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INSULIN-LIKE GROWTH FACTOR-I DEFICIENCY, INSULIN SENSITIVITY, AND GLUCOSE METABOLISM

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To Karin, Axel and Olof

ABSTRACT

In children and adolescents, growth hormone (GH) and insulin-like growth factor-I (IGF-I) act in concert to stimulate linear growth; however, the effects on glucose metabolism are in opposition. GH increases insulin resistance by lipolysis. In contrast, IGF-I stimulates glucose uptake and downregulates GH secretion, thus improving insulin sensitivity. Children with GH receptor mutations, severe primary IGF-I deficiency (PIGFD), lack both the growth-promoting and the metabolic effects of GH and IGF-I, and children with type 1 diabetes mellitus (T1DM), acquired IGF-I deficiency, have low portal insulin concentrations, increased IGFBP-1 levels, hepatic GH insensitivity, low circulating IGF-I, and increased GH secretion, i.e. mechanisms that increase insulin resistance and impair metabolic control (HbA1c).

The aims of this thesis were to study the effects of two different rhIGF-I preparations on growth and metabolism in severe PIGFD, and the effects of long-acting insulin glargine and continuous subcutaneous insulin infusion (CSII) on the GH/IGF-I axis as well as the direct effects of rhIGF-I on glucose disposal and tissue IGF-I levels in T1DM.

In **Paper I**, we studied the effects of rhIGF-I/rhIGFBP-3 for 17 months and thereafter rhIGF-I for 12 months in two siblings with a GH receptor mutation. We found decreased fat mass, increased lean body mass and improved linear growth in response to both preparations, although rhIGF-I was clearly more efficient. The data on insulin sensitivity (hyperinsulinemic euglycemic clamps) were incongruent. However, decreased overnight insulin secretion, most prominent after rhIGF-I, suggested improved insulin sensitivity. A diurnal rhythm of circulating IGF-I with higher mean levels and suppression of GH secretion was seen on rhIGF-I.

In **Paper II**, an observational study of 12 adolescents with T1DM, we studied the effects on the GH/IGF-I axis and metabolic control for up to 12 weeks after changing from NPH insulin to insulin glargine. We found decreased overnight IGFBP-1 levels and increased circulating IGF-I levels indicating a more efficient nightly insulin delivery thus suggesting improved hepatic insulin sensitivity and improved hepatic GH sensitivity which was associated with improved HbA1c.

In **Paper III**, a parallel multi-centre study lasting 24 months, 72 children and adolescents with newly diagnosed T1DM were randomized to multiple daily insulin injections (MDI) with NPH insulin or CSII and studied regarding the effects on the GH/IGF-I axis and endogenous insulin production. We found decreased fasting IGFBP-1, indicating a more efficient nightly insulin delivery with CSII and thus improved hepatic insulin sensitivity. In addition, the insulin doses were lower in the CSII group indicating improved insulin sensitivity.

In **Paper IV**, eight males with T1DM were studied in a randomized single-blind, placebo-controlled, cross-over study. We assessed the effects of a single subcutaneous rhIGF-I injection (120 µg/kg) or saline, during a normoinsulinemic euglycemic clamp, on glucose utilization and tissue levels of IGF-I in muscle and subcutaneous fat determined by microdialysis. We found an increase in whole body glucose disposal and a concomitant increase in tissue IGF-I levels during the second hour after injection.

In summary, this thesis demonstrates that rhIGF-I is superior to rhIGF-I/rhIGFBP-3 in promoting linear growth and also improves body composition and decreases insulin levels more efficiently. A more sustained insulin delivery profile of insulin glargine and CSII improves hepatic insulin sensitivity and insulin glargine increases circulating IGF-I and decreases HbA1c, and the thesis provides evidence that the microdialysis technique can be used to assess biological effects of IGF-I in tissues.

LIST OF PUBLICATIONS

This thesis is based on the following publications. The papers will be referred to in the text by their Roman numerals.

- I. **Ekström Klas**, Carlsson-Skwirut Christine, Ritzén E. Martin, Bang Peter.
IGF-I and IGFBP-3 Co-Treatment versus IGF-I Alone in Two Brothers with Growth Hormone Insensitivity Syndrome: Effects on Insulin Sensitivity, Body Composition and Linear Growth.
Hormone Research in Paediatrics, 2011, Nov;76(5): 355-366
- II. **Ekström Klas**, Salemyr Jenny, Zachrisson Ingmar, Carlsson-Skwirut Christine, Örtqvist Eva, Bang Peter.
Normalization of the IGF-IGFBP Axis by Sustained Nightly Insulinization in Type 1 Diabetes.
Diabetes Care, 2007, June; 30(6): 1357 – 1363
- III. **Ekström Klas**, Skogsberg Lars, Fors Hans, Carlsson-Skwirut Christine, Bang Peter.
Lower serum IGFBP-1 is a marker of increased hepatic insulin sensitivity in children on continuous subcutaneous insulin infusion therapy versus multiple daily insulin injections from onset of type 1 diabetes mellitus.
Manuscript
- IV. **Ekström Klas**, Pulkkinen Mari-Anne, Carlsson-Skwirut Christine , Brorsson Anna-Lena, Ma Zhulin, Frystyk Jan , Bang Peter.
Tissue Levels of IGF-I in Muscle and Subcutaneous Fat Determined by Microdialysis Reflect Whole-Body Glucose Utilization after a Subcutaneous rhIGF-I Injection in Adolescents with Type 1 Diabetes Mellitus.
Manuscript

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LIST OF ABBREVIATIONS

AGA	Appropriate for Gestational Age
ALS	Acid-labile Subunit
ASF	Abdominal Subcutaneous Fat
BMI	Body Mass Index
CSII	Continuous Subcutaneous Insulin Infusion
DCCT	The Diabetes Control and Complication Trial
GH	Growth Hormone
GHBP	GH Binding Protein
GHD	Growth Hormone Deficiency (Secondary IGF-I Deficiency)
GHIS	Growth Hormone Insensitivity Syndrome
GHR	GH Receptor
GnRH agonist	Gonadotropin Releasing Hormone Agonist
GIR	Glucose Infusion Rate
GLUT	Glucose Transporter
HGP	Hepatic Glucose Production
HSL	Hormone Sensitive Lipase
HtV	Height Velocity
IGF-I	Insulin-like Growth Factor-I
IGF-II	Insulin-Like Growth Factor-II
IGF-1R	Insulin-Like Growth Factor-1 Receptor
IGFBPs	Insulin-Like Growth Factor Binding Proteins
IR	Insulin Receptor
IRS	Insulin Receptor Substrate
JAK	Janus Kinase
KO	Knock Out
LID	Liver IGF-I Deficient Mouse
LMVL	Left Musculus Vastus Lateralis
MD	Microdialysis
md-IGF-I	Tissue IGF-I measured by microdialysis
MDI	Multiple Daily Insulin Injections
MMTT	Mixed Meal Tolerance Test
NEFA	Non-Esterified Fatty Acid
NPH	Neutral Protamine Hagedorn Insulin
PIGFD	Primary Insulin-like Growth Factor -1 Deficiency
PI3K	Phosphatidylinositol 3-Kinase
RCT	Randomized Controlled Trial
RMVL	Right Musculus Vastus Lateralis
rhIGF-I	Recombinant Human IGF-I
rhIGFBP-3	Recombinant Human IGFBP-3
SDS	Standard Deviation Score
SGA	Small for Gestational Age
SOCS	Suppressor of Cytokine Signaling
STAT	Signal Transducer and Activator of Transcription
T1DM	Type 1 Diabetes Mellitus

1 INTRODUCTION

In healthy children and adolescents growth hormone (GH) and insulin-like growth factor-I (IGF-I) act in concert to stimulate linear growth; however, the effects on glucose metabolism are in opposition. GH increases insulin resistance by stimulating lipolysis and hepatic glucose production. IGF-I, on the other hand, stimulates glucose uptake in muscles and downregulates GH secretion by negative feedback, thereby improving insulin sensitivity (Mauras and Haymond 2005; Kaplan and Cohen 2007).

In the first paper we studied two subjects with growth hormone insensitivity syndrome (GHIS) or Laron's syndrome (Laron, Pertzalan et al. 1966; Eshet, Laron et al. 1984; Enberg, Luthman et al. 2000; Laron, Ginsberg et al. 2006). The metabolic changes promoted by treatment with rhIGF-I have been studied in adults with GHIS, but until our report, no data have been published characterizing insulin sensitivity and body composition in adolescents with GHIS (Mauras, Martinez et al. 2000).

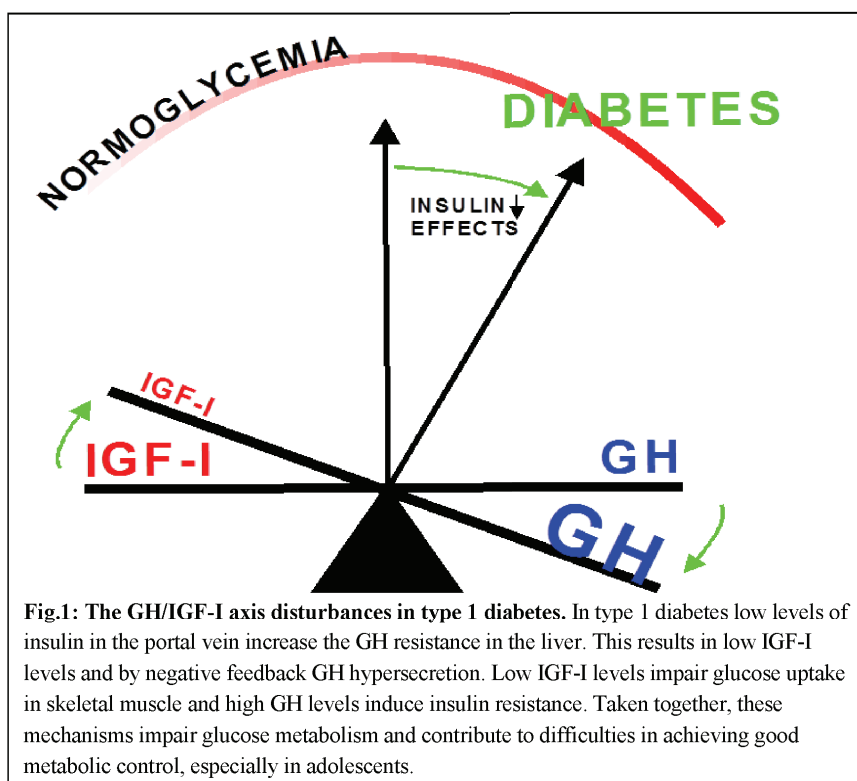
In papers II, III, and IV, "*acquired IGF-I deficiency*" in children and adolescents with type 1 diabetes (T1DM) were studied. Hepatic GH resistance and subsequent perturbations in the GH/IGF-I axis are due to low insulin concentrations in the portal vein. Low portal insulin concentrations impair hepatic GH receptor/post-receptor function (Daughaday, Phillips et al. 1976; Baxter, Bryson et al. 1980; Maes, Underwood et al. 1986; Clayton, Holly et al. 1994; Hanaire-Broutin, Sallerin-Caute et al. 1996), and decrease IGF-I gene expression and circulating IGF-I levels (Amiel, Sherwin et al. 1984; Lanes, Recker et al. 1985; Taylor, Dunger et al. 1988; Zachrisson, Brismar et al. 1997; Hedman, Frystyk et al. 2004), which, by negative feedback, increases GH secretion (Edge, Matthews et al. 1990; Batch and Werther 1992; Pal, Matthews et al. 1993; Halldin, Tylleskar et al. 1998). Furthermore, low portal insulin concentrations increase IGFBP-1 production (Hall, Johansson et al. 1989; Brismar, Fernqvist-Forbes et al. 1994).

Hypersecretion of GH stimulates lipolysis and increases hepatic glucose production (HGP); in addition, GH has direct negative effects on insulin signaling and thus increases insulin resistance Vijayakumar (Dominici, Argentino et al. 2005; Vijayakumar, Novosyadlyy et al. 2009; Clemmons 2012). IGF-I deficiency *per se* results in decreased glucose uptake in the muscles and insulin resistance (Guler, Zapf et al. 1987; Dohm, Elton et al. 1990; Russell-Jones, Bates et al. 1995; O'Connell and Clemmons 2002; Simpson, Jackson et al. 2004). Furthermore, portal insulin deficiency in T1DM increases HGP by direct and indirect mechanisms, the latter by increased glucagon secretion (Cherrington, Edgerton et al. 1998; Sindelar, Chu et al. 1998; Unger and Cherrington 2012). Moreover, the portal insulin deficiency increases IGFBP-1 production, thereby decreasing bioactive IGF-I in the circulation. These mechanisms act in concert and result in insulin resistance, which contributes to the deterioration of metabolic control during adolescence in T1DM.

Insulin treatment regimens using intermediate-acting neutral protamine hagedorn (NPH) insulin will not provide sufficient insulin effects late at night (Schmidt, Hadji-Georgopoulos et al. 1981; Edge, Matthews et al. 1990; Lepore, Pampanelli et al. 2000; Yagasaki, Kobayashi et al. 2010) thus leading to increased fasting blood glucose levels. In addition, the lack of insulin effects may also lead to increased IGFBP-1 levels and a

further reduction in circulating IGF-I levels and increased GH secretion, factors contributing to a deteriorating metabolic control. Hitherto, few randomized controlled studies from diagnosis of T1DM have addressed the effects of new long-acting basal insulin analogs or continuous subcutaneous insulin infusion (CSII) therapy on metabolic control, endogenous insulin secretion, and alterations in the GH/IGF-I axis (de Beaufort, Houtzagers et al. 1989; Pozzilli, Crino et al. 2003).

Administration of recombinant human IGF-I (rhIGF-I) as an adjunct to insulin has been shown to reduce GH hypersecretion and improve insulin sensitivity and HbA1c (Cheetham, Jones et al. 1993; Acerini, Patton et al. 1997; Thraikill, Quattrin et al. 1999; Saukkonen, Amin et al. 2004). More than 20 yrs ago Sonksen *et al.* proposed a hypothesis linking GH hypersecretion and low circulating IGF-I levels seen in T1DM to vascular complications (Sonksen, Russell-Jones et al. 1993). They referred to the imbalance between peripheral and portal insulin concentrations in type 1 diabetes. High peripheral insulin levels and GH hypersecretion may predispose to autocrine/paracrine overproduction of IGF-I, which has been shown to stimulate endothelial and smooth muscle cell proliferation in the capillary wall (Johansson, Chisalita et al. 2008), as well as to play a role in atherosclerosis (Clemmons, Maile et al. 2007). It has not yet been possible to study whether long-term rhIGF-I treatment, by reducing GH hypersecretion, also reduces tissue IGF-I levels due to the lack of a method to assess tissue levels of IGF-I. However, a method to determine IGF-I in peripheral tissues using a microdialysis technique was developed in our group (Berg, Gustafsson et al. 2006; Berg, Gustafsson et al. 2007). In paper 4, we attempted to establish that local tissue IGF-I measured by this method reflects the local actions of IGF-I in subjects with T1DM.



2 BACKGROUND

2.1 IGF-I

2.1.1 Historical aspects

In 1957 Salmon and Daughaday demonstrated that growth hormone (GH) stimulated incorporation of radiolabeled sulfate into cartilage by inducing a secondary growth-promoting substance which was described by its action, i.e. the “sulphation factor” (Salmon and Daughaday 1957). In 1972 Daughaday *et al.* proposed the name somatomedin, a mediator of the effects of somatotropin or growth hormone (Daughaday, Hall *et al.* 1972). Somatomedin was later demonstrated to consist of two forms, A and C. In the 1960s another group of researchers isolated a fraction of non-suppressible insulin-like activity from human serum, which they named NSILA and they proposed the name insulin-like factor I and II (Froesch, Muller *et al.* 1966; Van Wyk and Underwood 1975; Zapf, Rinderknecht *et al.* 1978). In 1978 Rinderknecht *et al.* demonstrated the amino acid sequence of human IGF-I (Rinderknecht and Humbel 1978). In 1983 somatomedin-C was called IGF-I and somatomedin-A insulin-like growth factor-II (IGF-II) (Klapper, Svoboda *et al.* 1983). In 1983 Li *et al.* managed to synthesize the polypeptide from amino acids, and then, following the isolation of the first human cDNA in 1983, the recombinant IGF-I (rhIGF-I) became available for *in vitro* and *in vivo* study (Jansen, van Schaik *et al.* 1983; Li, Yamashiro *et al.* 1983).

2.1.2 IGF-I related to insulin

IGF-I is a 70 amino acid single-chain polypeptide with a molecular size of ~7.6 kDa on SDS-PAGE coded from one gene at chromosome 12q23.2 and 48% sequence homology with human proinsulin (Rinderknecht and Humbel 1978). The major difference between IGF-I and insulin is that IGF-I keeps the connecting C-chain that is cleaved from proinsulin as the “connecting” C-peptide. This similarity supports the assumption that these molecules originated from a common ancestor gene and have similar metabolic and growth-promoting roles.

2.1.3 IGF-I – an endocrine, paracrine, and autocrine player

D'Ercole *et al.* (D'Ercole, Applewhite *et al.* 1980) demonstrated that IGF-I is produced in most fetal tissues in mice and speculated in autocrine/paracrine production of IGF-I in contradiction to the predominant view of the somatomedin hypothesis (section 2.1.1) (Salmon and Daughaday 1957). The widespread expression of IGF-I was later confirmed by Murphy *et al.*, who demonstrated IGF-I mRNA in virtually all tissues in the rat with a dominance of the liver (Murphy, Bell *et al.* 1987). Today, data from ample *in vivo* experiments in animals, as well as data from humans having rare gene defects in the GH/IGF-I axis, have more clearly defined the various endocrine versus paracrine/autocrine effects of IGF-I. It seems that paracrine/autocrine IGF-I expression is associated with growth while the endocrine effects of IGF-I are predominantly metabolic.

2.1.4 The IGFBPs

The majority of circulating IGF-I is produced in the liver due to GH stimulation. Liver-derived endocrine IGF-I has been shown to account for 75% of the circulating IGF-I in mice (Yakar, Liu *et al.* 1999; Le Roith, Bondy *et al.* 2001), and in humans IGF-I

mRNA is most abundant in the liver tissue; however, it is distributed in the whole body (Murphy, Bell et al. 1987). GH also stimulates the synthesis the acid-labile subunit (ALS) and IGF binding protein -3 (IGFBP-3). ALS is mainly of hepatic origin (Ueki, Ooi et al. 2000) and reflects hepatic GH responsiveness better than IGF-I and IGFBP-3, which are also produced in other tissues (D'Ercole, Applewhite et al. 1980; Jones and Clemmons 1995; Lee, Durham et al. 1997). The GH dependency is supported by very low IGFBP-3 and ALS levels in patients with GHR defects (Laron, Klingler et al. 1992; Labarta, Gargosky et al. 1997).

Approximately 95–99% of endocrine IGF-I is bound to IGFBPs (1- through 6) (Frystyk 2004; Clemmons 2012) and the complexes formed with IGFBP-3 and IGFBP-5 form large ternary complexes with ALS. To a lesser extent, IGF-I binds to IGFBP-1, 2, 4 and 6 and forms binary complexes. The unbound or free fraction of IGF-I in the circulation is estimated to amount to less than 1% of total IGF-I. The ternary complexes increase the half-life of IGF-I to 12–15 h, as compared to 6 h seen in untreated GHIS lacking almost all ternary complexes due to a deficiency of both IGFBP-3 and ALS (Grahnen, Kastrup et al. 1993; Jones and Clemmons 1995; Juul 2003). The IGFBPs serve to transport IGFs (both IGF-I and IGF-II), prolong their half-lives, and regulate clearance of the IGFs and modulate their bioactivity and the delivery to their cognate receptors. Data also support the view that there are specific actions of IGFBPs in the tissues which will not be further discussed in this thesis (Holly and Perks 2012).

In contrast to insulin, IGF-I is not stored and not delivered on demand; instead, the diurnal level is stable (Juul 2003). In serum IGF-I circulates in nanomolar concentrations, whereas the insulin concentrations are in the picomolar range. In human muscle *ex vivo*, glucose uptake is stimulated equipotently by IGF-I and insulin (Dohm, Elton et al. 1990), while the insulin-like activity of IGF-I after intravenous administration is approximately 7.5% of that of insulin (Guler, Zapf et al. 1987). Thus, in the presence of excess circulating IGFBPs, the glucose-lowering potency of IGF-I is largely decreased relative to that of insulin.

Moreover, measuring total circulating IGF-I has been a laboratory challenge, as IGF-I must be separated from IGFBPs in order to enable binding to specific antibodies in the immunoassay. Acidification is used to destroy ALS and also lowers the IGF-I affinity for IGFBPs. After neutralization to a physiological pH, reassociation of IGF-I (or labeled IGF-I in RIA analyses) can be prevented by adding excess IGF-II or by using labeled des (1-3) IGF-I with reduced IGFBP affinity (Bang, Eriksson et al. 1991). In the circulation and in the tissues, enhanced dissociation of IGF-I from IGFBPs is mediated by a specific cleavage of IGFBPs by IGFBP proteases (Bang, Brismar et al. 1994; Jones and Clemmons 1995; Holly and Perks 2012). Specific assays that assess the free fraction of IGF-I in serum have been developed (Bang, Ahlsen et al. 2001; Frystyk 2004).

2.1.5 IGF-I during childhood and puberty

Total IGF-I concentrations increase slowly in childhood (Juul, Bang et al. 1994). During puberty a steep increase in IGF-I concentrations, related to increased GH secretion, is seen with a maximum level at 14.5 yrs in girls (Tanner 3–4) and 15.5 yrs in boys (Tanner 4), thereby reflecting the different pubertal growth patterns in girls and boys. The IGF-I levels parallel the increase in height velocity until peak height is

attained, and thereafter IGF-I remains elevated for almost a year despite declining height velocity (Juul, Bang et al. 1994; Juul 2003). It is important to note that a significant variation with age occurs within each Tanner stage of puberty. In children starting puberty at an early age, higher IGF-I levels are seen at each Tanner stage, compared to those starting puberty later who have lower IGF-I levels at the same Tanner stage. Thus age, gender, and pubertal stage must be taken into account when comparing IGF-I levels in pubertal children, and models that convert endocrine IGF-I concentrations into SDS levels have been constructed (Juul, Bang et al. 1994; Lofqvist, Andersson et al. 2001).

2.1.6 IGF-I in growth and metabolism

IGF-I is an anabolic hormone that stimulates growth and acts in concert with GH in the proliferation and expansion of the epiphyseal chondrocytes in the growth plate (Isaksson, Lindahl et al. 1987; Le Roith, Bondy et al. 2001; Wang, Zhou et al. 2004; Kaplan and Cohen 2007). The additive effects of GH and IGF-I in promoting maximal growth are balanced by the opposing effects on carbohydrates. GH induces insulin resistance and thus an increasing demand for insulin; however, IGF-I opposes the effects of GH and acts in an insulin-like manner. These counteractive effects of IGF-I constitute a defense system enabling maximal growth without developing glucose intolerance and diabetes (Kaplan and Cohen 2007).

2.2 IGF-II

IGF-II shares 62% homology with IGF-I and 50% with proinsulin and consists of 67 amino acids (Rinderknecht and Humbel 1978; Daughaday and Rotwein 1989). It is transcribed from a paternally expressed imprinted gene on chromosome 11 (probably an evolutionary advantage) (Holly and Perks 2012). The circulating levels of IGF-II are 2 to 3-fold higher than those of IGF-I (Frystyk 2004). IGF-I and IGF-II bind to IGFBP-3 and subsequently associate with ALS to form a stable ternary complex that largely determines the total serum concentrations of IGF-I, IGF-II, and IGFBP-3. ALS is present in a two-fold excess in serum (Baxter 1990; Juul, Moller et al. 1998). Under normal physiological conditions, a given molar increase in IGF-I will be associated with a similar molar increase in IGFBP-3, while the relative increase in IGFBP-3 is smaller due to the presence of IGF-II (Juul 2003). Thus, the larger percentage increase in IGF-I relative to that of IGFBP-3 during puberty does not mean that a larger proportion of IGF-I is unbound. It is explained by the fact that IGF-II levels do not change during puberty. Overall, it is considered that IGF-II is most important as a paracrine/endocrine hormone involved in fetal growth and postnatal differentiation and regeneration (Wilson and Rotwein 2006; Holly and Perks 2012).

2.3 INSULIN

F. Banting, C. Best, J. Collip, and J. Macleod extracted insulin from the pancreas in 1921 and Banting and Macleod were awarded the Nobel Prize in 1923 (Rosenfeld 2002). The insulin gene is located at the distal part of the short arm on chromosome 11p15.5 (Harper, Ullrich et al. 1981) and insulin is synthesized in the β -cells of the islets of Langerhans in the endocrine pancreas and consists of two dissimilar polypeptide chains, A and B. Insulin is derived from proinsulin and consists of the A-chain (20 amino acids) and the B-chain (31 amino acids) linked by two disulfide bonds. Proinsulin is converted to insulin by removal of the connecting peptide (C-peptide), thus forming equimolar amounts. The entrance of glucose into the B-cell is facilitated

by GLUT2 transporters. Upon entrance, glucokinase (GK) initiates ATP production and a subsequent closing of the ATP-sensitive potassium channels. This in turn opens voltage-gated calcium channels and calcium flows into the cell. This stimulates insulin release into the portal vein (Hussain 2008). Roughly 50% of insulin is removed on its first passage through the liver where it binds the insulin receptor (IR) and stimulates glucose uptake and utilization (Saltiel and Kahn 2001).

