Insulin receptor and IGF-I receptor Bioactivity in Health and Disease

Aimee J. Varewijck

Stellingen behorend bij het proefschrift

Insulin Receptor and IGF-I Receptor Bioactivity in Health and Disease

1. Met de introductie van de insuline radio immunoassay (RIA) werd (en wordt nog steeds) de rol van non-supressible insulin-like activity in serum genegeerd.
   (Dit proefschrift)

2. De activering van de insuline receptoren door serum kan slechts ten dele met specifieke antilichamen gericht tegen insuline geblokkeerd worden. In serum aanwezige insuline-achtige groeifactoren lijken bij de activering van de insuline receptoren een niet te verwaarlozen rol te spelen.
   (Dit proefschrift)

3. Het meten van de concentratie van een insuline analoog met de huidige insuline immunoassays is onbetrouwbaar.
   (Dit proefschrift)

4. Er lijkt voor wat betreft de stimulatie van de IGF-I receptor geen duidelijk verschil te bestaan tussen het bloed van type 2 diabeten behandeld met insuline glargine of met humaan insuline.
   (Dit proefschrift)

5. Voor het stellen van de diagnose groeihormoon deficiëntie zijn metingen van de IGF-I receptor bioactiviteit waarschijnlijk meer informatief dan metingen van het immunoreactief totaal IGF-I.
   (Dit proefschrift)

6. In een subgroep van patiënten met Graves' Ophthalmopathie kunnen circulerende IGF-I receptor stimulerende antistoffen aangetoond worden. In hoeverre deze antistoffen een pathogenetische rol spelen bij Graves' Ophthalmopathie is op dit moment (nog) onduidelijk.
   (Dit proefschrift)

7. Kanker is een belangrijke complicatie van diabetes.

8. If the overall variation is mainly caused by narrow individual variation around dispersed individual set-points, then a population-based reference range is unlikely to detect minor deviations from the individual set-point.
   (Thyroid 2003 13 1069-1078)

9. Het verkorten van de opleiding tot internist is mogelijk als deze verkorting tenminste gepaard gaat met het tot een minimum beperken van de administratieve taken van de arts-assistent.

10. Optimism is the faith that leads to achievement.
    (Helen Keller)

11. If you wait for the perfect time to have children, you'll never have children.
    (Grey's Anatomy)

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Bioactiviteit in Gezondheid en Ziekte

Proefschrift

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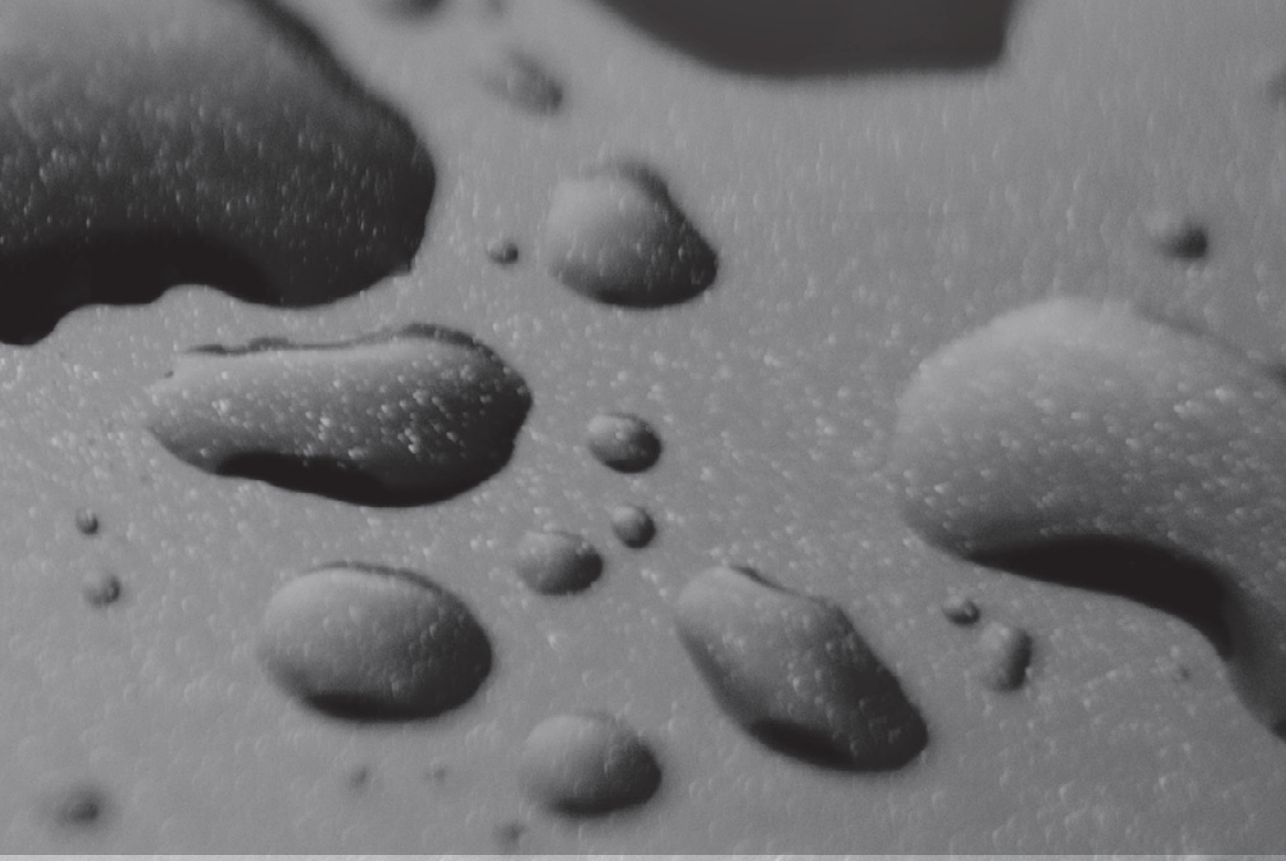

Introduction and aims of thesis

Aimee J. Varewijck, Joseph A.M.J.L. Janssen.

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I. GENERAL INTRODUCTION

Insulin bioassays and immunoassays laid the foundation for the discovery of the IGFs

"That which we call a rose by any other name would smell as sweet."

(Romeo and Juliet (II, ii, 1-2))

Insulin was discovered in 1921[1] by Banting and Best and its structure elucidated in 1955[2]. The first insulin bioassays appeared in the 1940s. First, rats were injected with a range of known concentrations of purified commercial or 'standard' insulin and the subsequent fall in blood glucose levels was measured. Then an unknown sample of human plasma was administered to a rat and its insulin concentration was assumed to be identical to the standard dilution that caused the same fall in glucose levels[3-4]. Due to poor correlations between measured blood glucose levels and calculated insulin levels, these bioassays were replaced by in vitro bioassays. Metabolic parameters, such as rate of glucose uptake in response to dose-response curves of known insulin concentrations were measured using isolated tissues, such as the hemidiaphragm or epididymal fat pad from the rat[5-7]. Also these in vitro bioassays for plasma insulin were not very successful due to high inter-assay variability, their laborious nature and due to a growing doubt that they were not specific for insulin[8]. Maybe even more importantly, in 1959 Leonards described a substance in normal human fasting serum that, like insulin, stimulated glucose oxidation and triglyceride synthesis in adipose tissue but that, unlike insulin, could not be extracted from plasma into acid-ethanol[9]. In 1963, Froesch et. al[10] found that serum from guinea pigs immunized against insulin, suppressed insulin action in fat tissue, but it had no effect on Leonards's insulin-like substance and so the term non-suppressible insulin like activity (NSILA) was born.

By the mid-1950s there was a clear need to measure plasma insulin by a different tool. In 1960, Berson and Yalow published a method for the measurement of plasma insulin by radioimmunoassay (RIA)[11]. The insulin RIA was based on the principle that the concentration of the unlabelled insulin (cold insulin) was proportional to the extent to which unlabelled insulin displaced radiolabelled insulin (hot insulin) from anti-insulin antibody[11]. In the final section of their paper[11], Berson and Yalow discussed the paradox that serum insulin concentrations determined by bioassay usually greatly exceeded insulin concentrations determined by RIA in the same specimens. In attempting to account for this discrepancy, they noted that Leonards's finding of NSILA in normal human fasting plasma 'has raised a serious question as to what part of the insulin action on fat tissue is due to insulin itself'. They then suggested that the 'much higher estimated plasma insulin concentration' determined by bioassay may indeed have been attributable to the presence of non-insulin substances with insulin-like activity[11]. The introduction of the RIA for insulin has greatly increased knowledge of the physiology of glucose

homeostasis and of the diverse causes of diabetes mellitus. However, its use discarded the notion of NSILA (as discussed above) and thereby delayed the identification of the insulin-like growth factors (IGFs)[12].

