations between longitudinal growth and bone mineralization in prepubertal short children during GH treatment. *Submitted.*

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LIST OF ABBREVIATIONS

AITT	Arginine–insulin tolerance test
Apo A-II	Apolipoprotein A-II
BA	Bone area
BMC	Bone mineral content
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BP	Binding protein
Col	Collagen
COMP	Cartilage oligomeric matrix protein
Cyr61	Cysteine-rich protein 61
CV	Coefficient of variation
DXA	Dual-energy X-ray absorptiometry
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FGF	Fibroblast growth factor
GH	Growth hormone
GHD	GH deficient
GH_{max}	Maximum peak GH secretion
GH_{max}24h	Maximum GH response during spontaneous 24-hour secretion
GHR	GH receptor
GHRH	GH-releasing hormone
HA	Height-adjusted
HDL	High-density lipoprotein
IGF-I	Insulin-like growth factor I
Ihh	Indian hedgehog
ISS	Idiopathic short stature
MMP	Matrix metalloproteinase
LC–MS/MS	Liquid chromatography–tandem mass spectrometry

FT/ICR MS	Fourier transform ion cyclotron resonance mass spectrometry
m/z	Mass/charge
LDL	Low-density lipoprotein
PCR	Polymerase chain reaction
PTHrP	Parathyroid hormone-related peptide
RIA	Radioimmunoassay
Runx	Runt-related transcription factor
SDS	Standard deviation scores
SELDI-TOF-MS	Surface-enhanced desorption/ionization time-of-flight mass spectrometry
SGA	Small for gestational age
S/N	Signal-to-noise ratio
SOX	SRY box
SS	Somatostatin
TGF-β	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
VLDL	Very-low-density lipoprotein
Wnt	Wingless

INTRODUCTION

Growth in children is a complex process depending on the interplay between environmental factors, nutrition, various signalling systems, transcription factors and hormones such as, growth hormone (GH) and insulin-like growth factor I (IGF-I). GH is secreted by the pituitary and GH therapy promotes longitudinal growth in a dose-dependent way in short prepubertal children with GH deficiency (GHD), as well as in children with idiopathic short stature (ISS). In addition to promoting longitudinal growth in humans, GH has important metabolic functions including a role in bone mineralization, anabolism and lipolysis (102). Biomedical research has provided great insights into human growth, although much remains to be learned about the exact mechanisms of interaction and regulation of longitudinal growth (99, 162).

A variety of different approaches have been used in attempts to identify novel factors involved in the regulation of longitudinal growth. Studies have looked at the impact of single factors and the simultaneous effects of multiple factors on growth (23, 24, 79). Investigations have included studies of genetic factors in families with inherited short stature (22, 28) and of biochemical factors such as adiponectin (167). Another approach has been to examine changes in gene expression in chondrocytes cultured with growth factors (23, 129).

In the series of studies described here, the focus has been to study GH-mediated regulation of longitudinal growth and bone mineralization using a wide range of techniques.

The first report of GH treatment in children was published in 1932 (51). This study, as well as later studies, supported the theory that pituitary extracts (including human GH) could enhance growth (113). In 1944, bovine GH was isolated from the pituitary (108) and in 1956 human GH was isolated (109). In 1979 the first recombinant human GH was produced, and in 1985, the first case of Creutzfeldt–Jakob disease was reported in a patient receiving pituitary-derived human GH (92). Following this finding, treatment with GH extracted from the human pituitary was stopped. In 1986

biosynthetic human GH became commercially available and research into its role in promoting growth subsequently intensified.

Genetic factors are the primary determinant of adult height when nutrition, psychological well-being and levels of growth-related hormones are sufficient. GH is the major hormonal regulator of growth from about 9 months of age onwards (136). During childhood, juvenility and puberty, the longitudinal growth of a child is largely dependent on the amount of available endogenous GH. One cause of childhood growth failure is GHD. GHD during childhood is defined as a maximum GH response (GH_{max}) of less than 10 μg/L during an arginine–insulin tolerance test (AITT) and has been estimated to occur with an incidence of between 1 in 4000 and 1 in 10 000 live births (114, 169). However, not all short children are GH-deficient regarding secretion. Some children may produce adequate levels of GH but have a poor response to GH at the target tissue level (94).

The individual growth rate is dependent on the balance between the secreted level of biological active GH and target organ sensitivity, or responsiveness. Responsiveness to GH in the different tissues/target cells can be regulated, both at the receptor and the post-receptor level (55, 145). GH exerts its stimulatory effect on longitudinal growth in a dose-dependent way. This is achieved by direct stimulation of the growth plate (endocrine) (75, 76), and by stimulating the production of IGF-I by the liver (dual effector theory), which in turn stimulates the growth plate, as well as by stimulating local tissue production of IGF-I (paracrine and autocrine) (77, 78).
The actions, half-life, and distribution of GH are modified by GH-binding proteins (GHBPs). These binding proteins may alter the distribution and biological activity of the different isoforms of GH (18, 112, 146). In addition to direct growth modulation by factors in the GH/IGF-I axis, several other hormones and locally produced factors are necessary for normal growth. Examples include thyroid hormone, insulin, sex steroids and signal substances from the immune system (42, 70, 181). In order to gain a better understanding of the complex system involved in the regulation of longitudinal growth and bone mineralization, there is a need to generate a clearer

picture of the underlying regulatory mechanisms. It is likely that a variety of factors contribute to the responsiveness of target tissues to GH.

To identify novel factors that play a role in GH responsiveness a combination of genomics and proteomics can be used. However, when using genomic techniques like DNA microarrays, the measured mRNA levels do not always reflect the corresponding protein levels due to alternative splicing of mRNA, mRNA breakdown and post-transcriptional modifications of the proteins (19, 67). This has lead to an increasing interest in using proteomics, the large-scale analysis of the protein complement of the genome, the proteome. The main benefit of using proteomics, compared with mRNA-based methods, is that a proteomic approach identifies the actual active substances (i.e. proteins) in the biochemical processes studied. The main drawbacks are that proteomics is more complex than mRNA-based methods such as microarray as it is affected by different levels of regulation and the number of different proteins present in the samples. Both genomic and proteomic technologies rapidly generate large quantities of data. Processing of the data will lead to useful predictive mathematical descriptions of biological systems which will hopefully permit rapid identification of novel biomarkers and possibly lead to the identification of new therapeutic targets (19, 67). Furthermore, proteomics has the advantage that factors of interest can be measured in the blood, which is easily available.

Bone physiology

Bone is a dynamic adaptive tissue which has several important functions in the body including providing support for locomotion and protecting vulnerable internal organs. Bones are composed of calcium, phosphorus and other minerals, as well as the protein collagen. Calcium makes the bones hard and allows them to support body weight. The amounts of available vitamins and minerals, especially vitamin D and calcium, directly affect how much calcium is stored in the bones (59). Bone growth and bone development are highly dependent on other organs like the intestines and kidneys, through which mineral and nutritional factors are absorbed, reabsorbed and

excreted. In addition, the hypothalamus, pituitary, parathyroid glands, gonads and liver produce hormonal factors that are of importance for bone growth and stability (59). GH has both direct and indirect effects on bone growth via IGF-I (127).

There are two main types of bone (Figure 1):

Cortical bone (compact bone) is the solid outside part of the bone. Its main function is to support the body, protect the organs, provide leverage for movement, and store and release chemical elements (mainly calcium and phosphorous). Holes and channels carrying blood vessels and nerves run through the cortical bone (59).

Trabecular bone (cancellous bone) is inside the compact bone. It is made up of a mesh-like network of tiny pieces of bone called trabeculae. The spaces in this network are filled with red marrow and yellow marrow. Red marrow is found mainly at the end of bones and is where most of the blood cells are made. Yellow marrow mostly consists of fat and is found throughout the bone (59).

A large increase in bone mass occurs during childhood and puberty via endochondral bone formation (127). About half of the adult bone mass is developed during the 3–4 years following the onset of puberty (59). A gradual increase in bone mass is then seen until peak bone mass is reached at 20–30 years of age (127).

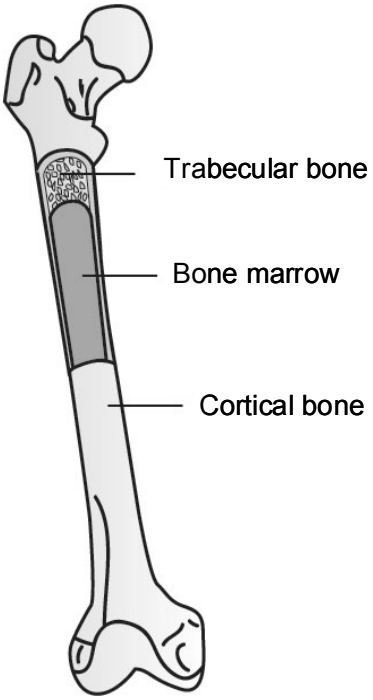

Figure 1. An overview of the bone showing the soft, spongy, trabecular bone and the hard cortical bone on the outside of the bones.

The growth plate

Children grow taller because their bones grow longer. The bones grow longer because they contain growth plates near the tip of both ends of the bone. The growth plate consists of cartilage within the epiphysis of the long bones. Within the growth plates, cells divide and enlarge, producing more cartilage which is subsequently converted into bone. This process causes the bones to elongate. The growth plate can be divided into several morphologically distinct zones (Figure 2).

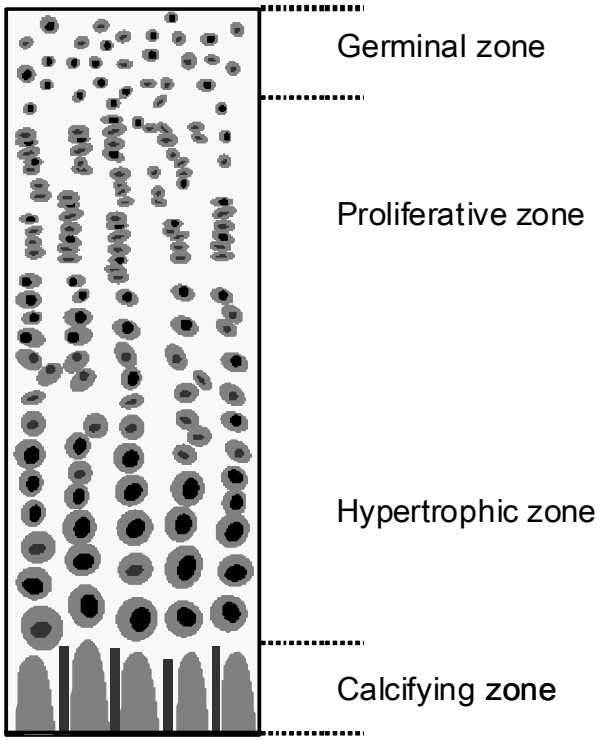

Figure 2. Schematic illustration of the zones of the growth plate. Bone formation begins with mesenchymal cells in the germinal zone which condense and then start to differentiate into chondrocytes. Thereafter, the chondrocytes rapidly proliferate and mature to become hypertrophic. After further maturation, the calcification process begins. This is characterized by breakdown of the extracellular matrix, vascularization and infiltration of osteoclasts Thereafter, osteoblasts start to ossificate the free space created by the breakdown of the extracellular matrix by the osteoclasts and apoptosis of the chondrocytes (65, 104, 132).

Chondrogenesis

Numerous factors are involved during chondrogenesis and endochondral bone formation, many of them probably still unknown (99, 139). The different stages of chondrogenesis are shown in Figure 2 and summarized in Table 1.

Chondrogenesis begins with the condensation of mesenchymal cells (Figure 2. Germinal zone). This process is characterized by a matrix rich in collagen (Col) type I, increased cell adhesion and the formation of gap junctions. At this stage, GH stimulates the prechondocytes to become chondrocytes, cartilage oligomeric matrix protein (COMP) is present and interacts with adhesion molecules to activate intracellular mechanisms that initiate the transition from chondroprogenitor cells to chondrocytes (58, 99). In the differentiation process of the chondroprogenitors, the

matrix is characterized by the expression of collagen II, IX, XI, aggrecan and COMP. At this stage some of the growth factors, IGF-I, fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) are controlling the differentiation process.

Chondrocyte proliferation (Figure 2. Proliferative zone) is the process by which the number of chondrocytes rapidly increases. The major regulators of the rate of proliferation are the BMPs and FGFs, as well as the parathyroid hormone-related peptide (PRHrP)/Indian hedgehog (Ihh) systems together with IGF-I.

Chondrocyte hypertrophy (Figure 2. Hypertrophic zone) is characterized by large increases in cell volume, the expression of the hypertrophic chondrocyte-specific marker type X collagen (Col10a1) and the expression of alkaline phosphatase. Another factor of importance is collagenase 3 (Table 2, Paper II), more commonly known as matrix metalloproteinase 13 (MMP-13). MMP-13 makes an important contribution to remodelling of the extracellular matrix (ECM), by degrading the ECM in the late hypertrophic zone. Remodelling of the ECM is considered to be a crucial step for angiogenesis and osteoblast recruitment during endochondral ossification.

Table 1. Summary of the different stages of chondrogenesis.

Process	Characterized by
Condensation	Condensation of mesenchymal cells. GH is active
Differention	Expression of collagens and COMP, IGF-I has effect
Proliferation	Number of chondrocytes rapidly increases
Hypertrophy	Large increase in cell volume, collagen X expressed

COMP, Cartilage oligomeric matrix protein; GH, Growth hormone; IGF-I, insulin-like growth factor 1.

Growth in children

Longitudinal growth can be divided into three different periods; infancy-childhood-puberty (ICP) (88). The mainly nutrition dependent infancy growth is characterized by a gradual deceleration of growth and last for approximately 9 months (115) until the growth rate abruptly increases. This defines the start of the childhood growth phase.

During this phase the relationship between GH and growth is dose dependent and the growth is affected by the balance between GH secretion and tissue sensitivity (8, 41). After the start of puberty the growth is dependent also on other hormones, besides GH, like the sex hormones.

In the northern and southern part of the globe the growth process is also affected by the time of year. Growth rates are higher during the spring and summer (the lighter periods of the year) than during the winter (56). A variety of factors can interfere with the rate of growth, including genetic factors, nutrition, general health and/or hormone levels. Physical well-being, exercise, sleep and diseases that affect longitudinal growth also have impact on the longitudinal growth (7, 25).

Growth hormone and bone

GH has clear effects on bone physiology and it has been demonstrated that children with GHD have decreased bone mineral density (BMD), both by areal and volumetric analysis (138). Therapy with GH increases BMD and also markers of bone formation such as bone-specific alkaline phosphatase, alkaline phosphatase and osteocalcin (102, 127). According to the 'the biphasic model' of growth (127), GH initially increases bone resorption with a concomitant loss of bone. This is followed by a phase of increased bone formation (127). After the point when bone formation is stimulated more than bone resorption (transition point), bone mass starts to increase. However, a net gain of bone mass may take some time (typically 12–18 months in adults receiving GH) (127). In children with ISS, BMD is typically decreased relative to controls of the same height and bone age. BMD increased significantly following 12 months of GH treatment and this was accompanied by increased bone turnover as measured by bone formation and resorption markers (102).

Growth hormone secretion and signalling

GH is the major hormone of the anterior pituitary and is secreted in a pulsatile pattern. The pulsatile pattern arises through the interaction between two peptides

secreted from the hypothalamus; somatostatin (SS) which inhibits the secretion of GH, and GH-releasing hormone (GHRH) which promotes GH secretion (Figure 3) (7, 25). The secretion of GH can vary with for example sleep (7), exercise and nutrition (93). The pituitary secretes different isoforms of GH. The major and the most potent isoform in serum is the 22 kDa isoform that constitutes approximately 70% of the GH in blood (17) The 22 kDa isoform is subjected to various post-translational modifications, including glycosylation, proteolysis and aggregation (106, 107). This isoform is produced commercially as recombinant biosynthetic human GH and is the only isoform given during GH replacement therapy. The other common isoforms are the alternative splice product 20 kDa and the proteolytic cleave products, 5 and 17 kDa, from the 22 kDa isoform (81, 106).

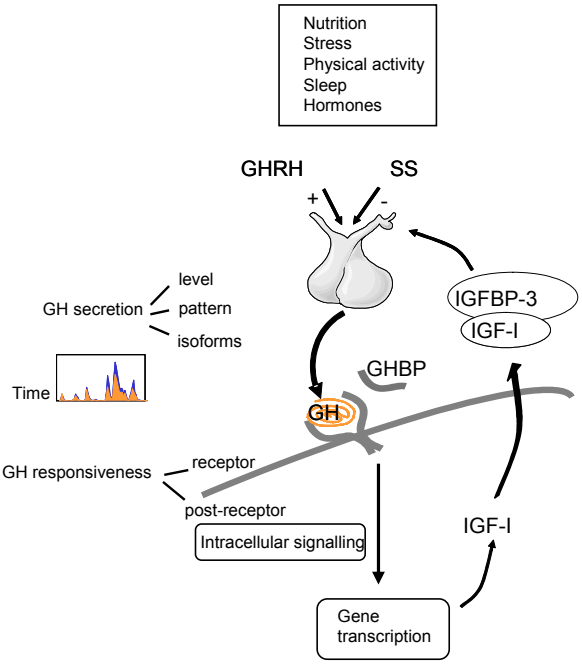

Figure 3. A schematic overview of the GH/IGF-I system. Pituitary secretion of GH is under the control of a negative feedback loop (IGF-I, IGFBP-3) caused by the GH-stimulated release of IGF-I. GH release is also under the control of GH-releasing hormone (GHRH), and somatostatin (SS).The level and pattern of GH secretion over the day is shown in the upper left hand corner of the figure. This illustrates that the amount of GH available at any time is variable. In the lower left hand corner of the figure it can be seen that GH responsiveness is controlled at different levels, both at the receptor level and post-receptor level.