2.4 GROWTH HORMONE

GH is synthesized in somatotrophs in the anterior pituitary (Oliveira, Salvatori et al. 2003) and promotes growth and has a major impact on metabolism. Regulation of GH secretion is complex and is due to hypothalamic factors (GH releasing hormone [GHRH], somatostatin, and ghrelin) and the negative feedback by endocrine IGF-I.

2.5 THE INSULIN AND IGF-I RECEPTORS

The insulin receptor (IR) and the insulin-like growth factor-I receptor (IGF-1R) belong to a family of growth factor receptors with tyrosine kinase activity (Rosen 1986; Jones and Clemmons 1995; White 1997; Dupont and LeRoith 2001; Clemmons 2012). The IR and the IGF-1R are heterotetrameric complexes embedded in the cell membrane. They consist of two α -subunits, which are entirely extracellular, and two β -subunits that penetrate through the plasma membrane and constitute the tyrosine kinase part, thus forming a ($\alpha_2\beta_2$) complex. The IGF-1R and IR share more than 50% overall homology and an even higher degree (84%) in the tyrosine kinase domain (Pandini, Frasca et al. 2002).

Insulin and IGF-I initiate their actions on metabolism and growth by binding to their cognate receptors. The affinity of IGF-I to the IGF-1R is 100 times higher than for insulin and, in a similar manner, insulin binds with a 100-fold higher affinity to the IR compared to IGF-I (Jones and Clemmons 1995; Back, Islam et al. 2012). The IR was identified more than 40 years ago and is widely distributed, although the concentration varies from very few up to 200,000 IRs on skeletal muscle cells, adipocytes, and hepatocytes (Kahn and White 1988; Ward and Lawrence 2011).

The IGF-1R was described almost 30 years ago (LeRoith, Werner et al. 1995; Juul 2003) and has not been recognized in mature adipocytes or hepatocytes, but otherwise it is generally distributed and highly abundant in other tissues, including skeletal muscles (Zapf, Schoenle et al. 1981; Bolinder, Lindblad et al. 1987; Caro, Poulos et al. 1988; Moller, Arner et al. 1991). In addition, IR- and IGF-1R-half-receptors can heterodimerize and form hybrid receptors. They are assemblies of one α/β part of the IR receptor and one α/β part of IGF-1R (Moxham, Duronio et al. 1989; Soos, Whittaker et al. 1990; Bailyes, Nave et al. 1997; Pandini, Frasca et al. 2002). Hybrid receptors are abundant in skeletal muscle, endothelial and vascular smooth muscle, and pre-adipocytes. Insulin has a low affinity for hybrids; in contrast, the affinity of IGF-I is high, thus the overall effects of IGF-I are thought to be mediated by both the IG-1R and the hybrid receptors (Soos, Field et al. 1993; Nitert, Chisalita et al. 2005; Arnqvist 2008).

2.5.1 The insulin/IGF-I signaling cascade

Binding of insulin or IGF-I to the alpha subunits induces conformational changes causing autophosphorylation of tyrosine sites on the β -subunits and thus stimulates its tyrosine kinase activity (Menting, Whittaker et al. 2013). The phosphorylation creates particular patterns (motifs) in the amino acid sequence for the insulin receptor

substrates (IRSs) (IRS-1, IRS-2, Shc) at the inner side of the cell membrane. When phosphorylated the IRSs initiate signal cascades that generates two different cellular responses. The primary effect of insulin (in some tissues also IGF-I) is metabolic (White 1997); in contrast, the primary effect of IGF-I is mitogenic (Dupont and LeRoith 2001). Termination of the signaling is mediated by internalization and dephosphorylation of the complexes (insulin-IR and IGF-I-IGF1R) by protein tyrosine phosphatases (PTPases) and the recycling of the IR and IGF-1R to the cell membrane (Carpentier 1994) (Fig.2).

2.5.2 The metabolic and mitogenic pathways

The IRS-PI3K-Akt pathway starts with the activation of IR/IGF-1R and results in glucose entering the cell and being metabolized by glycolysis or glycogen synthesis. IGF-I and, to a lesser extent, insulin also stimulate growth by the extracellular signal-related kinase (ERK) or mitogen-activated protein kinase (MAPK) pathway.

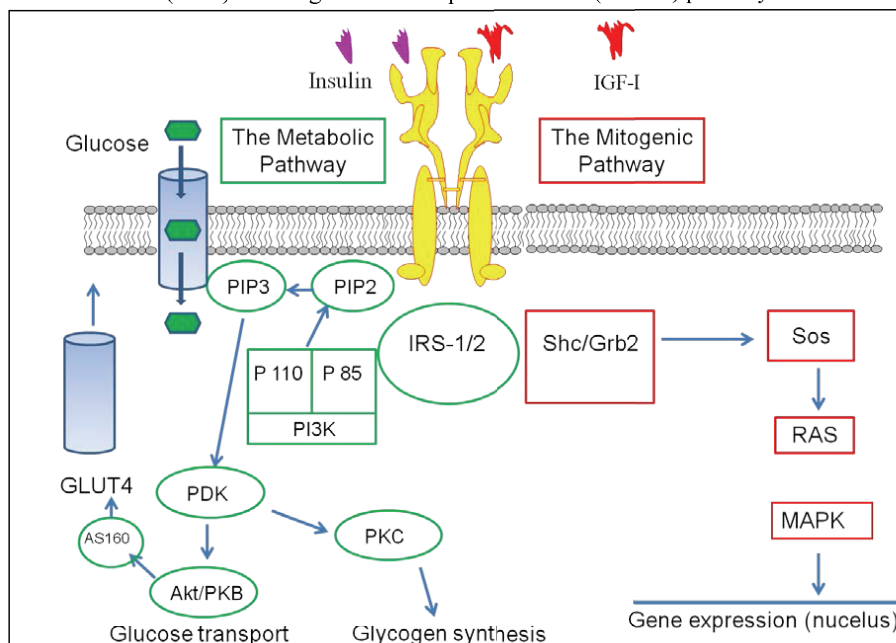


Fig.2. The metabolic and mitogenic pathways stimulated by insulin and IGF-I. The metabolic IRS-PI3K-Akt pathway starts with IR/IGF-1R activation and phosphorylation of the IRS proteins which act as an interface between downstream signaling proteins having the Src-homology-2 domain (SH2) in common (White 1997). Phosphatidylinositol 3-kinase (PI3K) is the major second messenger in cellular signaling (Di Paolo and De Camilli 2006) and IRS binds to the regulatory p85 subunit of PI3K. The catalytic subunit p110 of PI3K phosphorylates phosphatidylinositol 4, 5-biphosphate (PIP2) at the cell membrane to phosphatidylinositol 3,4,5-biphosphate (PIP3), which activates further downstream kinases, phosphoinositide-dependent protein kinase (PDK) and protein kinase C (PKC). PDK phosphorylates Akt/protein kinase B (PKB) (Bjornholm and Zierath 2005). Akt/PKB inhibits AS160 activity and thus GLUT4 translocates to the cell membrane (Tordjman, Leingang et al. 1989; Sakamoto and Holman 2008; Martins, Nachbar et al. 2012). PKC stimulates glycogen synthesis by inactivation of glycogen synthase kinase-3 (GSK-3) and activation of the glycogen synthase (GS). The mitogenic pathway involves tyrosine phosphorylation of Shc which in turn interacts with the growth factor receptor-bound protein-2 (Grb2), thus recruiting the son of sevenless (SOS) protein to the plasma membrane and thus activating Ras, which stimulates Raf and, finally, MAPK/ERK. Activated MAPK/ERKs translocate into the nucleus and initiate transcription, cell proliferation, and differentiation (Saltiel and Kahn 2001).

2.5.3 Metabolic or mitogenic response

It is still unexplained why activation of the IR results in mainly metabolic actions while the activation of the IGF-1R has mainly been considered to result in “mitogenic” (growth-promoting) actions, despite sharing the same signaling events. *In vitro* experiments demonstrated more than 20 yrs ago that IR and IGF-1Rs were similarly efficient in glucose transport; however, the IGF-1Rs were 10 times more active in stimulating DNA synthesis (Lammers, Gray et al. 1989). The differences might be due to extrinsic factors (tissue distribution, relative abundance) or intrinsic differences (Dupont and LeRoith 2001; Siddle 2012). This dichotomy has also been of particular interest in cancer research during the last few decades (Pollak 2008).

2.5.3.1 Extrinsic differences

The differences in metabolic versus mitogenic effects of insulin and IGF-I are related to the responding tissues and the distribution of IRs and IGF-1Rs. IRs are expressed in metabolically active tissues, including the liver, adipose tissue, and skeletal muscle. IGF-1R is largely expressed in the myotubes of skeletal muscle, where it has mainly metabolic effects, while IGF-1Rs are lacking in adipose tissue and the liver (Bolinder, Lindblad et al. 1987; Caro, Poulos et al. 1988). In skeletal muscle, the presence of both IGF-1R and the IR/IGF-I hybrid receptors mediate the metabolic effects of IGF-I (Dupont and LeRoith 2001; Le Roith 2007; Back, Islam et al. 2012; Siddle 2012).

2.5.3.2 Intrinsic differences

Other investigators have looked at differences in the ligand receptor affinities and associated long “off rates” with pronounced “mitogenic” responses on the IR (Hansen, Danielsen et al. 1996). This may also be explained by structural differences between the IR and IGF-1R in the beta-subunit, by differences in the alignment of different IRSs and *in vitro* data support the view that IRS-1 is responsible for the metabolic effects of insulin and Shc for the mitogenic effects of IGF-I. In addition, interactions with other signal pathways by suppressors of cytokine signaling (SOCS) 1-3 may also be important (Kalloo-Hosein, Whitehead et al. 1997; Dupont and LeRoith 2001; Dominici, Argentino et al. 2005; Lebrun and Van Obberghen 2008).

2.6 GH RECEPTOR AND SIGNALING

The human growth hormone receptor gene was cloned and characterized in the late 1980s (Leung, Spencer et al. 1987). Godowski *et al.* demonstrated a deletion in the GHR gene which explained the GH insensitivity in two patients with Laron’s syndrome (Godowski, Leung et al. 1989). The GHR gene is localized on chromosome 5p13 and consists of 10 exons (exons 1–7 corresponding to the extracellular domain) (de Vos, Ultsch et al. 1992) GHRs are present on most cells and belong to a family of cytokine receptors (Cosman, Lyman et al. 1990). After cleavage, the outer part of the GHR is separated into the circulation and becomes the circulating GH-binding protein (GHBP) (Baumann, Stolar et al. 1986). It was earlier believed that binding of GH to one part of the GHR promoted a dimerization; however, recent studies have shown that GH induces instead a conformational change in the GHR, resulting in a change in the preformed dimer and subsequently recruitment of cytoplasmic Janus kinases, JAK2s (Brown, Adams et al. 2005; Brooks and Waters 2010). Tyrosine phosphorylation of JAK2s and residues of the intracellular domains of the GHR provides docking sites for cytoplasmic signal pathways such as the signal transducer and activator of transcription

(STAT)5b, PI3K/Akt, and Ras/MAPK. The STAT5b pathway initiates gene transcription of IGF-I, IGFBP-3, and ALS (Rosenfeld 2006; Domene, Hwa et al. 2009). Termination of the GH signaling is regulated by SOCS2, a STAT5b-regulated gene acting in a negative feedback loop and thus downregulating the GHR signaling by direct ubiquitination (Vesterlund, Zadjali et al. 2011).

2.7 THE GH/IGF-I AXIS

GH is the main regulator of postnatal linear growth and the GH/IGF-I axis plays a key role in regulating somatic growth as seen in the rare overgrowth syndrome caused by a GH-producing adenoma in the pituitary (Eugster and Pescovitz 1999). In contrast, short stature is seen in GH deficiency leading to a secondary IGF-I deficiency and caused by defects in GH synthesis or release, or in defects of GH action leading to primary IGF-I deficiency (Rosenfeld 2003; Rosenfeld 2005; Rosenfeld 2006; Walenkamp and Wit 2006).

2.8 THE GH/ IGF-I AXIS AND GLUCOSE METABOLISM

The significance of the GH/IGF-I axis is not limited to aspects of growth. The magnitude of the interactions of GH, IGF-I, IGFBPs and insulin are important for understanding glucose and fat metabolism and the mechanisms behind normal insulin resistance in puberty, as well as the increased insulin resistance seen in type 1 diabetes and in GHR defects.

2.8.1 IGFBP-1

IGFBP-1 (~30 kDa) is synthesized in the liver and regulated by the portal insulin concentration (Brismar, Fernqvist-Forbes et al. 1994; Yki-Jarvinen, Makimattila et al. 1995; Wheatcroft and Kearney 2009). Low circulating IGFBP-1 levels increase measurements of free IGF-I concentrations in the circulation (Frystyk 2004). This has been suggested to impact on the bioactivity of IGF-I in the tissues. However, direct evidence for such an effect of physiological changes in IGFBP-1 concentrations is lacking. Infusion of IGFBP-1 in mice has been shown to increase glucose levels and is thus proposed to reduce the bioactivity of IGF-I (Lewitt, Denyer et al. 1991), and mice over-expressing IGFBP-1 have higher fasting glucose levels (Rajkumar, Barron et al. 1995; Crossey, Jones et al. 2000; Silha and Murphy 2002). Whether administration of IGFBP-1 causes inhibition of transport of endocrine IGF-I to the tissue site of action (low bioactive circulating IGF-I) or whether IGFBP-1 is transported to the tissues and exerts this effect by competing with IGF-1R interactions has not been clearly demonstrated.

2.9 INSULIN RESISTANCE

Insulin resistance is an important physiological mechanism in puberty and pregnancy and provides the prerequisites for optimal growth in puberty and fetal life. However, insulin resistance is also involved in such pathological states as obesity and diabetes and is defined as a subnormal glucose-lowering response to a given insulin concentration (Moller and Flier 1991; Lebovitz 2001). In T1DM (acquired IGF-I deficiency) GH hypersecretion and insulin resistance are well documented and are related to deteriorating metabolic control during puberty (Dunger and Acerini 1998;

Bereket, Lang et al. 1999; Acerini, Williams et al. 2001). It is important to note that not all of the biological effects of insulin are impacted in the insulin-resistant state. For example, the inhibitory action of insulin on IGFBP-1 production in the liver is not generally affected in T1DM. Furthermore, insulin resistance may affect some tissues/organs more than others and some tissues/organs may gain more sensitivity than others from a given treatment. For example, exercise particularly affects insulin sensitivity in skeletal muscle. In this context, it is also of interest that the levels of insulin in the portal, as opposed to the systemic, circulation are lower in patients with T1DM treated with subcutaneous insulin, CSII or multiple daily injections (MDI). This is particularly important for insulin actions on hepatic glucose production, on the inhibition of IGFBP-1, and in supporting GHR signaling, a major theme of this thesis. Insulin resistance is also a feature of untreated adult patients with GHR mutations (primary IGF-I deficiency) who lack both GH and IGF-I effects on glucose and fat metabolism (Laron, Avitzur et al. 1995).

2.9.1 Tissue differences in the response to insulin

Insulin sensitivity is a measure of insulin responsiveness in different tissues and is calculated during a constant insulin infusion in a clamp setting. The insulin sensitivity in different tissues (adipose tissue, liver and skeletal muscle) has a dose-response relationship that stretches from the low insulin concentrations that inhibit lipolysis by 50% (~10-15 $\mu\text{U/ml}$), via the somewhat higher concentrations that block HGP by 50% (~25 $\mu\text{U/ml}$) to the higher concentrations that increases glucose disposal in the muscles by 50% (~60 $\mu\text{U/ml}$) (Campbell, Mandarino et al. 1988; Groop, Bonadonna et al. 1989; Stumvoll and Jacob 1999). The skeletal muscles account for more than 90% of glucose disposal under maximal insulin-stimulated conditions (DeFronzo, Gunnarsson et al. 1985). The stepwise clamp procedure uses stable isotopes and identifies thresholds for the endogenous production of glucose and glycerol (Stumvoll and Jacob 1999). Insulin reduces HGP by both direct and indirect mechanisms. The direct effects are related to the portal insulin concentration and the indirect effects achieved at lower concentrations are mediated by inhibiting lipolysis (Lewis, Zinman et al. 1996; Sindelar, Chu et al. 1998; Cherrington 2005).

2.9.2 The glucose/fatty acid cycle

Insulin resistance is related to increased levels of non-esterified fatty acids (NEFA)s as seen in T1DM with GH hypersecretion and increased lipolysis. NEFAs are oxidized in the liver and skeletal muscles and block glucose utilization by inhibiting glycolytic enzymes and subsequent glucose uptake. This increases HGP from the liver and decreases glucose uptake in the muscles (Randle 1998; Dimitriadis, Mitrou et al. 2011; Martins, Nachbar et al. 2012). However, the enzymatic mechanisms described by Randle in 1963 have been challenged by experimental data from Roden *et al.* (Roden, Price et al. 1996; Shulman 2000) which supports the view that the mechanism is due instead to muscle/liver accumulation of NEFAs and interference with the insulin-signaling cascade leading to a reduction in GLUT4 translocation and glucose transport.

2.9.3 Puberty and insulin resistance

In both sexes, increasing levels of estrogen from the gonads (in boys, testosterone is aromatized to estrogen) during puberty increases GH secretion and subsequently endocrine IGF-I concentrations. Estrogen is thought to be permissive in allowing a concomitant increase in GH and IGF-I secretion by relaxing the negative-feedback axis (Mauras, Blizzard et al. 1987; Veldhuis, Metzger et al. 1997; Veldhuis and Bowers 2003). Increasing levels of GH and IGF-I are a prerequisite for maximal growth in puberty and increase insulin resistance in a physiological manner, which is further enhanced by a more efficient GHR function induced by compensatory hyperinsulinemia. IGF-I is important for both growth plate stimulation and opposing the diabetogenic effect of GH. GH secretion peaks at an earlier age and reaches higher levels in girls (Tanner stage 3–4) than in boys (Tanner stage 4) (Albertsson-Wikland, Rosberg et al. 1994). Several authors have demonstrated increasing insulin resistance during normal puberty, reaching a maximum in Tanner stages 3 and 4 and showing a close relationship to circulating IGF-I levels (Amiel, Sherwin et al. 1986; Amiel, Caprio et al. 1991; Caprio, Cline et al. 1994; Moran, Jacobs et al. 1999; Moran, Jacobs et al. 2002), which in turn reflects GH secretion.

2.9.4 GH, IGF-I, and insulin resistance

2.9.4.1 GH and insulin resistance

GH induces insulin resistance (Davidson 1987; Fowelin, Attvall et al. 1991; Moller, Jorgensen et al. 1991) and patients with acromegaly are insulin-resistant (Hansen, Tsalikian et al. 1986). GH increases insulin resistance by both direct and indirect mechanisms (Mauras, O'Brien et al. 2000; Yakar, Liu et al. 2001). The direct effects are thought to be mediated by increased lipolysis, which increases NEFAs and glycerol production (Williams, Amin et al. 2003; Salgin, Marcovecchio et al. 2009; Vijayakumar, Novosyadlyy et al. 2009), thus increasing HGP in the liver by the glucose-fatty acid cycle mechanisms. Bak *et al.* have also demonstrated inhibited glycogen synthase activity in healthy subjects receiving a GH infusion (Bak, Moller et al. 1991). Moreover, GH interacts with IR/IGF-1R signaling and the PI3K-Act pathway, thus inducing insulin resistance and HGP. Ueki *et al.* demonstrated reduced IR signaling mediated by SOCS-1/SOCS-3 and serine phosphorylation of IRS-1 (Ueki, Kondo et al. 2004; Dominici, Argentino et al. 2005).

2.9.4.2 IGF-I and insulin resistance

The effects of IGF-I in glucose metabolism oppose those of GH and may be divided into direct and indirect effects. IGF-1Rs are lacking in adipose tissues and the liver (Bolinder, Lindblad et al. 1987; Caro, Poulos et al. 1988) and, in a physiological setting, IGF-I stimulates glucose uptake in skeletal muscle. In experimental settings, IGF-I *per se* has been shown to improve glucose uptake (Guler, Zapf et al. 1987; Dohm, Elton et al. 1990; Crowne, Samra et al. 1998; O'Connell and Clemmons 2002; Simpson, Jackson et al. 2004), and the glucose lowering potency of IGF-I was demonstrated to be 13.5 times lower than that of insulin on an equimolar basis (Guler, Zapf et al. 1987). Thus, IGF-I acts similarly to insulin and may substitute for insulin actions rather than directly affecting the way that insulin signals for glucose clearance via its receptor. Whether IGF-I has effects on the IR signaling pathways that are shared

by IGF-1R has been less well studied. Many authors have demonstrated decreased HGP, improved glucose uptake, and reduced NEFAs in healthy controls given supraphysiological doses of rhIGF-I (Zenobi, Graf et al. 1992; Boulware, Tamborlane et al. 1994; Russell-Jones, Bates et al. 1995; Pratipanawatr, Pratipanawatr et al. 2002). The indirect IGF-I effects are mediated via negative feedback regulation of GH secretion. Short-term trials of rhIGF-I have demonstrated improved insulin sensitivity (Turkalj, Keller et al. 1992; Cheetham, Jones et al. 1993; Acerini and Dunger 2000; Saukkonen, Amin et al. 2004; Saukkonen, Shojaae-Moradie et al. 2006).

2.9.4.3 IGF-I deficiency in mouse and man

Transgenic mouse models have further elucidated the separate roles of GH and IGF-I in glucose metabolism. Liver IGF-I deficient (LID) mice lack endocrine IGF-I due to a liver-specific IGF-I gene deletion (endocrine IGF-I levels were reduced by 75%, GH secretion increased 4-fold and insulin levels 4-fold) and are insulin-resistant in the muscles. rhIGF-I replacement normalized endocrine IGF-I and GH levels and restored insulin sensitivity; however, by this approach, it was impossible to disclose the roles of IGF-I and GH *per se* on insulin resistance (Yakar, Liu et al. 2001). Therefore, treatment with a GH-releasing antagonist was carried out in the LID mouse. This reduced GH secretion and, despite the persistently low endocrine IGF-I levels, insulin sensitivity was improved, but not normalized, although indicating a direct role for circulating IGF-I in glucose uptake (Yakar, Liu et al. 2001). Furthermore, on mating the LID mouse with a strain lacking GH secretion, the GHa mouse, further reduced endocrine IGF-I levels (reduced paracrine/autocrine production) and insulin sensitivity was completely restored (Yakar, Setser et al. 2004). However, a specific role for IGF-I in insulin sensitivity cannot be excluded, knowing that patients with a GHR defect (Laron's syndrome) improve insulin sensitivity after rhIGF-I treatment (Laron, Avitzur et al. 1995). The first patient with an IGF-I gene deletion was described in 1996 (Woods, Camacho-Hubner et al. 1996) (further described in section 2.11.3). Evaluation of the GH/IGF-I axis demonstrated non-measurable IGF-I levels, GH hypersecretion, and substantially reduced insulin sensitivity; however, treatment with rhIGF-I reduced GH hypersecretion and normalized insulin sensitivity (Woods, Camacho-Hubner et al. 2000).

2.10 GLUCOSE METABOLISM

The monosaccharide glucose, $C_6H_{12}O_6$ (dextrose or grape sugar), is derived from plants and absorbed directly into the bloodstream. The normal amount of circulating glucose is 5-6 mmol/L and equals about two pieces of lump sugar (Szablewski 2011). The transport of glucose into the cells is mediated by glucose transporters (GLUTs) (Karim, Adams et al. 2012). GLUT 2 acts independently of insulin and is expressed in the liver, the β -cells, and the intestines and is capable of bidirectional fluxes of glucose, thus permitting HGP after glycogenolysis and gluconeogenesis. GLUT 4 is the insulin-dependent glucose transporter in skeletal muscle and adipose tissue (Birbaum 1989; James, Strube et al. 1989). IR or IGF-1R activation results in GLUT 4 transport from the intracellular vesicles to the cell membrane and glucose entrance (Karim, Adams et al. 2012) (Fig.2). In addition, exercise induces glucose-uptake in a non-insulin-dependent manner by GLUT 4 (Maarbjerg, Sylow et al. 2011). Glycogen synthase is stimulated by the IRS-PI3K- pathway and produces glycogen in the liver and muscles. Insulin blocks degradation of glycogen by reducing the activity of

glycogen phosphorylase and glucose-6-phosphatase, thus inhibiting HGP (Dimitriadis, Mitrou et al. 2011). Glucose-6-phosphatase is only present in the liver and explains why the glycogen storages in the muscles are not available for other tissues. Gluconeogenesis is an ATP-demanding (fatty acid oxidation) process that produces glucose from non-carbohydrate carbon substrates in the liver and kidneys. The sources are lactate, glucogenic amino acids, (alanine, glutamine), pyruvate and, to a lesser extent, glycerol (Gerich 2010; Szablewski 2011).

2.11 THE GH/IGF-I AXIS AND LINEAR GROWTH

The somatomedin hypothesis originally proposed that GH stimulated IGF-I synthesis in the liver and circulating/endocrine IGF-I was responsible for linear growth by binding to IGF-I receptors (IGF-1Rs) in the growth plate (Salmon and Daughaday 1957; Daughaday, Hall et al. 1972).