Growing awareness that not all ILA in serum was suppressible by insulin antibodies closely intertwined with the research that had begun in the mid-fifties by Salmon and Daughaday. They recognized that the failure of growth hormone (GH) to stimulate in vitro sulphate incorporation in cartilage was due to the existence of an intermediary factor induced by GH[13]. This factor was descriptively termed sulphation factor (SF)[13]. Later Salmon and DuVall showed that rat serum exhibited activities, besides SF activity, that resembled those of insulin and were not suppressible by insulin antibodies[14]. They suggested SF to be identical with one or more plasma insulin-like factors[14].

The observation that both SF and NSILA were GH dependent further raised the suspicion that these activities could be identical. In 1972 a more generic name for SF was proposed, namely somatomedin (SM)[15], since, besides stimulation of sulphate incorporation in cartilage, also protein and DNA synthesis in cartilage were stimulated by serum in a GH-dependent fashion and since SF was also active in muscle tissue. Subsequently, in the 'growth hormone field' different somatomedins were purified and termed SM-A, SM-B and SM-C respectively[16-18]. At the same time however, the 'metabolic field' pursued with the isolation of NSILA. In 1978 the primary structures of NSILA were identified and termed the insulin-like growth factor I (IGF-I) and II (IGF-II) due to their close similarity with proinsulin[19-20]. Years later it was finally shown that SM-A and SM-C were identical with IGF-I. Somatomedin B was found not to be GH-dependent and not to be a true growth factor[19-22]. IGF-II did not have a SM partner since it had not been purified by any of the groups using the SM nomenclature.

As for insulin, also for IGF-I and IGF-II, due to the laborious and unspecific nature of bioassays[23], these bioassays were quickly abandoned with the development of the first RIA for IGF-I in 1977[24] and for IGF-II in 1981[25].

The common origin of insulin, insulin-like growth factors and their receptors

Not only are there great functional similarities between insulin and the IGFs, there are also remarkable structural similarities. This has provided strong evidence that the genes encoding for insulin and IGFs are derived from a common ancestor gene that underwent duplication about 600 million years ago[26]. A following gene duplication may have taken place which led to the division into IGF-I and IGF-II about 300 million years ago, around the time mammals appeared on earth[26].

Insulin is formed from pre-pro insulin. Pre-pro insulin is processed into a single chain proinsulin and subsequently a connecting peptide (C-peptide) is cleaved of by a highly

specialized enzyme system resulting in an insulin molecule that contains a separate A- and B-chain, which are bound by two disulfide bridges[27-28] (Figure 1). In all vertebrates, the insulin molecule, like human insulin, is built from 51 amino acids and has a molecular weight of 5808 Da[26]. Both insulin and C-peptide are packaged in granules in the beta-cells of the pancreas, from which they are secreted into the interstitial space and hence into the blood by exocytosis[29-30]. Insulin concentrations in the blood increase rapidly and fall rapidly again according to the plasma glucose concentration and thus to the need of the organism, which can change from second to second[31].

The major structural differences between insulin and the IGFs, are that the IGFs are single chain polypeptides in which the C-peptide (C-domain) is not cleaved but conserved[26-27]. They also contain an additional D-domain, which extends from the C-terminal end of the A chain, which does not occur in insulin[27-28, 32] (Figure 1). IGF-I and IGF-II are 70 and 67- amino acid polypeptides with molecular weights of 7649 and 7479 Da, respectively[19]. In primary sequence, human insulin, IGF-I and IGF-II share 50% amino acid identity in the A- and B- domains[19]. Although IGFs are also processed from pre-pro and pro IGFs, the processing of these molecules is less complex compared to insulin[32]. In contrast to insulin, IGF-secretion is a steadier, slow process leading to a more constant level of IGFs in serum that is much higher than that of insulin. IGFs are not stored within

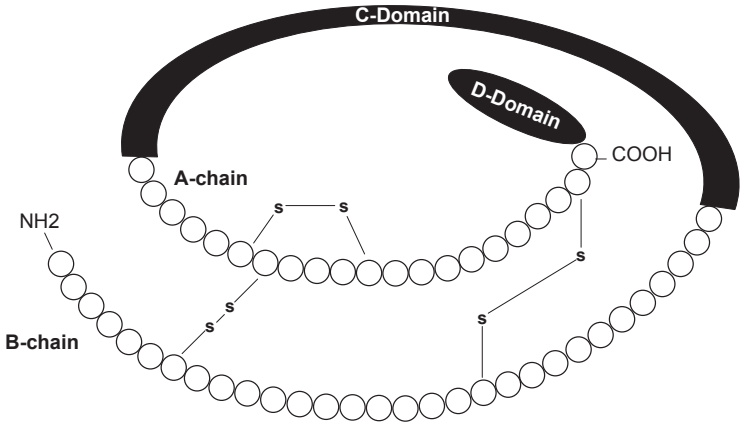


Figure 1.
Primary structure of human insulin is formed by 21 amino acids in the A-chain and 30 amino acids in the B-chain. At one end of each chain (the N terminal end) is an amino group, and at the other end (the C terminal end) is a carboxylic acid group.
The three-dimensional structure of insulin is further stabilised by disulphide bridges. These form between thiol groups (-SH) on cysteine residues. There are 6 cysteines, so 3 disulphide bridges are formed: 2 between the A and B chains and one within the A chain.
IGFs are single chain polypeptides in which the C-peptide (C-domain) is not cleaved but conserved (black). They also contain an additional D-domain (black), which extends from the C- terminal end of the A chain, which does not occur in insulin.

secretory granules within cells[31]. They are produced via a constitutive secretory pathway and directly released into the blood[31, 33]. When the IGFs are secreted into the blood, they immediately associate with soluble high affinity binding proteins, the IGFBPs, which compared to the IGFs are present in excess[31].

IGFs are widely expressed in many cell types in almost all tissues throughout the body and are considered key factors in intrauterine development and postnatal growth and metabolism[33-35].

The secretion of IGFs in uterus is largely independent of GH[36]. This was demonstrated in mice studies and in studies on patients with GHRH receptor and GH receptor mutations, in which only a slightly diminished intrauterine growth was observed[37]. In contrast, mice with a complete deficiency of IGF-I (IGF-I-null mice) created by homologous recombination, exhibited significant postnatal lethality and those who did survive showed substantial growth retardation (birth weight of approximately 60% of normal), infertility, and profound defects in the development of major organ systems[36-38]. Also IGF-II null mice had a birth weight of approximately 60% of normal. However, these mice showed normal postnatal growth rates, were fertile and had average life spans[36, 39].

In contrast to foetal life, in childhood and adult life, IGF-I secretion seems to be mainly controlled by GH. Circulating IGF-I is primarily produced in the liver. In mice specifically lacking IGF-I production by the liver, (induced via a Cre/loxP-induced conditional knockout system), circulating IGF-I levels were decreased by approximately 75%[40]. Despite the decrease in circulating IGF-I levels, these mice exhibited no defect in growth or development, suggesting that there is a considerable functional reserve capacity in the insulin-GH-IGF system and showing the importance of the autocrine/paracrine role of IGF-I. GH, age en developmental stage are not the only factors responsible for IGF-I production. Production also depends upon nutrition, adequate insulin secretion , the immune system, insulin, several tissue-specific factors such as gonadotrophins, sex steroids, cortisol, thyroid function and last but not least genetic factors[41-43].

In human foetal serum, IGF-I levels are relatively low and are positively correlated with gestational age[41, 44-45]. IGF-I levels in newborns are typically 30-50% of adult levels[25, 46]. IGF-I serum levels rise during childhood[25, 46]. During puberty, IGF-I levels rise to 2-3 times the adult range[25, 46], possibly indirectly due to the rise in gonadal steroids which give a rise in GH secretion and directly by augmenting liver synthesis and secretion of IGF-I[41]. After 20-30 years of age, like GH-levels, serum IGF-I levels gradually and progressively fall[41].

In contrast to the IGF-I gene, the IGF-II gene is imprinted with only the paternally expressed allele being active[47]. IGF-II mRNA expression is high in foetal life and declines postnatally. In human newborns, IGF-II levels are typically 50% of adult levels[25]. By one year of age, adult levels are attained and remain almost unchanged throughout life with quantities three-to four fold greater that that of IGF-I[25, 46]. These high levels of IGF-II in

adult life are in great contrast to IGF-II levels in rodents, in which levels greatly reduce after birth and remain reduced. The exact role of IGF-II in adult humans is at present not known[48]. In contrast to IGF-I production, IGF-II production seems to be much less dependent of GH[49]. In acromegalic patients normal IGF-II levels have been described[25, 50-52]. However, in contrast, in GH deficient patients, low IGF-II levels have been found[25, 50-54]. In addition, GH treatment of hypopituitary dwarfs has been found to return IGF-II levels within the normal range[49, 54-56]. This latter observation suggests that GH can stimulate IGF-II in GH-deficiency. So, a minimal concentration of GH is probably enough to maximally stimulate IGF-II production.