Human pituitary GH has two binding sites for the extracellular part of the GH receptor (GHR).

When one GHR molecule binds to site 1 of GH and a second GHR binds to site 2, a dimerization of the two GHR molecules occurs. However, today it is known that the GHR also exists as a constitutive dimer and is activated by a reorganization of receptor subunits as a result of asymmetric placement of two receptor binding sites on the hormone monomer (110, 111). GH exerts its effects on the target tissues through binding and dimerization of the GHR, and subsequent activation of several intracellular signalling pathways (69). The expression of GHR is under the control of the GH concentration in the blood and nutritional intake (151). The result is activation of a cascade of different intracellular signal pathways leading to the stimulation of IGF-I release (25, 128). The process starts by phosphorylation of the tyrosine residues in the intracellular domain of the GHR and phosphorylation of molecules downstream in the signalling pathway (69). The Janus kinase 2 (JAK2) and signal transducers and activators of transcription (STATs) pathway transduce signals to the cell nucleus, where activated STAT proteins induce gene expression. The JAK2–STAT5 pathway is believed to be the most important pathway for GH-mediated longitudinal growth. Another important signalling pathway is the MAP kinase pathway (69).

GHBP is present in the blood and is identical to the extracellular part of the GHR. In humans, GHBP is believed to be produced by proteolytic cleavage of the extracellular domain of the GHR (151). The physiological role of GHBP is unclear. However, *in vivo* it has been shown that GHBP can prolong the half-life of GH and increase the effects of GH on bone and growth (16).

Growth hormone treatment

Although GH has been available for clinical use since the late 1950s (140), it is still not known how to treat each individual short child optimally. In the early 1980s, daily GH injection was introduced to treat short children and is now the globally accepted treatment regimen. In 1986, the GH dose of 33 μg/kg/day was introduced, which is

still used today. It was shown that GH-deficient children as a group achieve normal height within 3 years after starting treatment (4), although the response to GH treatment varied widely within as well as between the different diagnostic groups (for example, children with short stature as a result of GHD and those with ISS). Today, it is clear that individualized GH treatment results in a much smaller range around the target height (94). For the purpose of individualizing treatment, validated multivariate regression models estimating GH responsiveness during long-term treatment have been developed.

Prediction models

Many short children will benefit from treatment with GH. The earlier that their short stature is detected, the greater the possibility to help them to achieve a normal stature. A lot of effort has been put into creating models that accurately predict growth in response to GH treatment in order to improve stature in these patients as efficiently as possible (3, 8, 41, 44, 101, 141). These evidence-based models for predicting growth in response to GH treatment provide an indirect measurement of the individual responsiveness to GH (95). The best models available today are able to explain up to 80% of the variation in growth response to GH, based on auxological data from the child and his/her parents(41), compared to 33% using traditional diagnostic criterias (97). Highly predictive variables that are used within these models include early growth data, the difference between the current height of the child and mid-parental height, and maximum spontaneous 24 h GH secretion (3, 8, 41, 44, 101, 141). As some parameters such as early growth data and details of parental height may be difficult or impossible to obtain, there are advantages to developing models that only include parameters that are easily attained at the start of the growth investigation at the paediatric unit. To achieve this goal it is necessary to identify novel markers of growth response, bone quality and metabolism to ensure optimal treatment for the individual child.

Individualized treatment

The idea of individualized treatment of many conditions has received considerable attention during the last few years due to potential advantages in terms of drug safety and effectiveness. Individualizing treatment will avoid the 'one size fits all' concept and ensure appropriate treatment for each patient (90). Today, around 10% of the patients receiving GH treatment do no benefit from treatment. This is either because they are poor responders, non-responders or because the drug is cleared too quickly from the circulation. As a result of the use of a standardized dose, this group may also be at increased risk of adverse effects (38, 137).

Complex conditions like cancer, asthma and growth retardation are comprised of numerous different subtypes among the affected patients. The different subtypes share signs, symptoms and risk factors (68, 120) and it is usually not possible to discriminate between them using traditional methods. To establish criteria's to discriminate between the subtypes, large-scale screening techniques like genomics or proteomics are most likely required. Besides allowing early detection, these techniques can improve the long-term treatment of patients suffering from the disease by providing tools for individualizing treatment, monitoring disease progression and adjusting the individual doses given depending on disease progression (79, 80).

Gene expression studies

During the last 100 years there have been many techniques used to identify and study factors involved in the regulation of longitudinal growth.

This section focus on the development of the techniques that paved the way for modern high-throughput techniques that made it possible to study the expression of multiple genes at the same time.

Traditionally, one studied the expression of mRNA from known candidate genes using somewhat labour-intensive, low-throughput techniques, like Northern blot and RNase protection assays. The discovery of the polymerase chain reaction (PCR), over 20 years ago, was a milestone in the study of gene expression (105). After this discovery, newer and more powerful technologies such as quantitative reverse transcription PCR (QRT-PCR) and DNA chips or microarrays were developed (105). Since 1995, when microarray methods were first used (150), this technology has been applied to a variety of different research fields and numerous statistical tools have been developed for evaluating the vast amount of data that are generated. The microarray technique has enabled the rapid and simultaneous comparison of the mRNA levels of thousands of genes in a wide range of biological samples using very low quantities of starting material. This process is generally referred to as genomics or transcriptomics. This has made microarray technology a suitable tool with which to explore the complex gene response patterns and regulatory pathways involved in human diseases (116).

There are many different microarray technologies but they all share the same basic principles. Unknown mRNA from the sample to be analysed is hybridized to an ordered array of DNA sequences corresponding to known genes or expressed sequence tags. A DNA microarray chip may contain DNA sequences corresponding to more than 30 000 human genes, ordered on a miniature glass slide (116). Every mRNA, or transcript, is labelled with a marker, for example, a fluorescent dye. After the processing of the chip they are placed in a reader where the location and intensity of the resulting signals give an estimate of the quantity of each transcript in the sample. It is thus possible to measure all transcripts in the sample (i.e. the

transcriptome) (116). Microarrays can also be used to search for DNA polymorphisms (single nucleotide polymorphisms, or SNPs) or to study protein interactions.
However, the mRNA levels measured do not always reflect the corresponding protein levels due to mRNA breakdown and the specific post-transcriptional modifications of the proteins (19, 67). In yeast it has been shown that the correlation between the expression levels of mRNAs and proteins are no higher than 0.54 (57, 61). Poor correlations between mRNA and protein abundance have also been found in the human liver (correlation coefficient, 0.48) (11).

Proteomics and protein regulation

The term proteome refers to the total set of proteins expressed by a cell, tissue or organism at a given time point (46). Proteomics is defined as the analysis of the entire protein complement expressed by a genome (46), and can be used to study the proteins expressed by the genes which are responsible for the biochemical processes taking place within the cell. This provides an advantage over studying the genome which does not reflect the dynamics that occur due to the different conditions that cells are exposed to. The proteome is highly dynamic and the same protein is expressed in different forms that cannot be predicted from mRNA analysis (12).

The development of new techniques has been a prerequisite for proteomics and has made it possible to shift focus from the time consuming study of single candidate proteins to the study of multiple proteins at the same time. It has also made it possible to move towards a better understanding of gene function.

Regulation of the proteome is highly complex and it can be modified at any of the many stages involved in the translation of genes into proteins (Figure 4). Modifications include alternative mRNA splicing, mRNA degradation, post-translational modifications at the protein level and associations with other proteins. All together this gives rise to numerous distinct proteins.

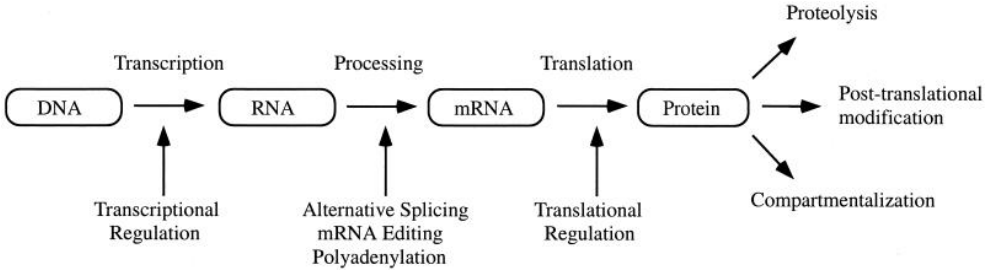

Figure 4: A schematic illustration of, 'the central dogma of molecular biology', the process from DNA to functional protein. Each step that can affect the protein end-product produced from the original gene is shown. The figure is used with permission from Graves et al. (60).

Biomarker discovery

The common goal of most proteomic studies has been to identify biomarkers which are related to a certain disease mechanism and can be used for screening (early detection), prognosis, monitoring of drug response or to assess disease progression/predisposition. Proteomics can also be used at an early stage in clinical studies of new drugs to examine clinical effects and toxicological responses (134). Proteomic technologies have successfully been used to identify novel biomarkers in a wide range of diseases (45, 62, 133).

Each cell type has a specific set of proteins at a specific time and condition. The set of proteins will change when the conditions change (e.g. in response to disease), making proteins promising targets in biomarker discovery. By using proteomics it is possible to study changes in protein expression patterns associated with a certain disease or in response to a certain drug. Typically, the changes observed involve either up or down regulation of one or more expressed proteins; however, sometimes a protein is no longer present or its pattern of post-translational modifications is altered (62, 133). There may also be expression of a previously non-transcribed protein.

Much of the focus in detecting clinically important biomarkers has centred on the use of readily available samples, such as serum, urine, amniotic fluid and cerebrospinal fluid. These fluids are like 'fingerprints', showing the status of the body at a given

time, and can be collected in sufficient quantities to allow protein profiling. Being easy to obtain they are suitable as samples for use in the early detection of lethal diseases like cancer.

The human plasma proteome

The plasma proteome is the largest and the most complex of the human proteomes (12). Serum samples, which are composed of plasma without fibrinogen or the other clotting factors, are used in many proteomic studies. Plasma and serum are often the first-choice samples when screening for biomarkers, because they are accessible and contain large amounts of protein (12). The blood has been estimated to contain over 10 000 of the proteins present in the human body (2, 13).

Plasma that circulates in the human body contains a myriad of proteins from different origins. However, only a small number of the proteins present in plasma are true plasma proteins that actually have functions in the circulation. Apart from the true plasma proteins, plasma contains proteins leaked by tissues, hormones, proteins fragments, etc. (Table 2).

The plasma proteins have a dynamic range higher than 10 orders of magnitude (9, 12), ranging from the most abundant protein, albumin, that constitute over half of the plasma proteome and is typically found in concentrations between 35 and 50 g/L, down to cytokines, with concentrations of just a few ng/L (Figure 5).

It has been calculated that 'true' plasma proteins (i.e. proteins that carry out their function in the plasma) probably would yield around 50 000 forms, but by the addition of all proteins contributing to the plasma proteome by tissue leakage, one will end up with a theoretical maximum number of around 500 000 protein forms in plasma, including essentially the entire human proteome (12).

Most of the instruments used today for proteomic analyses have limitations regarding the 10–12 orders of magnitude of the plasma proteome (9, 12). Mass spectrometry which is the most sensitive of the high-throughput techniques has the limitation of a dynamic range of three magnitudes. If used in combination with an HPLC system, the dynamic range can be increased to 10^4–10^6 (9, 12). Therefore, depletion and fractionation strategies are often used to decrease the diversity and complexity of the samples. Depletion of proteins in serum is usually performed using antibodies or beads designed against certain proteins. Fractionation is usually performed using different types of columns or beads with certain biochemical properties to which proteins bind and are subsequently eluted using individual buffers with different eluting properties (typically pH). Depletion and fractionation strategies are also used because the high dynamic range of plasma proteins tends to mask the presence of proteins or peptides with low abundance (9, 12). Most of the potential biomarkers are secreted into the bloodstream in low concentrations (9, 12). The most well-known biomarker for prostate cancer, prostate-specific antigen (PSA), is present in the low pg/mL concentrations (9, 12).

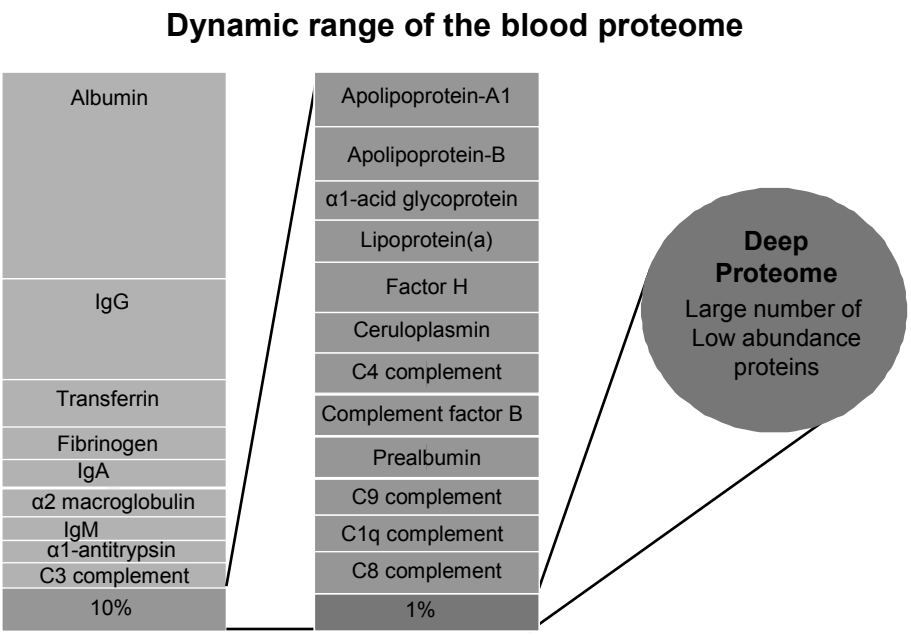

Figure 5: This figure demonstrates the high dynamic range of proteins present in a plasma sample. The figure was kindly provided by Bio-Rad.

Table 2. Putnam's classification of plasma proteins divided into functional groups, elaborated and described from a functional viewpoint by Anderson *et al.* (12).

Group	Description
Proteins secreted by tissues acting in plasma	Classical plasma proteins that are mostly secreted by the liver and intestines.
Immunoglobulins	Antibodies that circulate the blood stream.
Long distance receptor ligands	Peptide and protein hormones present in a range of sizes, for example insulin.
Local receptor ligands	Cytokines and other short mediators of cell responses that mediate local interactions between cells followed by dilution into plasma in low ineffective levels.
Temporary passengers	Non-hormone proteins that traverse plasma on their way to their primary site of function.
Tissue leakage products	Proteins that normally function inside cells but are released into plasma as a result of cell death or damage, e.g. lysosomal proteins
Aberrant secretions	Released from tumours or diseased tissue, presumably not as a result of a functional requirement.
Foreign proteins	Proteins from infectious organisms or parasites that are exposed to or released into the bloodstream.

Proteins and genes identified and studied in Papers I–V

Adiponectin

Besides being the major regulator of longitudinal growth, GH exerts direct effects on body composition through anabolic and lipolytic actions (82, 160). The function of adipose tissue was formerly considered to be mainly fat storage. Today, it is well established that adipose tissue produces and secretes a large number of growth factors and hormones called adipokines (20, 166). Many adipokines have profound effects on other tissues in the body (20), including bone (47, 131). Furthermore, it has been observed that many obese children have a normal longitudinal growth rate in spite of having very low circulating GH levels (135). This may suggest that adipose tissue-derived factors could affect GH responsiveness, or that adipokines may have direct effects on longitudinal growth.

The circulating levels of most adipokines, including leptin, are increased in obese subjects (65). However, the adipokine, adiponectin, that is studied in Paper I, is an exception to this rule. Levels of adiponectin, which is predominately expressed by the adipocytes in both subcutaneous and visceral fat (1), decrease as the amount of adipose tissue increases (65). Adiponectin influences glucose and lipid metabolism and is decreased in obese individuals and patients with type 2 diabetes (168, 176). In addition, adiponectin seems to play a role in cell adhesion and participates in the regulation of cell proliferation and growth, and tissue remodelling (132, 173, 177). It has also been shown that short children born small for gestational age (SGA) who show catch-up growth have significantly lower adiponectin levels than those who do not show catch-up growth (36). Furthermore, GH treatment in children with SGA leads to lower adiponectin levels (36, 72, 130). Adiponectin has also been shown to directly regulate GH secretion *in vitro* in pituitary cells by binding to the adiponectin receptor (144, 159). In turn, GH can induce adiponectin receptor 2 expression (53), whereas adiponectin decreases the expression of this receptor (31). These findings suggest the presence of a negative feedback loop between adiponectin and GH.

Cartilage oligomeric matrix protein

COMP is a secreted 550 kDa protein that is predominantly synthesized by chondrocytes and is found primarily in the extracellular matrix of cartilage, ligaments, and tendons (27, 29).

COMP is known to interact with collagen types I, II, and IX in a divalent cation-dependent manner. COMP and its proteolytic fragments are released into synovial fluid and serum on joint degradation, suggesting a possible role of COMP in the assembly and maintenance of the extracellular matrix (149). Mutations in the COMP gene are associated with pseudoachondroplasia and multiple epiphyseal dysplasia, conditions that are both characterized by short stature (27, 29).

High-density-lipoprotein-related markers

Apolipoprotein (Apo) A-I, A-II, C-I, and C-III, and serum amyloid A4 (SAA 4) and transthyretin (TTR), which are all markers of GH response in this study, are part of the high-density lipoprotein (HDL) (153, 157, 178). However, Apo A-II, Apo C-I, Apo C-III and SAA 4 have also been found in very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDLs) (10, 178).

HDL is known as the 'good' cholesterol because of its role binding unhealthy cholesterol from the blood and transporting it to the liver where the cholesterol is excreted or re-utilized (54).