However, the role of endocrine IGF-I as being solely responsible for growth stimulation was challenged in animal experiments by Isaksson *et al.* (Isaksson, Jansson et al. 1982), thereby indicating a direct role of GH in the growth plate. The somatomedin hypothesis was further questioned in transgenic mouse knockout studies (Le Roith, Bondy et al. 2001). In the liver IGF-I-deficient (LID) mouse model, with a lack of endocrine IGF-I due to a liver-specific gene deletion by the

Cre Lox system, the role of endocrine IGF-I in growth was further elucidated. The circulating IGF-I levels in the LID mice model were reduced by 75% and the GH secretion was increased 4-fold. However, in spite of the extensive reduction of endocrine IGF-I, the mice grew normally, indicating that autocrine/paracrine IGF-I production was responsible for postnatal growth (Sjogren, Liu et al. 1999; Yakar, Liu et al. 1999; Liu, Yakar et al. 2000). Moreover, GH hypersecretion did not increase local IGF-I mRNA levels in the heart, muscle, and fat. To further explore whether there is a threshold for the effects of endocrine IGF-I on growth, the LID mice were mated with the ALS knockout (ALSKO) mice (Yakar, Rosen et al. 2002). These double KO mice had further reduced endocrine IGF-I levels, almost 90% compared to WT; however, they demonstrated impaired longitudinal growth. This experiment demonstrates that a

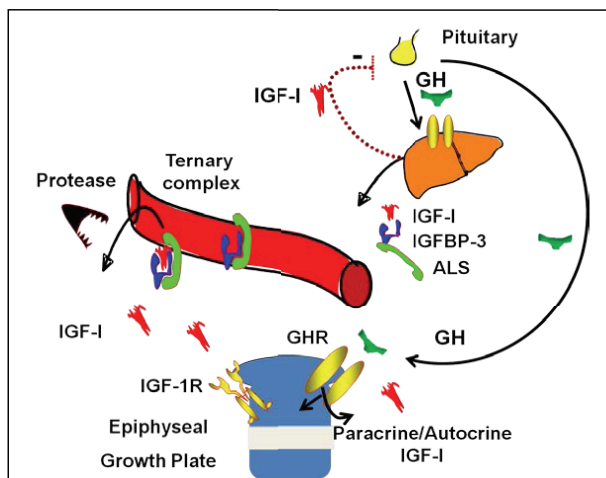


Fig.3. A schematic representation of the GH/IGF-I-axis in somatic growth .GH stimulates IGF-I, IGFBP-3 and ALS production in the liver which form the ternary complex in the circulation. IGF-I regulates GH secretion by a negative feedback mechanism. IGF-I is cleaved from the ternary complexes by proteases and the unbound IGF-I is biological active and binds to the IGF-1R in the growth plate and promotes growth. GH has both direct and indirect growth promoting effects in the growth plate and stimulates paracrine/ autocrine IGF-I production by an indirect effect.

threshold in the concentration of circulating IGF-I, necessary for normal growth, does exist.

These findings have been recognized as a paradigm shift and the original somatomedin hypothesis was revised by Leroith *et al.* in 2001 (Le Roith, Bondy *et al.* 2001). IGF-I is important for GH-stimulated postnatal body growth; however, endocrine IGF-I does not seem to be as essential for normal growth as earlier thought. Instead, local production of IGF-I, acting in a paracrine/autocrine manner, appears to mediate the GH-induced somatic growth, at least in the presence of functioning GH receptors (GHRs) (Fig.3).

2.11.1 Normal growth

Growth in childhood and adolescence is divided into three different stages according to the infancy-childhood-puberty model (Karlberg, Engstrom *et al.* 1987; Karlberg, Fryer *et al.* 1987). The infancy stage is mainly driven by nutrition and is the late part of the fetal growth phase. From the late infancy stage and the beginning of the childhood stage (between age 6 and 12 months), the GH/IGF-I axis becomes important in promoting linear growth. Height is normally distributed and can be expressed in a standard deviation score (SDS). Growth mirrors the socioeconomic situation and thus growth charts are country-specific (Wikland, Luo *et al.* 2002).

2.11.2 GH and IGF-I in the growth plate

The intrinsic regulation of the growth plate is not the focus in this thesis, although the actions of GH and IGF-I will be discussed briefly. GH and IGF-I act both directly and indirectly in the growth plate chondrocytes, as proposed by Isaksson and co-workers, who expanded the original dual effector hypothesis proposed by Green *et al.* (Green, Morikawa *et al.* 1985; Isaksson, Lindahl *et al.* 1987; Ohlsson, Bengtsson *et al.* 1998). The relative contributions of GH and IGF-I in postnatal growth have been elucidated in KO models in mice. In the lack of GH effects (GHRKO), a prominent reduction in postnatal growth of 65% was demonstrated. An absence of GH resulted in a reduction of the germinal zone and the numbers and rate of proliferation of the chondrocytes. Moreover, in the IGF gene KO (IGFKO), the postnatal growth was reduced by 35%, the numbers and rate of proliferation of chondrocytes were unchanged and a reduction in the hypertrophy of chondrocytes was seen, supporting a direct effect of IGF-I on the hypertrophy of chondrocytes (Lupu, Terwilliger *et al.* 2001; Wang, Zhou *et al.* 2004)

2.11.3 Effects on growth by IGF-I *per se*

The role of circulating IGF-I *per se* in linear growth must not be underestimated. Patients with GHR defects and the IGF-I deletion patients provide unique opportunities to disclose the different roles of GH and IGF-I *per se* regarding both linear growth and metabolism.

In humans with GHR defects, and thus a non-existing paracrine/autocrine IGF-I production, treatment with rhIGF-I has been shown to improve linear growth (Guevara-Aguirre, Rosenbloom *et al.* 1997; Chernausek, Backeljauw *et al.* 2007). However, linear growth is not completely restored, in contrast to children with GH deficiency. rhIGF-I treatment in patients with GHR defects increases endocrine IGF-I but is not able to replace the GH-stimulated paracrine/autocrine production of IGF-I or the direct growth-promoting effects of GH. Data from studies in patients with

GHR defects treated with rhIGF-I support the “dual effector” mechanism in the growth plate, indicating that both GH and IGF-I actions are necessary to achieve full catch-up (Isgaard, Nilsson et al. 1986; Isaksson, Lindahl et al. 1987).

The first patient with an IGF-I gene deletion was described by Wood *et al.* (Woods, Camacho-Hubner et al. 1996). At the time of diagnosis, he was 15.8 years old and presented with severe short stature, 119.1 cm (- 6.9 SDS), sensorineural deafness and microcephaly, and also mental retardation. He was born at term, but extremely small for gestational age (SGA) with a weight of 1.4 kg (-3.9 SDS) and a length of 37.8 cm (- 5.4 SDS). This supports the view that human growth is IGF-I- but not GH-dependent during fetal life (children with GHD or GHR defects are not growth retarded at birth). rhIGF-I replacement resulted in an increased height velocity from 3.8 to 7.3 cm/yr during mid-puberty (Woods, Camacho-Hubner et al. 2000). This is less than would be expected in a naïve GHD patient treated with rhGH and indicates that normal growth is dependent on autocrine/paracrine IGF-I production and further supports the “dual effector” mechanism. Furthermore, patients with an ALS deficiency have very low concentrations of endocrine IGF-I and IGFBP-3 and have GH hypersecretion. The lack of a ternary complex formation and a higher rate of clearance explain the low circulating hormone levels (Domene, Hwa et al. 2009). However, in spite of a profound circulating IGF-I deficiency, there is only a mild impact on postnatal growth, which is supposed to be attributable to preserved or perhaps even upregulated expression of locally produced IGF-I due to increased GH levels, which further supports the role of autocrine/paracrine IGF-I in linear growth.

2.12 PRIMARY IGF-I DEFICIENCY

2.12.1 From Laron’s syndrome to primary IGF-I deficiency (PIGFD)

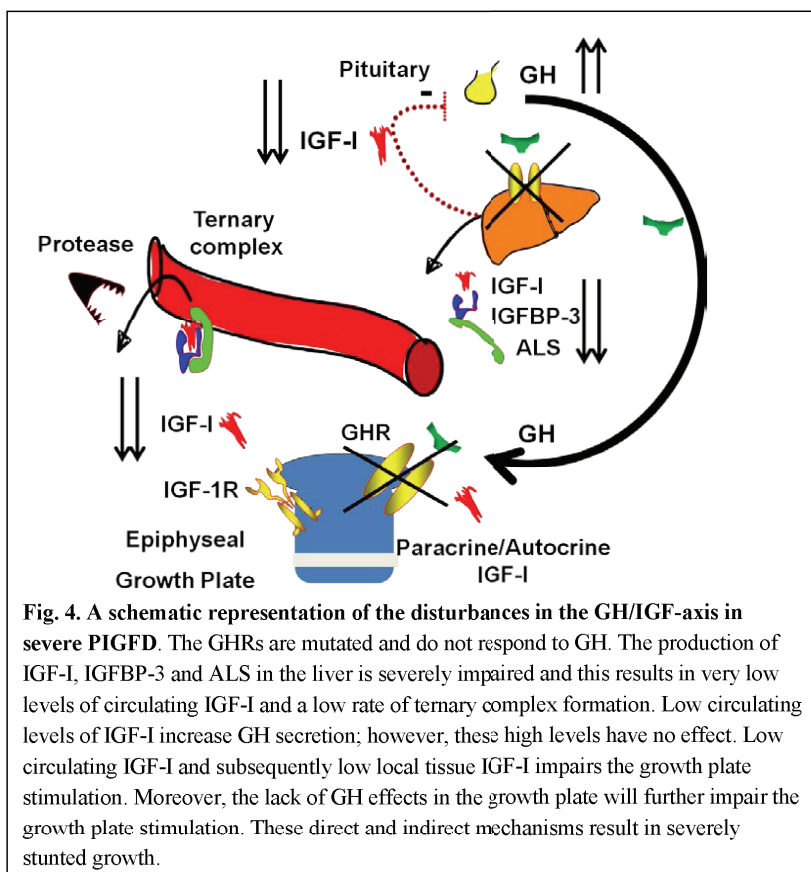
Laron’s syndrome stems from a defective GHR. GHIS is a wider term including all the different diseases showing unresponsiveness to GH. However, according to the identification of new molecular defects in the GH/ IGF-I axis, a new classification dividing the defects into primary IGF-I deficiency (PIGFD) and secondary IGF-I deficiency (SIGFD) has been proposed (Rosenfeld 2005; Rosenfeld 2006; Rosenfeld 2007). PIGFD includes diseases affecting the GH signal pathway from the GH receptor (outer part, transmembrane, and intracellular part) via the intracellular signaling cascade to defects in the IGF-I gene and IGF-1 receptor (IGF-1R), and SIGFD includes diseases involved in GH production and release.

2.12.2 Clinical appearance

Children with GHR defects have the same clinical features as children with severe GHD apart from high circulating GH levels and are characterized by short stature, frontal bossing, hypoplasia of the midface, and a pudgy appearance with increased subcutaneous fat tissue. In spite of high circulating GH levels, the circulating levels of IGF-I, IGFBP-3, and ALS are very low and GH treatment is ineffective in stimulating linear growth (Savage, Burren et al. 2001). The first patients (three siblings) were described by Laron and co-workers in 1966 (Laron, Pertzalan et al. 1966). In 1984 a GHR defect was demonstrated as the cause of the disease (Eshet, Laron et al. 1984) and in 1989 the molecular basis was elucidated in the form of a large deletion in the GHR

in two patients (Godowski, Leung et al. 1989). Today more than 250 patients with PIGFD have been described and ~ 60 different GHR mutations, most inherited in an autosomal recessive manner and a few in a dominant negative manner (Laron 2004; Rosenfeld 2007).

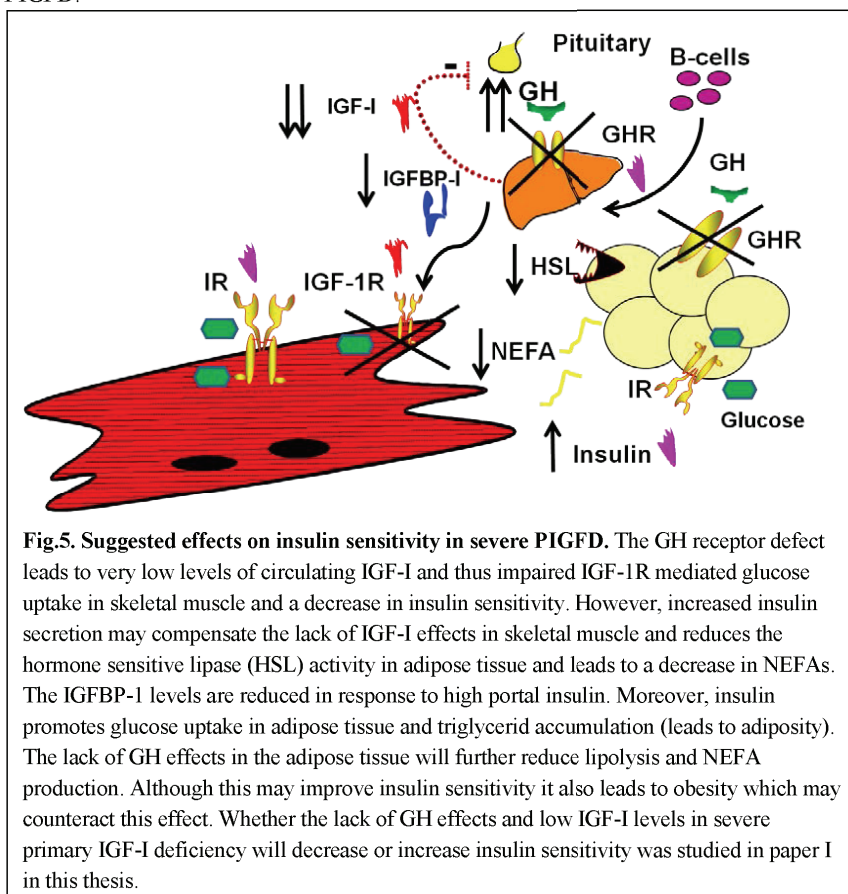
2.12.3 The GH/IGF-I axis in GHR defects



2.12.3.1 GHR defects and linear growth

Patients with GHR defects lack the growth-promoting (Fig.4) and metabolic actions (Fig. 5) mediated by growth hormone and the data support a fundamental role of GH that cannot be fully replaced by rhIGF-I (Isaksson, Lindahl et al. 1987; van der Eerden, Karperien et al. 2003; Wang, Zhou et al. 2004; Kaplan and Cohen 2007). At birth the length and weight of these infants are slightly below average; however, they rapidly lose growth velocity and, if untreated, their height deficit ranges between 4 and 10 SDS below the mean. Final heights (range) for untreated patients in the Israeli cohort were, males (116–142 cm) and females (108–136 cm), respectively (Laron, Lilos et al. 1993; Laron and Klinger 1994). Trials with rhIGF-I were started in the late 1980s (Rosenbloom 2007) and an initial growth response of 8.0 cm/yr during the first year, with a subsequent decline to approximately 5 cm/yr in the following years, has been

demonstrated in several trials (Ranke, Savage et al. 1995; Guevara-Aguirre, Rosenbloom et al. 1997; Backeljauw and Underwood 2001; Chernausek, Backeljauw et al. 2007). Unfortunately, most manufactures stopped production of rhIGF-I in the late 1990s due to the lack of a commercial market. In 2002 an equimolar complex of rhIGF-I and rhIGFBP-3 (rhIGF-I/rhIGFBP-3) became available for treatment in PIGFD patients on a research basis. The rationale was to obtain higher and stable IGF-I serum levels in the circulation (Camacho-Hubner, Rose et al. 2006), thus making once daily injections possible, avoiding post-injection hypoglycemia, and promoting better growth (Savage, Blum et al. 1993; Chernausek, Backeljauw et al. 2007; Williams, McDonald et al. 2008). However, few data have been reported on the clinical effect obtained (Tonella, Fluck et al. 2010) and, due to legal issues, rhIGF-I/rhIGFBP-3 was withdrawn from the field of growth research and since 2007 rhIGF-I has been approved for PIGFD.



2.12.3.2 GHR defects and insulin sensitivity

Whether the lack of direct and indirect effects (low IGF-I levels) of GH on the metabolism will increase or decrease insulin sensitivity has not been fully explored (Fig.5). A lack of GH effects (Yakar, Setser et al. 2004; Lebrun and Van Obberghen

2008; Vijayakumar, Novosyadlyy et al. 2009) decreases lipolysis and the availability of glycerol for gluconeogenesis and therefore should improve insulin sensitivity. However, at the same time, a loss of circulating IGF-I will decrease glucose utilization in skeletal muscles and decrease insulin sensitivity.

Infants and children with GHR defects are prone to have spontaneous hypoglycemia (Brain, Hubbard et al. 1998; Laron 2004). During puberty there is a poorly understood transition to adult abdominal obesity, hyperinsulinemia, and insulin resistance (Laron and Klinger 1993; Laron, Avitzur et al. 1995; Laron, Ginsberg et al. 2006). In adults with GHR defects rhIGF-I treatment decreased the insulin concentration. However, the reduction in insulin concentrations increased HGP and, in addition, increased lipolysis (less HSL activity) and subsequently further increased HGP. Protein synthesis was increased and, together, rhIGF-I improved body composition by increasing lean body mass and reducing fat mass (Mauras, Martinez et al. 2000). Moreover, Chernausek and co-workers have demonstrated reduced fat mass in children with GHR defects during short-term treatment, although the long-term effects of rhIGF-I may be the opposite (Backeljauw and Underwood 2001; Chernausek, Backeljauw et al. 2007).

2.13 TYPE 1 DIABETES (ACQUIRED IGF-I DEFICIENCY)

2.13.1 Background

Type 1 diabetes is an autoimmune disease (Bottazzo, Cudworth et al. 1978) that destroys the insulin producing β -cells in the pancreas. The scientific support of a multi-etiological origin is overwhelming, with a genetic predisposition as the predominant cause along with environmental risk factors (virus infections, psychosocial stress, dietary factors, gestational/perinatal factors, the hygiene hypothesis and, finally, early weight gain and increased linear growth (accelerator hypothesis) (Therlund, Dahlquist et al. 1995; Dahlquist, Patterson et al. 1999; Virtanen, Laara et al. 2000; Ilonen, Sjoroos et al. 2002; Yin, Berg et al. 2002; Viskari, Ludvigsson et al. 2005). The incidence rate of childhood type 1 diabetes has continued to rise across Europe by an average of approximately 3–4% per annum (Patterson, Gyurus et al. 2012), and the steep rise in children under than 5 years of age has been of particular concern (Gale 2002). However, a late report in 2011 from the Swedish Incidence Register shows a promising decline in the incidence in the youngest group since year 2000 (Berhan, Waernbaum et al. 2011).

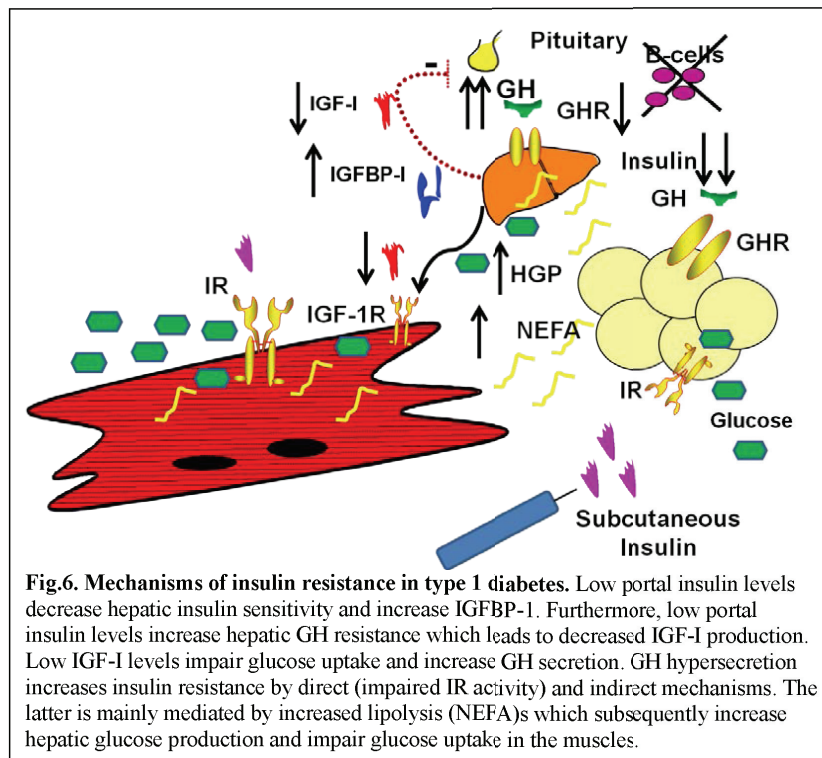
2.13.2 Perturbations in the GH/IGF-I axis in type 1 diabetes

Disturbances in the endocrine GH/IGF-I axis in children and adolescents with T1DM are well documented (Dunger and Cheetham 1996; Hanaire-Broutin, Sallerin-Caute et al. 1996; Bereket, Lang et al. 1999). Hepatic GH resistance and subsequent perturbations in the GH/IGF-I axis are due to low insulin levels in the portal vein in T1DM. Low portal insulin concentrations impair hepatic GH receptor/post receptor function (Daughaday, Phillips et al. 1976; Baxter, Bryson et al. 1980; Maes, Underwood et al. 1986; Clayton, Holly et al. 1994; Hanaire-Broutin, Sallerin-Caute et al. 1996) and decreases IGF-I gene expression and circulating IGF-I levels (Amiel, Sherwin et al. 1984; Taylor, Dunger et al. 1988; Zachrisson, Brismar et al. 1997; Hedman, Frystyk et al. 2004), which, by negative feedback, increases GH secretion in

T1DM (Edge, Matthews et al. 1990; Batch and Werther 1992; Pal, Matthews et al. 1993; Halldin, Tylleskar et al. 1998). Furthermore, portal insulin deficiency in T1DM increases hepatic IGFBP-1 production (Hall, Johansson et al. 1989; Brismar, Fernqvist-Forbes et al. 1994; Lepore, Pampanelli et al. 2000), which is suggested to decrease the bioactivity of endocrine IGF-I. IGFBP-1 has a circadian rhythm and the highest levels are seen in the morning (Yagasaki, Kobayashi et al. 2009).

Starting insulin therapy in new-onset T1DM will effectively increase circulating and free IGF-I levels, although not into the normal range (Bereket, Lang et al. 1995; Bereket, Lang et al. 1996). Intra-peritoneal insulin infusion has been shown to improve GHBP levels, indicating less GH resistance, and nearly normalized circulating IGF-I levels (Hanaire-Broutin, Sallerin-Caute et al. 1996). However, complete restoration of the GH/IGF-I axis disturbances has only been reported in an experimental setting using intraportal insulin administration (Shishko, Dreval et al. 1994).

2.13.3 Insulin resistance in type 1 diabetes



Hyperinsulinemic euglycemic clamp studies have demonstrated increased insulin resistance in T1DM (Amiel, Sherwin et al. 1986; Donga, van Dijk et al. 2013), whereas CSII or an addition of metformin resulted in improved insulin sensitivity (Simonson, Tamborlane et al. 1985; Samblad, Kroon et al. 2003).

Hypersecretion of GH in T1DM stimulates lipolysis and gluconeogenesis and increases HGP (Fig.6). In addition, GH has direct negative effects on insulin signaling, thus

increasing insulin resistance (Press, Tamborlane et al. 1984; Jones and Clemmons 1995; Dominici, Argentino et al. 2005; Vijayakumar, Novosyadlyy et al. 2009). IGF-I deficiency results in a reduced glucose uptake in the muscles and insulin resistance (Guler, Zapf et al. 1987; Dohm, Elton et al. 1990; O'Connell and Clemmons 2002; Simpson, Jackson et al. 2004). Furthermore, portal insulin deficiency increases HGP by direct and indirect mechanisms (Sindelar, Chu et al. 1998; Cherrington 2005). Moreover, the portal insulin deficiency increases IGFBP-1 production, and thereby decreases bioactive IGF-I in the circulation and, hypothetically, in the target tissues. These mechanisms act in concert and result in increased insulin resistance especially during adolescence.

2.13.4 HbA1c

HbA1c determinations (glycosylated hemoglobin) comprise an integrated measure mirroring mean blood glucose levels for approximately 3 months and provide a tool that makes inter- and intra-individual comparisons possible (Derr, Garrett et al. 2003; Dagogo-Jack 2010). The Diabetes Control and Complication Trial (DCCT) standard used to be the reference method; however, the standards differed between countries and thus presented an obstacle to comparisons. In Sweden, the Mono-S method was used until 2010 and the values were approximately 1% below the DCCT reference. However, as of the first of January, 2010, a new (IFCC) standard expressing HbA1c in mmol/mol was implemented (Landin-Olsson, Jeppsson et al. 2010).

2.13.5 Puberty and T1DM

HbA1c often deteriorates during puberty with an increased risk of both short- and long-term complications. This is of course related to psychological aspects of adolescence (Viklund and Wikblad 2009); however, the importance of the increased insulin resistance during puberty must not be underestimated.

2.13.6 Linear growth in T1DM

Stunted height used to be a common problem in children developing T1DM before completed linear growth (Tattersall and Pyke 1973). In its utmost state, patients developed Mauriac syndrome (short stature, obesity, and hepatomegaly) (Guest 1953). However, nowadays intensified insulin treatment has changed this scenario and children with T1DM reach their mean parental height (Lebl, Schober et al. 2003).