As insulin and the IGFs probably arose during evolution by gene duplication, there is the hypothesis that the insulin receptor (IR) and IGF-I receptor (IGF-IR) were also created by gene duplication of a common precursor receptor molecule[57]. Depending on which regions are being compared, the IR and IGF-IR have sequence similarities varying from 41-84%. Both the IR and IGF-IR are composed of two monomers, each comprising an extracellular alpha-subunit and a transmembranic beta-subunit which are linked by a disulfide bridge. They belong to the family of ligand-activated receptor kinases. Unlike other tyrosine receptor kinases, these receptors exist at the cell surface as homodimers composed of two identical alpha/beta monomers, or as heterodimers composed of two different receptor monomers (Figure 2). Binding of a ligand to the extra-cellular alpha-subunit induces the receptors to undergo a conformational change. This enables autophosphorylation of the intrinsic tyrosine kinase domains within the transmembranic beta-subunits, which is the first step in the intracellular signalling cascade.

In the human body, due to alternative splicing of exon 11 of the IR gene, two IR transcripts are generated, resulting in IR isoform A (IR-A) (lacking exon 11) and in IR isoform B (IR-B) (including exon 11)[58]. The IR-A is expressed ubiquitously, but is predominantly expressed in the central nervous system, haematopoietic cells and can also be substantially expressed in cancer tissues. IR-A is also expressed in the foetus where it may be activated by insulin and IGF-II for growth. IR-B is expressed predominantly in the liver and also in muscle and adipose tissue; the major target tissues for the metabolic effects of insulin[59-60].

The structures of the IR and IGF-IR resemble each other to such an extent that insulin and IGFs can interact with each others receptor, although with quite different affinities. The IR demonstrates high affinity binding to insulin ($K_d \sim 10^{-10}$ M), 10 fold lower affinity for IGF-II and a 50-100 fold lower affinity binding for IGF-I[61]. In this respect it is has been shown that there are differences between the IR-A and IR-B; IGF-II having a higher affinity for IR-A than for IR-B. On the other hand, the IGF-IR binds the IGFs with a high affinity ($K_d \sim 10^{-9}$- 10^{-10} M) but binds insulin with 100-fold lower affinity[61].

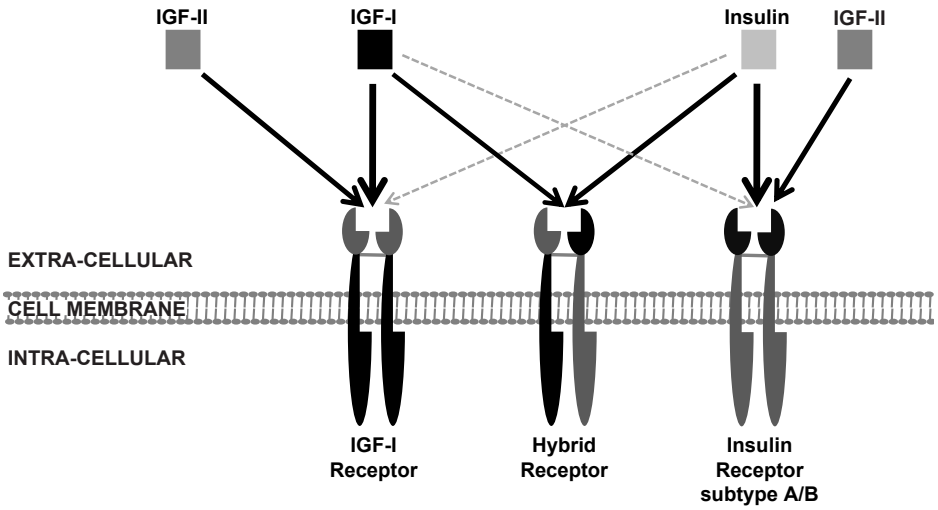


Figure 2.
Schematic overview of the receptors in the insulin-IGF system. Both the IR and IGF-IR are composed of two monomers, each comprising an extracellular alpha-subunit and a transmembranic beta-subunit which are linked by disulfide bridges (not shown). They exist at the cell surface as homodimers composed of two identical alpha/beta monomers, or as heterodimers composed of two different receptor monomers (so called hybrid receptors). Due to alternative splicing of exon 11 of the IR gene, two insulin receptor (IR) isoforms exist; isoform A (IR-A) (lacking exon 11) and in IR isoform B (IR-B) (including exon 11). With permission retrieved and modified from the thesis entitled 'IGF-I bioactivity in Aging, Health and Disease' by M.P. Brugts[166].

The IGF-IIR, or cation-independent mannose-6-phosphate receptor, is structurally unrelated to either the IGF-IR or IR as it consists of a single, primarily extracytoplasmic, polypeptide chain[62]. This receptor binds IGF-II with a greater affinity than IGF-I while it does not bind insulin[63]. It primarily acts in intracellular transport of lysosomal enzymes and sequesters IGF-II from potential receptor activating interactions by internalization and degradation[64]. Although the receptor does not contain a tyrosine kinase activity nor an autophosphorylation site, it does link to G-proteins which provides a potential mechanism for signal transduction[63].

What determines growth-promoting and metabolic effects of insulin and IGFs?

The conventional view regarding actions of insulin, the IGFs and their receptors is that insulin and the IR mainly mediate metabolic responses, whereas IGFs and the IGF-IR mediate growth promoting effects[65-66]. Structural differences of the beta-subunit and kinase domains of the IR and the IGF-IR leading to differences in substrate interactions have been suggested to be partly responsible for insulin-IGF specificity[67]. Moreover, the signal transduction by the receptors may not be limited to its activation at the cell

surface. It has been suggested that the activated ligand-receptor complex, initially at the cell surface, is internalised into endosomes. The lifetime of this complex within the endosomes might be an important factor in influencing the types of response produced by a particular receptor[68]. However, the role of ligand internalization and endosomal residence time as mechanism of selective signalling has yet never been confirmed. Recently, it was suggested that in vivo specificity of insulin and IGF-I reflects at least in part the levels and timing of the expression of IRs and IGF-IRs in target tissues in combination with ligand concentration and availability[69]. As Boucher stated: IR and IGF-IR act as identical portals for the regulation of gene expression, with differences between insulin and IGF-I effects due to a modulation of the amplitude of the signal created by the specific ligand-receptor interaction[69].

Nevertheless, evidence exists showing that insulin and IGF-I can mediate very similar responses[70]. IGF-I can exert acute metabolic effects like insulin, while insulin in turn, can substitute for IGF-I inducing growth-promoting and differentiation enhancing activities[70]. In addition, also the IR and IGF-IR share very similar intracellular signalling pathways[71] (Figure 3). Moreover, studies have suggested that differences between IR-A and IR-B in terms of receptor activation and signalling may result in different functions of each IR isoform[58].

In some tissues and cells where significant levels of both IRs and IGF-IRs are present, hybrids may be formed. These hybrids are heterodimeric receptors consisting of an IR alpha/beta monomer and an IGF-IR alpha/beta monomer linked by disulfide bonds. Such hybrids are probably formed during normal post- translational processing of both receptors[66] and are widely expressed on normal tissues and often aberrantly expressed in cancer cells[72]. Although the precise biological role of these hybrids is still unclear, it has been suggested that hybrid receptors may play a role in the overlapping functions of insulin and IGF-I[66]. Binding of insulin to a hybrid receptor would result in autophosphorylation of its own beta-subunit which, through subsequent transphosphorylation, activates the beta-subunit of the IGF-IR monomer resulting in a growth-promoting signal[66]. In the other way around, IGF binding to a hybrid receptor would result in autophosphorylation of its own beta-subunit which could activate the beta- subunit of the IR monomer by the same mechanism, thereby promoting metabolic actions[66]. Although this could explain why insulin under certain circumstances may induce cellular proliferation and IGF may stimulate metabolic functions, functional studies have demonstrated that hybrid receptors behave more like IGF-IRs than IRs[66]. So the general consensus is that the IR and IGF-IR besides their distinct functions also have overlapping functions.