Today, not much is known about the effects of GH on either the apolipoproteins, TTR or SAA 4.

There are contradictory results regarding the effects of GH treatment given as daily subcutaneous injections on the HDL which carries these proteins (71, 100, 104). This is discussed in more detail in the discussion section in Paper V. It was also seen that HDL decreased after a single large GH dose which created a GH plasma profile with a high peak after the injection (142, 143).

A short summary of the known functions of the HDL-related biomarkers identified:
Apolipoprotein A-I: The most common protein of the HDL molecule, Apo A-I promotes cholesterol efflux from tissues to the liver for excretion and may confer an important protective action against the accumulation of platelet thrombi at sites of vascular damage. (180).

Apolipoprotein A-II: Apo A-II is the second most common protein of the HDL molecule. Apo A-II-deficient mice showed improved insulin sensitivity, whereas transgenic mice over-expressing murine Apo A-II showed insulin resistance and obesity (32). Apo A-II has also been suggested as a candidate gene in type 2 diabetes (49, 63, 170).

Apolipoprotein C-I: Apo C-I is a major plasma inhibitor of cholesteryl ester transfer protein and appears to interfere directly with free fatty acid uptake (153). Hypertriglyceridaemia and increased atherosclerosis have been shown to be a direct consequence of over expression of Apo C-I (39). In obese mice, over expression of Apo C-I leads to insulin resistance (123). Furthermore, Apo C-I has been reported to increase HDL levels in blood (43).

Apolipoprotein C-III: Apo C-III is primarily expressed as a VLDL. Apo C-III is an inhibitor of lipolysis and its expression may contribute to the hypertriglyceridaemia and atherogenic lipoprotein profile observed after retinoid therapy (153, 171). High Apo C-III concentrations increase the risk of coronary heart disease associated with high triglyceride levels (73).

Serum amyloid A4: The function of SAA 4 is largely unknown. SAA 4 is a minor acute-phase reactant in humans (i.e. it does not change as much as other SAAs in response to inflammation). Furthermore, SAA 4 may be an indicator of nutritional state (179).

Transthyretin: Transthyretin is well known as a marker of nutrition (165). In addition to its functions as a carrier of serum thyroxine and triiodothyronine and a transporter of retinol (vitamin A) (52)

Haemoglobin beta

Haemoglobin is present in the red blood cells and is an iron-containing oxygen-transport metalloprotein. Haemoglobin transports oxygen from the lungs to the rest of the body where it releases the oxygen. In patients with thalassemia who have impaired haemoglobin beta function, BMD is decreased (48, 148). Furthermore, growth retardation and GH/IGF-I/IGFBP-3 hormone axis dysfunction, have been reported in patients with thalassemia major (over production of defective haemoglobin beta) (86). It has also been shown that haemoglobin beta is upregulated during GH-releasing hormone analogue treatment (147). Although the molecular mechanisms linking the proteins identified to the biological activity of GH and IGF-I remain to be clarified, the results suggest that they represent potential biomarkers of GH and/or IGF-I action.

AIM OF THE STUDY

The overall aim of the studies included in this thesis was to identify novel genes and proteins that could be used as markers for GH treatment response and that are of possible importance for both GH-mediated regulation of longitudinal growth in children and GH treatment response with respect to longitudinal growth and bone mineralization.

Specific aims

I. To investigate the relationship between growth response to GH treatment and adiponectin in short prepubertal children.

II. To use genomics to identify novel GH- and IGF-I-induced genes in a GH target tissue.

III–V. To elucidate if a pharmacy-proteomic approach can be used to identify novel serum markers of the growth response to GH treatment and changes in bone mineralization during GH treatment.

PATIENTS AND METHODS

The patients and methods used in this thesis are described in detail in the materials and methods sections of each individual paper. A shorter, more general, overview of the methods is presented below.

Ethical approvals

All studies were approved by the ethical boards of the University of Gothenburg (for patients from Gothenburg and Halmstad) and in Papers IV–V from the ethical boards in Umeå, Uppsala and Malmö, as well as the Medical Product Agency of Sweden. Written informed consent was obtained from all parents and from children if old enough. All trials included were performed in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Patients

This thesis includes patients from the Swedish National Registry for GH treatment and/or clinical trials. In Paper I 82 out of the 94 patients, and in Papers II and III all patients were included in the Swedish National Registry and the Swedish GH trials (14). There was an overlap between the study groups in Papers I–III; 25 patients are present in both Papers I and II, 20 patients are present in both Papers I and III, 27 patients are present in Papers II and III, and 8 patients are present in all three studies (illustrated in Figures 6 and 7). In Papers IV and V all 128 patients were included in the GH dose clinical trial (TRN 98-0198-003).

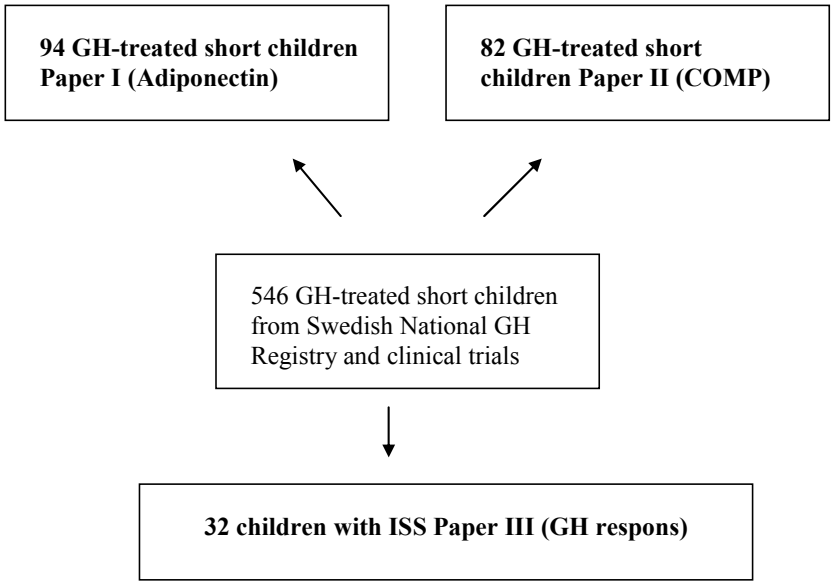

Figure 6: The procedure for study group selection in Papers I–III.

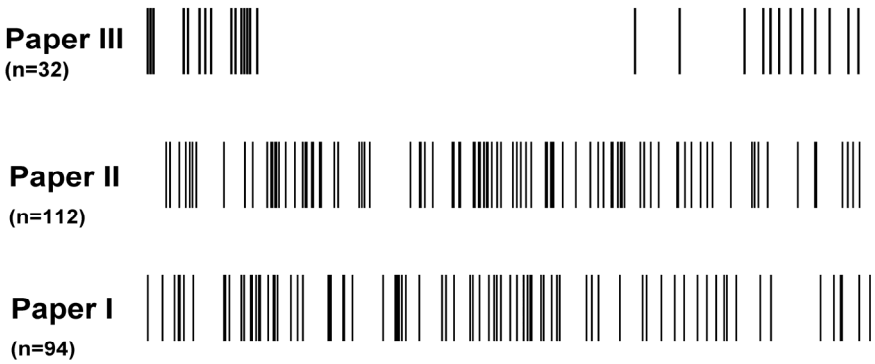

Figure 7: The number of children included in Papers I–III, sorted by GH_{max}24 h from lowest (left) to highest. Each vertical bar represents one child. 25 patients are present in both Papers I and II, 20 patients are present in both Papers I and III, 27 patients are present in Papers II and III, and 8 patients are present in all three studies.

Adiponectin study (Paper I)

To study the relationship between GH and adiponectin, 94 short prepubertal Swedish children (19 girls, 75 boys) were treated with daily injections of GH (dose range, 26–69 μg/kg/day) and followed for at least 1 year. The clinical characteristics of the patients are given in Table 1 of Paper I. Of the 94 children, 56 were diagnosed as having isolated GHD and 38 were short but did not have GHD. The children were well nourished, free from chronic disease and had no dysmorphic features.

Cartilage oligomeric matrix protein study (Paper II)

To verify the microarray analyses that COMP was regulated by GH, 113 short prepubertal Swedish children (14 girls, 99 boys) with a broad range of peak GH response (GH_{max}) during an AITT were treated with daily injections of GH (33 μg/kg/day) and followed for at least 1 year. The clinical characteristics of the patients are given in Table 2 of Paper II. Of the 113 children, 82 were diagnosed as having isolated idiopathic GHD and 31 were short but did not have GHD. The children were well nourished, free from chronic disease and had no dysmorphic features.

Growth hormone responder study (Paper III)

To identify serum biomarkers that could be used to discriminate between a good or poor growth response to GH treatment in short prepubertal children receiving GH treatment, the 40 children with the highest and the 40 children with the lowest first year growth response from within the cohort were selected. From this group of 80 children, 51 children with ISS and a maximum peak of GH secretion (GH_{max}) on the AITT > 5 μg/L were identified.

Growth hormone response and bone study (Papers IV–V)

To identify serum biomarkers that correlated with changes in growth, bone mineralization and bone volume in response to GH treatment in short prepubertal children, the per-protocol population from the GH dose clinical trial (TRN 98-0198-

003) which consisted of 128 short prepubertal children (94) was used. Study patients were randomized either to a group receiving an individualized (two-thirds of patients) or a standard GH dose (one-third of patients). The standard GH dose was 43 μg/kg/day. The individualized GH dose comprised one of six different doses (mean, 49 μg/kg/day; range, 17–100 μg/kg/day). The $GH_{max} \geq 32$ U/L (old 10 μg/L) on an AITT or of the spontaneous GH secretion over a 24 h period was used to classify the patients as having either ISS (n=89) or short stature due to GHD (n=39). Clinical data for the patient groups are presented in Table 1, Paper IV and V.

Study design

Pre-treatment investigations: Endocrine investigations were performed during the pre-treatment year. The children underwent both an AITT and a 24 h secretion profile (5, 26, 96). Blood samples were obtained for determination of hormone and protein concentrations.

Blood samples were taken at the start of the study, and 1 week, 1 month, 3 months and 1 year after the start of GH treatment. The samples were taken at health care units in Sweden approximately 24 h after the last GH injection. The samples were stored at –70 °C and were not thawed until the time of analysis.

Dual-energy X-ray absorptiometry (DXA) measurements and growth evaluations were performed at the paediatric units in the GH dose clinical trial (Papers IV and V) at the start of the study, and after 1 and 2 year of GH treatment.

Growth evaluation

The childhood component (87) of the Swedish population-based growth reference values (6) was used for the height-related inclusion criteria and to express the prepubertal height for weight (6) and body mass index (89) of the patients. Reference standards of newborns were used for standard deviation score (SDS) at birth (126).

Hormone and protein measurements

Adiponectin (Paper I): Concentrations of serum adiponectin were measured in duplicate by an ELISA (R&D Systems Inc., Minneapolis, MN). The assay has a detection limit of 1 ng/mL. All samples were analysed using the same assay batch, and samples from each patient were run in the same assay. In our laboratory, the assay had an interassay CV of 6.9% at 8.8 μg/mL and an intraassay CV of 3.6% at 14.5 μg/mL.

COMP (Paper II): Concentrations of serum COMP were measured in duplicate by an ELISA (Kamiya Biomedical Comp, Seattle, WA). The assay has a detection range of 10–80 ng/mL. All samples were analysed using the same assay batch, and samples from each patient were run in the same assay. Only COMP values with an intraassay CV below 15% were included. In our laboratory, the assay had an interassay CV of 10.0% at 1.0 μg/mL and 8.7% at 1.3 μg/mL.

GH: GH measurements were performed at the GP-GRC laboratory (SWEDAC accredited no 1899) at the University of Gothenburg using monoclonal (Wallac, Turkku, Finland),or polyclonal antibody-based immunoradiometric assay (Pharmacia Diagnostics, Uppsala, Sweden) (81).

IGF-I: Concentrations of serum IGF-I were measured by an IGFBP-blocked radioimmunoassay (RIA) without extraction and in the presence of an approximately 250-fold excess of IGF-II (Mediagnost GmbH, Tubingen, Germany) Published reference values were used to assess the results of analyses of IGF-I (117).

IGFBP-3: Serum IGFBP-3 concentrations were measured by a polyclonal antibody-based RIA (Mediagnost GmbH, Tubingen, Germany). Published reference values were used to assess the results of analyses of IGFBP-3 (118).

Dual-energy X-ray absorptiometry (Paper V)

To asses bone mineral content (BMC), bone area (BA) and BMD, whole body DXA scans were obtained using either a Lunar DPX-L scanner (GE Medical, Madison, WI) or a Lunar Prodigy (GE Medical). The results were comparable across DXA systems, and only small differences were detected between Lunar's Prodigy and DPX systems (74).

DXA scan reproducibility (CV) for the Lunar Prodigy system has been reported to be 0.18 to 1.97% for total body bone measures, and 0.96 to 6.91% for regional bone measures (121).

Cell culture (Paper II)

Cultured human primary chondrocytes were established from a surgically removed extra thumb from a 1-year-old boy. Cells were cultured in Dulbecco's modified Eagle's medium DMEM/F12 (1:1, v/v) (Gibco BRL, Paisly, UK) containing 10% (v/v) fetal calf serum (FCS; Bio Whittaker, Verviers, Belgium), Fungizone (500 μg/L; Gibco), gentamicin sulphate (50 mg/L; Biochrom KG, Berlin, Germany), L-glutamine (2 mmol/L; Gibco) and L-ascorbic acid (100 mg/L; Merck, Darmstadt, Germany) in a humidified 5% CO_2 atmosphere at 37 °C. Cells were routinely tested and found to be negative for mycoplasma infections. Cells in passage 3 were used for the experiment. Cells were grown to confluence and then rinsed twice with DMEM without phenol red (Gibco) before they were starved for 27 h in DMEM without phenol red and without serum. They were then stimulated with GH (50 ng/mL Genotropin, batch 28157B51; 36IE/KY (12 mg), supplied by Pharmacia) or IGF-I (50 ng/mL, IGF-I; lot 99H0295, Sigma, St. Louis MO) for 12 h before being harvested for RNA preparation.

Analysis of microarray data (Paper II)

Preparation of cRNA and microarray hybridization was according to standard protocols. After visual inspection for hybridization artefacts of the scanned output

files, Affymetrix software Microarray Suite 5.0 was used to analyse differences in gene expression between GH- or IGF-I-stimulated chondrocytes compared with controls. Samples from each treatment group and the control group were run in duplicate. The two GH and two IGF-I microarrays were compared separately with the two control microarrays, creating four comparison files for GH versus controls and four comparison files for IGF-I versus controls. Genes with different expression levels in chondrocytes cultured with GH or IGF-I versus controls were identified. A fold change of 1.5 was considered significant.

Serum denaturation and fractionation (Papers III–V)

Frozen serum samples were thawed on ice and spun at 10 000 rpm for 10 min at 4 °C. Each serum sample (10 µL) was denatured by the addition of 20 µL of U9 buffer (9 M urea, 2% CHAPS, 50 mM Tris-HCl, 1% DTT, pH 9.0) and vortexed at 4 °C for 30 min.

Sample fractionation was performed on a Q HyperD F resin plate (180 µl resin) (Ciphergen, Fremont, CA). The plate was prewashed and equilibrated with U1 solution (1 M urea, 0.2% CHAPS, 50 mM Tris-HCL, pH 9) prior to the addition of samples to the 96-well fractionation plate. The anion exchange fractionation included the following elution steps: (1) 50 µM Tris-HCL, 0.1% OGP (a nonionic detergent, b-D-glucopyranoside), pH 9; (2) 50 µM HEPES, 0.1% OGP, pH 7; (3) 100 µM Na Acetate, 0.1% OGP, pH 5; (4) 100 mM Na Acetate, 0.1% OGP, pH 4; (5) 50 µM Na Citrate, 0.1% OGP, pH 3; and (6) 33.3% isopropanol, 16.7% acetonitrile, 0.1% TFA (trifluoroacetic acid).

SELDI-TOF MS (Paper III-V)

To identify protein biomarkers of interest, the surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) technique was used (Figure 8).

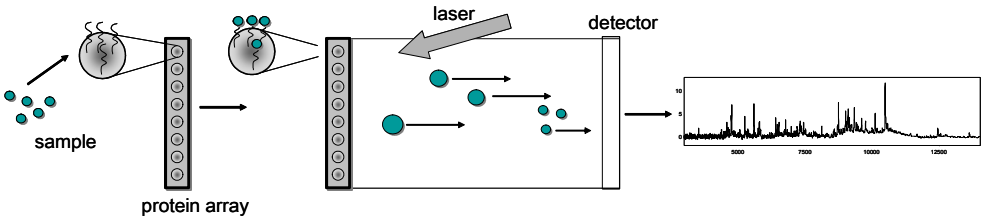

Figure 8: Schematic illustration of the SELDI-TOF technique. Samples are applied on the spots of the protein arrays. Thereafter, the arrays are loaded into the machine and laser is fired upon the spots. The proteins are eluted and travel towards the detector and mass over charge spectra (m/z) are created.

SELDI-TOF is an array-based high-throughput mass spectrometry-based technique, that has been successfully used to identify biomarkers/proteins in many different sample types and for many diseases (68, 84). SELDI-TOF technology is based on mass spectrometry, where retentate chromatography is performed on ProteinChip Arrays with varying chromatographic properties (Figure 9). Each array contains a chemically pre-activated surface (anion exchange, cation exchange, metal affinity and reverse phase) (Figure 9). The chemical surfaces are used to capture subsets of proteins for protein profiling analysis. Samples are applied on arrays which are loaded into the SELDI-TOF instrument. Proteins are eluted by laser desorption/ionization. Ionized proteins are detected and the mass is determined by TOF mass spectrometry, resulting in mass spectra reflecting the protein expression (Figure 8), where the peak intensities are proportional to the amount of the specific protein.