2.13.7 Treatment of T1DM in children and adolescents

The seminal trial in T1DM, the DCCT, included both adults and adolescents and demonstrated that any decline in HbA1c reduced the risk of microvascular complications in T1DM (DCCT 1993). Although advanced complications are rare in pediatric patients, the demonstration of a “glycemic memory” in follow-up studies mandates the striving for meticulous metabolic control from the start of treatment in children and adolescents (DCCT 1994; White, Cleary et al. 2001). Furthermore, intensive insulin treatment was shown to preserve endogenous insulin production (DCCT 1998). However, the major concern in the intensive insulin treatment group was a nearly threefold increase in severe hypoglycemia related to the shortcomings of intermediate-acting insulin such as NPH insulin (DCCT 1994).

2.13.7.1 *Intermediate-acting insulin, NPH*

NPH insulin consists of a non-covalent complex of human insulin and protamine, i.e. neutral protamine Hagedorn (NPH) insulin. A major disadvantage is the crystal suspension that has to be mixed homogeneously before injection, thus explaining the great day-to-day variability in effect. One of the main issues in NPH insulin treatment is to achieve a sufficient level of insulin in the morning hours in order to prevent the dawn phenomenon and at the same time avoid nocturnal hypoglycemia with the risk of unconsciousness (Edge, Matthews et al. 1990). The dawn phenomenon consists of high morning glucose levels related to late-night insulin resistance mediated by counterregulatory hormones such as GH (Schmidt, Hadji-Georgopoulos et al. 1981; Perriello, De Feo et al. 1990). Because of the fear of becoming unconscious, patients, and especially adolescents, are unwilling to increase the NPH insulin dose sufficiently. In addition, the waning insulin levels during the late night hours will decrease hepatic insulin sensitivity, increase IGFBP-1 levels, and further reduce free IGF-I (Lepore, Pampanelli et al. 2000; Yagasaki, Kobayashi et al. 2009). To sum up, the encouraging data from the DCCT underlined a need for better insulin regimens with long-acting insulin analogs or CSII in the efforts to further improve HbA1c without increasing the risk of hypoglycemia.

2.13.7.2 *Long acting analogs*

During the last decade two long-acting insulin analogs (insulin glargine and insulin detemir) have been approved for treatment in children (Rachmiel, Perlman et al. 2005). In comparison with NPH insulin, both have a more flattened and peakless action profile (Lepore, Pampanelli et al. 2000; Heise, Nosek et al. 2004; Regan and Dunger 2006). In addition, they show less day-to-day variation, comparable to CSII, and their working profiles allow once daily injection (Danne, Lupke et al. 2003). Moreover, studies have reported beneficial effects of insulin glargine and CSII on IGFBP-1, free IGF-I, and fasting glucose (Yagasaki, Kobayashi et al. 2009; Yagasaki, Kobayashi et al. 2010).

2.13.7.2.1 Insulin glargine structure

Insulin glargine is synthesized by a recombinant DNA technique and human insulin has been modified by adding two arginine molecules at the B-chain (position B30) and a substitution of glycine at the A-chain (position A21), thus changing the soluble properties (Rachmiel, Perlman et al. 2005). The acidic preparation (pH 4.0) is a solution and precipitates at neutral pH in the subcutaneous tissue, thus allowing a prolonged absorption with little peak activity (Lepore, Pampanelli et al. 2000). A limitation in studies on insulin glargine has been the lack of accurate insulin assays capable of discriminating different insulin analogs from each other and from human insulin.

2.13.7.2.2 Insulin glargine IR and IGF-1R binding

Concerns have been raised regarding the binding and activation properties of insulin glargine and IR and IGF-1R. Insulin glargine has been shown to bind and activate the IR and promote the same metabolic potency as human insulin (Kurtzhals, Schaffer et al. 2000; Ciaraldi, Carter et al. 2001). *In vitro* results in a malignant cell line raised concern as to whether insulin glargine, by prolonged activation of IGF-1R, induced mitogenic effects *in vivo* (Kurtzhals, Schaffer et al. 2000). However, when using

primary human cell cultures and “*in vivo* concentrations” of insulin glargine, the data do not support this view (Bahr, Kolter et al. 1997; Chisalita and Arnqvist 2004; Le Roith 2007). In a recent report, Sommerfeld *et al.* reported that insulin glargine is converted to a large extent to the main metabolites (M1 and M2) with similar mitogenicity to human insulin in malignant cell lines (Sommerfeld, Muller et al. 2010). However, in a report favoring a minor importance of glargine-IGF-1R interaction, Slawik *et al.* demonstrated in T1DM patients that insulin glargine did not suppress circulating IGF-I levels by activation of IGF-1R and downregulated GH secretion. By contrast, the IGF-I levels were increased (Slawik, Schories et al. 2006).

2.13.7.2.3 Insulin glargine - Clinical effects

A recently published meta-analysis disclosing the effect of long-acting insulin analogs (insulin glargine and insulin detemir) reported significant, but minor, effects on both HbA1c and a reduced risk of severe hypoglycemia (Monami, Marchionni et al. 2009). In children and adolescents, the reported effects of insulin glargine on HbA1c, hypoglycemia, and the body mass index (BMI) differ according to the study design (Rachmiel, Perlman et al. 2005). Retrospective trials in children and adolescents comparing insulin glargine and NPH insulin have reported a decline in HbA1c (Chase, Dixon et al. 2003; Hathout, Fujishige et al. 2003; Salemyr, Bang et al. 2011). In prospective RCT trials, a decrease in HbA1c has not been demonstrated while uncontrolled trials have suggested a lowering of HbA1c (Schober, Schoenle et al. 2002; Murphy, Keane et al. 2003; Alemzadeh, Berhe et al. 2005; Colino, Lopez-Capape et al. 2005; Chase, Arslanian et al. 2008). The most striking beneficial effect of insulin glargine treatment in children and adolescents, noted in some, but not all, trials, is a reduction in severe hypoglycemia (Murphy, Keane et al. 2003). Moreover, most studies do not report any significant changes in BMI in children and adolescents (Chase, Dixon et al. 2003; Alemzadeh, Ellis et al. 2004).

2.13.7.3 Continuous subcutaneous insulin infusion

Continuous subcutaneous insulin infusion (CSII) has been a treatment option in T1DM for more than 30 years and constitutes a unique opportunity to achieve an optimal insulin delivery according to the different physiological insulin needs in children and adolescents (Tamborlane, Sikes et al. 2006). CSII has been reported in some RCTs to improve metabolic control in children with type 1 diabetes mellitus (de Beaufort, Houtzagers et al. 1989; Doyle, Weinzimer et al. 2004), but not in other ones (Weintrob, Benzaquen et al. 2003; Fox, Buckloh et al. 2005; Skogsberg, Fors et al. 2008). A meta-analysis of RCTs in children reported a small positive effect on HbA1c in CSII vs. multiple daily insulin (MDI) therapy (Pankowska, Blazik et al. 2009). Furthermore, several studies in children and adolescents have reported a decreased incidence of severe hypoglycemia (Boland, Grey et al. 1999; Doyle, Weinzimer et al. 2004).

2.13.7.4 Recombinant human IGF-I treatment

RhIGF-I as an adjunct to insulin have been studied in several short and long-term trials demonstrating promising and favorable effects in T1DM. Long-term studies demonstrated significant improvement in HbA1c (Cheetham, Holly et al. 1995; Acerini, Patton et al. 1997; Quattrin, Thraillkill et al. 1997; Quattrin, Thraillkill et al. 2001), decreased insulin requirements (Cheetham, Holly et al. 1995; Carroll, Umpleby et al. 1997; Quattrin, Thraillkill et al. 1997), and decreased IGFBP-1 levels (Thraillkill, Quattrin et al. 1997). Short-term overnight studies demonstrated reduced GH secretion

(Cheetham, Clayton et al. 1994; Cheetham, Connors et al. 1997), reduced HGP (Acerini, Harris et al. 1998), and improved insulin sensitivity (Cheetham, Jones et al. 1993; Cheetham, Connors et al. 1997). However, the largest conducted rhIGF-I study used high doses (up to max. 140 µg/kg) and reported unacceptable side effects (Thraikill, Quattrin et al. 1999; Quattrin, Thraikill et al. 2001). Whether the worsening of retinopathy was a secondary phenomenon related to the “normoglycemic re-entry”, well known in patients starting intensified insulin regimens (DCCT 1993), or to a direct effect of IGF-I in the eye (Grant, Mames et al. 1993) has not been fully elucidated. Thus, despite the well- documented beneficial effects with a lower dose (40 µg/kg and day), there is a current hold on further exploration of this promising treatment for T1DM.

2.13.8 Complications

2.13.8.1 Acute complications

Short-term complications in T1DM include hypoglycemia and ketoacidosis. On a daily basis, mild hypoglycemia is frequent and unavoidable. However, the main worry and fear in many patients and families, apart from long-term vascular complications, is severe hypoglycemia, which may compromise the quality of life and worsen diabetic control (Clarke, Gonder-Frederick et al. 1998; Nordfeldt and Jonsson 2001).

2.13.8.2 Long-term complications and the GH/IGF-I axis

The well-known long-term microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular complications seen in T1DM are a tremendous burden on the patients. The role of the GH/IGF-I axis in the vascular disease is not fully understood. Patients with manifest T1DM and subsequent pituitary damage and GH deficiency show markedly improved retinopathy (Poulsen 1953). Whether the role of GH was direct or indirect and mediated by a decrease in tissue IGF-I, is not clear. Sonksen *et al.* presented a hypothesis 20 years ago linking GH hypersecretion and low circulating IGF-I levels seen in T1DM to vascular complications (Sonksen, Russell-Jones et al. 1993). They argued that the imbalance between peripheral-portal insulin concentrations is the crucial mechanism. High peripheral insulin levels (an inevitable consequence of subcutaneous insulin injections) in the state of GH hypersecretion may predispose to autocrine/paracrine overproduction of IGF-I and thus stimulate endothelial and smooth muscle cell proliferation in the capillary walls (Johansson, Chisalita et al. 2008). The expression of IGF-1R and hybrid receptors in capillary walls might constitute a link to the IGF-I effects on the proliferation and migration in states of hyperglycemia, as reported by Clemmons *et al.* (Clemmons, Maile et al. 2007). Hitherto, no method has been validated for determining tissue levels of IGF-I and thus further exploring the hypothesis.

3 HYPOTHESIS AND AIMS

Hypothesis

Severe primary IGF-I deficiency (in patients with a GHR defect) and acquired IGF-I deficiency (in patients with type 1 diabetes) affect glucose metabolism and are associated with decreased insulin sensitivity. Administration of rhIGF-I increases circulating and tissue levels of IGF-I and improves insulin sensitivity. In acquired IGF-I deficiency, treatment with the long-acting insulin analog glargine or continuous subcutaneous insulin infusion increases IGF-I, suppresses GH, decreases IGFBP-1 in the circulation (an inverse measure of hepatic insulin sensitivity), conserves endogenous insulin secretion, and improves HbA1c.

Specific aims

Paper I

To demonstrate that severe primary IGF-I deficiency is associated with insulin resistance, increased fat mass, decreased lean body mass, and poor linear growth and that rhIGF-I treatment improves insulin sensitivity, decreases fat mass, increases lean body mass, and increases height velocity. In addition, to study the pharmacokinetics and biological actions of rhIGF-I compared with rhIGF-I/rhIGFBP-3 combo administration.

Paper II

To demonstrate that treatment with insulin glargine in acquired IGF-I-deficient adolescents increases IGF-I, decreases GH and IGFBP-1, and improves HbA1c, as compared to treatment with NPH insulin.

Paper III

To demonstrate that treatment with continuous subcutaneous insulin infusion in acquired IGF-I-deficient children and adolescents from the initial diagnosis of type 1 diabetes increases circulating IGF-I, decreases IGFBP-1, and preserves endogenous insulin production as compared to treatment with NPH insulin.

Paper IV

To demonstrate that administration of rhIGF-I in acquired IGF-I-deficient young adult males increases tissue IGF-I in muscles and subcutaneous fat as determined by microdialysis and results in increased whole body glucose uptake and, in addition, to study the pharmacokinetics of rhIGF-I administration.

4 SUBJECTS AND METHODS

Paper	n	Age (yr)	HbA1c (%)	BMI (SDS)	Diabetes duration (yr)	Sex	Reported Insulin (IU/kg)	Pre-pubertal (n)	Pubertal (n)
I	2	AA:14.4 BB:12.2		AA 0.60 BB 0.25		2 boys		BB	AA
II	12	12.7(11.1-15.0)	8.3±0.6	-0.2(-0.51-0.51)	3.1(1.0-6.0)	8 girls 4 boys	1.1(0.5-2.1)		B2-B3 G2-G3
III	MDI (38) CSII (34)	12.3±4.5	8.4±0.5	0.45(-0.4-1.4)	0	16 girls 22 boys	0.85±0.15	15	23
		11.8±4.9	8.2±0.4	0.70(0.4-1.7)	0	14 girls 20 boys	0.74±0.10	12	22
IV	8	19.2(18.3-20.9)	8.0(6.7-10.4)	0.41(-0.49-2.34)	9.0(6.4-11.8)	8 boys	-		G5

Table 1. Subject characteristics at baseline. Data are given as the range (age and duration) and as mean values ± SEM when normally distributed, otherwise as the median and 25th–75th percentiles. HbA1c (MonoS standard).

4.1 SUBJECT SELECTION

4.1.1 Paper I

We studied two unique severe primary IGF-I deficient subjects who were compound heterozygous for two mutations in the extracellular GH binding part of the GHR (Enberg, Luthman et al. 2000) (Table 1.). They have been followed at our clinic since 1993 because of extreme short stature. Prior to this study, they were on rhIGF-I treatment for 8 years (1994 to 2002) with short periods off treatment due to a lack of drug supply. In the study, they were examined at the age of 11.6 and 13.8 yrs, and were then off treatment for a longer period of 8 months when a rhIGF-I preparation (rhIGF-I/rhIGFBP-3) became available through a compassionate use programme (section 2.12.3.1).

Few human studies have been able to assess the direct effects of IGF-I on glucose metabolism. Most studies of rhIGF-I therapy are confounded by IGF-I-negative feedback on GH, which in turn displays marked effects on glucose metabolism and insulin sensitivity. Dunger and co-workers (Crowne, Samra et al. 1998; Simpson, Jackson et al. 2004) have attempted to separate the effects of IGF-I and GH on glucose metabolism induced by IGF-I administration by blocking GH secretion by somatostatin infusion. In this study, we assumed that GH effects on glucose metabolism and insulin sensitivity would not be affected by changes in GH secretion induced by rhIGF-I treatment, given the lack of GH effects in patients with a GHR defect.

4.1.2 Papers II-IV

In Papers II–IV, subjects with T1DM (acquired IGF-I deficiency) were studied.

4.1.2.1 Subjects in Paper II

It has been demonstrated that the disturbances in the GH/IGF-I axis are more pronounced in pubertal children (Bereket, Lang et al. 1999; Acerini, Williams et al. 2001). We therefore expected that the effects of the long-acting insulin analog, insulin glargine (recently approved at the time of the study) on the GH/IGF-I axis would be most pronounced in T1DM adolescents in mid-puberty. At the time of the planning of this study (2002), studies on the effects of insulin glargine on the GH/IGF-I axis were

lacking. At the same time, the clinical use of insulin glargine in the adolescent population was rapidly evolving in our clinic, with only a limited number of patients still on NPH basal insulin. In order to determine that long-acting insulin had effects on the GH/IGF-I axis and HbA1c, an exploratory study was planned, although a randomized controlled trial (RCT) or a randomized cross-over study would have been preferable. We recruited 12 adolescents, four boys and eight girls, with T1DM from our clinic and the subject characteristics are shown in Table 1. One female subject had a suspected viral infection at the 6-week admission and was excluded from calculations at that point in time.

4.1.2.2 Subjects in Paper III

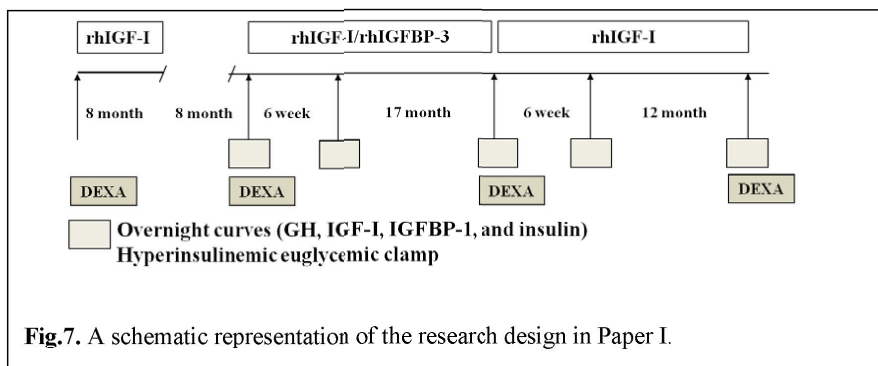
In Paper III, we studied subjects from the initial diagnosis of T1DM, who were randomized to MDI treatment with NPH insulin or CSII. Although the effects of different insulin regimens on the GH/IGF-I axis are expected to be more pronounced in children lacking endogenous insulin secretion, we wanted to examine the effects during the first years of treatment on HbA1c, as it has been reported to be of pivotal importance for metabolic control in subsequent years (White, Cleary et al. 2001). Data on HbA1c and treatment satisfaction were reported in 2008 by Skogsberg *et al.* (Skogsberg, Fors et al. 2008). In addition, we wanted to examine whether there were different effects of these treatments on endogenous insulin secretion and on the GH/IGF-I axis. In addition to pubertal subjects, prepubertal school children with T1DM were also studied. We considered that subjects ≥ 7 years of age were able to give informed consent. A total of 72 children and adolescents with newly diagnosed T1DM from 9 participating clinics were recruited (subject characteristics are shown in Table 1).

4.1.2.3 Subjects in paper IV

Although the major disturbances in the GH/IGF-I axis are seen in adolescence, the experimental and invasive nature of this study influenced us to study young adults. Males were chosen because it is easier to insert microdialysis catheters in males with a larger muscle mass and less subcutaneous fat than females. Eight young adult males with T1DM were enrolled in this study, all formerly followed at out unit. The baseline characteristics of the subjects are shown in Table 1. One subject withdrew at his own request after the first half of the study (after receiving saline) and his data were not included in the analysis.

4.2 METHODS – RESEARCH DESIGN

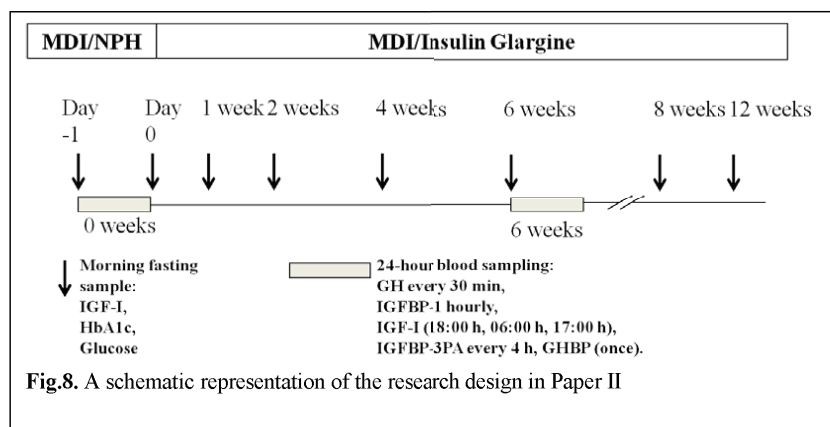
4.2.1 Research design in Paper I



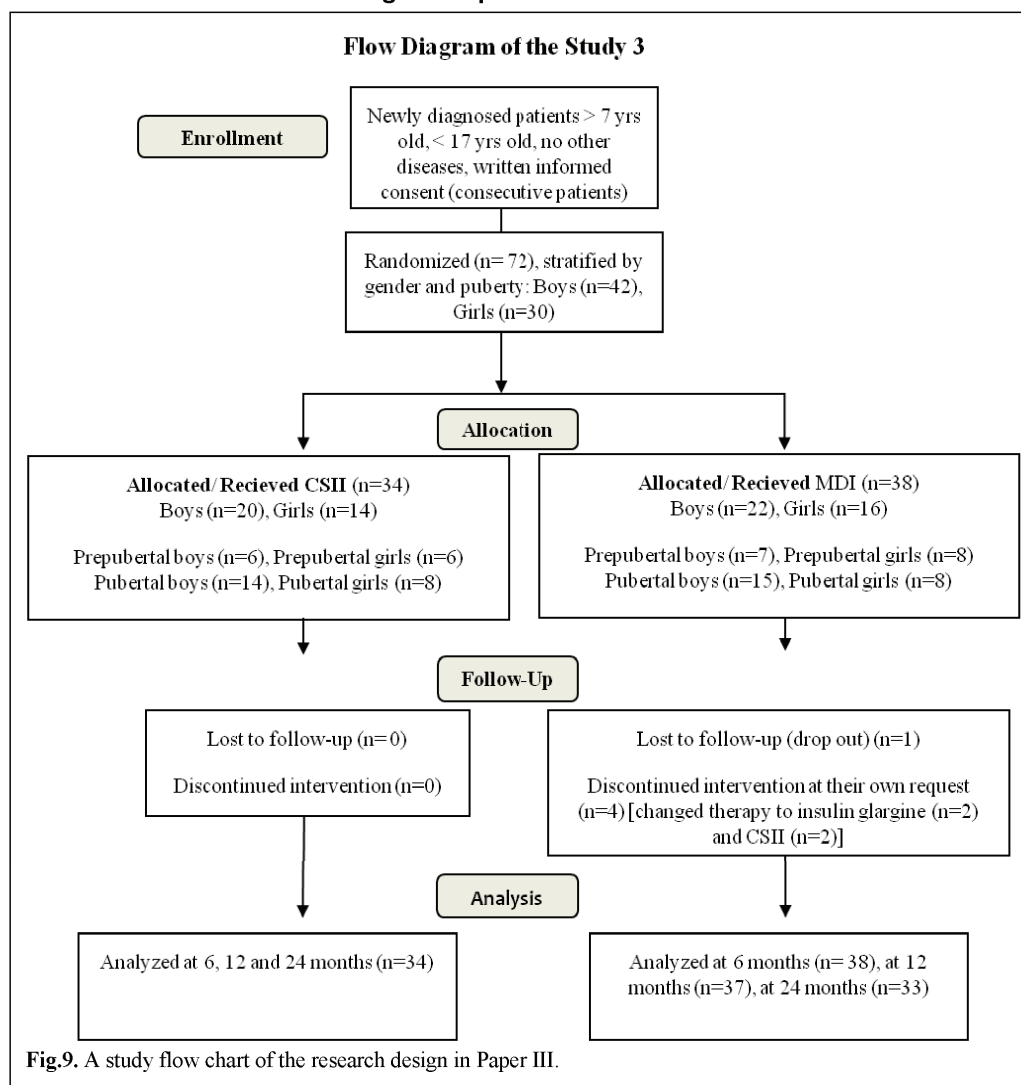
This was an observational study on linear growth, body composition, and insulin sensitivity in two subjects with a GHR mutation. After 8 months without treatment and at an age of 14.4 and 12.2 yrs, respectively, they were started on treatment with Somatokine®, a 1:1 molar complex of rhIGF-I and rhIGFBP-3 (rhIGF-I/rhIGFBP-3) given by s.c. injections once daily in a dose of $1 \text{ mg} \times \text{kg}^{-1} \times \text{day}^{-1}$ (equivalent to $200 \text{ } \mu\text{g} \times \text{kg}^{-1} \times \text{day}^{-1}$ of IGF-I) for 17 months. Three days after rhIGF-I/rhIGFBP-3 was stopped, treatment with s.c. rhIGF-I was started by s.c. injection twice daily in a dose of $200 \text{ } \mu\text{g} \times \text{kg}^{-1} \times \text{day}^{-1}$. Both subjects continued on rhIGF-I after the metabolic study period. Auxological data were obtained every 3 months. Bone age was assessed before and after 17 months on rhIGF-I/rhIGFBP-3 and after the following two and three years on rhIGF-I. Briefly, the subjects were admitted to our clinical research unit on five occasions for metabolic studies. Following admission, two indwelling catheters were inserted in the antecubital veins or on the dorsal side of the hand. Blood samples for 12-hour overnight hormone profiles were obtained every 30 minutes from 20:00 h until 07:30 h. The following morning a 2-hour hyperinsulinemic euglycemic clamp was performed. Body composition was determined by dual-energy X-ray absorptiometry (DEXA) on four occasions.

4.2.2 Research design in Paper II

This was an observational study in which each subject was his/her own control. A schematic representation of the study design is presented in Fig.8. Briefly, each subject was admitted to our clinical research unit during 26 h (16:00 h to 18:00 h + 1 day) before the start of insulin glargine (0 week) and after 6 weeks on insulin glargine. In addition, they were studied in the morning fasted state after 1, 2, 4, 8, and 12 weeks and advised to change the insulin doses to optimize their metabolic control. At the first admission, all subjects were on MDI (NPH insulin twice daily and insulin lispro or aspart at meals). Following admission, two indwelling catheters were inserted and blood samples were obtained at (18:00 h) and then every 30 minutes from 21:00 h to 17:00 h the next day. Hormone determinations between 00:00 h and 10:00 h were defined as overnight. The initial once daily insulin glargine dose was given before dinner after the first 24-hour admission.