IGF bioavailability

Whereas insulin circulates freely in the circulation, IGFs are found in complexes with IGF binding proteins (IGFBPs). There are, at present, six well characterized mammalian

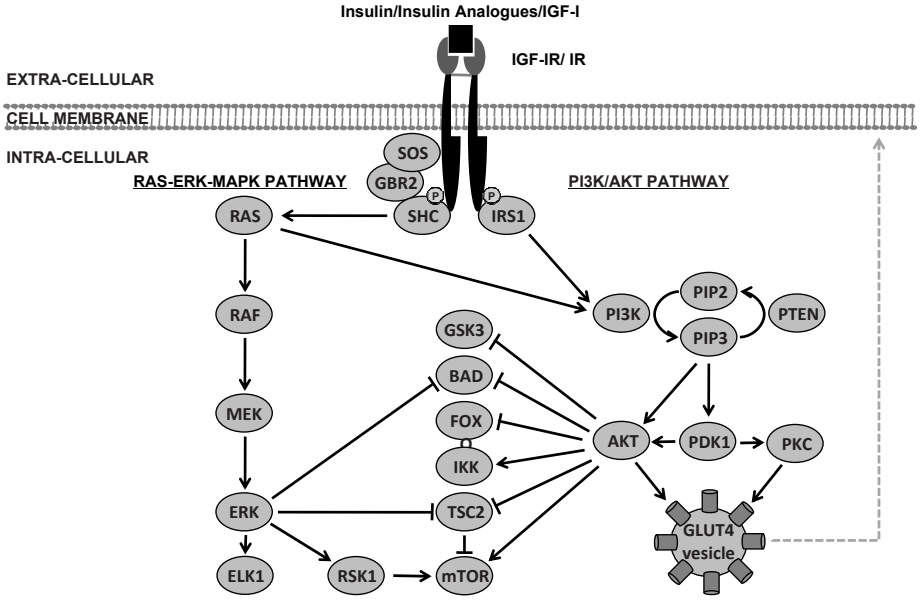


Figure 3.
Schematic overview of the IR and IGF-IR signalling pathways. After activation of the receptors by insulin, insulin analogues or IGF-I, autophosphorylation of kinase subunits leads to phosphorylation of adaptor proteins. The two main intracellular pathways, activated by both receptors, are the RAS-ERK-MAPK pathway and the PI3/AKT pathway. With permission retrieved and modified from the thesis entitled 'IGF-I bioactivity in Aging, Health and Disease' by M.P. Brugts[166].

IGFBPs, designated IGFBP-1 to -6[41, 73-74]. It was not until the mid 1980s to early 1990s that the six known IGFBPs were cloned and sequenced[49]. All six binding proteins share ~35% sequence identity with each other. IGFBPs have higher affinities for IGFs (K_d ~ 10^{-10}- 10^{-11}M) than the IGF-IR. Therefore, IGFBPs not only act as carrier proteins for IGFs, thereby prolonging half-life of the IGFs, but also modulate IGF bioavailability and activity[73]. Moreover, various studies have shown that some IGFBPs may mediate their effects on target cells by IGF-independent factors, e.g. direct association of IGFBPs with a variety of extracellular and cell surface molecules[75-76]. It has been estimated that around 1% of circulating IGF is present in its free form[77]. IGFBP-3, which is a GH-dependent IGFBP, is the most abundant IGFBP in the circulation with levels more than 10-fold higher than the other IGFBPs[49]. The major pool of IGFs (75-80%) circulate in human serum as 150-200kDa complexes consisting of IGF, IGFBP-3 (38-43 kDa) and an acid labile subunit (ALS) (80 kDa)[49]. The remaining IGFs circulate within a ~50 kDa complex consisting of one of the IGFs and one of the other IGFBPs. The ALS does not bind the IGFs but increases the molecular mass of the IGF-IGFBP-3 complex (ternary complex). In the ternary complex circulating IGFs cannot leave the circulation and can therefore not be transported to the

extracellular fluid or various tissues. In this way the availability of the IGFs to the tissues is limited and controlled.

From bioassays to immunoassays and back?

As mentioned above, IGF bioactivity nowadays is estimated primarily by the measurement of total (i.e. extractable) IGF-I and IGF-II due to the lack of reliable bioassays[78-79]. Moreover, these bioassays often lacked specificity and were labour intensive. Many of the methods currently used for measurement of circulating total IGFs are hampered by interferences of IGF-binding proteins (IGFBPs) remaining after extraction[80]. On the other hand, by extracting IGFBPs the modifying effects of these proteins on IGF-I action are ignored. A bioassay for assessing IGF bioactivity should be easily quantifiable, highly sensitive, and based on a signal specifically transmitted by the IGF-IR[81]. In 2003, a Kinase Receptor Activation (KIRA) bioassay was developed by Chen et al. in order to measure IGF bioactivity at physiological conditions[78]. The principle of this assay is based on quantification of IGF-IR activation after stimulation with serum in vitro[23]. In this way bioavailable IGF-I is quantified while taking into account the modifying effects of IGFBPs. The IGF-IR KIRA assay directly targets the activated IGF-IR, requires only small volumes of serum, has a short incubation time, is sensitive to the modifying influences of circulating IGFBPs and IGFBP-proteases, and has an overall precision that is comparable with the traditional IGF-I immunoassays[23, 78, 82].

Insulin immunoassays measure only immunoreactive insulin and by doing so they ignore at least the potential insulin-like effects of the IGFs in blood. Moreover, insulin immunoassays do not assess potential biological effects of circulating insulin-like factors on the IR-A and the IR-B.

First aim of the thesis: To develop a KIRA bioassay specific for the IR-A and IR-B in order to determine the potential biologic actions of ligands and serum on the IR-A and the IR-B and to assess the relative contribution of circulating IGFs in this respect. The results of this study are described in chapter 2.

II. INSULIN ANALOGUES AND THE INSULIN-IGF SYSTEM

The discovery of insulin represented a milestone in clinical medicine. It has saved the lives of many who would otherwise have died, but its unforeseen effect was to transform an acute, rapidly fatal illness into a chronic disease with serious long-term complications[83].

In the late eighties of the past century methods were developed that allowed insulin to be synthesized in the laboratory. These methods have permitted the production of limitless quantities of human insulin for therapeutic use.

In the body insulin exists as monomers, dimers and as hexamers (consisting of six monomers which self associate in conjunction with zinc ions). An adaptation of beta-cell insulin production is the self association of insulin molecules, at high concentrations, in conjunction with zinc ions into hexamers[84]. This process provides efficient spatial storage within the beta-cell vesicles, but dilution upon exocytosis ensures immediate dissociation into dimers (association of two insulin molecules) and finally into monomers. Monomers are the biological active forms that bind to the IR.

Insulin complexed to zinc ions dissociates only slowly into insulin monomers. Therefore these preparations are used to maintain basal insulin levels (i.e. levels required in a fasting state). During a meal more rapid-acting monomeric insulin is needed to provide meal-related increased insulin requirements.

The first available insulin preparations failed to simulate physiological insulin profiles. However, through genetic engineering of DNA, the amino acid sequence of natural insulin could be changed in such a way that alterations were made in absorption, distribution, metabolism and excretion characteristics of this molecule. Interestingly, although these modified molecules are more commonly referred to as insulin analogues, the U.S. Food and Drug Administration (FDA) refers to these also as "insulin receptor binding agonists"[85]. Two main groups of insulin analogues can be distinguished in 1] short-acting insulin analogues, genetically engineered in such a way that they dissociate more rapidly following injection and in 2] long-acting insulin analogues which show a delayed absorption or a prolonged duration of action (see below).

Binding of insulin analogues to the IR and the IGF-IR

Structural modification of the insulin molecule may result in altered binding affinities and activities to the IR and/or the IGF-IR. As a consequence insulin analogues may have an increased/decreased metabolic action and an increased/decreased mitogenic action than human insulin.

The amino acid residues in the insulin molecule that are essential for binding to the IR have been identified[86]. Especially modifications at positions in the B26-B30 region i.e. the C-terminus of the B-chain, do not seem to significantly influence insulin binding to the IR[87-88] (Figure 4). However, this region is important for at least 2 reasons. First, these aminoacids are important for insulin dimerisation[89-90]. Modification of this latter region reduces the stability of monomer-monomer interactions and this effect has been used to generate monomeric insulin analogues with only slight changes in affinity for the IR. Secondly, substitutions of amino acids in the B- chain result in insulin molecules which show increased structural homology with IGF-I and as a consequence have an increased affinity for the IGF-IR[86-87]. Proline at position 28 and lysine at position 29 is the natural sequence which is present in the B- chain of human insulin (Figure 4). The number and position of basic or acid residues in this region seems very important for IGF-IR bind-

ing[86]. Substitution of position B28 with basic residues increases the relative affinity to the IGF-IR approximately 1.5-2-fold (ArgB28ProB29> OrnB28ProB29=LysB28ProB29)[86]. In contrast, substitution with acidic residues (CyaB28ProB29=GluB28ProB29>AspB2 8ProB29) reduces relative affinity for the IGF-IR approximately 2-fold. Combination of aspartic acid substitution at B10 with a modification in position B28-29 (e.g. AspB10, LysB28, ProB29) increases the affinity for the IGF-IR 2-fold. Addition of arginine residues at position B31-32 (B31B32diArg) increases the affinity for the IGF-IR 10-fold compared to human insulin, while the same addition in combination with aspartic acid substitution at B10 increases the affinity to the IGF-IR even 28-fold. In general, analogues with substantially increased IGF-IR affinity are more potent in stimulating proliferation of cells. This could be of clinical importance since most primary tumours and malignant cells show an increased expression of IGF-IRs[91].

On the other hand, Hansen et al. have shown that an increased mitogenic potency of insulin analogues may not only be due to an increased affinity for the IGF-IR but may also result from slow ligand dissociation from the IR[92]. A slow IR dissociation rate is associated with a sustained activation of the IR tyrosine kinase and phosphorylation of the intracellular located Shc protein.