In the TOF analyser the ions are accelerated by an electric field prior to entering the field-free flight tube. 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.

Figure 9: Various ProteinChip array surfaces. Chromatographic surfaces are composed of reversed-phase (H50), ion exchange (CM10 and Q10), immobilized metal-affinity capture (IMAC30) or normal-phase (NP20) chemistries. The figure was provided by Bio-Rad.

Serum samples were thawed, denatured and fractionated using anion-exchange beads in a serum fractionation kit (Bio-Rad Laboratories, Hercules, CA) according to protocols provided by Bio-Rad Laboratories. The fractionated serum samples were analysed on weak anion-exchange (CM10), immobilized metal-affinity capture (IMAC30) and reversed-phase (H50) arrays according to the protocols described in Papers III–V.

In brief, the samples were bound to the arrays and SPA matrix was added. The arrays were analysed using the SELDI-TOF instrument generating TOF. To minimize experimental variation, all samples were randomized and analysed concurrently within 1 week by the same operator. In addition, one reference serum sample was randomly applied on each array and evaluated. The mass accuracy was calibrated in the molecular range of 5–18 kDa using external calibrators from Bio-Rad Laboratories.

Data pre-processing

Data handling was performed using ProteinChip Data Manager (Bio-Rad Laboratories). All spectra were baseline-subtracted and normalized according to total ion current. Settings for peak identification and clustering of peaks across multiple spectra were first pass signal-to-noise ratio (S/N) > 5 in 15% of all spectra and second pass S/N > 3 in Paper III and (S/N) > 3 in 15% of all spectra and second pass S/N > 2 in Papers IV–V, with a cluster mass window of 0.3% of the mass.

Spectra were visually inspected and 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).

Only patients for whom there were two high-quality mass spectra for the relevant array and time point were included in further statistical analyses.

Protein quantification (Papers III–IV)

To use the Microcon filtration cut-off membranes (YM30, Millipore, USA) and to perform the ACN precipitation in preparation of the 1D SDS-PAGE analysis it is of importance to know how much protein was loaded.

The protein concentration of a 1 μL sample was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) according to manufacturer's instructions. The protein concentrations were measured at 280 nm.

In some cases the protein concentration was measured using the DC Protein Assay (Bio-Rad, Munich, Germany) in a microplate assay mode. The standard curve was made up of four concentrations of bovine serum albumin (Bio-Rad), ranging from 0.1 to 1.5 mg/mL. The assay was performed according to manufacturer's instructions and the UV-absorbance was measured at 750 nm.

Protein purification strategy (Papers III–IV)

To prepare the fractionated serum for 1D SDS-PAGE analysis and to get as good a resolution as possible in the low mass region, the following two strategies were used:

1. Microcon filtration cut-off membranes (YM30, Millipore, USA) with a cut-off of 30 kDa were used according to manufactures instructions to concentrate the fractionated serum approximately 100 times.

The filtrate obtained was analysed on NP20 (hydrophilic normal phase) arrays in the SELDI-TOF machine to test if the filtration had been successful.

2. ACN precipitation of major serum protein components. Human serum (20mL) was transferred to a LoBind micro-centrifuge tube (Eppendorf, Cambridge, UK), diluted

with 40 mL of 18.2MV water and vortexed to mix. ACN (90mL) was added and the sample sonicated for 10 min in a U300 ultrasonic water bath (Ultrawave Ltd., Cardiff, UK). The sample was vortexed briefly then sonicated for a further 10 min. The protein precipitate was pelleted by centrifugation at 12 000 g for 10 min at room temperature in a fixed rotor EBA 12-R centrifuge (Hettich, Tuttlingen, Germany). The supernatant was transferred to a clean LoBind tube and evaporated to dryness in an HT-4 centrifugal evaporator (Genevac, Ipswich, UK) with no heating (91).

1D SDS-PAGE analysis (Papers III–V)

Fractionated and purified serum was loaded onto the gel to separate the proteins. The 1D SDS-PAGE was run under denaturing 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 proteins 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, San Diego, CA, USA) to keep the proteins in a reduced state. Gels were run at 200 V for 35 min using a POWER PAC 1000 (Bio-Rad, Hercules, CA, USA) power supply. Staining was performed using Colloidal Blue Staining Kit (Novex, San Diego, CA, USA). Precision Plus Protein Unstained Standards were used as the molecular weight standard and the gels were scanned using a Fluor-S MultiImager and Quantity One Software, all purchased from Bio-Rad (Hercules, CA, USA).

Passive elution (Papers III–V)

To be able to use mass spectrometry analyses 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 were washed with 50% acetonitrile (ACN)/50 mM Ambic (3 x 15 min or until 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 NP20 array to confirm that the same m/z identity and peak pattern could be seen as in the original SELDI-TOF spectra profile.

Protein identification

Immunodepletion (Papers III–V)

To confirm the identity of the biomarkers of interest, immunodepletion was performed using antibodies and dynabeads (Invitrogen, Carlbad, CA) coated with anti-rabbit immunoglobulin G (IgG). Dynabeads were washed twice in 1 mL PBS (PAA Laboratories GmbH, Pasching, Austria) and resolved in 0.5 mL PBS. Four µg of antibody was added per 50 µL dynabeads and incubated over night 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 resolved in 50 µL PBS. Twenty µL of fractionated serum were then added followed by incubation for 1 h at 4 °C on a rotation mixer and two washes in 1 mL PBS. The depleted serum was analysed on the same arrays as the biomarkers were found on.

Destaining of proteins and in-gel protein digestion (Paper III)

To prepare the gel pieces corresponding to the protein biomarkers of interest for protein identification, destaining of the Coomassie-stained gel pieces was necessary. Destaining of the excised gel pieces was performed by adding 100 µL of 100 mM ammonium bicarbonate/acetonitrile (1:1, vol/vol) and the samples were incubated for 30 min on a shaker. Thereafter, the samples were dehydrated by adding 500 µL of acetonitrile. The samples were incubated at room temperature on a shaker until the gel pieces had shrunk and became white. Finally, the acetonitrile was removed (154).

Trypsin digestion was done by adding trypsin buffer to cover the dry gel pieces (typically, 50 μL or more, depending on the volume of the gel matrix) and the sample was put in a fridge for 120 min. Approximately 10–20 μL of ammonium bicarbonate buffer was added to cover the gel pieces and keep them wet during enzymatic cleavage. The tubes containing the gel pieces were placed into an air circulation thermostat and incubated for 30 min at 55 °C (154). Finally, 100 μL of extraction buffer (1:2 (vol/vol) 5% formic acid/acetonitrile) was added to each tube and the samples were incubated for 15 min at 37 °C on a shaker (154).

Nanoflow liquid chromatography/tandem MS (LC -MS/MS) Fourier transform ion cyclotron MS (FT/ICR MS) (Papers III–IV)

Two-microliter sample injections were made with an HTC-PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) connected to an Agilent 1100 binary pump (Agilent Technologies, Palo Alto, CA, USA). The peptides were trapped on a precolumn (45 × 0.075 mm i.d.) packed with 3 μm C18-bonded particles and separated on a reversed phase column, 200 × 0.050 mm. Both columns are packed in-house with 3 μm Reprosil-Pur C18-AQ particles. A 40 min gradient 10–50% CH3CN in 0.2% COOH was used for separation of the peptides.

Nanoflow LC-MS/MS was performed on a hybrid linear ion trap-FTICR mass spectrometer (LTQ-FT, Thermo Electron, Bremen, Germany). The spectrometer was operated in data-dependent mode, automatically switching to MS/MS mode. MS-spectra were acquired in the FTICR, while MS/MS-spectra were acquired in the LTQ-trap. All the tandem mass spectra were searched by MASCOT (Matrix Science, London, UK) against the SwissProt 5.16 database. The search parameters were set to: MS accuracy 5 ppm, MS/MS accuracy 0.5 Da, one missed cleavage by trypsin allowed, fixed propionamide modification of cysteine and variable modification of oxidized methionine.

Statistical methods

Statistical analyses were performed using SPSS (version 11.5 and 12.0.1, SPSS, Inc., Chicago, IL), Origin™ (version 5.0, Microcal™ Software, Inc., Northampton, MA) and Matlab software (version 7.7.0 R2008b, The Mathworks). Data are presented as means ± SD. The Pearson product-moment correlation coefficient was used to calculate correlations at the individual level when the data were normal distributed, otherwise a Mann–Whitney–Wilcoxon non-parametric test was used. Independent *t*-tests were used to test significance at the group level and one way analysis of variance (ANOVA) was used to study changes within the groups between different time points.

To identify potential biomarkers of interest, serum proteomic profiles were compared before and after 1 year of treatment. In addition, changes in profiles over 1 year of treatment were analysed. Proteomic data from the different array surfaces were analysed individually as well as merged and analysed together. The peak intensity data were analysed either directly after pre-processing or log transformed. The peak data were thereafter analysed with multidimensional scaling (MDS) to explore the relationships between the peak data from the different spectra, and to identify outlying spectra/patients.

Non-linear adjustments

The DXA-derived parameters of BMC and BA were strongly correlated with height (Paper V, Figure 2) and therefore underwent non-linear correction for height. BMD was not correlated with height and therefore the raw values were used in the data analyses.

Multivariate statistics

Multivariate data analysis was performed with Matlab software on the mean intensity levels of the duplicate samples. Cross-validated stepwise regression was computed to find subsets of peaks that correlated with the delta height SDS 0–2 years, BMD, height adjusted BMC (BMC_{HA}) and height adjusted bone area (BA_{HA}). Final selection of reliable subsets of predictive peaks was based on a random permutation test.

Between-duplicate variation

To estimate the reliability of the peaks compared with their biological range, the ratio of the between-duplicate variation and the total variation was computed, giving the proportion of variance explained by duplicates. A low value for a certain peak meant that there was relatively little variation between the duplicates compared with the total expected biological and instrumental variation.

Stepwise regression

Subsets of peaks were selected by stepwise regression using Matlab software, for predicting the delta height SDS 0–2 year, BMC_{HA}, BA_{HA} and BMD. Sets of potential regression models were generated using 1 to a maximum of 15 peaks. For the regression analyses, either traditional stepwise regression analyses or a modified version of the SparseLab Matlab packages was used (158) (see Paper V for details).

Random permutation tests

Random permutation tests were performed on the stepwise regression results to test the stability of the results. For each number of peaks, we tested for 999 permutations if the permuted cross-validated R^2 was equal to or above 90% of the calculated true cross-validated R^2. In other words, we assessed if there was a significant gap (10%) between the calculated true cross-validated R^2 and the distribution of all permuted cross-validated R^2. Random permutation tests resulting in a p-value < 0.05 were considered significant.

RESULTS AND DISCUSSION

Adiponectin as a marker of GH-treatment

Growth hormone has direct effects on both longitudinal growth and adipose tissue (82, 160), and it has been observed that many obese children have a normal longitudinal growth rate in spite of very low circulating GH levels (135). This suggests that adipose tissue-derived factors could affect GH responsiveness, or that factors produced by adipose tissue, adipokines, directly effect longitudinal growth. Therefore we wanted to investigate adiponectin as a possible marker of GH treatment response.

In this study it was found that the decrease in adiponectin correlated with growth in response to GH treatment after 3 months and 1 year ($r = –0.38$; $p < 0.0001$ and $r = –0.47$; $p < 0.0001$, respectively) (Paper I, Figure 2). Furthermore, mean serum adiponectin levels decreased significantly relative to baseline after the start of GH treatment; from 14.5 ± 5.71 to 13.1 ± 5.22 μg/mL after 1 week ($p < 0.0001$), to 10.3 ± 4.82 μg/mL after 3 months ($p < 0.0001$), and to 12.5 ± 5.34 μg/mL after 12 months ($p < 0.0001$) (Figure 10). One-way analysis of variance of the changes in adiponectin levels during the first year of GH treatment also showed significant differences between the different time points.

Multivariate analysis: multiple linear forward regression analysis was performed using SPSS (version 12.0.1, SPSS) on the auxological data and values from the protein and hormone analysis. The first year growth response was the dependent variable. A model with the following predictors was derived (beta coefficients are given in parentheses): the logarithmic value of GH_{max} during a 24 h profile (–0.429), age at the start of GH treatment (–0.354), GH dose (0.315), adiponectin level at baseline (0.194) and a constant. This model accounted for 41.6% of the variation in the growth response. In ANOVA analyses, the model had an F value of 15.9 and a p value of < 0.001.

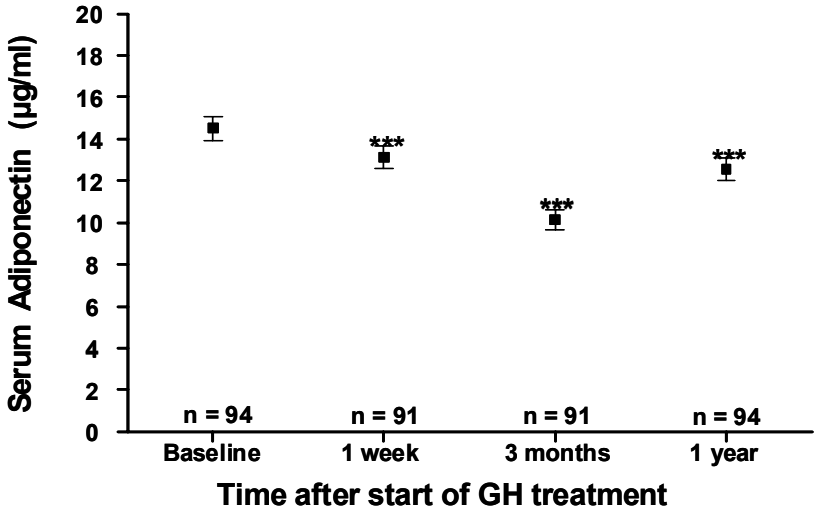

Figure 10: Mean levels of serum adiponectin at the start of GH treatment and after 1 week, 3 months and 1 year of treatment. Values are means ± SEM. *** $p < 0.0001$ compared with baseline.

Levels of adiponectin decreased during the first 3 months of GH treatment before starting to increase (Figure 10). However, adiponectin levels after 1 year of treatment were still significantly lower than at baseline. The finding that adiponectin levels decreased during GH treatment could have been related to the known association between increased insulin levels, lowered insulin sensitivity and decreased adiponectin levels (122, 130). However, no correlation between increased insulin levels and decreased adiponectin levels was seen in this study.

It is still not known whether or not adiponectin is involved in the regulation of longitudinal growth, or if changes in adiponectin levels are the indirect result of the effects of GH on adipose tissue. However, there are data supporting the hypothesis that adiponectin is involved in the regulation of longitudinal growth. Firstly, adiponectin and its receptors are expressed in bone-forming cells (53, 155). Adiponectin can stimulate osteoblast proliferation and suppress osteoclasts (21, 119, 131), and both leptin and adiponectin inhibit bone formation *in vitro*. The inhibitory effect of adiponectin on bone formation is negated by insulin (47, 155). It has also

been shown that children born SGA who experience catch-up growth have significantly lower adiponectin levels than those who do not show catch-up growth (36). In addition, GH treatment in children with SGA leads to lower adiponectin levels (36, 72, 130). Furthermore, GH exerts its lipolytic effects on adipose tissue through the GHR, which is expressed both in pre-adipocytes and adipocytes, and it has been shown that GH can stimulate pre-adipocyte proliferation and inhibit differentiation into adipocytes (172). Children with GHD have fewer fat cells than healthy children, and the cells that they do have are enlarged. After treatment with recombinant human GH there is an increase in the number of fat cells and a reduction in cell volume (30, 172). This finding suggests a direct link to adiponectin as it has been established that large adipocytes release less adiponectin than small adipocytes (37, 66). It has been speculated that the low adiponectin levels that are observed in obese patients, who generally have increased visceral and decreased subcutaneous fat, are the result of enlarged subcutaneous fat cells (161). Therefore, one would have expected that adiponectin levels would have increased during GH treatment as a direct consequence of the decreased fat mass. However, adiponectin has been shown to regulate GH secretion directly in *in vitro* studies of pituitary cells by binding to the adiponectin receptor (144, 159). In turn, GH can induce adiponectin receptor 2 expression (53), whereas adiponectin decreases expression of the adiponectin receptor 2 (31). These findings suggest the existence of a negative feedback loop between adiponectin and GH.

Conclusion: This study showed that there was a relationship between growth response to GH treatment and adiponectin in short prepubertal children. It was found that adiponectin decreased during GH treatment and that this decrease was correlated with the growth response.

Genomics to identify novel GH- and/or IGF-I-induced genes (Paper II)

To identify GH- and/or IGF-I-induced genes in a GH target tissue, primary cultured human chondrocytes from the growth plate were stimulated with GH and/or IGF-I and gene expression was analysed using Affymetrix microarray technique.

In total, 25 genes were found to be regulated by GH (21 were up-regulated and 4 were down-regulated), 93 genes were regulated by IGF-I (72 up-regulated and 21 down-regulated) and 11 genes were found to be regulated by both GH and IGF-I (all were up-regulated). These 11 up-regulated genes (Paper II, Table2,) were evaluated further. By combining the microarray ranking with a literature search, the *COMP* gene was identified as a candidate gene responsible for the regulation of longitudinal growth because of its association with conditions known to be associated with short stature (29, 156). Mutations in the *COMP* gene are associated with both pseudoachondroplasia and multiple epiphyseal dysplasia, both of which are characterized by short stature (27, 29).