4.2.3 Research design in Paper III



Study III was an open, randomized, parallel, multicenter study with nine participating pediatric departments in Sweden. A study flowchart is presented in Fig. 9. Briefly, newly diagnosed children and adolescents with T1DM were enrolled at a median (range) time of 11 days (1–21) from the diagnosis. At inclusion and after 6, 12, and 24 months, morning fasting blood samples for IGFBP-1, IGF-I and C-peptide determinations were obtained.

4.2.4 Research design in Paper IV

This was a randomized single-blind, placebo-controlled cross-over study of the effects of s.c. rhIGF-I administration on the glucose infusion rate (GIR), a measure of

whole-body glucose utilization, and interstitial muscle and adipose tissue IGF-I concentrations under euglycemic clamp conditions (Fig.10) Briefly, each subject was studied twice and randomized to receive a s.c. injection of rhIGF-I (120 µg/kg) or saline (0.1 ml). The s.c. injection was given in the abdominal fat tissue. Strenuous exercise was avoided and glucose control was optimized by CSII for at least 48 h before each study day. The two study periods were separated by a median interval of 12 (7–43) days. Subjects were admitted to the hospital at 08.00 h after over-night fasting (from 10.00 h). After application of a local anesthetic cream, two indwelling cannulas were inserted, one into the distal forearm vein for blood sampling and one into the antecubital fossa vein for continuous insulin and glucose infusion. Three microdialysis catheters were inserted as outlined below. CSII was stopped and a normoinsulinemic euglycemic clamp was started and monitored by frequent bedside glucose measurements. A one-hour run-in period was allowed to reach euglycemia (target plasma glucose, 5.0 mmol/l) and calibrate the microdialysis catheters. Collection of the microdialysate was then commenced, using 1-h fractions, whereas blood was sampled every 30 min for determinations of IGF-I, insulin, and GH (every 30 min), and IGFBP-1 and cortisol (hourly). After completing the study day, subjects started their usual insulin therapy and received a meal. They were observed for at least one more hour and instructed to perform frequent self-monitoring of blood glucose.

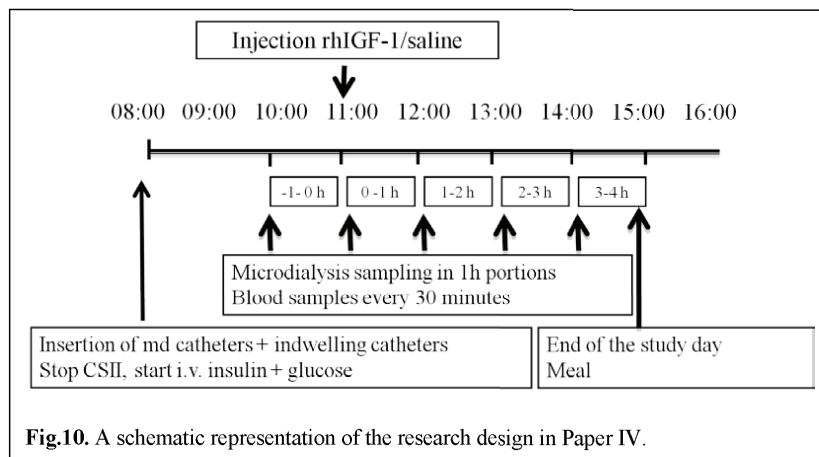


Fig.10. A schematic representation of the research design in Paper IV.

4.2.5 Methods - Analytic approaches

4.2.5.1 Insulin sensitivity

The clamp technique assesses *in vivo* insulin sensitivity. However, it is invasive, costly and labor-intensive and thus often not applicable in large-scale studies. Therefore, simple surrogate estimates such as the Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) and the Quantitative Insulin Sensitivity Check Index (QUICKI) measuring fasting indices of insulin sensitivity have been developed (Rossner, Neovius et al. 2010). These measures cannot be used in T1DM. The minimal model analysis of the frequently sampled intravenous glucose tolerance test (FSIVGTT-MMOD) may have been an alternative in Study 1, but is not usable in T1DM where endogenous insulin production is lacking (Bergman, Prager et al. 1987)

Therefore, we used the hyperinsulinemic-euglycemic clamp technique in Study I and a modified normoinsulinemic version in Study IV. The hyperinsulinemic-euglycemic clamp method is considered to be the golden standard for determining insulin sensitivity and offers several advantages. It has a high reproducibility and, by clamping insulin at a high level, maximal peripheral glucose disposal can be determined, i.e. maximal insulin sensitivity. Insulin sensitivity is thus a measure of insulin responsiveness in different tissues and is calculated as glucose disposal ($\text{mg}/\text{kg} \cdot \text{min}^{-1}$) during a constant insulin infusion in a clamp setting.

Furthermore, both hepatic and peripheral insulin sensitivity in different tissues can be measured if stable isotopes of glucose and glycerol are used in a step-wise hyperinsulinemic clamp procedure (Arslanian 2005). In retrospect, the use of stable isotopes would have been an advantage in Paper I by providing data on the separate effects on lipolysis, hepatic insulin sensitivity, and maximal glucose disposal obtained by rhIGF-I treatment.

4.2.5.1.1 Hyperinsulinemic euglycemic clamp (Paper I)

In Paper I we performed a 2-hour hyperinsulinemic euglycemic clamp to determine insulin sensitivity by peripheral glucose disposal as originally described by DeFronzo *et al.* (DeFronzo, Tobin *et al.* 1979), with some modifications according Veening *et al.* (Veening, Van Weissenbruch *et al.* 2002). The glucose infusion rate (GIR) was used as a measure of insulin sensitivity and was calculated from the amount of glucose infused per minute during steady state between 60 and 120 min divided by body weight ($\text{mg} \times \text{kg}^{-1} \times \text{min}^{-1}$).

4.2.5.1.2 Normoinsulinemic euglycemic clamps (Paper IV)

In Paper IV we performed a normoinsulinemic euglycemic clamp. A primed infusion of insulin, 0.5 $\text{mU}/\text{kg} \times \text{min}$, Actrapid®, 1 IU/ml, was given. The lower rate of insulin infusion (0.72 IU/kg \times day) as compared to Paper I was chosen to slightly exceed the normal insulin needs in well-controlled post-pubertal type 1 diabetic patients, thus allowing euglycemia (5 mmol/l) to be maintained with a low variable glucose infusion (200 mg/ml). The basal glucose infusion is higher in an “original clamp” setting (1 $\text{mU}/\text{kg} \times \text{min}$). A rather low basal glucose infusion rate opened up space for the expected increase in glucose demand after the rhIGF-I injection and we assumed that this had not been possible to achieve in the original clamp setting (DeFronzo, Tobin *et al.* 1979; Veening, Van Weissenbruch *et al.* 2002) with much higher insulin concentrations and perhaps a saturated maximal glucose disposal. The advantage of studying T1DM subjects in the context of an IGF-I injection is that insulin levels are kept constant with the insulin infusion and in the absence of endogenous insulin (Guler, Zapf *et al.* 1987; Boulware, Tamborlane *et al.* 1994).

4.2.5.1.3 Insulin doses

The total insulin dose is a simple indirect measure of insulin sensitivity. In Paper II total insulin doses were recorded at each overnight admission (at baseline and after 6 w). In Paper III mealtime and basal insulin doses were obtained from a pump download (CSII group) and reported by the subjects in the MDI group. This is a limitation since the reported doses in the NPH group may be under- or more likely overestimated. Patients with high blood glucose levels and high HbA1c may over-report their doses

(have insulin omissions) and higher insulin doses are reported in registries for these groups (SWEDIABKIDS 2011). Moreover, high insulin doses may reflect increased insulin resistance and it is well known that improved metabolic control will decrease the required insulin doses.

4.2.5.1.4 IGFBP-1 (Papers I-IV)

We used IGFBP-1 as an indirect marker of hepatic insulin sensitivity (Paper II, and III). IGFBP-1 is directly regulated by portal insulin (Brismar, Fernqvist-Forbes et al. 1994) and has been used in a number of studies as marker of hepatic insulin effects in T1DM (Hall, Johansson et al. 1989; Lepore, Pampanelli et al. 2000). IGFBP-1 is also influenced by endogenous insulin secretion and hepatic action of injected insulin. Therefore, in T1DM, insulin doses need to be considered when interpretation of changes in IGFBP-1 is made. In Paper I the increase in IGFBP-1 was interpreted to be caused by the lower endogenous insulin secretion resulting in lower insulin levels. In papers II and III the suppression of IGFBP-1 was considered to be a result of an increased hepatic insulin sensitivity in the absence of endogenous insulin and in the face of a lower insulin dose with insulin glargine (tendency in paper II) and CSII (paper III).

4.2.5.1.5 IGF-I and hepatic GH insensitivity (Papers II, and III)

Serum IGF-I concentrations reflect the liver production of IGF-I and are regulated by portal insulin via its potentiation of the GHR number and post-receptor signaling (Daughaday, Phillips et al. 1976; Maes, Underwood et al. 1986; Hanaire-Broutin, Sallerin-Caute et al. 1996; Hedman, Frystyk et al. 2004). In Papers II and III we regarded IGF-I as an indirect measure of the GH sensitivity in the liver and assumed that an improved insulin delivery by the long-acting insulin glargine or CSII mediate an improved GHR function and increases circulating IGF-I. Therefore, in analogy with IGFBP-1, IGF-I is a marker of hepatic insulin sensitivity. However, IGFBP-1, is directly insulin-regulated (production and clearance) (Brismar, Fernqvist-Forbes et al. 1994), while the IGF-I production is indirectly dependent on hepatic insulin action and thus may be a less sensitive marker of hepatic insulin sensitivity.

4.2.5.2 *Overnight curves (Papers I, and II)*

Extended (24-h or overnight) hormone determinations are necessary to assess hormones with random secretory patterns, such as GH, or more reproducible diurnal rhythms, such as insulin and IGFBP-1. Circulating IGF-I does not display a diurnal rhythm in healthy children (Juil 2003); however, in patients with a GHR defect, the lack of IGFBP-3 and ALS decreases the half-life of circulating IGF-I (Grahnen, Kastrup et al. 1993). Therefore, in severe PIGFD patients treated with rhIGF-I the serum profiles of IGF-I vary markedly after injection. In Paper I we assessed overnight levels of GH, IGFBP-1, IGF-I, insulin, IGF-II, IGFBP-3, and ALS (IGF-II, IGFBP-3, and ALS were only determined on the first two occasions).

4.2.5.3 *Determinations of IGF-I (Papers I-IV)*

Total serum concentrations of IGF-I were measured in Papers I-IV and expressed either as absolute concentrations or SDS scores (Juil, Bang et al. 1994). We determined IGF SDS scores according to sex and age (Paper III) and to sex, age, and

pubertal stage (Papers I and II). In this thesis the total serum concentration of IGF-I is also referred to as circulating IGF-I. In Paper I we measured circulating IGF-I to determine the pharmacokinetic profiles of rhIGF-I/rhIGFBP-3 and rhIGF-I, and in Paper IV circulating IGF-I concentrations were compared to md-IGF-I. Determinations of serum free IGF-I (also referred to as free dissociable, bioavailable or bioactive IGF-I) are a research tool often reported in the literature. It measures the fraction of IGF-I that is not associated with binding proteins (or is loosely bound and released during the assay procedure). Various assays employing ultrafiltration, immunoassays with antibodies identifying only unbound IGF-I, or assays using cell IGF-1R phosphorylation (KIRA) have been developed and used (Frystyk 2004). Although performed in serum, these measurements have been claimed to reflect local tissue levels of IGF-I, but only circumstantial and no direct *in vivo* evidence for this has been presented.

4.2.5.4 Measurements of insulin concentrations (Papers I, and IV)

We measured insulin concentrations in papers I and IV during the euglycemic clamps to determine whether steady-state levels were achieved. Furthermore, in Paper I the overnight insulin concentrations were used as an indirect measurement of insulin sensitivity and the insulin effects on the liver were mirrored by the effects on IGFBP-1. In Paper II we evaluated an in-house insulin assay and its ability to cross-react with different insulin analogs (Bang, unpublished results). Large differences in potency among the insulin analogs and, even more problematic, a lack of parallelism of dilutions of these various analogs prevented us from performing such measurements. More recently, assay procedures to determine insulin analogs and their metabolites have been reported. However, there are no assay kits that fit all needs (insulin analogs).

4.2.5.5 Measurements of C-peptide (Paper III)

Mixed-meal tolerance test (MMTT) area under the curve C-peptide (AUC) is the gold standard measure of endogenous insulin production in T1DM. We were not able to determine endogenous insulin production by the MMTT in Paper III (too resource-demanding) and used fasting C-peptide as a proxy. However, in a recent study, Besser et al. demonstrated a strong correlation between AUC and fasting C-peptide (Besser, Shields et al. 2012).

4.2.5.6 Microdialysis (Paper IV)

In Paper IV, tissue IGF-I concentrations were determined by microdialysis (md IGF-I) in skeletal muscle and adipose fat tissue. We used a 20 kDa cut-off probe previously demonstrated to give reproducible and sufficiently high recovery of IGF-I in the microdialysate (md) ($16 \pm 6\%$ *in vitro* and $11 \pm 1\%$ *in vivo*) under resting conditions (Berg, Gustafsson et al. 2007). There have been no previous studies that assess how local tissue concentrations of IGF-I reflect biological actions. Insulin has been studied using the 20 kDa probes and both delivery and local measurements have been examined (Jansson, Fowelin et al. 1993). One potential problem with insulin and IGF-I is their adherence to plastic. In this study, as well as in previous studies, human albumin was added to the perfusion fluid in an attempt to diminish unspecific adherence of IGF-I to the probe and tubings. The advantage of the 20 kDa probe is that, hypothetically, only unbound IGF-I passes through the probe. We have previously demonstrated that only unbound IGF-I crosses the membrane of the md

catheter and that neither IGF-BPs nor their fragments are detectable in the microdialysate (Berg, Gustafsson et al. 2006) (Fig. 12). Tissue IGF-I concentrations (md-IGF-I) determined by microdialysis in the left and right musculus vastus lateralis (LMVL, RMVL) have been reported to agree (Berg, Gustafsson et al. 2007). A correlation was also found in Paper IV (Fig. 11) and there was a good correlation with abdominal subcutaneous fat (ASF) levels (previously unpublished data).

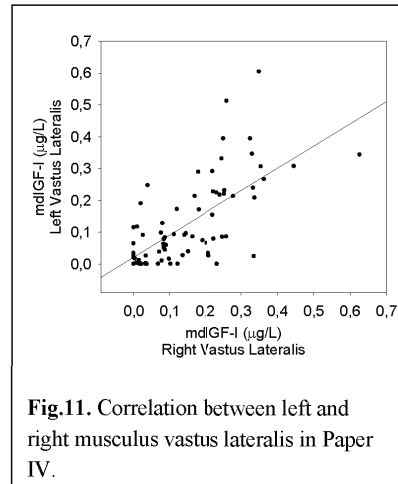
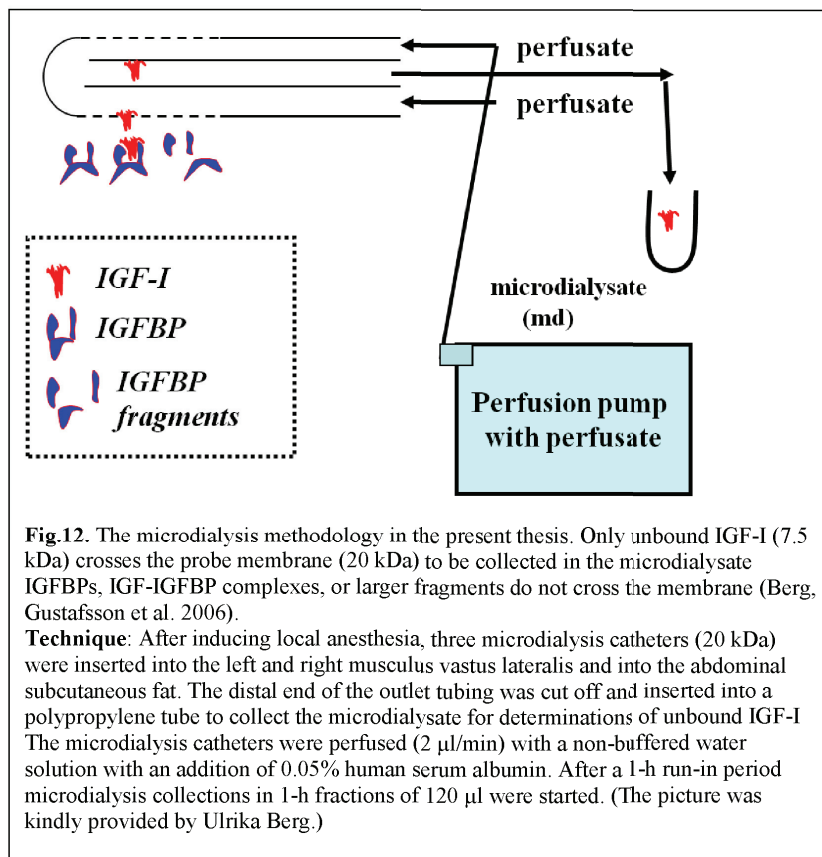


Fig.11. Correlation between left and right musculus vastus lateralis in Paper IV.



4.2.5.7 Blood analyses

A detailed description of the different assays is given in each paper.

4.3 ETHICS

All subjects and, if they were under 18 years of age, their parents gave their informed and signed consent. Studies I and II were approved by the Local Ethics Committee of the Karolinska Institute, Sweden. Study III was approved by the Local Ethics Committee at Uppsala University, Sweden, and registered as ISRCTN, trial no: CCT-NAPN-16461. Study IV was approved by the Central Ethical Review Board, Stockholm, and registered as Eudra CT no. 2011-000233-37. Studies I and IV were approved by the Swedish Medical Products Agency.

4.4 STATISTICS

Only descriptive statistics are presented in Paper I. In Papers II, III and IV data are given as the mean value and SEM when normally distributed (Paper II), otherwise as the median and 25th and 75th percentiles (Papers III and IV). Outcome variables with a skewed distribution were log-transformed (Paper III). Follow-up data were analyzed by one-way ANOVA using pairwise multiple comparison procedures (Student-Newman-Keul method) or Student's paired t-test was used when appropriate, and correlations were analyzed by linear regression (Paper II). Follow-up data were analyzed by two-way repeated measures, ANOVA (one factor repetition) with all pairwise multiple comparison procedures (Holm-Sidak method) (Papers III and IV); correlations were analyzed by multiple linear regression (Paper III). Sigma Stat 2.0 (Paper II) and Sigma Plot 11.0 (papers I, III, and IV), (SYSTAT Software, Inc, London, UK). P values < 0.05 were considered significant.

Paper	Descriptive statistics	T-test	ANOVA One way	ANOVA Two way	Linear regression	Multiple linear regression
I.	Baseline characteristics					
II.	Baseline characteristics	GH, IGFBP-1, BP-3PA, GHBP, Insulin-dose, Height, Weight, BMI	HbA1c, IGF-I, IGF-I SDS		IGF-I vs HbA1c, IGF vs insulin dose	
III.	Baseline characteristics			IGFBP-1, C-peptide, IGF-I SDS, Insulin dose		Log C-peptide vs IGF-I, Log C-peptide vs IGFBP-1
IV.	Baseline characteristics			md-IGF-I, IGF-I, GIR, Glucose, Insulin, IGFBP-1, GH, Cortisol		

Table 2: Statistic methods

5 RESULTS

5.1 PAPER I

5.1.1.1 *Linear Growth*

During an 8-month period without treatment, both subjects had a very low height velocity (HtV), (AA 0.7 cm/yr and BB 1.6 cm/yr). The annualized first-year HtV during rhIGF-I/rhIGFBP-3 was 3.5 cm/yr for AA and 2.8 cm/yr for BB, i.e. slightly improved compared to baseline. Compared to the height reference, there was a further loss in Ht SDS in both subjects. During the first year on rhIGF-I the HtV was almost doubled in both subjects (5.3 cm/yr in AA and 5.5 cm/yr in BB) compared to the first year on rhIGF-I/rhIGFBP-3, despite an equal weight-based dosing of IGF-I. At the time when paper VI was finalized (2009), AA had stopped treatment at the age of 20.3 years and a near-final height of 155.7 cm and BB (18.1 years old, HtV was 6.2 cm/yr and 154.3 cm) continued on rhIGF-I treatment (¹Paper I, Table 1 and Fig.1). At present (2013) both boys have stopped their treatment (AA 2009, and BB 2012) and achieved a final height of 156.6 and 160.6 cm, respectively.

5.1.1.1.1 Puberty and bone age

At study start, AA was 14.4 years old and showed signs of early puberty (¹Paper I, Table 3). Already after 6 weeks on rhIGF-I/rhIGFBP-3, progression of puberty with a doubling of morning testosterone was seen. AA started GnRH agonist therapy (¹Paper I, Table 1), which was continued during the study period and withdrawn at age 16 and 10 months. BB was 12.2 years old and prepubertal at study start and showed signs of early puberty after 17 months on rhIGF-I/rhIGFBP-3 (¹Paper I, Table 3) and was started on GnRH agonist treatment, which was continued throughout the remaining study period. GnRH agonist treatment was stopped at age 16. Bone age became more delayed in both boys during the study, probably due to the GnRH agonist therapy (¹Paper I, Fig.1).

5.1.1.2 *Hormonal changes*

5.1.1.2.1 IGF-I

Serum total IGF-I concentrations are presented in Fig.13. Mean 12-hour overnight levels are given in ¹Paper I, Table 3. The IGF-I levels were undetectable or very low in both subjects before starting rhIGF-I/rhIGFBP-3. After 6 weeks on treatment, stable levels of IGF-I were obtained throughout a 12-hour post-injection period (mean IGF-I corresponding to an IGF-I SDS of -3.1 in AA and -3.3 in BB). After 17 months on therapy, omitting injections of rhIGF-I/rhIGFBP-3 for 3 days caused IGF-I to drop back to pretreatment levels. After 6 weeks on rhIGF-I, peak levels of IGF-I were obtained approximately 2 h after s.c. injections and the mean 12-hour concentrations were ~ 50 to 60% of the peak levels. Peak IGF-I levels were 2 to 5-fold higher and mean levels were 2 to 3-fold higher, corresponding to an IGF-I SDS of -1.34 (AA) and -1.65 (BB) on rhIGF-I, compared to those on rhIGF-I/rhIGFBP-3. This was observed despite the equal daily dosing. The IGF-I profiles after 1 year on rhIGF-I were almost identical to those after 6 weeks.

¹ Paper I, published version

5.1.1.2.2 GH

Overnight serum GH concentrations are presented in Fig.13. The mean 12-hour overnight levels are presented in ¹Paper I, Table 3. Before starting rhIGF-I/rhIGFBP-3, both subjects had elevated baseline GH levels at all time points. AA had a more pronounced elevation of peaks and a higher mean 12-hour concentration than BB. After starting rhIGF-I/rhIGFBP-3, no major suppression of baseline GH levels was observed, although AA had slightly attenuated peak heights and decreased mean 12-hour GH levels. After 17 months on rhIGF-I/rhIGFBP-3, three days without treatment markedly elevated baseline and peak GH levels and mean 12-hour levels were increased to levels similar to those prior to starting rhIGF-I/rhIGFBP-3. After 6 weeks and 12 months on rhIGF-I, GH peaks were suppressed and baseline levels were undetectable in both subjects.

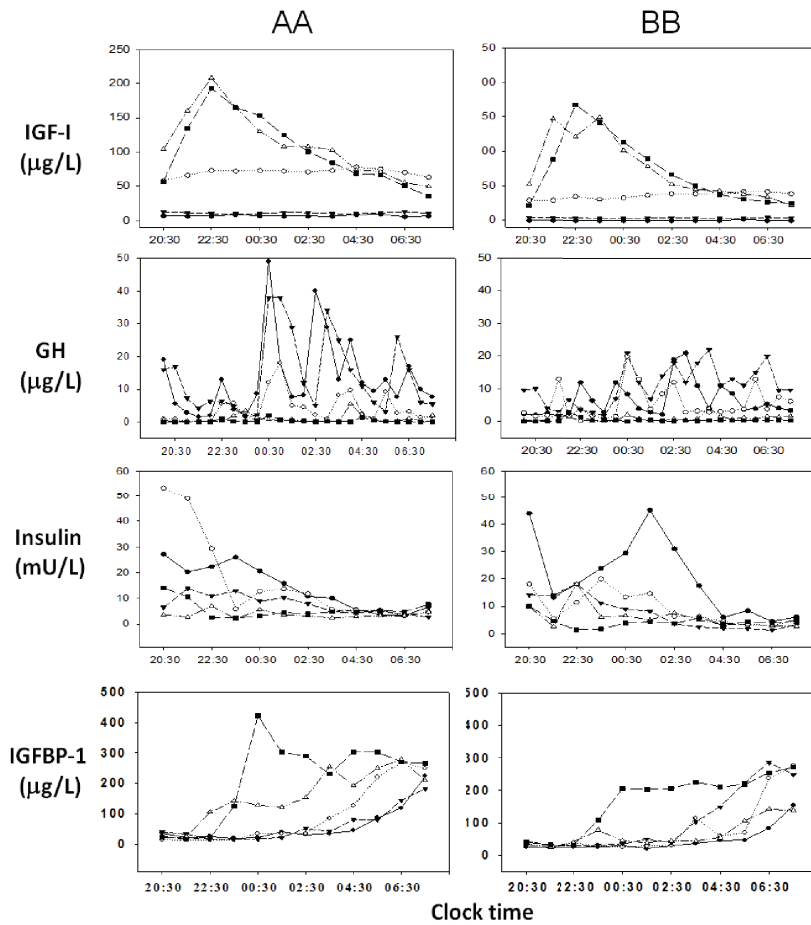


Fig.13. Overnight serum IGF-I, GH, insulin, and IGFBP-1 levels without treatment at study start (solid dots), after 6 weeks of IGF-I/IGFBP-3 co-treatment (open circles), before start of rhIGF-I (solid triangles), after 6 weeks of rhIGF-I (open triangles) and after 1 year of rhIGF-I (filled squares).