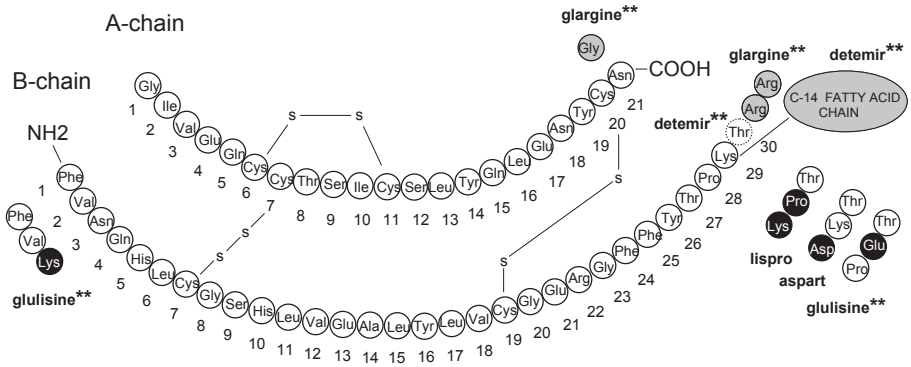


Figure 4.

Modifications made on the insulin backbone to produce short-acting insulin analogues are indicated in black, modifications made to produce long-acting insulin analogues are indicated in grey. For insulin lispro, proline at the position 28 and lysine at position 29 in the B-chain of human insulin are interchanged. For insulin aspart, the proline at position 28 in the B-chain is replaced by aspartic acid. For insulin glulisine, the asparagine at position 3 and lysine at position 29 in the B-chain are replaced by lysine and glutamic acid. For insulin glargine, aspartic acid at position 21 in the A-chain has been replaced for glycine and the B-chain contains two extra amino acids (two argenines) at position 31 and 32. For insulin detemir, threonine at position 30 of the B- chain is removed and a 14-carbon fatty acid chain is added to position 29 of the B-chain. ** Two changes have been made.

So, there seem to be at least two mechanisms by which analogues may have an increased mitogenic potency; either through a higher affinity for the IGF-IR and/or by a slower dissociation after binding to the IR.

The story of the first rapid-acting analogue Insulin X10

The first rapid-acting insulin analogue was developed by replacing a histidine residue for the negatively charged aspartic acid at position B10 (insulin X10 or B10Asp)[93]. The applied genetic modification led to disrupt the ability of insulin molecules to self-associate as hexamers. Therefore, after subcutaneous injection of insulin X10, a much higher and earlier insulin peak was reached. Although clinical results were quite promising[94] further development of this analogue was discontinued when a dose-dependent increase in the occurrence of mammary tumours was observed in female Sprague-Dawley rats that were treated with supraphysiologic doses of insulin X10[95].

Insulin X10 has been shown to induce enhanced mitogenic effects due to the activation of both the IRs and the IGF-IRs[96]. In most studies, its binding affinity for the IR has been found to be 200-400% higher than that of human insulin[93]. Although it has an identical "on-rate", it has a much slower "off-rate" from the IR than human insulin[97]. In addition, its affinity for the IGF-IR has been found to be increased compared to human insulin although fairly lower compared to IGF-I[97]. Both effects may have resulted in an increased mitogenicity of this insulin analogue.

Currently Available Insulin analogues

Currently there are three commercially available rapid-acting insulin analogues (insulin lispro, insulin aspart, insulin glulisine) and two commercially available long-acting insulin analogues (insulin glargine and insulin detemir).

A] Rapid-acting insulin analogues

Insulin lispro (LysB28, ProB290 human insulin) was the first clinically available insulin analogue (Figure 4). In insulin lispro the natural amino acid sequence of the B chain is reversed at position 28 and 29. As a consequence there is a lysine at position 28 and a proline at position 29, like in IGF-I. This amino acid sequence reduces the ability of insulin to self-associate leading to a significantly higher absorption and elimination rate than that of human insulin after subcutaneous injection. For insulin aspart (AspB28) another strategy is used to reduce self-association (Figure 4). Insulin aspart is obtained by changing proline at position B28 by the negatively charged amino acid aspartic acid. The pharmacokinetic and pharmacodynamic characteristics of insulin aspart resemble and are very similar to that of insulin lispro. For both insulin analogues the affinities for the IR and the IGF-IR have been reported to be similar to that of human insulin[66, 98-99].

Insulin glulisine has been developed by substituting aspartic acid at position B3 with lysine and lysine at position B29 with glutamine (Figure 4). These changes also reduce the self-association when injected subcutaneously and thereby provide a quick biological availability after injection. Insulin glulisine has similar or slightly less binding affinity for the IR than human insulin. In addition, it has been suggested that IGF-IR binding affinity is significantly lower than of human insulin[100].

B] Long-acting insulin analogues

Two strategies to protract absorption by genetically modifying the insulin molecule have been tested clinically[101].

The first principle was to shift the isoelectric point of the molecule towards neutrality to provide reduced solubility at physiological pH values. This principle has been used for insulin glargine (GlyA21, ArgB31, ArgB32 human insulin); so it is injected as an acid solution (pH 4.0) and forms a slowly absorbed precipitate in the neutral environment of the subcutis. This property means that it cannot be mixed with neutral formulations of other insulins. Insulin glargine has been produced by substituting asparagine with glycine in the A chain at position 21 and by adding two arginine residues to the B chain at position 30 (Figure 4).

In vitro studies using rat fibroblasts showed similar binding characteristics for insulin glargine and human insulin. The IGF-IR binding affinity in vitro on cardiac myocytes has been reported to be stronger than that for human insulin[98]. However, native insulin glargine and its metabolites M1 and M2 are released from the subcutaneous depot and M1 has been found to be the most abundant in the circulation and is therefore most likely to be the biologically active form[102]. This ultimately makes it even more difficult to interpret in vitro results of insulin glargine.

Another strategy to protract absorption has been to acylate fatty acid species to the insulin molecule to allow reversible albumin-insulin binding in an attempt to protract the time action profile while retaining the practical advantages of a neutral liquid preparation. This strategy has been applied to insulin detemir (LysB29 (N-tetradecanoyl) des (B30) human insulin). In insulin detemir, the ε-amino group on the side-chain of lysine at position B29 is acylated, while threonine at position B30 is removed (Figure 4). After subcutaneous injection, insulin detemir binds to albumin through this fatty acid chain[98-99]. This binding prolonged half-life in pigs to 14.3 hrs compared with 10.5 h with neutral protamine Hagedorn (NPH) insulin and reduces the biological availability of free insulin detemir making it more predictable in terms of the risk for hypoglycaemic episodes[101, 103-105].

The affinity of insulin detemir for the IR has been found to be reduced, both in vitro and in vivo[104]. In vivo, 4-fold higher doses were required tot obtain a similar blood glucose lowering effect as observed after regular insulin injections or injections with other

insulin analogues. Therefore, the commercially available insulin detemir has a 4-fold concentrated formulation in order to match biological potency of human insulin[106].

Binding affinity of insulin detemir to IGF-IR is as low as its affinity to the IR. The ratio between IR affinity/IGF-IR affinity is therefore approximately the same as for human insulin[107].

In vitro/ex vivo cell culture systems

Insulin analogues have been tested for their binding affinity to the IR and the IGF-IR, their metabolic potency, their mitogenic potency and their dissociation rate from the IR.

In vitro, all at present commercially available insulin analogues have lower affinities for the IR (Table 1). In contrast, insulin X10 has significantly higher affinity for the IR than human insulin.

All in vitro studies have documented that insulin X10 and the long-acting insulin analogue insulin glargine have a higher affinity for the IGF-IR than human insulin (Table 2). Affinity for the IGF-IR for all available insulin analogues is significantly lower than that for IGF-I (Table 2).

Table 1. Binding affinity and off rate of commercially available insulin analogues for the insulin receptor

Analogue	Insulin Receptor binding affinity (%)					Insulin Receptor off rate (%)
Human insulin	100[1]	100[4a]	100[4b]	100[8a]	100[8b]	100[1]
X10	205[1]	817[4a]	271[4b]	148[8a]	245[8b]	14[5]
Aspart	92[1]	ND	ND	67[8a]	70[8b]	81[5]
Lispro	84[2]	ND	ND	93[8a]	91[8b]	100[2]
Glargine	86[1]	59[4a]	53[4b]	43[8a]	84[8b]	152[1]
Glargine M1	ND	48[4a]	42[4b]	ND	ND	ND
Glargine M2	ND	53[4a]	52[4b]	ND	ND	ND
A21Gly	78[1]	ND	ND	ND	ND	162[1]
B31B32diArg	120[1]	ND	ND	ND	ND	75[1]
Detemir	46[3]	ND	ND	44[8a]	3.5[8b]	204[1]
Glulisine	ND	ND	68[7]	66[8a]	94[8b]	Comparable to human insulin[6]
IGF-I	ND	0.8[4a]	0.3[4b]	0.6[8a]	<0.5[8b]	ND

All values relative to human insulin.