To verify *in vivo* the results of the microarray study and to assess whether COMP was upregulated by GH, serum COMP levels were measured by ELISA. Serum COMP levels increased significantly relative to baseline after the start of GH treatment; from 1.58 ± 0.28 to 1.83 ± 0.28 μg/mL after 1 week, to 1.91 ± 0.28 μg/mL after 1 month, to 1.78 ±0.28 μg/mL after 3 months, and to 1.70 ± 0.24 μg/mL after 12 months (Figure 11). However, there was no correlation between the first year growth response (expressed as change in height SDS from the start of GH treatment) and COMP levels at any time-point.

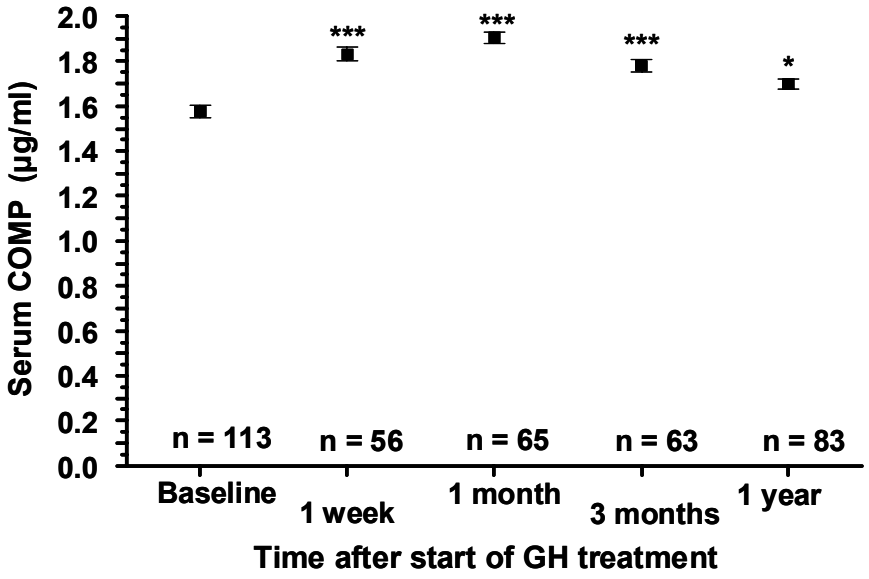

Figure 11: Mean levels of serum COMP at the start of GH treatment and after 1 week, 1 month, 3 months, and 1 year of treatment. Values are means ± SEM.*, $P < 0.05$, ***, $P < 0.0001$, compared with baseline.

The mechanisms by which GH and IGF-I influence COMP expression and serum levels are unknown. Levels of COMP increased rapidly during the first month of GH treatment before starting to decrease progressively over time. Nevertheless, COMP levels after 1 year of treatment were still significantly higher than at baseline. No correlation was found between COMP levels in serum and the growth response. However, COMP may still be of importance for growth as GH treatment in children with pseudoachondroplasia appears to have a negative effect on growth rate (85). This negative effect may be caused by increased apoptosis of growth plate chondrocytes, that leads to a decrease in growth plate elongation (64).

Many factors may influence the correlation between serum levels of COMP and the response to GH. For example, in conditions such as rheumatoid arthritis and osteoarthritis, increased COMP levels most likely result from the destruction of cartilage. In addition, COMP levels are elevated in marathon runners, suggesting that physical activity influences serum COMP levels in healthy subjects, possibly via the increase in GH secretion that occurs during or after exercise (50, 83, 124). The release

of COMP into the blood during certain conditions may make it hard to detect changes in serum levels of COMP induced by GH treatment.

Conclusion: Microarray techniques could be used to identify novel GH-regulated genes in a GH target tissue. COMP was shown to be up regulated during GH treatment in short prepubertal children.

Proteomics to identify novel GH responsive serum biomarkers (Papers III–V)

In Papers III–V a pharmacoproteomic approach was used in order to identify novel GH-regulated biomarkers. SELDI-TOF-MS was used to analyse serum protein expression profiles during the first year of GH treatment in two different patient materials.

At start of treatment it would be advantageous to be able to identify very poor or very good responders to GH treatment, because these groups require either individualized GH treatment in order to achieve an appropriate growth response, or do not benefit enough from GH treatment and should remain untreated. The goal of Paper III was to use proteomics to identify novel protein biomarkers that could be used to discriminate between good and poor responders to GH treatment.

Overall, 82% of children with ISS (Paper III, Table 2) were correctly classified as having either a good or poor growth response based on changes in the intensities of the protein peaks, TTR and Apo A-II (13.8 kDa and 17.1 kDa, respectively) between baseline and 1 year on GH treatment. The 13.8 kDa peak, TTR, decreased in the high-responder group and increased in the low-responder group, whereas the 17.1 kDa peak, Apo A-II, decreased in the high-responder group and remained unchanged in the low-responder group during 1 year of treatment (Figure 3, Paper III). Furthermore, it was seen that Apo A-II was associated with the GH/IGF-I axis and TTR was associated with the auxological axis (Paper III, Figure 2). In Table 2 (Paper III), it can be seen that the difference between including 2 and 15 peaks in the

regression models was small in terms of the change in R^2. When more peaks are used, the lower the cross-validated R^2 became. This indicated that the Apo A-II and TTR have the most predictive power and that including more peaks only adds noise to the model.

In Papers IV and V serum samples from a study group of 128 children, including both children with ISS and GH-deficient children, from the clinical trial (TRN 98-0198-003) were analysed to identify GH-regulated proteins that could be used to predict growth response and changes in bone mineralization and bone volume during GH treatment.

In Paper IV the aim was to identify GH-regulated proteins that could be used to predict the 2-year growth response to GH treatment in short prepubertal children.
For the GH-deficient group of children it was possible to predict the 2-year growth response at both the start and after 1 year of GH treatment (Table 3). After 1 year of treatment it was also possible to predict the 2-year growth response in the total group and in the ISS group. The majority of the proteins identified from the protein profiles were different apolipoproteins; Apo A-I, Apo A-II, Apo C-I and Apo C-III. Other proteins identified were TTR and serum amyloid A4.

Conclusion: These results verified previous findings (Paper III) suggesting that Apo A-II and TTR may have a role in determining GH sensitivity. It was also possible to predict the 2-year growth response in short prepubertal children on GH treatment.

Table 3. The most predictive peaks identified by regression analysis at the start of GH treatment, and during and after 1 year of GH treatment. For each model the R^2-value and cross-validated R^2 are presented.

Timepoint	Group	Patients (n=)	Peaks (n=)	R^2	CV R^2	p-value	Peak m/z value (kDa)
At GH start	GHD	39	7	0.73	0.61	0.032	3.160, 3.318, 8.767 (Apo A-II), 9.135, 9.642, 12.872 (TTR), 17.390 (Apo A-II)
1 year	GHD	39	4	0.64	0.53	0.017	4.408 (Apo A-II), 8.696 (Apo A-II), 9.019 (Apo A-II), 17.146 (Apo A-II)
1 year	ISS	89	8	0.47	0.35	0.015	3.160, 4.470, 6.857 (Apo C-I), 8.767 (Apo A-II), 8.875 (Apo A-II), 9.425 (Apo C-III), 12.607 (SAA 4), 12.872 (TTR)
1 year	Total	128	8	0.38	0.28	0.003	4.628, 4.470, 4.793, 8.817, 8.875, 9.019, 12.872, 17.146
0-1 years	GHD	39	4	0.59	0.48	0.026	4.138, 8.817 (Apo A-II), 9.019 (Apo A-II), 17.262 (Apo A-II)
0-1 years	Total	128	8	0.35	0.24	0.003	4.138, 8.636 (Apo A-II), 8.875 (Apo A-II), 9.135, 9.425 (Apo C-III), 14.055 (TTR), 28.090 (Apo A-I), 29.003

Apo, apolipoprotein; CV, cross-validated; Da, Dalton; m/z, mass over charge; SAA 4, serum amyloid A4; TTR, transthyretin.

GH has other important functions in the human body besides regulation of longitudinal growth. One of these functions is a role in bone mineralization. We therefore wanted to test the hypothesis that longitudinal growth, bone mineralization and 'bone volume' were partly regulated by different and partly by the same mechanism (Paper V, Figure 1).

The same approach was used as in Paper IV to study changes in bone mineralization and bone volume during GH treatment. It has previously been shown that children with GHD and ISS have lower BMD than children in the general population and that GH therapy increased markers of bone formation. In this study the DXA-derived variables BMD, bone area and BMC were used as outcome variables. It was possible to predict changes in bone mineralization in terms of BMD, BMC_{HA} and BA_{HA} during the first year of GH treatment using SELDI-TOF data (Table 4). These results were compared to those found for longitudinal growth. From comparing the protein peaks included in all of the significant regression models for longitudinal growth, BMD, BMC_{HA} and BA_{HA} it was possible to conclude that there is a partial dissociation between mechanisms involved in longitudinal growth and bone mineralization because out of a total of 50 unique peaks found in the regression models, 23 were only present in models related to bone mineralization, and 17 peaks were only present in models related to height (Paper V, Table 4). As in Paper IV, the majority of the proteins identified from the protein profiles were different apolipoproteins; Apo A-I, Apo A-II, Apo C-I and Apo C-III. However, different isoforms of the proteins were in detected some cases. Other proteins identified were TTR, serum amyloid A4 and haemoglobin beta.

Table 4. The most predictive peaks identified from serum protein profiling by regression analysis at start of and during the first year of GH treatment.

Group	DXA		SELDI					
	Time point	Predicted Variable	Time point	Peaks (n=)	R^2	CV R^2	p-value	Predictors Peak m/z value (kDa)
GHD	0 – 1 year	BMD	at start	5	0.64	0.52	0.038	3890I, 4156C, 7967I, 12453C, 15860I
	0 – 2 year			5	0.64	0.48	0.018	3881I, 3890I, 4156C, 6676I (Apo C-I), 17586C (Apo A-II)
	1 year	BMC_{HA}		4	0.63	0.47	0.047	4408C (Apo A-II), 6629I (Apo C-I), 14675I, 15164C
	2 year			3	0.61	0.52	0.017	4641I (Apo A-II), 8817C (Apo-AII), 15363C
	1 year	BA_{HA}		2	0.44	0.35	0.05	6940H (Apo C-I), 15164C
	2 year			4	0.66	0.56	0.04	6478I (Apo C-I), 6940H (Apo C-I), 15164C, 16063C
	0 – 1 year	BMD	0 – 1 year	3	0.61	0.51	0.047	3083I, 3890I, 12607C (SAA 4)
	0 – 2 year			6	0.76	0.61	0.04	3160C, 3890I, 8920C (Apo A-II), 9193I, 12607C (SAA 4), 29003C
	0 – 2 year	BMC_{HA}		4	0.67	0.54	0.016	8567H (Apo A-II), 9012H, 17146C (Apo A-II), 18030H
ISS	0 – 2 year	BA_{HA}	at start	6	0.4	0.28	0.049	2750I, 3219C, 4297I, 6629H (Apo C-I), 7923I,12453C
Total	0 – 1 year	BA_{HA}		10	0.42	0.29	0.008	2856I, 4297I, 7634C, 8642C (Apo A-II), 8767C (Apo A-II), 8875C (Apo A-II), 8920C (Apo A-II), 9012H, 12607C (SAA 4), 17586C (Apo A-II)
	0 – 2 year		at start	8	0.35	0.22	0.017	3019C, 3083I, 3219C, 6629H (Apo C-I), 8636C (Apo A-II), 8696C (Apo A-II), 8817C (Apo A-II), 17262C (Apo A-II)
	2 year			10	0.4	0.26	0.024	2856I, 3314H, 4466I (Apo A-II), 7771C, 8696C (Apo A-II), 12453C, 13890C (TTR), 17382H (Apo A-II), 17586C (Apo A-II), 17792H (Apo A-II)

BA, bone age; BMC, bone mineral content; BMD, bone mineral density; DXA, dual-energy X-ray absorptiometry; GHD, GH-deficient; HA, height-adjusted; ISS, idiopathic short stature; CV, cross-validated.

To ensure that the data were valid, we tested all variables entered into the regression models thoroughly. When assessing the data obtained from DXA we found a clear relationship between height and both BMC and BA. Therefore, both of these variables were corrected for height (Paper V, Figure 2).

In this study, BMC_{HA} significantly decreased in the ISS and the total group during the first year of GH treatment, whereas during the second year there was a significant increase in BMC_{HA} (Paper V, Table 2). After 2 years of treatment BMC_{HA} was greater than at start of treatment. This is in line with 'the biphasic model' (103, 127) showing that GH initially increases bone resorption with a resultant reduction in bone,. This is followed by a phase of increased bone formation. After a transition point, where bone formation is stimulated more than bone resorption, bone mass is increased. In general, it will take some time for GH treatment to induce a net gain in bone mass (typically 12–18 months in adults) as the initial deficit in bone mass must first be replaced (127). Alternatively, one can speculate that the process of bone tissue formation and the process of bone mineralization occur in parallel but at different rates (i.e. bone tissue formation is more rapid than bone mineralization).

The results of this study supported our hypothesis that longitudinal growth, bone mineralization and bone volume (represented by BA_{HA}) are partly regulated by different and partly by the same mechanisms. At start of treatment, regression models identified a number of unique protein peaks which correlated with changes in longitudinal growth and bone mineralization in the GH-deficient group of children. However, no dissociations between longitudinal growth and delta BA_{HA} were found, as no significant correlations between protein peak data and bone volume were found in the GH-deficient group.

A lot of the focus during the work in Papers III–IV was on developing robust statistical methods to ensure that the data analysed and the results presented were reliable. In Papers IV and V, SELDI-TOF data were analysed using multidimensional scaling (MDS) to explore the relationships between the protein peaks or patients. Three patients with outlying values were identified using this method (Figure 12). For these patients, several parts of the spectra deviated from the spectra for the majority

of patients, indicating haemolysis in the samples. This was confirmed after visual inspection of the protein profile and the blood sample. These spectra, from the IMAC 30 surface, were therefore excluded from further statistical analyses (Figure 13).

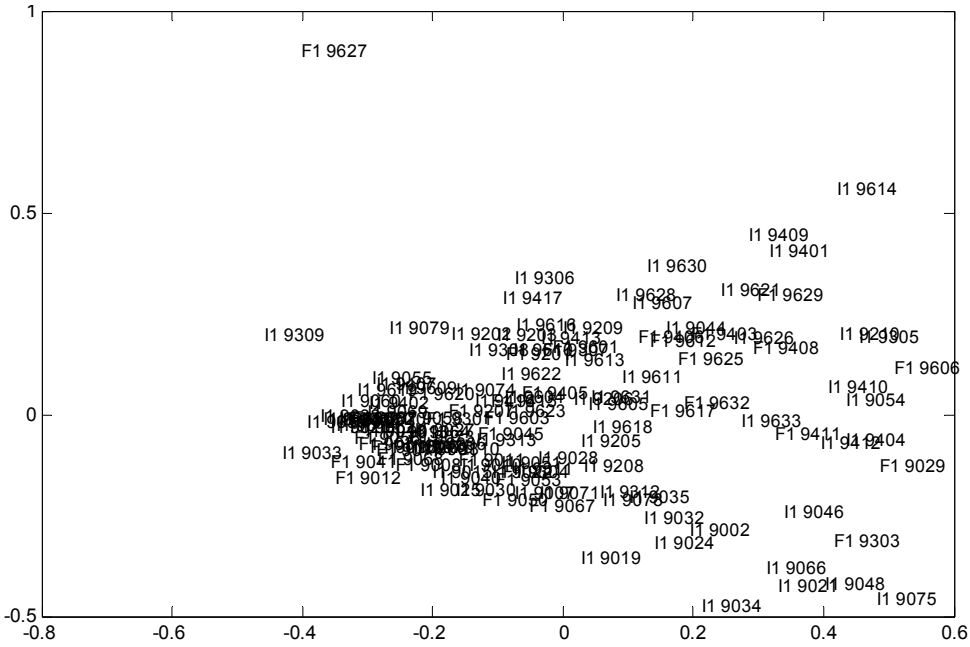

Figure 12: Multidimensional scaling. This figure gives an example of how multidimensional scaling can be used to detect outlying values. In this example, the sample in the upper part of the figure from the patient identified as 9627 is an outlier

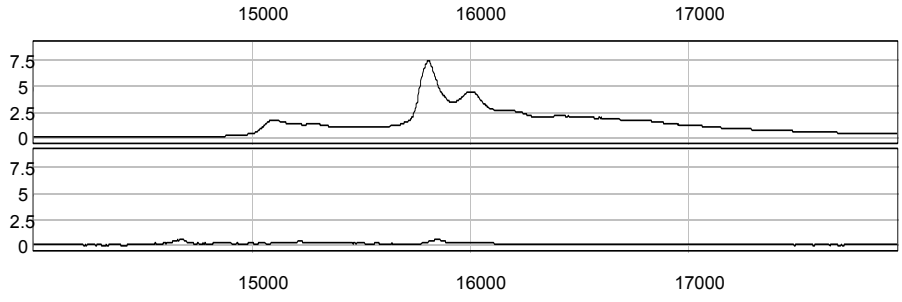

Figure 13: Haemolysis. This figure shows the serum profile from patient 9627 (upper panel) compared with normal serum (bottom panel). Visual inspection of the profile shows that the sample from patient 9627 that was identified as an outlier by multidimensional scaling had in fact undergone haemolysis.

Conclusion: These studies demonstrated that high-throughput proteomic techniques can be used to identify biomarkers of growth response to GH treatment and bone mineralization. Of great interest was the observation that all proteins detected were bound to, or were part of, the HDL. The relationship between these biomarkers and the regulation of growth and bone mineralization is unclear.

GENERAL DISCUSSION

GH action on growth in children is a complex process that depends on the interplay between environmental, genetic and hormonal factors, and nutrition. GH therapy promotes longitudinal growth and bone mineralization. The mechanisms responsible for the regulation of the factors involved and the interactions between the factors themselves are incompletely understood. There is a need to improve our understanding of this complex system.