5.1.1.2.3 IGF-II, IGFBP-3 and ALS

Data were available only from the first part of the study (¹Paper I, Table 3). There was no significant diurnal variation in IGF-II, IGFBP-3, and ALS before or after starting rhIGF-I/rhIGFBP-3 (not shown). After starting rhIGF-I/rhIGFBP-3, the mean IGF-II level decreased by ~ 50% in AA but only by ~ 25% in BB, who also showed a smaller increase in IGF-I. The mean IGFBP-3 levels were slightly increased or unchanged (¹Paper I, Table 3). Given this finding, it should be noted that IGF-I and IGFBP-3 were administered in a 1:1 molar ratio and that the basal molar concentrations of IGFBP-3 were markedly higher than the increase in IGF-I molar concentrations. Basal ALS concentrations were very low and decreased by ~ 50% in both subjects after administration of IGF-I/IGFBP-3.

5.1.1.3 Insulin sensitivity

5.1.1.3.1 Hyperinsulinemic clamps

Results from four of the planned five hyperinsulinemic euglycemic clamps in each subject are presented in Fig.14 (see also ¹Paper I, Table 4). AA displayed slightly improved insulin sensitivity on rhIGF-I while treatment with rhIGF-I/rhIGFBP-3 did not appear to affect GIR. BB had improved peripheral insulin sensitivity after 17 months on rhIGF-I/rhIGFBP-3, even though injections had been stopped three days earlier. AA was relatively insulin resistant compared to pubertal children born appropriate and small for gestational age (AGA, SGA) and normal children in puberty BB initially had a GIR (M-value) within the normal range (Fig.14).

(Moran, Jacobs et al. 2002; Veening, Van Weissen-bruch et al. 2002)

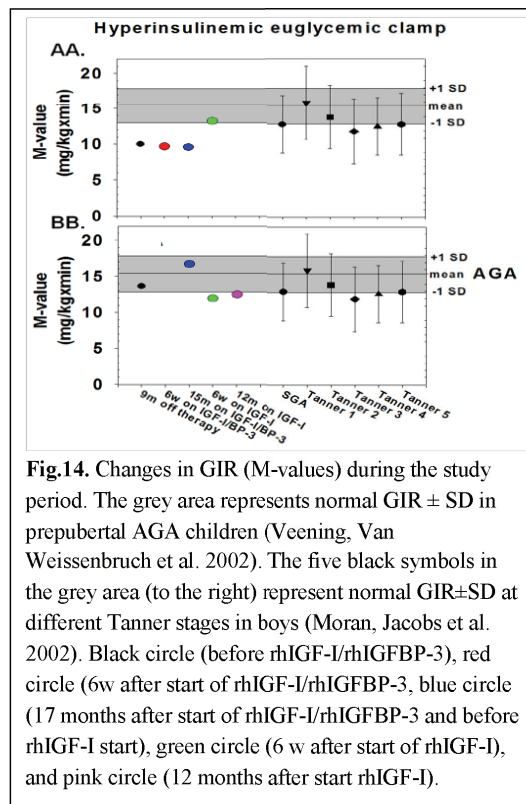


Fig.14. Changes in GIR (M-values) during the study period. The grey area represents normal GIR \pm SD in prepubertal AGA children (Veening, Van Weissenbruch et al. 2002). The five black symbols in the grey area (to the right) represent normal GIR \pm SD at different Tanner stages in boys (Moran, Jacobs et al. 2002). Black circle (before rhIGF-I/rhIGFBP-3), red circle (6w after start of rhIGF-I/rhIGFBP-3), blue circle (17 months after start of rhIGF-I/rhIGFBP-3 and before rhIGF-I start), green circle (6 w after start of rhIGF-I), and pink circle (12 months after start rhIGF-I).

5.1.1.3.2 Insulin

Serum insulin concentrations are presented in Fig.13 and the mean overnight fasting levels are given in ¹Paper I, Table 3. The mean insulin levels were decreased 6 weeks after starting rhIGF-I/rhIGFBP-3. In BB, the marked decrease in insulin may be at least partly explained by high basal insulin levels due to suspected snacking during the first night (Fig.13). Long-term mean insulin levels were reduced in both subjects. In BB a high level of physical activity the days before sampling may explain the low mean

insulin level at 17 months. During IGF-I alone, sustained low mean insulin levels were observed in both subjects.

5.1.1.3.3 IGFBP-1

Serum IGFBP-1 concentrations are presented in Fig.13 and the mean overnight fasting levels are given in ¹Paper 1, Table 4. Mean IGFBP-1 levels were increased by almost 100% in both subjects 6 weeks after starting rhIGF-I/rhIGFBP-3. After 17 months a decrease in mean IGFBP-1 was observed in AA, followed by an increase in IGFBP-1 when rhIGF-I treatment was started. In accord with the changes in insulin, BB showed a further increase in IGFBP-1 despite the interruption of rhIGF-I/rhIGFBP-3 for 3 days, and IGFBP-1 decreased after 6 weeks on rhIGF-I treatment. After 12 months on rhIGF-I treatment, both subjects had the highest mean overnight IGFBP-1 levels consistent with their low insulin levels at this point in time.

5.1.1.4 Body composition

The percentages of trunk fat mass are presented in ¹Paper 1 (Table 4) and in Fig.15. Total body fat (AA, 27.2%, and BB, 30.9%) was markedly elevated in both subjects 16 months before starting the study in spite of the ongoing IGF-I treatment at that time (van der Sluis, de Ridder et al. 2002). Their BMIs were only slightly above zero SDS. At study start, after 8 months without treatment, BMI SDS had increased only slightly while the relative trunk fat mass was markedly increased by 15.2% and 15.6%, respectively (Fig.15). After 17 months of rhIGF-I/rhIGFBP-3 BB decreased his relative trunk fat mass by 22% and increased his relative lean body mass by 7.5%, resulting in

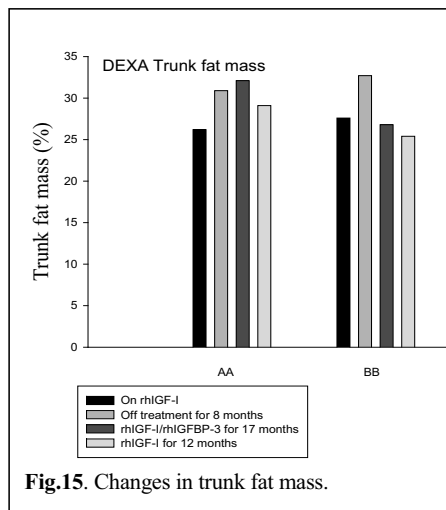


Fig.15. Changes in trunk fat mass.

a reduced BMI. In contrast, AA, who was started on a GnRH agonist due to progression of puberty (¹Paper 1, Table 1), had a minor increase in BMI SDS and relative trunk fat mass while lean body mass was unchanged. After 12 months on rhIGF-I, both subjects increased their relative lean body mass (AA, 5.5%, and BB, 2%) and decreased their relative trunk fat mass (AA, 10.3%, and BB, 5.5%) (Fig.15).

5.2 PAPER II

5.2.1.1.1 HbA1c

Treatment with insulin glargine significantly decreased HbA1c (Mono-S standard) from $8.3 \pm 0.6\%$ to a nadir of $6.9 \pm 0.3\%$ at 6 weeks ($P < 0.002$, $n = 11$). The improvement in HbA1c was already significant at 2 weeks ($7.5 \pm 0.4\%$, $P < 0.008$) and was sustained at the end of the 12-week study period ($7.3 \pm 0.3\%$, $P < 0.008$) in all patients (Fig. 16).

5.2.1.1.2 IGF-I

The mean total IGF-I level increased already after 1 week from $231 \pm 19 \mu\text{g/L}$ to $309 \pm 17 \mu\text{g/L}$ ($P < 0.001$) and was further increased by $44 \pm 7\%$ at 4 weeks ($P < 0.001$) (Fig. 16). At 6 weeks, on the fasting morning, IGF-I was $274 \pm 25 \mu\text{g/L}$ ($P = 0.022$) or $17 \pm 9\%$ over baseline. Subsequently, the increase consolidated with a peak value of $347 \pm 25 \mu\text{g/L}$ ($P < 0.001$) or $54 \pm 9\%$ over baseline at 12 weeks.

The changes in IGF-I mirrored HbA1c changes as shown in Fig. 16. During both admissions a significant diurnal variation in IGF-I levels was observed, with the lowest values in the morning samples. On NPH insulin, IGF-I was $249 \pm 24 \mu\text{g/L}$ at 18:00 h, $231 \pm 19 \mu\text{g/L}$ at 06:00 h and $248 \pm 23 \mu\text{g/L}$ at 17:00 h ($P < 0.05$).

After 6 weeks on insulin glargine, IGF-I was $307 \pm 27 \mu\text{g/L}$, $274 \pm 25 \mu\text{g/L}$, and $286 \pm 24 \mu\text{g/L}$, respectively ($P = 0.002$). The individual mean IGF-I SDS was markedly subnormal

on NPH insulin at 0 weeks (-1.8 ± 0.4 SDS) with five patients having values below -2 SDS and only one patient having IGF-I SDS above 0 SDS (1.2 SDS)

(Fig. 17). Individual IGF-I SDS correlated inversely with HbA1c ($r = -0.64$, $P = 0.025$) at 0 weeks. Although 12 weeks of treatment markedly increased the mean IGF-I SDS by $54 \pm 9\%$ to -0.55 ± 0.3 SDS ($P > 0.001$), IGF-I SDS was in the lower normal range in all patients except one (Fig. 17).

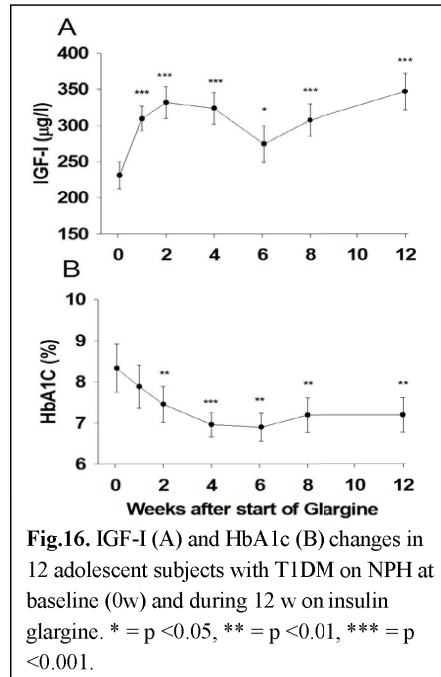


Fig.16. IGF-I (A) and HbA1c (B) changes in 12 adolescent subjects with T1DM on NPH at baseline (0w) and during 12 w on insulin glargine. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

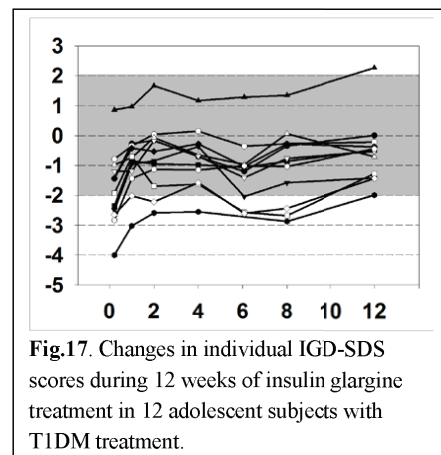


Fig.17. Changes in individual IGD-SDS scores during 12 weeks of insulin glargine treatment in 12 adolescent subjects with T1DM treatment.

5.2.1.1.3 IGFBP-1

In the majority of patients, IGFBP-1 displayed a diurnal rhythm with higher levels during the night and early morning. On insulin glargine, the excursions of IGFBP-1 to higher levels in the early morning were suppressed. The mean overnight IGFBP-1 concentration decreased significantly from 127 ± 21 ng/mL to 90 ± 12 ng/mL, ($P = 0.035$) but did not reach significance when evaluated over the total admission periods ($P = 0.065$)(Fig.18).

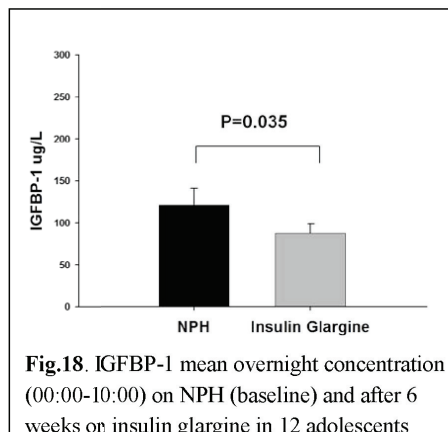


Fig.18. IGFBP-1 mean overnight concentration (00:00-10:00) on NPH (baseline) and after 6 weeks on insulin glargine in 12 adolescents

5.2.1.1.4 GH, GHBP, and IGFBP3-PA

The individual GH levels and secretory patterns of GH were similar before and after glargine. The GH peaks appeared to be equally distributed during the day and night, although some patients displayed more normal rhythms with more peaks during the night. There were no significant changes in measures of GH secretion (mean, mean overnight, AUC) or the GH secretory pattern (peak number, peak height, peak length, or inter-peak interval) after 6 weeks on glargine (pulsar analysis, data not shown). The mean GHBP determined at 0 weeks and 6 weeks did not significantly differ (523 ± 95 vs. 488 ± 80 pmol/L). The mean IGFBP-3-PA on insulin glargine was significantly lower than that on NPH insulin (35.2 ± 1.2 vs. 33.3 ± 1.3 , $P < 0.001$). Day mean values were significantly higher than night mean values on both NPH insulin and insulin glargine ($P = 0.003$ and 0.002 , respectively).

5.2.1.1.5 Insulin dosage

The mean total insulin dose given during the first admission was 1.21 ± 0.12 IU/kg. After 6 weeks the mean total insulin dose given was 1.05 ± 0.11 IU/kg or $89 \pm 6\%$ of the initially given total dose, although this did not reach significance ($P = 0.13$). Interestingly, the change in the total insulin dose and the change in IGF-I were positively correlated ($r = 0.61$, $P = 0.046$).

5.2.1.1.6 Auxology

Body weight increased from 46.8 ± 2.5 kg to 48.3 ± 2.5 kg ($P = 0.002$), height from 157.8 ± 2.0 cm to 158.7 ± 2.0 cm ($P = 0.002$), and BMI from 18.7 ± 0.8 kg/m² to 19.1 ± 0.9 kg/m² ($P = 0.03$) in the 11 patients studied at 6 weeks.

5.3 PAPER III

5.3.1.1.1 IGFBP-1

At 12 and 24 months serum IGFBP-1 levels were significantly lower in the CSII-treated group than in the MDI-treated group (²Table 3 and Fig. 19). In the CSII group there was no significant difference over time, while in the MDI group IGFBP-1 was increased at 24 months (vs. 0 months [$P < 0.001$] and vs. 6 months [$P = 0.004$]) (Fig. 19).

5.3.1.1.2 IGF-I

There were no significant differences in IGF-I SDS (²Table 3) or total IGF-I levels between the CSII and MDI groups. In both the CSII group and the MDI group, IGF-I SDS levels decreased significantly over time, $P < 0.001$ at all intervals (0 and 24 months, 6 and 24 months, and 12 and 24 months).

5.3.1.1.3 C-peptide

There were no significant differences in C-peptide levels between the CSII and MDI groups (²Table 3). In both groups, C-peptide levels were unchanged at 6 months and then decreased

over time (²Table 3). At 12 and 24 months a significant positive linear correlation between log C-peptide and IGF-I was seen, which was independent of treatment modality ($P = 0.032$ and $P = 0.023$, respectively). At 6 months, apart from a positive linear correlation between log C-peptide and IGF-I in both treatment regimes ($P > 0.001$), a significant difference between the MDI and CSII groups was seen ($P = 0.008$), (Fig. 20). There were also significant negative linear correlations, regardless of treatment modality, between log C-peptide and IGFBP-1 at 6, 12 and 24 months ($P > 0.001$, $P = 0.002$ and $P < 0.001$, respectively). In addition, at 6 months a significant

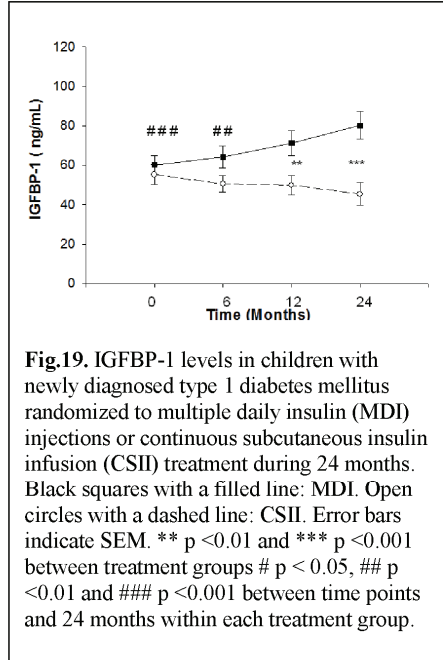


Fig.19. IGFBP-1 levels in children with newly diagnosed type 1 diabetes mellitus randomized to multiple daily insulin (MDI) injections or continuous subcutaneous insulin infusion (CSII) treatment during 24 months. Black squares with a filled line: MDI. Open circles with a dashed line: CSII. Error bars indicate SEM. ** $p < 0.01$ and *** $p < 0.001$ between treatment groups # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ between time points and 24 months within each treatment group.

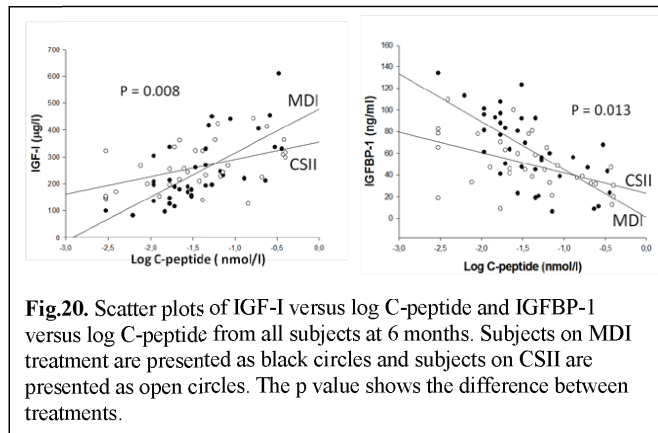


Fig.20. Scatter plots of IGF-I versus log C-peptide and IGFBP-1 versus log C-peptide from all subjects at 6 months. Subjects on MDI treatment are presented as black circles and subjects on CSII are presented as open circles. The p value shows the difference between treatments.

difference between the MDI and CSII groups was seen ($P = 0.013$) (Fig.20). At 12 and 24 months the C-peptide levels were very low in the majority of subjects and no differences were seen between the treatment groups.

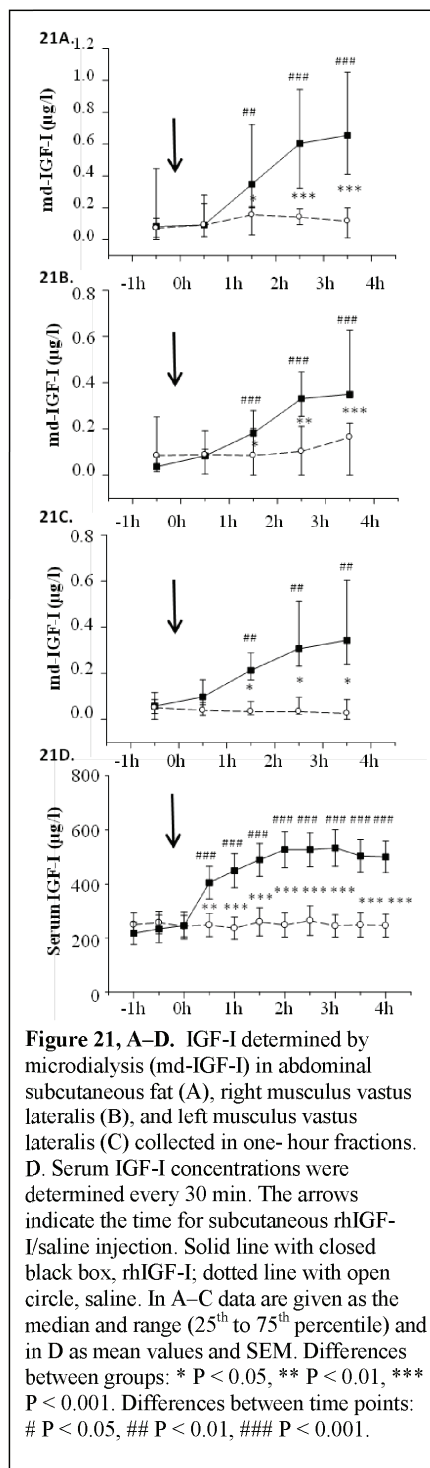
5.3.1.1.4 Insulin doses

In paper III the insulin doses did not differ between the treatment groups at 6 month. In both the CSII and the MDI groups, mealtime insulin doses and basal insulin doses decreased significantly between baseline and 6 months ($P = 0.007$ and $P = 0.01$, respectively, $P = 0.003$ and $P = 0.007$, respectively). At 12 and 24 months both mealtime insulin doses and basal insulin doses were significantly lower in the CSII group ($P < 0.001$ and $P < 0.001$, respectively). Between 6 and 24 months a significant increase in the basal insulin doses was seen in both treatment groups ($P < 0.001$ and $P < 0.001$, respectively). The mealtime insulin doses in the CSII group remained unchanged between 6 and 24 months, which was in contrast to a significant increase in the MDI group ($P < 0.001$).

5.4 PAPER IV

5.4.1.1.1 Tissue IGF-I in muscle and subcutaneous fat (md-IGF-I)

Median levels of md-IGF-I in the left and right musculus vastus lateralis (LMVL, RMVL) and abdominal subcutaneous fat (ASF) increased significantly and in parallel in all compartments during the second and third hour after rhIGF-I treatment and then remained elevated (Fig.21 A-C): in the LMVL, from 0.058 (0.003–0.111) to 0.343 (0.256–0.551) $\mu\text{g/L}$, in the RMVL, from 0.037 (0.026–0.068) to 0.350 (0.335–0.559) $\mu\text{g/L}$, and in ASF from 0.082 (0.025–0.126) to 0.655 (0.455–1.004) $\mu\text{g/L}$, with all P values ≤ 0.001 . In contrast, there was no change in md-IGF-I after saline. In all three compartments, the median levels of md-IGF-I were significantly higher after rhIGF-I vs. saline, starting from the second hour after injection. The md-IGF-I levels in fat and muscle did not differ over time and were undetectable in 17.1% of the measurements in muscle and in 11.4% of



those in subcutaneous fat, but only after saline.

5.4.1.1.2 Circulating IGF-I

Serum-IGF-I increased significantly during the first 30 min after rhIGF-I injection from 246 ± 50 to $405 \pm 61 \mu\text{g/l}$ ($P < 0.001$), reached a maximum level 3 h post injection ($533 \pm 67 \mu\text{g/l}$) and remained increased throughout the study. There was no change in serum IGF-I after saline (Fig. 21D).

5.4.1.1.3 Glucose infusion rate

Normoinsulinemic euglycemic clamps were associated with stable insulin and glucose levels from (-1 to 0 h) before rhIGF-I/saline injection until the end of the study (Fig. 22A, 22B). The baseline (-1 to 0 h) mean GIR was $2.22 \pm 0.54 \text{ mg/kg} \times \text{min}$ and did not change during the first hour after the rhIGF-I injection. The mean GIR then increased significantly during the second hour (1 to 2 h) and the third hour (2 to 3 h) and reached $5.11 \pm 0.92 \text{ mg/kg} \times \text{min}$, ($P < 0.001$) (3 to 4 h) (Fig. 22C) There was no change in the mean GIR after saline injection. The mean GIR was significantly elevated after rhIGF-I, as compared to saline, at the second, third, and fourth hour.

5.4.1.1.4 Glucoregulatory hormones

IGFBP-1 decreased steadily in response to the insulin infusion but was not affected by the injection of rhIGF-I, as compared with saline (Fig. 23). The mean GH levels did not change over time and did not differ between the rhIGF-I and saline groups. The initially higher morning cortisol levels decreased steadily during both study days) without any difference between saline and rhIGF-I. (³Paper IV, Fig. 4)

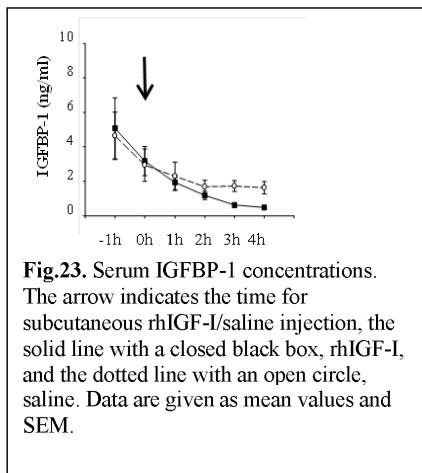


Fig.23. Serum IGFBP-1 concentrations. The arrow indicates the time for subcutaneous rhIGF-I/saline injection, the solid line with a closed black box, rhIGF-I, and the dotted line with an open circle, saline. Data are given as mean values and SEM.