1. Data from Kurtzhals et al.[87]

2. Data from Slieker et al.[86]

3. Data from Markussen et al.[104]

4a. Data from Sommerfeld et al. (IR-A)[167]

4b. Data from Sommerfeld et al. (IR-B)[167]

5. Data from Hansen et al. [110]

6. Data from Hennige et al.[168]

7. Data from Stammberger et al.[100]

8a. Data from Sciacca et al. (IR-A)[108]

8b. Data from Sciacca et al. (IR-B)[108]

ND= not determined

Table 2. Binding affinity of insulin, insulin analogues and IGF-I for the IGF-I receptor

Analogue	IGF-I Receptor binding affinity (%)			
Human insulin	100[1]	100[2]	100[4]	100[5]
X10	587[1]	277[2]	364[4]	≈250[5]
Aspart	81[1]	ND	ND	≈100[5]
Lispro	156[1]	-	144[4]	≈100[5]
Glargine	641[1]	457[2]	ND	≈333[5]
Glargine M1	ND	45[2]	ND	ND
Glargine M2	-	68[2]	ND	ND
A21Gly	42[1]	ND	ND	ND
B31B32diArg	2049[1]	ND	2275[4]	ND
Detemir	16[1]	ND	ND	≈333[5]
Glulisine	ND	68[3]	ND	≈100[5]
IGF-I	ND	32472[2]	75428[4]	>44843[5]

All values relative to human insulin

1. Data from Kurtzhals et al.[87]

2. Data from Sommerfeld et al.[167]

3. Data from Stammberger et al.[100]

4. Data from Slieker et al.[86]

5. Data from Sciacca et al.[108]

ND= not determined

Until now insulin X10 is the only insulin analogue which has been shown to be metabolically more potent in vitro than human insulin (Table 3). All other currently commercially available insulin analogues show equal or less metabolic potency than human insulin (Table 3).

Both insulin X10, B31B32diArg and insulin glargine have higher mitogenic potencies in vitro than human insulin, while most commercially available insulins show less mitogenic activity in vitro than human insulin (Table 3). However, in all circumstances mitogenic potency of insulin analogues was significantly lower than of IGF-I. A recent paper compared insulin analogues for mitogenic effects (cell proliferation and colony formation) in engineered cells expressing only one receptor type (IR-A, IR-B or IGF-IR) in order to analyze the individual contribution of each receptor type[108]. They found that human insulin and short-acting insulin analogues induced significant cell proliferation in IR expressing cells, whereas both long-acting insulin analogues induced significant cell proliferation not only in IR expressing cells but also in IGF-IR expressing cells. Interestingly, in terms of colony formation, all insulin analogues (except insulin X10) showed less mitogenic activity through the IGF-IR than human insulin, while all insulin analogues (except insulin glargine) had more mitogenic activity through IR-A than human insulin (Table 4).

Table 3. In vitro metabolic potency and mitogenic potency of commercially available insulin analogues

Analogue	Metabolic Potency (%)		Mitogenic Potency (%)		
Human insulin	100[1]	100[2]	100[1]	100[2]	100[3]
X10	207[1]	145[2]	975[1]	806[2]	340[3]
Aspart	101[1]	ND	58[1]	ND	ND
Lispro	82[1]	ND	66[1]	ND	89[3]
Glargine	60[1]	68[2]	783[1]	760[2]	ND
Glargine M1	ND	32[2]	ND	75[2]	ND
Glargine M2	ND	52[2]	ND	68[2]	ND
A21Gly	88[1]	ND	34[1]	ND	ND
B31B32diArg	75[1]	ND	2180[1]	ND	ND
Detemir	27[1]	ND	11[1]	ND	ND
Glulisine	ND	ND	ND	ND	Comparable to human insulin[4]
IGF-I	ND	0.02[2]	ND	5568[2]	5700[3]

All values relative to human insulin
[1]. Data from Kurtzhals et al.[87]
[2]. Data from Sommerfeld et al.[167]
[3]. Data from Slieker et al.[108]
[4]. Data from Stammberger et al.[100]
ND= not determined

Table 4. In vitro anchorage-independent cell growth (in terms of colony formation) in engineered cells expressing only one receptor type.

Analogue	Mitogenic potency (%)		
	IR-A	IR-B	IGF-IR
Human insulin	100	100	100
X10	173	100	118
Aspart	134	100	64
Lispro	112	89	91
Glargine	97	75	91
Detemir	127	94	91
Glulisine	103	94	82
IGF-I	89	8.3	509

All values relative to human insulin
Data from Sciacca et al.[108]

As previously discussed, occupancy time at the IR has been correlated with mitogenic potential. Insulin X10, insulin aspart and B31B32diArg have a lower insulin off-rate than human insulin, while the other insulin analogues (like insulin glargine) show a higher IR off-rate (Table 1). Whether the same phenomenon plays a role for the IGF-IR has not yet been studied and is therefore still unknown.

There is conflicting evidence as to whether or not the mitogenic effects of insulin and insulin analogues at high doses are mediated via the IR and/or the IGF-IR. The ratio of IGF-IRs/ IRs may play a role in the sensitivity of cells to insulin and insulin analogues in vitro[109]. Differences in this ratio between different cell lines may explain at least partly the observed differences in mitogenic potencies of insulin analogues; for example an increased potency of insulin glargine is only seen in cells which a relatively high proportion of IGF-IR[110] (Table 5). However, also this is not a consistent finding. Staiger et al. failed to detect any increased mitogenicity of insulin glargine in MC7-cells, despite the fact that these cells express 4-fold more IGF-IRs compared to IRs[111]. They suggested that a certain ratio of IGF-IRs/IRs is needed before insulin glargine induces a mitogenic response[110]. On the other hand, as mentioned before, Sciacca et al. compared insulin analogues for mitogenic effects in engineered cells expressing only one receptor type (IR-A, IR-B or IGF-IR) and found that mitogenic effects were also induced in cells not expressing the IGF-IR[108].

Furthermore, IR and IGF-IR expression may vary in a tissue-specific manner and inter-individual differences in the levels of proteins of the IGF-IR system may function as a critical determinant of the mitogenic potency of insulin analogues[112].

Thus the question remains whether all these in vitro observations may have any clinical implications. There are several uncertainties which make it very difficult to answer the question decisively[110]. There seems consensus that insulin and insulin analogues may have growth promoting activity. Moreover, insulin and insulin analogues have no

Table 5. Receptor expression in experimental cell lines and observed response after stimulation with insulin glargine[110]

Cell lines	IGF-I receptor/ insulin receptor ratio	IGF-I receptor affinity	mitogenicity
SaosB101	Predominantly IGF-I receptor	↑	↑
HMEC2	Predominantly IGF-I receptor	↑	↑
Rat-1 over expressing IR3	Predominantly IGF-I receptor	ND	↔
MCF-7 cells 4	4:1	ND	↔
MCF-10 4	1:1	ND	↔
MCF-7-cells5	7:1	ND	↔
SKBR-3 cells 5	1:1	ND	↔

1. Data from Kurzhals et al.[87]
2. Data from Kohn et al.[169]
3. Data from Berti et al.[170]
4. Data from Staiger et al.[111]
5. Data from Liefvendahl et al.[171]

ND= not determined. ↑= increased compared to human insulin, ↔= no difference with human insulin.

carcinogenic activity (cell transformation) and are not a co-carcinogen when evaluated in special toxicology[113]. Although Giorgino et al. found that supraphysiological overexpression of IRs does favour ligand-dependent cell transformation in vitro, which underlines the potency of insulin to do so[114], it certainly does not mean that this occurs in vivo. On the other hand, one should keep in mind that increased mitogenic activity per se may increase the chances of mutations thereby initiating tumour formation. However, circulating concentrations of injected insulin analogues are normally quite low compared to the levels needed to elicit a mitogenic response[110].

As previously discussed, it has been suggested that the IR and IGF-IR act at identical portals to the regulation of gene expression, with differences between insulin and IGF-I effects due to a modulation of the signal created by the specific ligand-receptor interactions[69]. As a consequence it is almost impossible in most in vitro cell lines to disentangle the individual contribution of each type receptor to the final downstream event. In this respect, Sciacca et al. have made a great effort when they compared analogues for binding, post-receptor signalling and mitogenic effects in mouse embryonic fibroblasts expressing only the human IR-A, IR-B or the human IGF-IR[108].

Second aim of the thesis: 1. To compare insulin analogues with human insulin in their potency to activate the human IGF-IR, IR-A and IR-B in vitro by using KIRA bioassays specific for the human IGF-IR, IR-A and IR-B, respectively. 2. To compare serum IGF-IR bioactivity in type 2 diabetic patients after long term treatment with either insulin analogues or human insulin. 3. To compare serum IR-A and IR-B bioactivity in type 2 diabetic patients after long term treatment with either insulin analogues or human insulin. The results of these studies are described in chapter 3, 4 and 5.