For this reason, the main aim of this thesis was to identify genes and proteins that are regulated by GH and may therefore be important in the regulation of longitudinal growth and bone mineralization in children. This will lead to better understanding of the molecular basis of GH action in the human body.

To achieve this several different approaches were used. Firstly, we performed a single factor study (Paper I) where the relationship between adiponectin and the growth response was studied. Thereafter, we employed a genomic approach using a microarray technique to identify genes that respond to changes in GH and IGF-I in primary cultured human chondrocytes from the growth plate, where it is known that both GH and IGF-I have direct effects (Paper II). A high-throughout proteomic technique, SELDI-TOF, was used in Papers III–V to identify novel GH-related proteins in serum from short prepubertal children. Robust statistical methods were developed and used to ensure that proteomic data were valid and that the results were reliable.

Methodological part

In this thesis several different methods have been used to identify novel GH-regulated factors and markers. Each has its advantages and drawbacks. In Paper I, which used a hypothesis testing approach, a single factor study was performed using an ELISA technique. Using ELISA it was demonstrated that decreases in adiponectin were negatively correlated with the growth response. The main advantages of ELISA are; it is typically reproducible, easy to use and has a high throughput. The drawbacks are

that it is only possible to study one factor at a time using a standard ELISA, and if there is no commercially available assay then it is time consuming to develop a new one for your candidate marker.

Papers II–V used a system biology approach to generate hypotheses. In Paper II, a microarray technique was used and several novel GH-regulated genes were identified. These observations will generate new hypothesis which need to be investigated further in future studies. The main advantage of using microarray is that one can quickly look at thousand of genes simultaneously, providing that it is possible to isolate RNA from the sample. Another advantage compared to many proteomic techniques is that the identities of almost all of the genes that can be detected are known. The main drawback is that the gene expression levels do not always reflect the actual levels of proteins in the sample studied. Microarray is also an expensive technique. Although the COMP was found to be upregulated in vivo, it was necessary to perform an ELISA study to verify the COMP gene was GH-regulated.

The next step after using microarray was to study protein expression in serum using proteomics. Proteins secreted from many of the largest tissues and organs in the human body will be represented in serum. The large quantity of proteins found in serum, in combination with the ease of obtaining samples, makes serum one of the most attractive samples to study when searching for biomarkers. Papers III–V showed that there was great potential in using proteomic methods to identify markers of GH response. In Papers III–V the proteomics technique SELDI-TOF was used to identify novel GH-related proteins in serum from short prepubertal children.

Compared to the most widely used proteomic technique two-dimensional gel electrophoresis (2-DE), SELDI-TOF has both advantages and disadvantages. 2-DE was the starting point of the proteomic era when it was invented in 1970s. 2-DE was the first technique that made it possible to study a large number of proteins simultaneously with, at the time, a high resolution. The name 2-DE originates from the fact that the proteins are separated into two dimensions, the first using isoelectric focusing and the second in terms of protein mass.

2-DE has evolved over time and today several thousands of proteins can be detected in a single serum sample (60). However, some of the spots found on the gel represent the same protein as do peaks in the SELDI-TOF spectra.

Although 2-DE offers high resolution, its need for protein staining and subsequent sample handling results in limited sensitivity. Furthermore, 2-DE is highly laborious which makes it unsuitable for large-scale studies. There is also a limitation in that large or hydrophobic proteins will not enter the gel in the first dimension, and the technique has difficulties resolving proteins with extreme pI or low molecular weight (below 15 kDa) (60).

Compared to 2-DE, SELDI-TOF requires small amounts of sample and is particularly effective in characterising both hydrophobic and low molecular weight proteins (< 20kDa).

During the last few years, novel proteomic techniques have been developed and are used along side with SELDI-TOF and 2-DE. Mass spectrometry has so far mainly been used in studying and determining the identity of single proteins, often in combination with gel electrophoresis techniques like Western blot or 2-DE. However, the technique has rapidly evolved and today new mass spectrometry-based techniques using Fourier-transform mass spectrometry (FT-MS) like the Orbitrap (174) offers the potential to detect many proteins simultaneously from one single sample. In combination with extensive pre-fractionation, up to 6000 proteins can today be detected from one serum or plasma sample (152). The main drawbacks are the expensive equipment, not especially high-throughput (depending on the extensive pre-fractionation), and last but not least, the huge amount of data generated by each run, which require both computer power and advanced bio-informatic tools.

In Paper III, an initial pilot study in order to identify the most promising conditions for the identification of biomarkers was performed. Using a pilot study to find the optimal settings has several benefits including saving time, lowering the costs,

decreasing the workload and the amount of data produced. The main drawback is that there is a risk of missing important data because only the selected settings have been run due to prioritisations. There is also a risk that the study group chosen for the pilot study did not reflect the total study group.

Furthermore, in this study where relatively few patients were included (n = 32) a problem called over-fitting can occur. This happens when a model is excessively complex (i.e. has too many degrees of freedom in relation to the amount of data available) (163). In this case, there can be too much peak data compared to the number of patients. Therefore, we put a lot of effort into developing and testing different methods in order to ensure that our results were reliable and robust.

The first step was to test univariate analyses in the pilot study. Several protein peaks were identified as possible candidates that could discriminate between good and poor responders in terms of growth in the pilot study. However, using the total study group these candidates were no longer valid because of overlap between the subgroups of good and poor responders to GH. After searching the literature and conducting discussions within the group, partial least-squares regression (PLS-regression) in combination with cross-validated regression analyses were chosen since these methods were well suited for discriminant analyses and visually detected clusters of peaks giving the same information (Paper III, Figure 2). PLS-regression is related to the more well-known principal component analysis (PCA). The main difference is that PLS uses a linear model that is projected in a plane. PLS-regression is particularly suited when the predictors are more than the observations, and when there is multicollinearity among the values (175).

One of the main concerns was to be sure that the data analysed were reliable and of high quality. A lot of effort was put into finding methods that were suitable for detecting system biases and bad peak data. Here PCA and linear regression were suitable tools for identifying correlations between peak data and systematic parameters. Only a correlation with storage time was found, which was corrected for with linear regression. This shows that the SELDI-TOF system is a very stable and robust system and that one should avoid long-term storage of samples if at all possible.

In Papers III–IV, we put a lot of effort into the further development and testing of statistical methods to ensure that the data analysed were valid and the results presented were robust. Different approaches were used depending on the question asked. In Paper IV, a random permutation test was added to ensure that the results are true, also MDS was introduced to detect outliers. However, this procedure became very slow and was unpractical for data sets as large as ours, therefore, the SparseLab Matlab toolbox was used. The SparseLab toolbox uses different kinds of regression analyses and is very fast compared with the previous analyses without losing accuracy. In comparison to the analyses performed in Paper IV which took several hours, the analyses in Paper V only took a few minutes.

Paper V used DXA which is the most widely used technique for bone measurements. BMC, BA and BMD can be determined. Bone volume is not available from DXA measurements and therefore height adjusted BA (BA_{HA}) was used as a proxy measure of bone volume in this study. In growing children, BMC, BMD and BA are closely related to age and bone maturation (15) and BMD can be falsely low in short children due to short bones and falsely high in tall children because of longer bones (125). In Paper V, strong correlations were found between height and both BMC and BA and therefore these data was corrected for height. In this study, there was no correlation between BMD and height, most likely because BMD is in a way already corrected for height (BMC/BA), whereas BA depends on the length of the bones. It is also important to bear in mind that BMD gives an areal density (BMDarea) and not a measure of true bone density (15). Volumetric BMD is a function of BMC per volume of bone. This can not be measured with DXA; peripheral quantitative computed tomography (pQCT) or magnetic resonance imaging (MRI) have to be used. However, approximations have been done for lumbar trabecular bone (98). In this study, height was a more suitable way to correct the BMA and BA than age. This was most likely because the children were small for their age and, therefore, if the data should have been corrected for age, bone age should probably have been more appropriate to correct for than biological age. This has previously been shown in prepubertal children with chronic renal insufficiency (102). BMD of these patients was

low at baseline when compared with healthy age-matched controls, but normal when compared with height- and bone-age-matched controls.
Using SELDI-TOF to study changes in bone mineralization is just one part of the puzzle in understanding the complex regulation of bone mineralization, as the mineralization process is governed by multiple factors that interplay with each other, for instance the availability of vitamin D, calcium and a number of limiting proteins.

The main advantages of proteomics techniques like SELDI-TOF are that they are high throughput and study the active products of the genes. A drawback is that the identities of the proteins are not available and other techniques have to be used to be able to identify the proteins of interest. For SELDI-TOF and other proteomic techniques a major limitation is that many samples, like serum, is so complex that no technique can identify every protein in the samples and compromises have to be made. One of the challenges in proteomic analyses of serum is the broad range of expression levels between proteins with low and high abundance (12, 40, 164). In order to partly overcome this problem, we used fractionated serum that was analysed on different array surfaces in order to detect proteins in a larger area of the proteome. Alternative strategies are to use antibodies or different bead-based techniques, such as the Proteominer (Bio-Rad), to deplete the most abundant proteins. In the work of discovering protein biomarkers relevant to a condition or disease one would like to study the actual tissue instead of serum. However, it is often very difficult to get samples from the actual tissue, especially in children.

Physiological part

In the studies included in this thesis, HDL-related proteins were found to be markers of GH action. Not much is known about the relationship between GH and the HDL-related biomarkers found in Papers III–V; the apolipoproteins, TTR, SAA 4 and haemoglobin beta. There are contradictory results regarding the effects of GH treatment given as daily subcutaneous injections on the HDL (71, 100, 104).

From these studies, it is not possible to draw any conclusions whether the markers identified are actually involved in the regulation of longitudinal growth or if they are indirect markers of the effects of GH on the target tissues. Interpretation of the results is also complicated by the findings of different regulated isoforms and cleavage products of Apo A-II as seen if Figures 1, 2 and 3 in Paper IV. The physiology behind this will be an interesting topic to evaluate further in future studies. The functions of the different isoforms and interpretation of having found these is not clear. However, it is easy to speculate that different isoforms have different biological functions as seen for GH (18, 112, 146).

In the field of GH research, proteomics has not been used frequently. In a small study of healthy adults with GHD, SELDI-TOF was used and it was found that haemoglobin alpha-chain was up-regulated during GH treatment (34). In a later study by the same group, IGF-I was found to be up-regulated (33). The same group have also shown that the pro-inflammatory calgranulins were down-regulated in response to GH treatment, and were therefore proposed to be markers of GH action (35). Using 2-DE in combination with MS/MS, preliminary results indicated that isoforms of transthyretin, clusterin, Apo E and Apo-AI were differentially expressed during GH treatment and may be potential biomarkers for initiating studies using recombinant human GH (45) This is in line with our findings that TTR and Apo AI are regulated during GH treatment.

The importance of identification of novel GH-regulated factors

Detailed understanding of the molecular mechanisms behind growth failure and GH action in its target tissues opens possibilities for the development of better diagnostic tools and improved selection criteria for drug treatment. It is also possible to develop better prediction models for longitudinal growth, which lead to treatment with individualized GH dosages with positive impact on the child and gives economical benefits for public medical services. Furthermore, it may contribute to the

identification of some causes of growth failure in the selected groups of children and increase the possibility to monitor the progression of GH treatment and to evaluate the efficiency of the GH treatment.

CONCLUSIONS

The main aim of the studies included in the thesis was to identify novel GH-responsive genes and proteins of importance for the regulation of longitudinal growth and bone mineralization in children.

To achieve these goals several different approaches were successfully used; including traditional methods such as ELISA and cell culture experiments in combination with genomic and proteomic techniques. Using and developing robust statistical methods were a prerequisite to ensure that proteomic data were valid and that the results were reliable.

I. There is a relationship between growth response to GH treatment and adiponectin in short prepubertal children. It was found that adiponectin decreases during GH treatment and that the decrease is correlated with the growth response.

II. A genomic approach can be used to identify novel GH-regulated genes in a GH target tissue. COMP was found to be up-regulated during GH treatment in short prepubertal children using ELISA.

III. A pharmacoproteomic approach can be used to identify protein biomarkers of growth response to GH treatment and bone mineralization. All biomarkers detected except for haemoglobin beta are bound to, or are part of, the HDL.

FUTURE PERSPECTIVES

These studies show that there is great potential in using proteomics, genomics and system biology studies in general, in the GH research area. Further characterization and evaluation of the biomarkers of GH response that were identified will improve knowledge about the mechanism(s) involved in the regulation of the growth response. It is hoped that in the future these data will be incorporated within prediction models for longitudinal growth that will also include targets related to gaining optimal bone quality and metabolism. Protein peak data could be used to replace missing pre-treatment data that is included in current prediction models. Using proteomic data to predict changes in bone quality may help to determine when it is appropriate to discontinue GH treatment after final height in order to ensure that peak bone mass has been reached.

The use of system biology, which is a hypothesis generating approach, will increase in the research on multifactorial diseases in general, as well as in the GH research field. Different types of protein arrays, where large-scale studies of protein–protein interactions, transcription factors and enzymes can be studied, will be used more extensively in the future. In general, system biology generates a lot of candidate biomarkers leading to an increasing need for techniques that can be used to verify the findings as knock-out animal experiments and short interference RNA studies there genes (typically) in cell cultures are silenced. These techniques can be used to study direct effects of GH on its target tissues. It would also be of great interest to use a system biology approach in combination with microdialysis in GH target tissues to study the local GH response.

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REFERENCES

1. Absenger Y, Hess-Stumpp H, Kreft B, Kratzschmar J, Haendler B, Schutze N, Regidor PA, Winterhager E. Cyr61, a deregulated gene in endometriosis. Mol Hum Reprod 10 (6): 399-407, 2004.
2. Adkins JN, Varnum SM, Auberry KJ, Moore RJ, Angell NH, Smith RD, Springer DL, Pounds JG. Toward a human blood serum proteome: analysis by multidimensional separation coupled with mass spectrometry. Mol Cell Proteomics 1 (12): 947-55, 2002.
3. Albertsson-Wikland K, Aronson AS, Gustafsson J, Hagenas L, Ivarsson SA, Jonsson B, Kristrom B, Marcus C, Nilsson KO, Ritzen EM, Tuvemo T, Westphal O, Aman J. Dose-dependent effect of growth hormone on final height in children with short stature without growth hormone deficiency. J Clin Endocrinol Metab 93 (11): 4342-50, 2008.
4. Albertsson-Wikland K, Aronson S, Nilsson KO, Ritzen M, Tuvemo T, Westgren U, Westphal O, Perheentupa J, Sipila I, Wilton P. Recombinant somatropin in treatment of growth hormone deficient children in Sweden and Finland. Acta Paediatr Scand Suppl 347: 176-9, 1988.
5. Albertsson-Wikland K, Jansson C, Rosberg S, Novamo A. Time-resolved immunofluorometric assay of human growth hormone. Clin Chem 39 (8): 1620-5, 1993.
6. Albertsson-Wikland K, Luo ZC, Niklasson A, Karlberg J. Swedish population-based longitudinal reference values from birth to 18 years of age for height, weight and head circumference. Acta Paediatr 91 (7): 739-54, 2002.
7. Albertsson-Wikland K, Rosberg S, Karlberg J, Groth T. Analysis of 24-hour growth hormone profiles in healthy boys and girls of normal stature: relation to puberty. J Clin Endocrinol Metab 78 (5): 1195-201, 1994.
8. Albertsson Wikland K, Kristrom B, Rosberg S, Svensson B, Nierop AF. Validated multivariate models predicting the growth response to GH treatment in individual short children with a broad range in GH secretion capacities. Pediatr Res 48 (4): 475-84, 2000.
9. Aldrich S. Protein depletion for plasma and serum proteomic analysis http://www.bio-medicine.org/biology-technology/Protein-Depletion-for-Plasma-and-Serum-Proteomic-Analysis-1497-1/.
10. Allard L, Lescuyer P, Burgess J, Leung KY, Ward M, Walter N, Burkhard PR, Corthals G, Hochstrasser DF, Sanchez JC. ApoC-I and ApoC-III as potential plasmatic markers to distinguish between ischemic and hemorrhagic stroke. Proteomics 4 (8): 2242-51, 2004.
11. Anderson L, Seilhamer J. A comparison of selected mRNA and protein abundances in human liver. Electrophoresis 18 (3-4): 533-7, 1997.
12. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 1 (11): 845-67, 2002.
13. Anderson NL, Polanski M, Pieper R, Gatlin T, Tirumalai RS, Conrads TP, Veenstra TD, Adkins JN, Pounds JG, Fagan R, Lobley A. The human plasma

proteome: a nonredundant list developed by combination of four separate sources. Mol Cell Proteomics 3 (4): 311-26, 2004.
14. Ankarberg-Lindgren C, Norjavaara E, Albertsson-Wikland K. Short boys treated with growth hormone show normal progression of testicular size and achieve normal serum testosterone concentrations. Eur J Endocrinol 146 (5): 681-5, 2002.
15. Baroncelli GI, Bertelloni S, Ceccarelli C, Saggese G. Measurement of volumetric bone mineral density accurately determines degree of lumbar undermineralization in children with growth hormone deficiency. J Clin Endocrinol Metab 83 (9): 3150-4, 1998.
16. Baumann G. Growth hormone binding protein 2001. J Pediatr Endocrinol Metab 14 (4): 355-75, 2001.
17. Baumann G. Growth hormone heterogeneity: genes, isohormones, variants, and binding proteins. Endocr Rev 12 (4): 424-49, 1991.
18. Baumann G, Amburn KD, Buchanan TA. The effect of circulating growth hormone-binding protein on metabolic clearance, distribution, and degradation of human growth hormone. J Clin Endocrinol Metab 64 (4): 657-60, 1987.
19. Bensmail H, Haoudi A. Postgenomics: Proteomics and Bioinformatics in Cancer Research. J Biomed Biotechnol 2003 (4): 217-230, 2003.
20. Berg AH, Combs TP, Scherer PE. ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. Trends Endocrinol Metab 13 (2): 84-9, 2002.
21. Berner HS, Lyngstadaas SP, Spahr A, Monjo M, Thommesen L, Drevon CA, Syversen U, Reseland JE. Adiponectin and its receptors are expressed in bone-forming cells. Bone 35 (4): 842-9, 2004.
22. Binder G, Schwarze CP, Ranke MB. Identification of short stature caused by SHOX defects and therapeutic effect of recombinant human growth hormone. J Clin Endocrinol Metab 85 (1): 245-9, 2000.
23. Bjarnason R, Andersson B, Kim HS, Olsson B, Swolin-Eide D, Wickelgren R, Kristrom B, Carlsson B, Albertsson-Wikland K, Carlsson LM. Cartilage oligomeric matrix protein increases in serum after the start of growth hormone treatment in prepubertal children. J Clin Endocrinol Metab 89 (10): 5156-60, 2004.
24. Bjarnason R, Boguszewski M, Dahlgren J, Gelander L, Kristrom B, Rosberg S, Carlsson B, Albertsson-Wikland K, Carlsson LM. Leptin levels are strongly correlated with those of GH-binding protein in prepubertal children. Eur J Endocrinol 137 (1): 68-73, 1997.
25. Bluet-Pajot MT, Epelbaum J, Gourdji D, Hammond C, Kordon C. Hypothalamic and hypophyseal regulation of growth hormone secretion. Cell Mol Neurobiol 18 (1): 101-23, 1998.
26. Boguszewski M, Rosberg S, Albertsson-Wikland K. Spontaneous 24-hour growth hormone profiles in prepubertal small for gestational age children. J Clin Endocrinol Metab 80 (9): 2599-606, 1995.