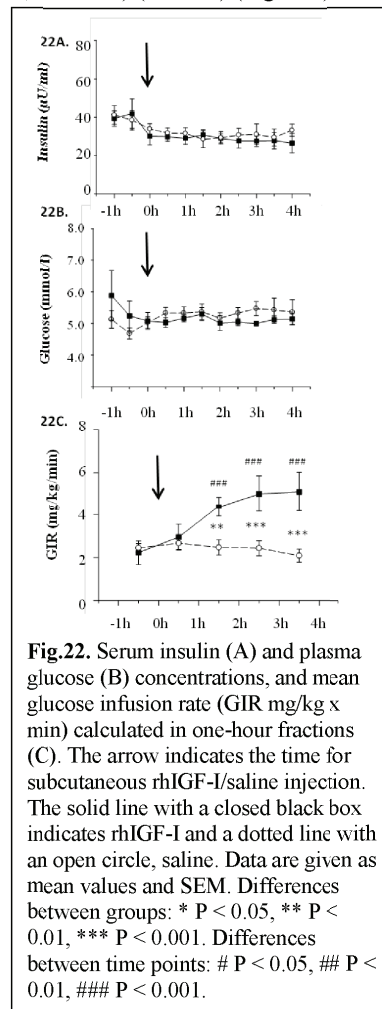


Fig.22. Serum insulin (A) and plasma glucose (B) concentrations, and mean glucose infusion rate (GIR mg/kg x min) calculated in one-hour fractions (C). The arrow indicates the time for subcutaneous rhIGF-I/saline injection. The solid line with a closed black box indicates rhIGF-I and a dotted line with an open circle, saline. Data are given as mean values and SEM. Differences between groups: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Differences between time points: # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$.

³ Paper IV, manuscript

GENERAL DISCUSSION

5.5 PRIMARY IGF-I DEFICIENCY – PIGFD (PAPER I)

5.5.1 Growth

In Paper I the HtV on rhIGF-I alone was superior to that of rhIGF-I/rhIGFBP-3 and close to the expected second- (or subsequent-) yr height velocity reported in PIGFD patients (Chernausek, Backeljauw et al. 2007). The superior effect of rhIGF-I on linear growth as compared to rhIGF-I/rhIGFBP-3 has also been reported by Tonella *et al.* (Tonella, Fluck et al. 2010). In our study, the 2-fold higher height velocity on rhIGF-I given twice daily was associated with a diurnal variation in circulating IGF-I. Furthermore, mean levels were 2- to 3-fold higher and peak levels 2- to 5-fold higher as compared to rhIGF-I/rhIGFBP-3 administration.

In our severe PIGFD patients, treatment with rhIGF-I alone provided a better growth response, although it was below that seen in GHD patients treated with GH. Furthermore, despite rhIGF-I treatment during 8 prepubertal years (with short interruptions due to a shortage of drug supply) our patients did not normalize height and were still severely growth stunted. This lack of catch-up growth may be related to the low IGF-I SDS levels obtained on rhIGF-I alone and the substantially low levels on rhIGF-I/rhIGFBP-3.

In addition, IGF-I is not likely to mediate all the actions of GH on human growth. This was also concluded in the study by Chernausek *et al.* (Chernausek, Backeljauw et al. 2007), demonstrating that rhIGF-I treatment of a larger cohort of patients with severe PIGFD (of which some had genetically proved GHR defects) did not completely restore growth. GH is thought to have direct IGF-I independent effects in the growth plate (Le Roith, Bondy et al. 2001; Kaplan and Cohen 2007). As mentioned, the low levels of circulating IGF-I may also be of importance. To this end, it has been thought that the lack of IGFBP-3 and ALS in the circulation and the decreased ternary complex formation seen in GHR defects (decreasing the half-life of rhIGF-I), contribute to the lack of circulating IGF-I and growth restitution. ALS deficiency also results in severe PIGFD with a similar deficiency of circulating IGF-I, IGFBP-3, and ALS (which is often absent). However, short stature is moderate in ALS deficiency compared to GHR defects (Domene, Hwa et al. 2009). Furthermore, preliminary data from a clinical trial of rhIGF-I/rhIGFBP-3 in severe PIGFD (Savage, Underwood et al. 2006) indicated a lower growth potency of that preparation as compared to rhIGF-I. This trial (not finalized due to a legal dispute) as well as our data strongly challenges the hypothesis that replacement therapy with rhIGF-I/rhIGFBP-3 is superior to rhIGF-I alone in promoting linear growth.

One question raised by our study is why an equivalent dose of rhIGF-I results in markedly lower average levels of circulating IGF-I than when given in combination with rhIGFBP-3. When on rhIGF-I/rhIGFBP-3, the serum IGF-I concentrations were stable after injection in accord with previous reports (Camacho-Hubner, Rose et al. 2006; Tonella, Fluck et al. 2010). This stabilization of circulating IGF-I has been suggested to depend on the rhIGFBP-3 binding and stabilization of rhIGF-I by

formation of complexes in the circulation. However, the finding of more or less unchanged IGFBP-3 levels 6 weeks after starting rhIGF-I/rhIGFBP-3 and markedly decreased ALS does not support the view that non-glycosylated rhIGFBP-3 reaches the circulation and stabilizes IGF-I. Rather, rhIGF-I may be kept in complexes at the tissue injection site. The stable levels as well as the lower quantities may be explained by a slow and steady release from the complexes into the circulation. This view is further supported by the finding that already after 3 days without treatment the concentrations were back to pre-treatment levels in accord with the shorter half-life of IGF-I in patients with GHR defects (Grahnen, Kastrup et al. 1993). Our results regarding IGFBP-3 levels are in accord with the report by Camacho-Hübner *et al.* (Camacho-Hübner, Rose et al. 2006) and in contrast to the marked increases in IGFBP-3 levels after rhIGF-I/rhIGFBP-3 administration in the study by Tonella *et al.* (Tonella, Fluck et al. 2010).

5.5.2 Insulin sensitivity and body composition

Our aim was also to demonstrate that severe PIGFD is associated with insulin resistance, increased fat mass, and decreased lean body mass and that rhIGF-I treatment improves insulin sensitivity, decreases fat mass, increases lean body mass, and increases height velocity. In Paper I short-term metabolic effects of rhIGF-I/IGFBP-3 were observed in both subjects and included increased IGFBP-1, which was in accordance with decreased insulin levels at 17 months and an insulin-sparing effect. Despite of these observations we failed to demonstrate consistent improvement in peripheral insulin sensitivity by the clamp studies. When on rhIGF-I, the mean serum IGF-I levels were almost twice those on rhIGF-I/rhIGFBP-3 and sustained suppression of insulin as well as the highest overnight levels of IGFBP-1 were observed. In line with the suppression of overnight insulin levels by both IGF-I preparations in our study, similar results have been reported in adolescents with T1DM (Cheetham, Jones et al. 1993; Cheetham, Connors et al. 1997; Acerini, Harris et al. 1998; Saukkonen, Amin et al. 2004).

Patients with GHR defects and untreated GHD are obese (Laron and Klinger 1993; Mauras and Haymond 2005; Garten, Schuster et al. 2012). In the presence of a functioning GHR, chronic GH treatment will increase lipolysis and decrease fat mass (Tanner and Whitehouse 1967; Mauras and Haymond 2005; Vijayakumar, Novosyadlyy et al. 2009). However, patients with GHR defects lack both the lipolytic action of GH and the insulin-sparing effects of IGF-I and consequently their insulin levels are increased, and these mechanisms may act in concert and increase lipogenesis and adipose tissue (Laron and Klinger 1993). IGF-1Rs are lacking in mature adipocytes (DiGirolamo, Eden et al. 1986; Bolinder, Lindblad et al. 1987; Back and Arnqvist 2009) and, considering the low affinity of IGF-I to the IR (100-fold less than insulin), it is unlikely that IGF-I should stimulate adipocytes under physiological conditions (Clemmons 2012). The role of IGF-I in lipid metabolism is believed to be mainly indirect by reducing GH secretion. However, GHR-deficient adults treated with rhIGF-I showed increased lipolysis and lipid oxidation, which was assumed to be an effect of decreased insulin levels (Mauras, Martinez et al. 2000). In non-physiological doses, rhIGF-I, given to healthy controls, showed decreased NEFA levels, assumed to be an effect of reduced GH secretion (Boulware, Tamborlane et al. 1994; Pratipanawatr, Pratipanawatr et al. 2002). In contrast, healthy volunteers given rhIGF-I infusion for 5 days showed increased lipid oxidation and increased NEFA levels, probably related to a decrease in insulin concentrations (Hussain, Schmitz et al. 1993).

In our study a decrease in fat mass and an increase in lean body mass were observed in both subjects on rhIGF-I, which are in accord with a more pronounced insulin-sparing effect, while rhIGF-I/rhIGFBP-3 did not result in consistent changes. Although the results were not completely congruent, we speculate that the suppression of insulin secretion by IGF-I may be the major driving force for the observed loss in fat mass. Such a mechanism was suggested by studies in rhIGF-I-treated adults with a GHR defect (Mauras, Martinez et al. 2000). The concomitant increase in lean body mass, most clearly seen during rhIGF-I treatment in our study, may be the result of increased protein synthesis by IGF-I (Mauras, Martinez et al. 2000). The pronounced effect of IGF-I alone is also supported by the marked changes in body composition observed during the 8 months without treatment prior to this study, which resulted in substantially decreased lean body mass and increased fat mass.

By blocking GH effects, the metabolic effects of IGF-I *per se* have been studied and showed improved glucose uptake and unchanged lipid metabolism (Crowne, Samra et al. 1998; O'Connell and Clemmons 2002; Simpson, Jackson et al. 2004). In Paper I we attempted to demonstrate the effects of IGF-I *per se* on glucose metabolism by performing hyperinsulinemic euglycemic clamps. Veening *et al.* (Veening, Van Weissenbruch et al. 2002) measured insulin sensitivity in prepubertal children by using a protocol similar to the one we used and our data were also compared to the reference material on insulin sensitivity in healthy prepubertal and pubertal children by Moran *et al.* (Moran, Jacobs et al. 2002). The GIR results in our subjects were inconsistent. BB demonstrated high insulin sensitivity before starting the study and remained in the upper normal range during treatment, while AA had lower insulin sensitivity with some increase on rhIGF-I. However, the inconsistent findings must be interpreted with caution. Apart from there being only two subjects, they did not perform all five clamp studies (only three at the same time). Another shortcoming is that we did not use the two-step clamp technique with stable isotopes of glucose and glycerol and therefore we have not been able to detect whether the decreased insulin concentrations, seen after both rhIGF-I/rhIGFBP-3 and rhIGF-I, increased lipolysis and HGP. Such potential effects of rhIGF-I may tend to increase insulin resistance and counteract the beneficial effects on glucose disposal and insulin sensitivity (Guler, Zapf et al. 1987; Hussain, Schmitz et al. 1993; Mauras, Martinez et al. 2000; Simpson, Jackson et al. 2004). The hyperinsulinemic euglycemic clamp technique determines the maximal glucose disposal at very high insulin levels. An additional problem in using this approach may be that less prominent effects of IGF-I on glucose disposal are masked.

We found major effects of both rhIGF-I preparations on IGFBP-1, a marker of hepatic insulinization (Brismar, Fernqvist-Forbes et al. 1994). Overnight mean IGFBP-1 levels increased after 6 weeks on rhIGF-I/rhIGFBP-3 and were most elevated after 12 months on rhIGF-I. We considered these changes in IGFBP-1 as dictated by a lower endogenous insulin secretion rather than a result of a change in hepatic insulin sensitivity. This view is supported by our finding that the overnight insulin levels decreased. The lowering of insulin secretion suggests an increase in overall insulin sensitivity. The effects of IGF-I on insulin sensitivity and glucose metabolism have been largely attributed to its negative feedback suppression of GH secretion (Acerini, Harris et al. 1998; Yakar, Setser et al. 2004). However, in the absence of a functional GHR, we propose that the effects observed in our patients are direct effects of IGF-I. Apart from being a case study, there were other limitations. The long-term metabolic effects measured after 17 months on rhIGF-I/rhIGFBP-3 may be influenced by being off treatment for 3 days (end of drug supply). Furthermore, it is possible that physical

activity (we did not ask for this to be stopped before the metabolic studies) and/or pubertal development have an impact on insulin sensitivity which overrides the effects of IGF-I on GIR. With regards to the possible role of puberty, Arslanian *et al.* did not report any effect of testosterone treatment on GIR in boys with delayed puberty (Arslanian and Suprasongsin 1997).

5.6 ACQUIRED IGF-I DEFICIENCY, TYPE 1 DIABETES (PAPERS II, III, AND IV)

Subcutaneous insulin administration aims to keep the blood sugar levels in a desired range in children and adolescents with T1DM. However, despite an efficient subcutaneous insulin delivery, the portal insulin concentration will be far below the normal and cause disturbances in the GH/IGF-I axis. Low portal insulin levels result in high levels of IGFBP-1, increased hepatic GH resistance and decreased IGF-I production (Zachrisson, Brismar *et al.* 1997; Hedman, Frystyk *et al.* 2004). Low circulating levels of IGF-I will increase GH secretion and induce insulin resistance (Amiel, Sherwin *et al.* 1984; Amiel, Sherwin *et al.* 1986; Dunger and Acerini 1998; Bereket, Lang *et al.* 1999). These disturbances have an impact on the deteriorating metabolic control seen in older children and adolescents (Dunger and Acerini 1998).

The aims in Paper II were to demonstrate that treatment with insulin glargine in adolescents with T1DM increases IGF-I, decreases GH and IGFBP-1, and improves HbA1c, as compared to treatment with NPH insulin. In Paper III the aims were to demonstrate that treatment with CSII in children and adolescents from the initial diagnosis of T1DM increases circulating IGF-I, decreases IGFBP-1, preserves endogenous insulin production, and improves insulin sensitivity, as compared to treatment with NPH insulin. The aims in Paper IV were to demonstrate that administration of rhIGF-I in young adult males with T1DM increases tissue IGF-I in muscle and subcutaneous fat, as determined by microdialysis, and results in increased whole body glucose uptake and, lastly, to study the pharmacokinetics of rhIGF-I

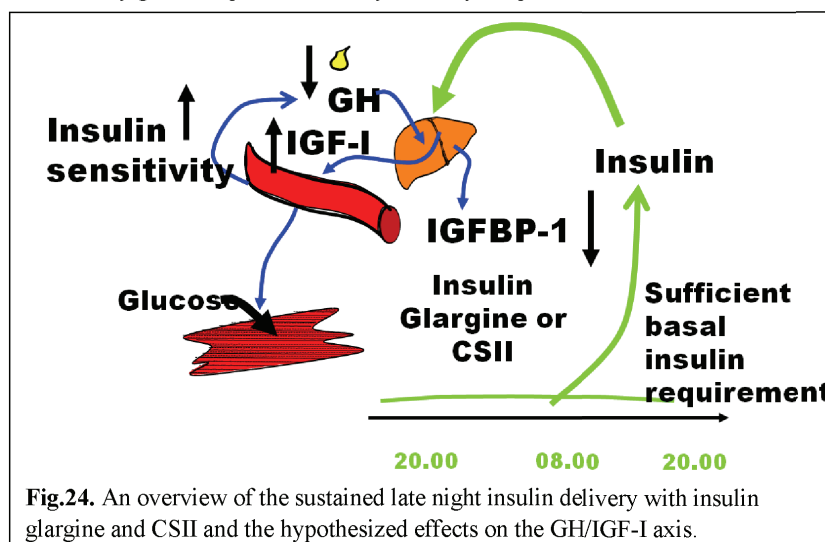


Fig.24. An overview of the sustained late night insulin delivery with insulin glargine and CSII and the hypothesized effects on the GH/IGF-I axis.

5.6.1 Hepatic insulin sensitivity

Insulin treatment regimens using intermediate-acting insulin, NPH, will not (especially in adolescents) support sufficient insulin concentrations in the late night, and increased IGFBP-1 levels and low free IGF-I levels in the early morning have been demonstrated (Edge, Matthews et al. 1990; Lepore, Pampanelli et al. 2000; Yagasaki, Kobayashi et al. 2009; Yagasaki, Kobayashi et al. 2010).

In Paper II, we demonstrated decreased overnight IGFBP-1 levels after 6 weeks on insulin glargine. However, we did not see any effects on the 20-hour mean IGFBP-1 levels. In Paper III, we demonstrated that fasting IGFBP-1 levels were lower at 12 and 24 months in the CSII-treated group. These findings indicate that both CSII and insulin glargine, as compared to NPH insulin, improve hepatic insulin sensitivity by a more sustained late-night insulinization and thus affect IGFBP-1 suppression more during the night. A limitation in Paper II is that we were not able to determine the insulin concentrations in the overnight profiles due to a lack of specific assays for different insulin analogs. However, the advantage of using IGFBP-1 is that it integrates the combined effects of all insulin analogs and, in accord with other studies, we have considered overnight IGFBP-1 levels and fasting IGFBP-1 to be an indirect marker of late-night insulinization and a measure of hepatic insulin sensitivity (Lepore, Pampanelli et al. 2000; Yagasaki, Kobayashi et al. 2009; Yagasaki, Kobayashi et al. 2010).

In the newly diagnosed subjects in Paper III, we assessed the possible role of endogenous insulin production (C-peptide) on IGFBP-1 production. The C-peptide levels peaked at 6 months in both groups and decreased rapidly thereafter, although without any differences between the treatments. However, inverse correlations between log C-peptide and IGFBP-1 were found in both treatment groups at 6, 12, and 24 months. Interestingly, at 6 months we demonstrated a higher increase in IGFBP-1 for a given decrease in log C-peptide in the MDI group, indicating a more pronounced dependence on endogenous insulin. One obvious limitation in Paper III was that fasting, rather than stimulated, C-peptide was used as a marker of endogenous insulin production. However, it has recently been shown that the correlation between fasting C-peptide and AUC during a MMTT is better than previously assumed (Besser, Shields et al. 2012).

IGFBP-1 binds to IGF-I and lowers circulating IGF-I activity (Bereket, Lang et al. 1999) and, in adolescents with type 1 diabetes, elevated IGFBP-1 has been suggested to further inhibit IGF-I activity (Zachrisson, Dahlquist et al. 2000). In animal studies, IGFBP-1 co-administration or over-expression inhibits the glucose-lowering effects of IGF-I (Lewitt, Denyer et al. 1991; Crossey, Jones et al. 2000). The lower levels of IGFBP-1 on insulin glargine in Paper II and on CSII in Paper III may have positively affected the insulin-like actions of IGF-I, which are suggested to be related to increased bioactivity of circulating IGF-I and may have contributed to the lower insulin requirements seen in Paper III and the beneficial effects on HbA_{1c} in Paper II. An increase in circulating IGFBP-1 has been shown to reduce free IGF-I in serum (Frystyk 2004). We have already discussed (4.2.5.3) that data are lacking to directly demonstrate that free IGF-I in serum reflects local tissue levels or local tissue action of IGF-I. It may therefore be questioned whether changes in free IGF-I have any physiological

significance (Bang, Ahlsen et al. 2001). The approach that we have taken in this thesis to further explore local levels and actions of IGF-I is to measure local tissue levels by microdialysis, as discussed in 6.2.2.4.

5.6.2 Hepatic GH insensitivity

In Paper II we showed that insulin glargine, by providing a more physiological insulin delivery during late night, increased circulating IGF-I, which is in accord with Slawik *et al.* (Slawik, Schories et al. 2006). The portal insulin effects were improved, which is supported by our observation of lower IGFBP-1, and we suggest that increased hepatic GH sensitivity resulted in increased circulating IGF-I levels.

During the first days after the diagnosis of T1DM in children and adolescents, a fast and marked increase in circulating IGF-I was seen after the initiation of insulin treatment (Bereket, Lang et al. 1996). Paper III did not involve this early phase and, in Paper II, patients with a long duration of the disease were studied. Only intra-portal or intra-peritoneal insulin delivery in T1DM normalizes, or nearly normalizes, circulating IGF-I levels (Shishko, Dreval et al. 1994; Hanaire-Broutin, Sallerin-Caute et al. 1996; Frystyk, Ritzel et al. 2008) by facilitating hepatic GHR expression and signaling (Daughaday, Phillips et al. 1976; Maes, Underwood et al. 1986).

In Paper II we demonstrated that insulin glargine treatment resulted in markedly increased circulating IGF-I. The role of a sufficient insulin delivery for improved GH sensitivity was supported by the positive correlation between the relative changes in total insulin dose and serum IGF-I, suggesting the importance of maintaining the insulin dose to support an efficacious insulin delivery and maintain the improved hepatic GH sensitivity. Given the lack of significant changes in the total insulin dose, GH secretion, and GHBP on insulin glargine, the suggested improved hepatic insulinization appears to increase GH sensitivity and IGF-I generation by post-receptor mechanisms (Maes, Underwood et al. 1986; Ueki, Kondo et al. 2004).

Although the suppression of IGFBP-1 in the CSII-treated group in Paper III indicates an improved insulin action on the liver, it was not sufficient to facilitate GHR stimulation and increase circulating IGF-I levels significantly. The lower insulin doses in the CSII group at 12 and 24 months indicate improved overall insulin sensitivity; however, the potential effects on hepatic GH sensitivity of an improved insulin delivery may, to some extent, have been lost when the dose was reduced and thus prevented an increase in IGF-I and improved metabolic control. In addition, improved hepatic insulin actions may have more direct and pronounced effects on IGFBP-1 inhibition than are required to interact with the GH signaling pathways. This may also involve other signaling pathways, an area that is still open for further exploration.

Early short-term studies of CSII (Tamborlane, Hintz et al. 1981; Amiel, Sherwin et al. 1984) showed an improvement in circulating IGF-I levels. However, these studies were not conducted in newly diagnosed patients with persisting endogenous insulin production, and changes in the GH/IGF-I axis may be more pronounced when patients on CSII are compared with those treated with 1–2 daily insulin injections (Tamborlane, Hintz et al. 1981; Amiel, Sherwin et al. 1984; Shishko, Dreval et al. 1994). In addition, interactions of IGFBPs with IGF-I measurements in these older studies may also cause problems in the interpretation of the results (Bang, Baxter et al. 1994).

Although we reported an approximately 1.2-fold increase in circulating IGF-I levels at 6 weeks and a marked 1.5-fold increase at 12 weeks in Paper II, the IGF-I levels were still subnormal and close to -1 SDS. The lack of suppression of GH secretion at 6 weeks suggests that IGF-I needs to reach a normal “setpoint” to establish the negative feedback on GH. This is in accord with the findings of Saukkonen *et al.*, who demonstrated that a 1.5-fold increase in IGF-I concentrations was required to reduce overnight GH secretion (Saukkonen, Amin *et al.* 2004), although IGF-I SDS was not reported in that study.

The serum IGF-I levels were between -1 SDS and -2 SDS in prepubertal boys and girls (Paper III, Fig. 2) and even more subnormal in pubertal children (Paper II, Fig. 17, and Paper III, Fig. 2) with almost half of the patients below -2 SDS. This is in line with a previous report from our group (Zachrisson, Brismar *et al.* 1997). In addition, in Paper III, we found correlations between log C-peptide and IGF-I in both treatment groups at 6, 12, and 24 months, which underlines the importance of hepatic insulin actions on IGF-I generation. This is also in accord with results in adults with T1DM (Hedman, Frystyk *et al.* 2004). Interestingly, at 6 months, when the C-peptide levels peaked in both groups, a difference in the correlation lines was demonstrated, suggesting that subjects on CSII are less dependent on endogenous insulin to maintain their circulating IGF-I levels toward the normal range. Again, this suggests that the mode of insulin delivery results in different abilities to meet hepatic insulin needs.

5.6.3 Peripheral insulin sensitivity

There is a close association between peripheral insulin sensitivity and metabolic control (Simonson, Tamborlane *et al.* 1985). In Paper II we demonstrated lower HbA1c on insulin glargine (from 2 weeks to the end of the study at 12 weeks), with a nadir at 6 weeks. Although the total insulin dose was unchanged at 6 weeks, there was a trend toward a lower dose; taken together, these findings indicate an improved insulin sensitivity on insulin glargine.

Retrospective studies on insulin glargine treatment have reported improved HbA1c (Chase, Dixon *et al.* 2003; Hathout, Fujishige *et al.* 2003; Salemyr, Bang *et al.* 2011). However, in prospective trials (RCT and observational), the decrease in HbA1c has been less prominent or not obtained (Schober, Schoenle *et al.* 2002; Murphy, Keane *et al.* 2003; Chase, Arslanian *et al.* 2008). A recently published meta-analysis reported a significant, but minor, effect of long-acting insulin analogs on HbA1c (Monami, Marchionni *et al.* 2009).