III. ADULT GROWTH HORMONE DEFICIENCY AND THE IGF-SYSTEM

Adult growth hormone deficiency (GHD) comprises patients with adult-onset (AO) GHD and those with childhood-onset (CO) GHD persisting into adulthood. There are only few data on incidence rate of GHD. CO GHD has been estimated to occur in 3.3 per 100 000 people per year[115] while AO GHD has been estimated to occur in 1.2-1.65 per 100 000 adults a year[116-117]. In about one fourth of the adult patients, GHD is of CO.

Aetiology

CO GHD may be the result of structural pituitary disease (e.g. craniopharyngioma), genetic mutations (Transcription factor defects, GHRH receptor gene defects, GH secretagogue receptor gene defects, GH gene defects, GH receptor/post receptor defects[118-120]) or irradiation but, in contrast to AO GHD, is often a consequence of a partial deficiency

of growth hormone releasing hormone. From this latter group, 30-70% establish normal GH responses upon provocative testing at completion of linear growth[121]. Therefore it is essential that provocative GH testing is undertaken when linear growth is complete.

Pituitary adenoma is the most frequent cause of AO GHD followed by craniopharyngioma, which together account for 66% of cases. Table 6 shows a spectrum of causes of adult GHD based on data derived from KIMS, a multinational, pharmacoepidemiological surveillance database for adult hypopituitary patients receiving GH replacement[122].

Table 6. Causes of GH deficiency in adult patients.

Diagnosis	Percentage
Functioning pituitary adenoma	22%
Non-functioning pituitary adenoma	31%
Craniopharyngeoma	13%
Iatrogenic	3%
Trauma	2%
Idiopatic	13%
Other	16%

Based on data derived from KIMS[122]

Symptoms

Nowadays, GHD is recognized as an important (metabolic) syndrome[123]. The main features of GHD are shown in table 7. They have abnormal body compensation due to increased fat mass and reduced muscle mass, decreased energy and an impaired quality of life (QoL)[124]. These symptoms are not pathogenomic and overlap with a wide variety of other conditions[123, 125]. The accuracy of hormonal testing highly depends on the pretest probability of GHD[124] and therefore it is mandatory that it should only be undertaken against the right clinical background (Table 7).

Diagnosis

GH is considered to be the main regulator of circulating IGF-I[35]. Circulating total (extractable) IGF-I is therefore routinely used for diagnosing and monitoring treatment of adult GHD[126-127]. With increasing age, the contribution of GH to circulating total IGF-I levels appears to diminish, making total IGF-I a less useful diagnostic marker of GHD[128]. It has been proven that the diagnosis of GH disorders cannot solely rely on determination of total IGF-I. In a substantial fraction of patients diagnosed as GH deficient, total IGF-I levels remain within the normal range, especially in patients above 40 yr of age[129-130]. Therefore, current diagnostic testing uses provocative tests of GH secretion[124]. The endocrine society guideline recommends the insulin tolerance test (ITT) and the GHRH-arginine test (unless hypothalamic causes of suspected GHD where GHRH-arginine

Table 7. Clinical features of GHD in adults.

Background	*Need for GH treatment as a child*
	- genetic conditions (PIT-1, PROP-1 mutations)
	- structural lesions of hypothalamic-pituitary region
	Adults
	- With structural lesions of hypothalamic-pituitary region
	- Following surgery to the hypothalamic-pituitary region
	- Following cranial irradiation
	- Evidence of other pituitary hormone deficiencies
	- Following moderate to severe traumatic brain injury and subarachnoid haemorrhag
Symptoms	Abnormal body composition Reduced strength and exercise capacity Impaired psychological well-being
Tests	Stimulated GH level below 3 mg/L (after ITT) Low or low-normal serum IGF-I Elevated serum lipids, particularly LDL cholesterol Reduced lean body/increased fat mass Reduced bone mineral density

Table modified from Carroll et al.[125]

may be misleading) to establish the diagnosis of GHD after other hormone deficiencies have been adequately replaced[124]. An increasing number of additional pituitary deficits increases the probability of GHD[131]. Therefore, in isolated GHD 2 provocative tests are recommended for the diagnosis of GHD, whereas patients with multiple deficiencies (2 additional deficiencies or more) together with a low serum IGF-I do not need confirmation by provocative testing. Moreover, in CO GHD due to genetic causes or structural lesions, a decline in total IGF-I after 1 month off-GH therapy is sufficient documentation of persistent adult GHD without provocative testing.

As mentioned previously, many of the methods currently used for measurement of circulating total IGFs are hampered by interferences of IGF-binding proteins (IGFBPs) remaining after extraction[80]. On the other hand, by extracting IGFBPs the modifying effects of these proteins on IGF-I action are ignored. The IGF-IR KIRA assay quantifies bioavailable IGF-I while taking into account the modifying effects of IGFBPs.

Third aim of the thesis: To investigate the diagnostic value of IGF-IR bioactivity in patients with proven GHD. The results of this study are discussed in chapter 6.

Treatment (consequences)

Several parameters have been found to be positively affected by GH treatment among patients with GHD; a reduction in fat mass (and to a lesser extend increase in muscle mass), an anabolic effect on bone mass, beneficial changes in cardiovascular risk factors

(increase flow mediated dilatation, reduce arterial stiffness, increase HDL and decrease LDL and total cholesterol) and QoL[124]. Although several studies have shown that adults with hypopituitarism have increased mortality compared with non hypopituitary populations adjusted for age and sex, it cannot be concluded that this premature mortality can be attributed solely to GHD.

During GH therapy, GH dose is titrated against total IGF-I levels and the consensus guidelines advise that "values should be kept in the age-related normal range"[124]. Thus, the values of total IGF-I can be normal to begin with and yet that is the goal of therapy[132].

Fourth aim of the thesis: 1. To investigate the relationship between IGF-IR bioactivity and QoL during GH treatment of GH deficient patients. 2. To investigate the value of IGF-IR bioactivity for monitoring GH therapy. The results of these studies are discussed in chapter 7 and 8.

IV. ACROMEGALY AND THE IGF-SYSTEM

Acromegaly is an insidious disease that results from excessive GH secretion. It is a rare disease which affects probably 86-240 individuals per million of the general population[133].

Aetiology

Acromegaly is most often caused by GH-producing tumour of the anterior pituitary gland. More than 95% of cases occur sporadically due to pituitary adenoma in patients without genetic traits that are associated with endocrine tumours. Approximately 1% of cases are caused by familial or inherited endocrine diseases as multiple endocrine neoplasia type I (MEN-I). GH-secreting pituitary carcinomas are very rare. The remaining cases of acromegaly are due to GHRH hypersecretion from a site in the hypothalamus or ectopically that can induce pituitary hyperplasia.

Symptoms

The physical features of a patient with a well-established acromegaly are virtually pathognomonic for the diagnosis. Almost all patients report acral growth and changes in facial features, although important but non specific complaints such as headache, decreased libido, erectile dysfunction, arthritis and fatigue are also prominent. However, in contrast, early acromegaly may be characterized by nonspecific complaints, such as increased sweating and mild soft tissue swelling.

Diagnosis

Acromegaly, like all diseases of insidious onset, requires a high index of clinical suspicion if it is to be detected early. The diagnosis of acromegaly is composed of three elements; identification of clinical signs and symptoms, performance of endocrine testing according to modern consensus criteria, and neuroradiological grading of the pituitary tumour using magnetic resonance imaging. Yet, to date, it has not been possible to identify a reliable sign/symptom (score)/biochemical marker with good test characteristics that reflects disease activity[134].

The best single test for the diagnosis of acromegaly is measurement of serum total IGF-I. Unlike GH, serum IGF-I concentrations do not vary from hour to hour according to food intake, exercise or sleep, but instead reflect integrated GH secretion during the preceding day or longer. The results must be interpreted according to the patient's age.

All patients with acromegaly have increased GH secretion. Unlike normal subjects, the patient's serum GH concentrations change little during the day or night, and in most patients do not change in response to stimuli such as food or exercise. However, the random serum GH concentration is often in the range of 2 to 10 ng/mL during much of the day, values that can be found in normal subjects. A random taken GH value is therefore not very informative.

The oral glucose tolerance test (OGTT) is the most specific dynamic test for establishing the diagnosis of acromegaly. In normal subjects, serum GH concentrations fall to 1 ng/mL or less within two hours after ingestion of 75 g glucose. In contrast, the post-glucose values are greater than 2 ng/mL in over 85 percent of patients with acromegaly[135].

Discordance between GH and total IGF-I levels has been noted in active acromegaly and in those following treatment[134].

In acromegaly, QoL has been reported to be significantly reduced in active untreated disease[134]. QoL in acromegaly can be quantified by using several disease-specific questionnaires and more general health related questionnaires[134].