27. Briggs MD, Chapman KL. Pseudoachondroplasia and multiple epiphyseal dysplasia: mutation review, molecular interactions, and genotype to phenotype correlations. Hum Mutat 19 (5): 465-78, 2002.
28. Briggs MD, Hoffman SM, King LM, Olsen AS, Mohrenweiser H, Leroy JG, Mortier GR, Rimoin DL, Lachman RS, Gaines ES, Cekleniak JA, Knowlton RG, Cohn DH. Pseudoachondroplasia and multiple epiphyseal dysplasia due to mutations in the cartilage oligomeric matrix protein gene. Nat Genet 10 (3): 330-6, 1995.
29. Briggs MD, Mortier GR, Cole WG, King LM, Golik SS, Bonaventure J, Nuytinck L, De Paepe A, Leroy JG, Biesecker L, Lipson M, Wilcox WR, Lachman RS, Rimoin DL, Knowlton RG, Cohn DH. Diverse mutations in the gene for cartilage oligomeric matrix protein in the pseudoachondroplasia-multiple epiphyseal dysplasia disease spectrum. Am J Hum Genet 62 (2): 311-9, 1998.
30. Brook CG. Effect of human growth hormone treatment on adipose tissue in children. Arch Dis Child 48 (9): 725-8, 1973.
31. Caminos JE, Nogueiras R, Gallego R, Bravo S, Tovar S, Garcia-Caballero T, Casanueva FF, Dieguez C. Expression and regulation of adiponectin and receptor in human and rat placenta. J Clin Endocrinol Metab 90 (7): 4276-86, 2005.
32. Castellani LW, Goto AM, Lusis AJ. Studies with apolipoprotein A-II transgenic mice indicate a role for HDLs in adiposity and insulin resistance. Diabetes 50 (3): 643-51, 2001.
33. Chung L, Baxter RC. Detection of growth hormone responsive proteins using SELDI-TOF mass spectrometry. Growth Horm IGF Res, 2009.
34. Chung L, Clifford D, Buckley M, Baxter RC. Novel biomarkers of human growth hormone action from serum proteomic profiling using protein chip mass spectrometry. J Clin Endocrinol Metab 91 (2): 671-7, 2006.
35. Chung L, Nelson AE, Ho KK, Baxter RC. Proteomic profiling of growth hormone responsive proteins in human peripheral blood leukocytes. J Clin Endocrinol Metab, 2009.
36. Cianfarani S, Martinez C, Maiorana A, Scire G, Spadoni GL, Boemi S. Adiponectin levels are reduced in children born small for gestational age and are inversely related to postnatal catch-up growth. J Clin Endocrinol Metab 89 (3): 1346-51, 2004.
37. Cnop M, Havel PJ, Utzschneider KM, Carr DB, Sinha MK, Boyko EJ, Retzlaff BM, Knopp RH, Brunzell JD, Kahn SE. Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. Diabetologia 46 (4): 459-69, 2003.
38. Cohen P, Rogol AD, Howard CP, Bright GM, Kappelgaard AM, Rosenfeld RG. Insulin growth factor-based dosing of growth hormone therapy in children: a randomized, controlled study. J Clin Endocrinol Metab 92 (7): 2480-6, 2007.
39. Conde-Knape K, Bensadoun A, Sobel JH, Cohn JS, Shachter NS. Overexpression of apoC-I in apoE-null mice: severe hypertriglyceridemia due to inhibition of hepatic lipase. J Lipid Res 43 (12): 2136-45, 2002.

40. Corthals GL, Wasinger VC, Hochstrasser DF, Sanchez JC. The dynamic range of protein expression: a challenge for proteomic research. Electrophoresis 21 (6): 1104-15, 2000.
41. Dahlgren J, Kristrom B, Niklasson A, Nierop AF, Rosberg S, Albertsson-Wikland K. Models predicting the growth response to growth hormone treatment in short children independent of GH status, birth size and gestational age. BMC Med Inform Decis Mak 7 (1): 40, 2007.
42. De Benedetti F, Alonzi T, Moretta A, Lazzaro D, Costa P, Poli V, Martini A, Ciliberto G, Fattori E. Interleukin 6 causes growth impairment in transgenic mice through a decrease in insulin-like growth factor-I. A model for stunted growth in children with chronic inflammation. J Clin Invest 99 (4): 643-50, 1997.
43. de Haan W, Out R, Berbee JF, van der Hoogt CC, van Dijk KW, van Berkel TJ, Romijn JA, Jukema JW, Havekes LM, Rensen PC. Apolipoprotein CI inhibits scavenger receptor BI and increases plasma HDL levels in vivo. Biochem Biophys Res Commun 377 (4): 1294-8, 2008.
44. de Ridder MA, Stijnen T, Hokken-Koelega AC. Validation and calibration of the Kabi Pharmacia International Growth Study prediction model for children with idiopathic growth hormone deficiency. J Clin Endocrinol Metab 88 (3): 1223-7, 2003.
45. Ding J, List EO, Okada S, Kopchick JJ. Perspective: proteomic approach to detect biomarkers of human growth hormone. Growth Horm IGF Res 19 (4): 399-407, 2009.
46. Dove A. Proteomics: translating genomics into products? Nat Biotechnol 17 (3): 233-6, 1999.
47. Ducy P, Amling M, Takeda S, Priemel M, Schilling AF, Beil FT, Shen J, Vinson C, Rueger JM, Karsenty G. Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. Cell 100 (2): 197-207, 2000.
48. Dundar U, Kupesiz A, Ozdem S, Gilgil E, Tuncer T, Yesilipek A, Gultekin M. Bone metabolism and mineral density in patients with beta-thalassemia major. Saudi Med J 28 (9): 1425-9, 2007.
49. Elbein SC, Hoffman MD, Teng K, Leppert MF, Hasstedt SJ. A genome-wide search for type 2 diabetes susceptibility genes in Utah Caucasians. Diabetes 48 (5): 1175-82, 1999.
50. Eliakim A, Brasel JA, Cooper DM. GH response to exercise: assessment of the pituitary refractory period, and relationship with circulating components of the GH-IGF-I axis in adolescent females. J Pediatr Endocrinol Metab 12 (1): 47-55, 1999.
51. Engelbach W. The growth hormone. Endocrinology 16:1–19, 1932.
52. Escher N, Kaatz M, Melle C, Hipler C, Ziemer M, Driesch D, Wollina U, von Eggeling F. Posttranslational modifications of transthyretin are serum markers in patients with mycosis fungoides. Neoplasia 9 (3): 254-9, 2007.
53. Fasshauer M, Klein J, Kralisch S, Klier M, Lossner U, Bluher M, Paschke R. Growth hormone is a positive regulator of adiponectin receptor 2 in 3T3-L1 adipocytes. FEBS Lett 558 (1-3): 27-32, 2004.

54. Florentin M, Liberopoulos EN, Wierzbicki AS, Mikhailidis DP. Multiple actions of high-density lipoprotein. Curr Opin Cardiol 23 (4): 370-8, 2008.
55. Frank SJ. Growth hormone signalling and its regulation: preventing too much of a good thing. Growth Horm IGF Res 11 (4): 201-12, 2001.
56. Gelander L, Karlberg J, Albertsson-Wikland K. Seasonality in lower leg length velocity in prepubertal children. Acta Paediatr 83 (12): 1249-54, 1994.
57. Gershon D. Proteomics technologies: probing the proteome. Nature 424 (6948): 581-7, 2003.
58. Goldring MB, Tsuchimochi K, Ijiri K. The control of chondrogenesis. J Cell Biochem 97 (1): 33-44, 2006.
59. Grabowski P. Physiology of Bone. Endocr Dev 16: 32-48, 2009.
60. Graves PR, Haystead TA. Molecular biologist's guide to proteomics. Microbiol Mol Biol Rev 66 (1): 39-63; table of contents, 2002.
61. Gygi SP, Rochon Y, Franza BR, Aebersold R. Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19 (3): 1720-30, 1999.
62. Hanash S. Disease proteomics. Nature 422 (6928): 226-32, 2003.
63. Hanson RL, Ehm MG, Pettitt DJ, Prochazka M, Thompson DB, Timberlake D, Foroud T, Kobes S, Baier L, Burns DK, Almasy L, Blangero J, Garvey WT, Bennett PH, Knowler WC. An autosomal genomic scan for loci linked to type II diabetes mellitus and body-mass index in Pima Indians. Am J Hum Genet 63 (4): 1130-8, 1998.
64. Hashimoto Y, Tomiyama T, Yamano Y, Mori H. Mutation (D472Y) in the type 3 repeat domain of cartilage oligomeric matrix protein affects its early vesicle trafficking in endoplasmic reticulum and induces apoptosis. Am J Pathol 163 (1): 101-10, 2003.
65. Havel PJ. Control of energy homeostasis and insulin action by adipocyte hormones: leptin, acylation stimulating protein, and adiponectin. Curr Opin Lipidol 13 (1): 51-9, 2002.
66. Havel PJ. Update on adipocyte hormones: regulation of energy balance and carbohydrate/lipid metabolism. Diabetes 53 Suppl 1: S143-51, 2004.
67. He QY, Chiu JF. Proteomics in biomarker discovery and drug development. J Cell Biochem 89 (5): 868-86, 2003.
68. Hellgren G, Andersson B, Nierop AF, Dahlgren J, Hochberg Z, Albertsson-Wikland K. A proteomic approach identified growth hormone dependent nutrition markers in children with idiopathic short stature. Proteome Sci 6 (1): 35, 2008.
69. Herrington J, Carter-Su C. Signaling pathways activated by the growth hormone receptor. Trends Endocrinol Metab 12 (6): 252-7, 2001.
70. Hervas F, Morreale de Escobar G, Escobar Del Rey F. Rapid effects of single small doses of L-thyroxine and triiodo-L-thyronine on growth hormone, as studied in the rat by radioimmunoassy. Endocrinology 97 (1): 91-101, 1975.
71. Hilczer M, Smyczynska J, Stawerska R, Lewinski A. Effects of one-year low-dose growth hormone (GH) therapy on body composition, lipid profile and carbohydrate metabolism in young adults with childhood-onset severe GH deficiency confirmed after completion of growth promotion. Endokrynol Pol 59 (4): 292-300, 2008.

72. Ibanez L, Fucci A, Valls C, Ong K, Dunger D, de Zegher F. Neutrophil count in small-for-gestational age children: contrasting effects of metformin and growth hormone therapy. J Clin Endocrinol Metab 90 (6): 3435-9, 2005.
73. Ikeda T, Ohtani I, Fujiyama K, Hoshino T, Tanaka Y, Tekeuchi T, Mashiba H. Apolipoprotein levels in non-insulin-dependent diabetes mellitus with clinical macroangiopathy. Diabete Metab 17 (3): 373-8, 1991.
74. Ioannidou E, Padilla J, Wang J, Heymsfield SB, Thornton JC, Horlick M, Gallagher D, Pierson RN, Jr. Pencil-beam versus fan-beam dual-energy X-ray absorptiometry comparisons across four systems: appendicular lean soft tissue. Acta Diabetol 40 Suppl 1: S83-5, 2003.
75. Isaksson OG, Jansson JO, Gause IA. Growth hormone stimulates longitudinal bone growth directly. Science 216 (4551): 1237-9, 1982.
76. Isaksson OG, Lindahl A, Nilsson A, Isgaard J. Mechanism of the stimulatory effect of growth hormone on longitudinal bone growth. Endocr Rev 8 (4): 426-38, 1987.
77. Isgaard J, Carlsson L, Isaksson OG, Jansson JO. Pulsatile intravenous growth hormone (GH) infusion to hypophysectomized rats increases insulin-like growth factor I messenger ribonucleic acid in skeletal tissues more effectively than continuous GH infusion. Endocrinology 123 (6): 2605-10, 1988.
78. Isgaard J, Moller C, Isaksson OG, Nilsson A, Mathews LS, Norstedt G. Regulation of insulin-like growth factor messenger ribonucleic acid in rat growth plate by growth hormone. Endocrinology 122 (4): 1515-20, 1988.
79. Jain KK. Personalized medicine. Curr Opin Mol Ther 4 (6): 548-58, 2002.
80. Jain KK. Role of pharmacoproteomics in the development of personalized medicine. Pharmacogenomics 5 (3): 331-6, 2004.
81. Jansson C, Boguszewski C, Rosberg S, Carlsson L, Albertsson-Wikland K. Growth hormone (GH) assays: influence of standard preparations, GH isoforms, assay characteristics, and GH-binding protein. Clin Chem 43 (6 Pt 1): 950-6, 1997.
82. Johannsson G, Rosen T, Lonn L, Bengtsson BA. Effects of recombinant human growth hormone on adipose tissue in adults with growth hormone deficiency. Acta Paediatr Suppl 406: 60-3; discussion 64, 1994.
83. Jorgensen JO, Krag M, Kanaley J, Moller J, Hansen TK, Moller N, Christiansen JS, Orskov H. Exercise, hormones, and body temperature. regulation and action of GH during exercise. J Endocrinol Invest 26 (9): 838-42, 2003.
84. Jr GW, Cazares LH, Leung SM, Nasim S, Adam BL, Yip TT, Schellhammer PF, Gong L, Vlahou A. Proteinchip(R) surface enhanced laser desorption/ionization (SELDI) mass spectrometry: a novel protein biochip technology for detection of prostate cancer biomarkers in complex protein mixtures. Prostate Cancer Prostatic Dis 2 (5/6): 264-276, 1999.
85. Kanazawa H, Tanaka H, Inoue M, Yamanaka Y, Namba N, Seino Y. Efficacy of growth hormone therapy for patients with skeletal dysplasia. J Bone Miner Metab 21 (5): 307-10, 2003.
86. Karimi M, Karamifar HA. Short stature in beta-thalassemia minor subjects. Med Sci Monit 10 (11): CR603-5, 2004.

87. Karlberg J. On the construction of the infancy-childhood-puberty growth standard. Acta Paediatr Scand Suppl 356: 26-37, 1989.
88. Karlberg J. On the modelling of human growth. Stat Med 6 (2): 185-92, 1987.
89. Karlberg J, Luo ZC, Albertsson-Wikland K. Body mass index reference values (mean and SD) for Swedish children. Acta Paediatr 90 (12): 1427-34, 2001.
90. Katsanis SH, Javitt G, Hudson K. Public health. A case study of personalized medicine. Science 320 (5872): 53-4, 2008.
91. Kay R, Barton C, Ratcliffe L, Matharoo-Ball B, Brown P, Roberts J, Teale P, Creaser C. Enrichment of low molecular weight serum proteins using acetonitrile precipitation for mass spectrometry based proteomic analysis. Rapid Commun Mass Spectrom 22 (20): 3255-60, 2008.
92. Koch TK, Berg BO, De Armond SJ, Gravina RF. Creutzfeldt-Jakob disease in a young adult with idiopathic hypopituitarism. Possible relation to the administration of cadaveric human growth hormone. N Engl J Med 313 (12): 731-3, 1985.
93. Kraemer WJ, Ratamess NA. Hormonal responses and adaptations to resistance exercise and training. Sports Med 35 (4): 339-61, 2005.
94. Kristrom B, Aronson AS, Dahlgren J, Gustafsson J, Halldin M, Ivarsson SA, Nilsson NO, Svensson J, Tuvemo T, Albertsson-Wikland K. Growth hormone (GH) dosing during catch-up growth guided by individual responsiveness decreases growth response variability in prepubertal children with GH deficiency or idiopathic short stature. J Clin Endocrinol Metab 94 (2): 483-90, 2009.
95. Kristrom B, Dahlgren J, Niklasson A, Nierop AF, Albertsson-Wikland K. The first-year growth response to growth hormone treatment predicts the long-term prepubertal growth response in children. BMC Med Inform Decis Mak 9: 1, 2009.
96. Kristrom B, Jansson C, Rosberg S, Albertsson-Wikland K. Growth response to growth hormone (GH) treatment relates to serum insulin-like growth factor I (IGF-I) and IGF-binding protein-3 in short children with various GH secretion capacities. Swedish Study Group for Growth Hormone Treatment. J Clin Endocrinol Metab 82 (9): 2889-98, 1997.
97. Kristrom B, Karlberg J, Albertsson-Wikland K. Prediction of the growth response of short prepubertal children treated with growth hormone. Swedish Paediatric Study Group for GH treatment. Acta Paediatr 84 (1): 51-7, 1995.
98. Kroger H, Kotaniemi A, Vainio P, Alhava E. Bone densitometry of the spine and femur in children by dual-energy x-ray absorptiometry. Bone Miner 17 (1): 75-85, 1992.
99. Kronenberg HM. Developmental regulation of the growth plate. Nature 423 (6937): 332-6, 2003.
100. Kuromaru R, Kohno H, Ueyama N, Hassan HM, Honda S, Hara T. Long-term prospective study of body composition and lipid profiles during and after growth hormone (GH) treatment in children with GH deficiency: gender-specific metabolic effects. J Clin Endocrinol Metab 83 (11): 3890-6, 1998.