A major limitation when interpreting the improved HbA1c in Paper II is the lack of a randomized design, and this study does not allow us to separate the effects of intensified treatment with frequent visits and supervision from a specific role of insulin glargine on improved HbA1c and increased IGF-I. No matter which way the increase in IGF-I is established, the demonstrated effects of rhIGF-I on glucose disposal (Guler, Zapf *et al.* 1987; Dohm, Elton *et al.* 1990; Crowne, Samra *et al.* 1998; O'Connell and Clemmons 2002; Simpson, Jackson *et al.* 2004) may suggest that the IGF-I increase in Paper II was important for the improvement in HbA1c. A beneficial role of rhIGF-I on metabolic control has also been demonstrated in long-term trials (4-24 weeks) (Cheetham, Holly *et al.* 1995; Acerini, Patton *et al.* 1997; Quattrin, Thraillkill *et al.* 1997; Quattrin, Thraillkill *et al.* 2001), although some of these effects may be related to suppression of GH secretion (Cheetham, Clayton *et al.* 1994; Cheetham, Connors *et al.* 1997; Acerini, Harris *et al.* 1998). In Paper II the GH secretion was unchanged at 6

weeks, supporting the view that the insulin resistance mechanisms (lipolysis, gluconeogenesis) induced by GH were unchanged.

CSII has been found to improve metabolic control in children with T1DM mellitus in some randomized controlled trials (RCTs) (de Beaufort, Houtzagers et al. 1989; Doyle, Weinzimer et al. 2004), but not in others (Weintrob, Benzaquen et al. 2003; Fox, Buckloh et al. 2005). A meta-analysis of RCTs in children reported a small positive effect on HbA1c in CSII vs. MDI therapy (Pankowska, Blazik et al. 2009). In a previous report on the study population in Paper III, no effect on HbA1c was demonstrated (Skogsberg, Fors et al. 2008). Furthermore, studies in children and adolescents have reported lower insulin requirements in the CSII group (Boland, Grey et al. 1999; Wiegand, Raile et al. 2008; Pankowska, Blazik et al. 2009). Apart from our study, there are two RCTs in children and young adults that compare CSII with MDI or conventional therapy (one or two injections daily) from the onset of type 1 diabetes up to 24 months, and they reported conflicting data on HbA1c and insulin requirements, but no differences in C-peptide levels (de Beaufort, Houtzagers et al. 1989; Pozzilli, Crino et al. 2003).

It is well documented that portal insulin concentrations are important in determining HGP in humans (Lewis, Zinman et al. 1996; Cherrington, Edgerton et al. 1998). Whether CSII and insulin glargine treatment through improved nightly insulin delivery, despite non-portal administration, more effectively reduces HPG and thus improves whole-body insulin sensitivity was not determined in the present studies.

Although we did not assess GH secretion in Paper III, it is unlikely that the improved insulin sensitivity in the CSII group, reflected by lower requirements, is related to decreased GH secretion in the absence of higher IGF-I levels. Instead, the improved insulin sensitivity may at least partly be explained by improved nightly insulin delivery. Although this may correspond to a more pronounced effect on hepatic insulin sensitivity in the morning in the CSII group (Yagasaki, Kobayashi et al. 2009), our finding of lower meal insulin requirements supports the view that the effect on hepatic insulin sensitivity is more long-lasting and maybe mediated by other mechanisms. This is supported by Simonson *et al.* (Simonson, Tamborlane et al. 1985), who demonstrated improved overall insulin sensitivity after CSII. Our finding of lower insulin requirements in the CSII group are in accord with the large observational study by Wiegand *et al.* (Wiegand, Raile et al. 2008); however, an obvious limitation in Paper III is the self-reported insulin doses in MDI as compared to downloaded doses in CSII.

In vitro studies support a direct role of IGF-I on glucose uptake (Dohm, Elton et al. 1990); however, administration of rhIGF-I to healthy subjects causes a marked suppression of insulin secretion (Guler, Zapf et al. 1987; Boulware, Tamborlane et al. 1994) and does not allow a clear interpretation of the glucose-lowering potential of IGF-I. Another obstacle in studies of IGF-I effects *per se* is the close relationship to GH secretion, which is well known to affect insulin sensitivity (Yakar, Liu et al. 2001; Vijayakumar, Novosyadlyy et al. 2009); therefore, by blocking GH effects, the direct effects of rhIGF-I have been elucidated and improved glucose uptake and improved insulin sensitivity have been demonstrated (Crowne, Samra et al. 1998; O'Connell and Clemmons 2002; Simpson, Jackson et al. 2004; Yakar, Setser et al. 2004). Improved HbA1c has been demonstrated in long-term studies on rhIGF-I (Cheetham, Holly et al. 1995; Acerini, Patton et al. 1997; Quattrin, Thrailkill et al. 1997) but, although not significant, a reduction in GH secretion was demonstrated

(Cheetham, Holly et al. 1995; Thrailkill, Quattrin et al. 1997), making the interpretation of the role of IGF-I *per se* difficult.

5.6.4 Tissue IGF-I and glucose disposal

In order to validate determinations of tissue IGF-I (md-IGF-I) by microdialysis and to further disclose the role of IGF-I on glucose metabolism, we conducted Study IV. We circumvented the issue of interference of endogenous insulin by studying subjects with T1DM and a lack of endogenous insulin production. The clamp provided a continuous insulin infusion and insulin levels were constant. The distribution of IGF-1R and IGF-1R/IR-hybrid receptors suggests that the actions of IGF-I are almost entirely restricted to skeletal muscles and not to the liver or adipose tissue (Zapf, Schoenle et al. 1981; Bolinder, Lindblad et al. 1987; Caro, Poulos et al. 1988; Moller, Arner et al. 1991; Furling, Marette et al. 1999; Back and Arnqvist 2009). We demonstrated that muscle and subcutaneous fat IGF-I levels, determined by md (md-IGF-I), directly reflected the action of IGF-I on glucose metabolism and are thus a valid method for detecting tissue IGF-I levels. We also demonstrated that a s.c. injection of a high dose (120 µg/kg) of rhIGF-I induced a sustained increase in glucose utilization with a simultaneous increase in tissue md-IGF-I levels for at least 4 hours. Circulating IGF-I increased already after 30 min, demonstrating that time is required before s.c. injected IGF-I is taken up by the circulation, in accord with the observation that an i.v. injection of rhIGF-I momentarily increases circulating IGF-I. After an i.v. injection of rhIGF-I, the glucose-lowering effect is observed after 30 min (Guler, Zapf et al. 1987), indicating that injected IGF-I is retained in the circulation by the IGF-BPs. This is in line with the observation that the increase in md-IGF-I levels was detected in muscle and subcutaneous fat during the second hour after the s.c. injection of rhIGF-I. It is noteworthy that the GIR increased with the same delay, indicating that glucose uptake and utilization correlate with the local changes in tissue IGF-I levels. We did not determine any effect on GH and cortisol (glucagon was not measured) suggesting that, under the present study conditions, the glucose-lowering effects of rhIGF-I are direct and not mediated via suppression of these glucoregulatory hormones.

During the insulin clamp, IGFBP-1 decreased rapidly and to the same extent after saline and rhIGF-I injection. This would be expected to increase the bioactivity of IGF-I (free IGF-I) in the circulation (Yagasaki, Kobayashi et al. 2009). Interestingly, we did not find any increase in glucose disposal or in md-IGF-I levels after saline injection, in spite of this marked decrease in IGFBP-1. Thus, our study has the potential to determine the validity of measurements of free IGF-I in serum as predictors of tissue IGF-I effects, but we have to await such determinations to fully settle this matter. However, we can draw the conclusion that the biological effects on glucose metabolism and on tissue IGF-I of such a marked suppression of IGFBP-1 are lacking. In further support of our observation is the study in transgenic mice by Yakar *et al.* (Yakar, Liu et al. 1999) which demonstrates an unchanged free IGF-I level in the LID mice and, at the same time, a markedly (75%) decreased total IGF-I level which was associated with a 4-fold increase in GH secretion, supporting the view that the effects on the IGF-1R in the pituitary are not reflected by circulating free IGF-I.

Local muscle IGF-I levels remained elevated and appeared to level off toward the end of this study (4 h). Our study did not determine whether there was a slow release to the tissues of circulating stores of IGF-I bound in the ternary complex with IGFBP-3 and ALS, or whether IGF-I was stored locally bound to IGF-BPs and the extracellular

matrix. However, the finding of a sustained increase in circulating IGF-I for up to 4 h supports the former explanation. In addition, a more prolonged increase in glucose utilization was seen after injecting this high dose of rhIGF-I (120 µg/kg), compared with the peak after approximately 2 h after injection that we observed in our severe PIGFD subjects, who have a compromised ternary complex formation. Our observation in severe PIGFD patients is in accord with previous reports (Grahnen, Kastrup et al. 1993).

The md-IGF-I levels did not differ between muscle compartments and subcutaneous fat levels at baseline, but they increased to slightly higher levels in subcutaneous fat. Whether this observation is explained by a higher access of circulating IGF-I to subcutaneous fat, perhaps related to higher blood flow, by low expression of the IGF-IR in subcutaneous fat and thereby a decreased internalization and clearance, or by other factors, was not investigated.

We have previously evaluated the 20 kDa cut-off microdialysis probe and demonstrated that the *in vitro* recovery of IGF-I was approximately 10%, whereas neither intact nor fragmented IGF-BPs crossed the probe (Berg, Gustafsson et al. 2006; Berg, Gustafsson et al. 2007). Although the IGF-I assay was slightly modified in the current study, we found that baseline values were comparable with those of our previous studies in healthy subjects. Thus, so far, our hypothesis that tissue levels of IGF-I are unchanged or even elevated in T1DM despite the low circulating IGF-I levels is supported, although this observation needs to be confirmed in the same study. Also the peak md-IGF-I levels in interstitial muscle tissue after rhIGF-I injection were comparable to peak md-IGF-I levels in muscle after exercise (Berg, Gustafsson et al. 2006). We believe that we have obtained physiologically relevant, although not exact, tissue IGF-I levels. Desvigne *et al.* (Desvigne, Barthelemy et al. 2005) used a 60-kDa cut-off probe and reported free IGF-I concentrations in the muscle tissue that were 10 times higher than the levels reported by us and also 10 times higher than serum free IGF-I levels in their own study. The existence of an IGF-I gradient from muscle (high) to circulation (low) would imply that endocrine IGF-I does not significantly contribute to muscle glucose utilization and this notion contrasts with our current findings.

The md technique has a potential for assessing paracrine/autocrine IGF-I abnormalities in type 1 diabetes and, in this study, we demonstrated for the first time that tissue IGF-I levels determined by md are closely linked to local actions of IGF-I on glucose metabolism. Tissue IGF-I may help us to understand the involvement of IGF-I in the development of diabetic micro- and macrovascular complications (Sonksen, Russell-Jones et al. 1993; Clemmons, Maile et al. 2007).

We used the highest recommended dose of rhIGF-I (120 µg/kg) approved in PIGFD (Chernausek, Backeljauw et al. 2007) to increase the likelihood of obtaining detectable levels of md-IGF-I. However, previous studies exploring the potential role of rhIGF-I as an adjunct to intensive insulin treatment in children and adolescents with type 1 diabetes have shown that lower doses (40 µg/kg daily) are safe and effective for lowering GH secretion, HbA1c, and insulin requirements over 4–12 weeks (Cheetham, Holly et al. 1995; Acerini, Patton et al. 1997). However, despite the well-documented beneficial effects of a lower dose (40 µg/kg and day), there has been a hold for almost a decade on further exploration of this promising treatment for T1DM.

6 SUMMARY AND CONCLUSIONS

Paper I was the first, and is still, the only report presenting data on insulin sensitivity and body composition in adolescents with a GHR defect (severe PIGFD) treated with either rhIGF-I/rhIGFBP-3 or rhIGF-I alone. We found decreased fat mass, increased lean body mass, and improved linear growth in response to rhIGF-I administration, although rhIGF-I alone appeared to be more efficient. The data on changes in insulin sensitivity assessed by hyperinsulinemic euglycemic clamps were not congruent. However, decreased overnight insulin secretion, most prominent on rhIGF-I alone, suggested that insulin sensitivity was improved. rhIGF-I alone resulted in a clear diurnal rhythm of circulating IGF-I, a higher average circulating IGF-I level, and more pronounced suppression of GH, which was associated with faster linear growth and, to some extent, more pronounced metabolic effects.

Paper II was the first report on changes in the GH/IGF-I axis in adolescents with T1DM (acquired IGF-I deficiency) changing from NPH insulin to long-acting insulin glargine. The finding of decreased overnight IGFBP-I levels and increased circulating IGF-I levels indicated a more efficient nightly insulin delivery, thus suggesting improved hepatic insulin sensitivity and improved hepatic GH sensitivity, which was associated with improved HbA1c.

Paper III is the first and only report on changes in the GH/IGF-I axis in children and adolescents randomized to CSII or NPH insulin from the initial diagnosis of T1DM (acquired IGF-I deficiency). The finding of decreased fasting IGFBP-1 indicated a more efficient nightly insulin delivery by CSII and thus improved hepatic insulin sensitivity and, in addition, decreased insulin levels in the CSII group, indicating improved whole body insulin sensitivity.

Paper IV is the first, and only report demonstrating that administration of rhIGF-I to young adult males with T1DM (acquired IGF-I deficiency) increases tissue IGF-I in muscle and subcutaneous fat as determined by microdialysis and, concomitantly, increased whole body glucose disposal, presumably by increasing glucose uptake in skeletal muscle.

Taken together, this thesis demonstrates that in children and adolescents with T1DM (acquired IGF-I deficiency), treatment with insulin glargine or CSII improves hepatic insulin sensitivity by virtue of a more beneficial insulin delivery profile. Under certain circumstances, insulin glargine does increase circulating IGF-I, presumably by affecting hepatic GHR function, and it also lowers HbA1c. We did not demonstrate an effect of CSII on endogenous insulin production. Suppression of GH hypersecretion requires a marked increase in circulating IGF-I, which was not obtained by the insulin regimens tested. Most probably, this requires IGF-I administration as this was shown to efficiently suppress GH in the two patients with severe PIGFD. Although this thesis suggests that IGF-I has positive effects on insulin sensitivity which are independent of GH, the major effect of IGF-I administration, as previously shown in T1DM, is mediated by suppression of GH. This thesis presents evidence that the microdialysis technique can be used to assess biological effects of IGF-I at the tissue levels. This may be crucial in future studies of insulin and IGF-I co-treatment in T1DM for monitoring tissues levels and testing the hypothesis that local IGF-I remains unchanged or is even lowered if GH hypersecretion and peripheral hyperinsulinemia can be prevented.

7 FUTURE PERSPECTIVES

The subjects in Paper I have reached their final height (AA 156.6 cm and BB 160.6 cm) and stopped rhIGF-I treatment. Currently, there is no approved indication for rhIGF-I treatment in adult severe PIGFD. However, the findings in this thesis suggest that there are beneficial effects on body composition and decreased overnight insulin secretion, in accord with the metabolic effects reported in adults (Laron, Avitzur et al. 1995; Mauras, Martinez et al. 2000). This strongly suggests that rhIGF-I treatment should be considered for “metabolic” reasons in the adult. In GHD patients, GH treatment is approved to normalize metabolism in adulthood. I would suggest that an international multicenter RCT on IGF-I treatment in adults with severe PIGFD should be conducted.

The main target in the treatment of T1DM in children and adolescents should be to optimize metabolic control by active education, psychosocial support, and intensive insulin treatment. However, this thesis suggests that normalization of the obvious hormonal disturbances in the GH/IGF-I axis may be important in this respect, and even beyond that.

The beneficial effects of CSII and insulin glargine on the GH/IGF-I axis should be further explored in adolescents beyond remission in an RCT (cross-over design) to elucidate the unanswered questions regarding the mechanisms behind improved hepatic and peripheral insulin sensitivity.

Furthermore, whether MDI therapy with the new long-acting basal insulin analogs from the onset of T1DM in children and adolescents is superior to NPH insulin and/or CSII with respect to metabolic control and changes in the GH/IGF-I axis remains to be demonstrated in randomized trials.

In the future, we hope for a permanent cure of T1DM by either primary or secondary prevention or halting of the destruction of the β -cells. While awaiting such a breakthrough, effective tertiary strategies for optimizing glucose control and preventing micro- and macrovascular complications must be the focus.

This thesis questions whether normalization of the GH/IGF-I axis can be achieved by new insulin analogs, CSII, or with closed-loop systems. We believe that adjunctive therapy with rhIGF-I should be further explored in the absence of other current pharmacological substances capable of improving the GH resistance in the liver. Given that rhIGF-I treatment is available and has been shown to have the expected beneficial effects in T1DM and is approved in severe PIGFD, it is surprising that there are no currently ongoing studies of rhIGF-I treatment in T1DM.

At the time when exploration of rhIGF-I as adjunctive therapy in T1DM was discouraged by the medical agencies due to the fear that rhIGF-I therapy would accelerate complications, our understanding of these processes and the involvement of IGF-I was limited. It is clear that IGF-I is involved in the progression of complications. However, this thesis raises the question of whether local tissue levels of IGF-I may be increased rather than decreased in T1DM and has provided and validated the microdialysis tool for studying this in the future.

The goal for adjunctive rhIGF-I treatment in T1DM is improved insulin sensitivity achieved by increasing circulating IGF-I levels and reduced GH hypersecretion. In future trials it will be of interest to explore whether adolescents with type 1 diabetes show similar or even higher tissue IGF-I levels, compared to those of healthy adolescents. If a proven safe dose of rhIGF-I (40 µg/kg/day), through improved insulin sensitivity and decreased subcutaneous insulin dosages, will reduce peripheral hyperinsulinemia and thus suppress, rather than increase, tissue IGF-I levels, must be examined. Moreover, in a life-long perspective, these considerations may help to understand the involvement of tissue IGF-I in the development of diabetic micro- and macrovascular complications

I hope that, in the future, we will be given the opportunity to further explore the actions of rhIGF-I and elucidate their effects on metabolic control, tissue IGF-I levels, and the systemic level of the GH/IGF-I axis. In this context, I suggest that we should study patients before or at the onset of puberty so that the pathophysiological changes that are the basis for the development of complications are prevented. That, instead of treating patients in a phase where IGF-I treatment may have completely opposite effects in already established pathological lesions, may be the most important factor for success. In the currently ongoing studies on proliferative retinopathy of the premature, such a strategy has been employed.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Bakgrund: GH/IGF-I axeln beskriver samspelet mellan tillväxthormon, GH, och den insulinlika tillväxtfaktorn, IGF-I. Dessa stimulerar längdtillväxten men motverkar varandra i glukosmetabolismen. Under puberteten ökar GH och IGF-I för att maximera tillväxten och kroppen blir ”insulin resistent”. Det betyder att mer insulin behövs för att ta hand om samma mängd glukos. För att avlasta insulin stimuleras glukosupptaget i muskulaturen också av IGF-I (insulinlik). GH bildas i hypofysen och insöndras till blodet där det transporteras till dockningsstationer (receptorer) på kroppens celler. GH stimulerar tillväxt genom direkta effekter men också via stimulering av IGF-I bildning både i vävnaderna och i levern. IGF-I balanserar GH insöndringen från hypofysen genom ”negativ återkoppling”, dvs låga nivåer av IGF-I ökar GH insöndringen till blodet. Barn med svår primär IGF-I brist (ca: 250 fall i världen) saknar fungerande receptorer för tillväxthormon (Larons syndrom) och har extremt låga nivåer av IGF-I. Utan behandling blir de kortvuxna (vuxenlängd ~120-130 cm) och feta. De saknar GH:s effekter på tillväxt och ämnesomsättning (fettnedbrytande) och bristen på IGF-I kompenseras med ökad insulininsöndring som driver fettbildningen. Typ 1 diabetes är en autoimmun sjukdom där den egna insulinbildningen försvinner och som behandlas med insulininjektioner i huden. Insulinnivåerna i leverblodkärlet blir därför låga och GH receptorena i levern reagerar trögt på GH stimulering (lokal GH resistens) och endast små mängder IGF-I bildas (förvärvad brist på IGF-I). Lågt IGF-I medför sämre glukosupptag i musklerna men också ökad GH insöndring från hypofysen. Hos pubertala ungdomar leder detta till sämre sockerbalans, högre blodsockervärden och sämre metabol kontroll (högt HbA1c) och ökad risk för långtidsskador på blodkärlen (syn, njurar, nerver).

Avhandlingens syfte

-att studera hur behandling med rhIGF-I eller i kombination med rhIGFBP-3 påverkar tillväxt, insulinkänslighet och kroppssammansättning vid svår primär brist på IGF-I (Larons syndrom).

-att studera hur behandling med den långverkande insulinanalogen insulin glargin respektive insulinpump påverkar GH/IGF-I axeln, insulinkänslighet och HbA1c samt att studera effekten av rhIGF-I på glukosupptag och vävnadsnivåer av IGF-I mätt med mikrodialys vid förvärvad brist på IGF-I (typ 1 diabetes).

I **arbete I** studerade vi ett brödrapar med en GH receptor mutation. De har av och till behandlats med rhIGF-I sedan 1994. I vår studie undersöktes de första gången efter att ha varit utan läkemedel i 8 månader (rhIGF-I tillverkades ej), därfter insattes behandling med rhIGF-I/rhIGFBP-3. Utöver tillväxt undersöktes kroppssammansättning och insulinkänslighet efter 6 veckor respektive 17 månader. Vi kunde därefter byta till rhIGF-I och nya undersökningar gjordes efter 6 veckor respektive 12 månader. Vi fann att båda behandlingarna gav minskad fettmassa, ökad muskelmassa och bättre tillväxt men rhIGF-I var mest effektiv. Resultaten avseende insulinkänslighet, mätt med s.k klamptechnik, gick åt olika håll men en sänkt nattlig insöndring av insulin sågs efter båda behandlingarna och talar för att insulinkänsligheten förbättrades.

I arbete II studerade vi effekten på HbA1c och GH/IGF-I axeln före och efter byte till det nya långverkande insulin glargin som har en bättre nattlig insulineffekt i jämförelse med NPH insulin. Vi undersökte 12 st pubertala ungdomar (4 pojkar) med typ 1 diabetes i en observationsstudie under 12 veckor där de var sina egna kontroller. Vid studiens start och efter 6 veckor var de inlagda på sjukhus under 26 timmar och vi mätte hormonella dygnsprofiler för GH och IGFBP-1. Uppföljande läkarbesök och provtagning gjordes vid 2, 4, 8 och 12 veckor. Vi fann att insulin glargin gav minskade nivåer av IGFBP-1, en markör på förbättrad insulinkänslighet i levern, ökade nivåer av IGF-I, som tecken på bättre leverinsulinerings och sänkt GH resistens i levern, samt förbättrat HbA1c.

I arbete III. studerade vi effekterna på GH/IGF-I axeln och ev. skillnader i förmåga att bevara den egna insulinbildningen med insulinpump eller NPH (måltidsinsulinregim) från debut av typ 1 diabetes. I en randomiserad studie undersöktes 72 barn och ungdomar (34 insulinpump). Vid 0, 6, 12 och 24 månader togs fastande blodprov för IGF-I, IGFBP-1, C-peptid och insulindoserna dokumenterades. Vi fann att behandling med insulinpump gav minskade nivåer av IGFBP-1, en markör på förbättrad insulinkänslighet i levern och sänkta insulindoser indikerande en förbättrad insulinkänslighet men ingen skillnad i IGF-I eller egen insulinbildning.

I arbete IV studerade vi effekten av rhIGF-I på glukosupptag och vävnadsnivåer av IGF-I mätt med mikrodialys teknik. Vi undersökte 8 st män (~19 år) med typ 1 diabetes i en randomiserad, placebokontrollerad och enkelblindad studie. De undersöktes med s.k. insulinklamp och gavs en kontinuerlig insulininfusion och samtidigt en glukosinfusion som varierades för att hålla blodsockret på 5 mmol/l. Mikrodialyskaterar placerades i vardera lårmuskeln och i underhuds fett på magen. Katetern är ett membranföretsett plaströr (3 cm) med små hål genom vilka fritt IGF-I kan passera. Via en pump flyter vätska genom katetern och det IGF-I som mäts i uppsamlingsröret är en spegel av det fria IGF-I i vävnaden. Efter ca 2 timmars stabilisering gavs en engångsdos av rhIGF-I (120 µg/kg) eller koksalt i underhuden. Mikrodialysat insamlades i timfraktioner och blodprover togs var 30:e minut. Vi fann under den andra timmen en klar ökning av glukosupptaget och parallellt en ökning av IGF-I i mikrodialysaten men IGF-I i cirkulationen ökade redan efter 30 minuter.

Slutsatserna i avhandlingen är att rhIGF-I är effektivare än rhIGF-I/rhIGFBP-3 för att stimulera längdtillväxt hos barn med svår primär IGF-I brist men båda behandlingarna förbättrar kroppsammansättningen och sänker insulinsekretionen vilket skulle kunna tyda på bättre insulinkänslighet även om klamparna inte visade detta. Vidare att vid typ 1 diabetes ger insulin glargin respektive insulinpump en mer effektiv nattlig insulinerings och förbättrar leverns insulinkänslighet mätt som lägre IGFBP-1. Insulin glargin ger också ökade IGF-I nivåer och förbättrad metabol kontroll, HbA1c. Avhandlingen visar också att mikrodialys kan användas för att mäta fritt IGF-I i muskel och fett samt att fritt IGF-I speglar en direkt biologiska effekt (glukosupptag).

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