101. Land C, Blum WF, Shavrikova E, Kloeckner K, Stabrey A, Schoenau E. Predicting the growth response to growth hormone (GH) treatment in prepubertal and pubertal children with isolated GH deficiency--model validation in an observational setting (GeNeSIS). J Pediatr Endocrinol Metab 20 (6): 685-93, 2007.
102. Lanes R. Growth velocity, final height and bone mineral metabolism of short children treated long term with growth hormone. Curr Pharm Biotechnol 1 (1): 33-46, 2000.
103. Lanes R, Gunczler P, Esaa S, Weisinger JR. The effect of short- and long-term growth hormone treatment on bone mineral density and bone metabolism of prepubertal children with idiopathic short stature: a 3-year study. Clin Endocrinol (Oxf) 57 (6): 725-30, 2002.
104. Lanes R, Soros A, Gunczler P, Paoli M, Carrillo E, Villaroel O, Palacios A. Growth hormone deficiency, low levels of adiponectin, and unfavorable plasma lipid and lipoproteins. J Pediatr 149 (3): 324-9, 2006.
105. Lauerman LH. Advances in PCR technology. Anim Health Res Rev 5 (2): 247-8, 2004.
106. Lewis UJ. Variants of growth hormone and prolactin and their posttranslational modifications. Annu Rev Physiol 46: 33-42, 1984.
107. Lewis UJ, Sinha YN, Haro LS. Variant forms and fragments of human growth hormone in serum. Acta Paediatr Suppl 399: 29-31; discussion 32, 1994.
108. Li C, Evans H. The Isolation of Pituitary Growth Hormone. Science 99 (2566): 183-184, 1944.
109. Li C, Papkoff A. Preparation and properties of growth hormone from human and monkey pituitary glands. Science 124:1293–1294, 1956.
110. Lichanska AM, Waters MJ. How growth hormone controls growth, obesity and sexual dimorphism. Trends Genet 24 (1): 41-7, 2008.
111. Lichanska AM, Waters MJ. New insights into growth hormone receptor function and clinical implications. Horm Res 69 (3): 138-45, 2008.
112. Lim L, Spencer SA, McKay P, Waters MJ. Regulation of growth hormone (GH) bioactivity by a recombinant human GH-binding protein. Endocrinology 127 (3): 1287-91, 1990.
113. Lindholm J. Growth hormone: Historical notes. Pituitary 9 (1): 5-10, 2006.
114. Lindsay R, Feldkamp M, Harris D, Robertson J, Rallison M. Utah Growth Study: growth standards and the prevalence of growth hormone deficiency. J Pediatr 125 (1): 29-35, 1994.
115. Liu YX, Jalil F, Karlberg J. Growth stunting in early life in relation to the onset of the childhood component of growth. J Pediatr Endocrinol Metab 11 (2): 247-60, 1998.
116. Lobenhofer EK, Bushel PR, Afshari CA, Hamadeh HK. Progress in the application of DNA microarrays. Environ Health Perspect 109 (9): 881-91, 2001.
117. Lofqvist C, Andersson E, Gelander L, Rosberg S, Blum WF, Albertsson Wikland K. Reference values for IGF-I throughout childhood and

adolescence: a model that accounts simultaneously for the effect of gender, age, and puberty. J Clin Endocrinol Metab 86 (12): 5870-6, 2001.
118. Lofqvist C, Andersson E, Gelander L, Rosberg S, Hulthen L, Blum WF, Albertsson-Wikland K. Reference values for insulin-like growth factor-binding protein-3 (IGFBP-3) and the ratio of insulin-like growth factor-I to IGFBP-3 throughout childhood and adolescence. J Clin Endocrinol Metab 90 (3): 1420-7, 2005.
119. Luo XH, Guo LJ, Yuan LQ, Xie H, Zhou HD, Wu XP, Liao EY. Adiponectin stimulates human osteoblasts proliferation and differentiation via the MAPK signaling pathway. Exp Cell Res, 2005.
120. Malik G, Ward MD, Gupta SK, Trosset MW, Grizzle WE, Adam BL, Diaz JI, Semmes OJ. Serum levels of an isoform of apolipoprotein A-II as a potential marker for prostate cancer. Clin Cancer Res 11 (3): 1073-85, 2005.
121. Margulies L, Horlick M, Thornton JC, Wang J, Ioannidou E, Heymsfield SB. Reproducibility of pediatric whole body bone and body composition measures by dual-energy X-ray absorptiometry using the GE Lunar Prodigy. J Clin Densitom 8 (3): 298-304, 2005.
122. Mauras N, Haymond MW. Are the metabolic effects of GH and IGF-I separable? Growth Horm IGF Res 15 (1): 19-27, 2005.
123. Muurling M, van den Hoek AM, Mensink RP, Pijl H, Romijn JA, Havekes LM, Voshol PJ. Overexpression of APOC1 in obob mice leads to hepatic steatosis and severe hepatic insulin resistance. J Lipid Res 45 (1): 9-16, 2004.
124. Neidhart M, Muller-Ladner U, Frey W, Bosserhoff AK, Colombani PC, Frey-Rindova P, Hummel KM, Gay RE, Hauselmann H, Gay S. Increased serum levels of non-collagenous matrix proteins (cartilage oligomeric matrix protein and melanoma inhibitory activity) in marathon runners. Osteoarthritis Cartilage 8 (3): 222-9, 2000.
125. Nielsen SP. The fallacy of BMD: a critical review of the diagnostic use of dual X-ray absorptiometry. Clin Rheumatol 19 (3): 174-83, 2000.
126. Niklasson A, Ericson A, Fryer JG, Karlberg J, Lawrence C, Karlberg P. An update of the Swedish reference standards for weight, length and head circumference at birth for given gestational age (1977-1981). Acta Paediatr Scand 80 (8-9): 756-62, 1991.
127. Ohlsson C, Bengtsson BA, Isaksson OG, Andreassen TT, Slootweg MC. Growth hormone and bone. Endocr Rev 19 (1): 55-79, 1998.
128. Olney RC. Regulation of bone mass by growth hormone. Med Pediatr Oncol 41 (3): 228-34, 2003.
129. Olney RC, Wang J, Sylvester JE, Mougey EB. Growth factor regulation of human growth plate chondrocyte proliferation in vitro. Biochem Biophys Res Commun 317 (4): 1171-82, 2004.
130. Ong K, Beardsall K, de Zegher F. Growth hormone therapy in short children born small for gestational age. Early Hum Dev 81 (12): 973-80, 2005.
131. Oshima K, Nampei A, Matsuda M, Iwaki M, Fukuhara A, Hashimoto J, Yoshikawa H, Shimomura I. Adiponectin increases bone mass by suppressing osteoclast and activating osteoblast. Biochem Biophys Res Commun 331 (2): 520-6, 2005.

132. Pajvani UB, Du X, Combs TP, Berg AH, Rajala MW, Schulthess T, Engel J, Brownlee M, Scherer PE. Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications fpr metabolic regulation and bioactivity. J Biol Chem 278 (11): 9073-85, 2003.
133. Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, Mills GB, Simone C, Fishman DA, Kohn EC, Liotta LA. Use of proteomic patterns in serum to identify ovarian cancer. Lancet 359 (9306): 572-7, 2002.
134. Petricoin EF, Zoon KC, Kohn EC, Barrett JC, Liotta LA. Clinical proteomics: translating benchside promise into bedside reality. Nat Rev Drug Discov 1 (9): 683-95, 2002.
135. Phillip M, Moran O, Lazar L. Growth without growth hormone. J Pediatr Endocrinol Metab 15 Suppl 5: 1267-72, 2002.
136. Preece M. Prepubertal and pubertal endocrinology., in Human growth. A comprehensive treatise., F.F.T.J.M., Editor. Plenum Press: New York: 211-24, 1986.
137. PriceWaterhouse. Personalized medicine February 2005.
138. Procter AM, Phillips JA, 3rd, Cooper DN. The molecular genetics of growth hormone deficiency. Hum Genet 103 (3): 255-72, 1998.
139. Provot S, Schipani E. Molecular mechanisms of endochondral bone development. Biochem Biophys Res Commun 328 (3): 658-65, 2005.
140. Raben MS. Treatment of a pituitary dwarf with human growth hormone. J Clin Endocrinol Metab 18 (8): 901-3, 1958.
141. Ranke MB, Lindberg A, Chatelain P, Wilton P, Cutfield W, Albertsson-Wikland K, Price DA. Derivation and validation of a mathematical model for predicting the response to exogenous recombinant human growth hormone (GH) in prepubertal children with idiopathic GH deficiency. KIGS International Board. Kabi Pharmacia International Growth Study. J Clin Endocrinol Metab 84 (4): 1174-83, 1999.
142. Robinson IC. The growth hormone secretory pattern: a response to neuroendocrine signals. Acta Paediatr Scand Suppl 372: 70-8; discussion 79-80, 1991.
143. Robinson IC, Gevers EF, Bennett PA. Sex differences in growth hormone secretion and action in the rat. Growth Horm IGF Res 8 Suppl B: 39-47, 1998.
144. Rodriguez-Pacheco F, Martinez-Fuentes AJ, Tovar S, Pinilla L, Tena-Sempere M, Dieguez C, Castano JP, Malagon MM. Regulation of pituitary cell function by adiponectin. Endocrinology 148 (1): 401-10, 2007.
145. Rosenfeld RG, Hwa V. The growth hormone cascade and its role in mammalian growth. Horm Res 71 Suppl 2: 36-40, 2009.
146. Rosenfeld RG, Hwa V, Wilson L, Lopez-Bermejo A, Buckway C, Burren C, Choi WK, Devi G, Ingermann A, Graham D, Minniti G, Spagnoli A, Oh Y. The insulin-like growth factor binding protein superfamily: new perspectives. Pediatrics 104 (4 Pt 2): 1018-21, 1999.
147. Sackmann-Sala L, Ding J, Frohman LA, Kopchick JJ. Activation of the GH/IGF-1 axis by CJC-1295, a long-acting GHRH analog, results in serum

protein profile changes in normal adult subjects. Growth Horm IGF Res, 2009.
148. Salama OS, Al-Tonbary YA, Shahin RA, Eldeen OA. Unbalanced bone turnover in children with beta-thalassemia. Hematology 11 (3): 197-202, 2006.
149. Saxne T, Heinegard D. Cartilage oligomeric matrix protein: a novel marker of cartilage turnover detectable in synovial fluid and blood. Br J Rheumatol 31 (9): 583-91, 1992.
150. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270 (5235): 467-70, 1995.
151. Schwartzbauer G, Menon RK. Regulation of growth hormone receptor gene expression. Mol Genet Metab 63 (4): 243-53, 1998.
152. Service RF. Proteomics. Proteomics ponders prime time. Science 321 (5897): 1758-61, 2008.
153. Shachter NS. Apolipoproteins C-I and C-III as important modulators of lipoprotein metabolism. Curr Opin Lipidol 12 (3): 297-304, 2001.
154. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1 (6): 2856-60, 2006.
155. Shinoda Y, Yamaguchi M, Ogata N, Akune T, Kubota N, Yamauchi T, Terauchi Y, Kadowaki T, Takeuchi Y, Fukumoto S, Ikeda T, Hoshi K, Chung UI, Nakamura K, Kawaguchi H. Regulation of bone formation by adiponectin through autocrine/paracrine and endocrine pathways. J Cell Biochem, 2006.
156. Song HR, Lee KS, Li QW, Koo SK, Jung SC. Identification of cartilage oligomeric matrix protein (COMP) gene mutations in patients with pseudoachondroplasia and multiple epiphyseal dysplasia. J Hum Genet 48 (5): 222-5, 2003.
157. Sousa MM, Berglund L, Saraiva MJ. Transthyretin in high density lipoproteins: association with apolipoprotein A-I. J Lipid Res 41 (1): 58-65, 2000.
158. SparseLab. Website: http://sparselab.stanford.edu/. 2009.
159. Steyn FJ, Boehme F, Vargas E, Wang K, Parkington HC, Rao JR, Chen C. Adiponectin regulate growth hormone secretion via adiponectin receptor mediated Ca(2+) signalling in rat somatotrophs in vitro. J Neuroendocrinol 21 (8): 698-704, 2009.
160. Strobl JS, Thomas MJ. Human growth hormone. Pharmacol Rev 46 (1): 1-34, 1994.
161. Taksali SE, Caprio S, Dziura J, Dufour S, Cali AM, Goodman TR, Papademetris X, Burgert TS, Pierpont BM, Savoye M, Shaw M, Seyal AA, Weiss R. High visceral and low abdominal subcutaneous fat stores in the obese adolescent: a determinant of an adverse metabolic phenotype. Diabetes 57 (2): 367-71, 2008.
162. Tanaka T. Postnatal growth: An overview., in Pediatrics & Perinatology., P.G.M. Heymann, Editor. Arnold: London: 304-09, 1996.

163. Tetko IVL, D. J.; Luik, A. I. . Neural network studies. 1. Comparison of overfitting and overtraining. J. Chem. Inf. Comput. Sci. 35: 826-833, 1995.
164. Thadikkaran L, Siegenthaler MA, Crettaz D, Queloz PA, Schneider P, Tissot JD. Recent advances in blood-related proteomics. Proteomics 5 (12): 3019-34, 2005.
165. Thissen JP, Underwood LE, Ketelslegers JM. Regulation of insulin-like growth factor-I in starvation and injury. Nutr Rev 57 (6): 167-76, 1999.
166. Trayhurn P. Endocrine and signalling role of adipose tissue: new perspectives on fat. Acta Physiol Scand 184 (4): 285-93, 2005.
167. Trujillo ME, Scherer PE. Adiponectin--journey from an adipocyte secretory protein to biomarker of the metabolic syndrome. J Intern Med 257 (2): 167-75, 2005.
168. Tsao TS, Lodish HF, Fruebis J. ACRP30, a new hormone controlling fat and glucose metabolism. Eur J Pharmacol 440 (2-3): 213-21, 2002.
169. Vimpani GV, Vimpani AF, Lidgard GP, Cameron EH, Farquhar JW. Prevalence of severe growth hormone deficiency. Br Med J 2 (6084): 427-30, 1977.
170. Vionnet N, Hani EH, Dupont S, Gallina S, Francke S, Dotte S, De Matos F, Durand E, Lepretre F, Lecoeur C, Gallina P, Zekiri L, Dina C, Froguel P. Genomewide search for type 2 diabetes-susceptibility genes in French whites: evidence for a novel susceptibility locus for early-onset diabetes on chromosome 3q27-qter and independent replication of a type 2-diabetes locus on chromosome 1q21-q24. Am J Hum Genet 67 (6): 1470-80, 2000.
171. Vu-Dac N, Gervois P, Torra IP, Fruchart JC, Kosykh V, Kooistra T, Princen HM, Dallongeville J, Staels B. Retinoids increase human apo C-III expression at the transcriptional level via the retinoid X receptor. Contribution to the hypertriglyceridemic action of retinoids. J Clin Invest 102 (3): 625-32, 1998.
172. Wabitsch M, Braun S, Hauner H, Heinze E, Ilondo MM, Shymko R, De Meyts P, Teller WM. Mitogenic and antiadipogenic properties of human growth hormone in differentiating human adipocyte precursor cells in primary culture. Pediatr Res 40 (3): 450-6, 1996.
173. Wang Y, Lam KS, Xu JY, Lu G, Xu LY, Cooper GJ, Xu A. Adiponectin inhibits cell proliferation by interacting with several growth factors in an oligomerization-dependent manner. J Biol Chem 280 (18): 18341-7, 2005.
174. Wikipedia.org. Orbitrap mass spectrometer, Website: http://en.wikipedia.org/wiki/Orbitrap 2009.
175. Wikipedia.org. Partial least squares regression, Website: http://en.wikipedia.org/wiki/Partial_least_squares_regression 2009
176. Wolf G. Adiponectin: a regulator of energy homeostasis. Nutr Rev 61 (8): 290-2, 2003.
177. Wong GW, Wang J, Hug C, Tsao TS, Lodish HF. A family of Acrp30/adiponectin structural and functional paralogs. Proc Natl Acad Sci U S A 101 (28): 10302-7, 2004.
178. Yamada T, Miida T, Itoh Y, Kawai T, Benson MD. Characterization of serum amyloid A4 as a plasma apolipoprotein. Clin Chim Acta 251 (1): 105-12, 1996.

179. Yamada T, Miyake N, Itoh K, Igari J. Further characterization of serum amyloid A4 as a minor acute phase reactant and a possible nutritional marker. Clin Chem Lab Med 39 (1): 7-10, 2001.
180. Yui Y, Aoyama T, Morishita H, Takahashi M, Takatsu Y, Kawai C. Serum prostacyclin stabilizing factor is identical to apolipoprotein A-I (Apo A-I). A novel function of Apo A-I. J Clin Invest 82 (3): 803-7, 1988.
181. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature 372 (6505): 425-32, 1994.