Fifth aim of the thesis: To investigate the value of circulating IGF-IR bioactivity in the evaluation of patients with untreated acromegaly. The results of these studies are discussed in chapter 9.

Treatment

When complete surgical removal of GH-secreting tumours is achieved, this results in improvement of soft tissue changes and hormonal control of acromegaly, with results varying between 40-98% depending on tumour size[136]. Especially in patients with larger tumours (macroadenomas), less hormonal control is achieved and approximately 40–60% of these patients are unlikely to be controlled with surgery alone[136]. An option

for such tumours is medical therapy. Currently, there are three drug classes available for the treatment of acromegaly: dopamine agonists (DAs), somatostatin receptor ligand antagonists (SRLs), and a GH receptor antagonist (GHRA). The dopamine agonists have only little efficacy in acromegaly; monotherapy is effective in less than 10% of patients[136]. In contrast, long-term studies indicate that approximately 70% of patients receiving SRLs have GH levels below 2.5 ng/ml and normalized IGF-I concentrations[136]. GHRAs are indicated in those patients that have persistently elevated IGF-I concentrations despite maximal therapy with other SRLs[136]. GHRA as monotherapy or in combination with a SRL induces biochemical in more than 90% of all acromegalic patients[136].

V. GRAVES' OPHTHALMOPATHY AND THE IGF-SYSTEM

Graves' disease (GD) occurs approximately 7-10 fold times more frequent in women compared to men and usually occurs in patients between 20 and 50 years of age[137].The classical signs and symptoms of GD, include hyperthyroidism, diffuse goitre, ophthalmopathy (Graves' ophthalmopathy (GO)), and rarely, dermopathy.

Aetiology

GD is an autoimmune thyroid disorder in which immunoglobulins are produced that directly stimulate the TSH-receptor (TSH-R) in the thyroid gland leading to hyperthyroidism. In addition to stimulating immunoglobulins directed against the TSH-R, those blocking the TSH-R have also been detected in patients with hyperthyroidism and a shift in the balance between these two types of antibodies can result in hypothyroidism occurring in around 15% of patients[138].

The TSH-R stimulating immunoglobulins may not only influence thyroid function but have also been recognized as the target autoantigen that mediates extrathyroidal manifestations of GD, in particular GO. Not only is there a close relationship between serum levels of anti-TSH-R immunoglobulins and GO-activity[139-140], anti-TSH-R immunoglobulin levels also influence GO prognosis[139] and these antibodies can be detected in serum of the vast majority of patients with euthyroid GO[141]. Moreover, TSH-Rs have been found to be expressed in (differentiated) orbital fibroblasts and orbital preadipocytes[142-144], with an elevated expression in orbital tissues from patients with GD[145-146].

In thyreotoxicosis, serum total IGF-I levels have been found to be normal to high and IGF-I bioactivity to be low[147-149] (it has been suggested that this discrepancy may be explained by changes in IGFBPs[148, 150]). This may be explained by an increase in the number and size of spontaneous GH secretory bursts, although the response to GHRH is attenuated and delayed[147, 151-153]. Moreover, thyroid hormones have direct actions

at the level of the pituitary, both by changing the number of somatotroph cells and influencing expression of the GH gene[152]. In addition, thyroid hormones have shown to stimulate hepatic GH binding and to potentiate the GH-induced synthesis of IGF-I mRNA and peptide in the liver. Although elevated serum levels of IGF-I and of IGFBPs have also been found in patients with hyperthyroidism due to GD, they decreased after antithyroid (methimazole) treatment[154]. In line with these findings it has been reported that in euthyroid Graves' patients with active GO, serum IGF-I and IGF-II (including total and free fractions) and IGFBPs are not increased[155] compared to healthy controls.

A role for increased IGF (signalling) in the pathogenesis of GO has long been studied. The fraction of IGF-IR expressing fibroblasts cultured from the orbit, skin and thyroid of patients with GD has been found to be increased[156-158]. Also in retrobulbar tissues increased IGF-levels have been described[159]. So, the increased IGF-I levels in retrobulbar tissues appear to be independent of serum IGFs concentrations and probably represent an autocrine and/or paracrine activity[155].

More than 20 years ago it was reported that immunoglobulins produced in GD had the ability to immunoprecipitate with the IGF-IR[160]. In addition, it was found that these Graves' immunoglobulins could displace radiolabelled IGF-I from its binding sites on orbital fibroblasts[161]. Smith et al. previously reported that Graves' immunoglobulins could stimulate the production of the T-cell chemoattractants interleukin (IL)-16, RANTES (regulated upon activation normal T-cell expressed and secreted) and hyaluronan by orbital fibroblasts retrieved from patients with GD[156, 162]. These effects were blocked by monoclonal antibodies directed against the IGF-IR, suggesting that these actions of Graves' immunoglobulins were mediated through pathways independent of the TSH-R. These findings have raised the hypothesis that in (a subset of) Graves' patients immunoglobulins may stimulate the IGF-IR which may contribute to GO and suggest that the IGF-IR may be another important aetiologic autoantigen in GO.

Sixth aim of the thesis: To study relationships between TSH binding inhibitory immunoglobulins (TBII) and serum IGF-IR stimulating activity in relationship to age in patients with GO. The results of this study are described in chapter 10.

Symptoms and Treatment

Eye signs do not always occur at the same time as hyperthyroidism and goiter[163]. Approximately 25-50% of patients with GD develop GO, whereas 10% of patients with GO do not develop hyperthyroidism[164]. The course of GO is biphasic with an initial phase of active and progressive disease (characterized by orbital and periorbital inflammation targeting connective tissue and fat which translates into proptosis, conjunctival injec-

tion, chemosis, diplopia, corneal ulceration and rarely loss of site due to optic nerve compression), followed by a static phase (with resolution of inflammation associated with clinical improvement). The activity of GO can be scored by using the clinical activity score (CAS) based on four of the five well-known classical signs of inflammation (pain, redness, swelling and impaired function)[165]. This score has a predictive value for the outcome of immunosuppressive therapy (such as corticosteroids or retrobulbar external irradiation)[165] which are effective in the active phase, while in the static phase surgical intervention can be performed.

REFERENCES

1. Banting FG & Best CH. The internal secretion of the pancreas. 1922. *Indian J Med Res* 2007 **125** 251-266.
2. Sanger F, Thompson EO & Kitai R. The amide groups of insulin. *Biochem J* 1955 **59** 509-518.
3. Anderson E, Lindner E & Sutton V. A sensitive method for the assay of insulin in blood. *Am J Physiol* 1947 **149** 350-354.
4. Bornstein J & Lawrence RD. Plasma insulin in human diabetes mellitus. *Br Med J* 1951 **2** 1541-1544.
5. Groen J, Kamminga CE, Willebrands AF & Blickman JR. Evidence for the presence of insulin in blood serum; a method for an approximate determination of the insulin content of blood. *J Clin Invest* 1952 **31** 97-106.
6. Martin DB, Renold AE & Dagenais YM. An assay for insulin-like activity using rat adipose tissue. *Lancet* 1958 **2** 76-77.
7. Vallance-Owen J & Hurlock B. Estimation of plasma-insulin by the rat diaphragm method. *Lancet* 1954 **266** 68-70.
8. Blumenthal S. The insulin immunoassay after 50 years: a reassessment. *Perspect Biol Med* 2009 **52** 343-354.
9. Leonards JR. Insulin-like activity of blood. What is it? *Fed. Proc.* 1959 **18** 272.
10. Froesch ER, Buergi H, Ramseier EB, Bally P & Labhart A. Antibody-Suppressible and Nonsuppressible Insulin-Like Activities in Human Serum and Their Physiologic Significance. An Insulin Assay with Adipose Tissue of Increased Precision and Specificity. *J Clin Invest* 1963 **42** 1816-1834.
11. Yalow RS & Berson SA. Immunoassay of endogenous plasma insulin in man. *J Clin Invest* 1960 **39** 1157-1175.
12. Kahn CR & Roth J. Berson, Yalow, and the JCI: the agony and the ecstasy. *J Clin Invest* 2004 **114** 1051-1054.
13. Salmon WD, Jr. & Daughaday WH. A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. *J Lab Clin Med* 1957 **49** 825-836.
14. Salmon WD, Jr. & DuVall MR. In vitro stimulation of leucine incorporation into muscle and cartilage protein by a serum fraction with sulfation factor activity: differentiation of effects from those of growth hormone and insulin. *Endocrinology* 1970 **87** 1168-1180.
15. Daughaday WH, Hall K, Raben MS, Salmon WD, Jr., van den Brande JL & van Wyk JJ. Somatomedin: proposed designation for sulphation factor. *Nature* 1972 **235** 107.
16. Sievertsson H, Fryklund L, Uthne K, Hall K & Westermark B. Isolation and chemistry of human somatomedins A and B. *Adv Metab Disord* 1975 **8** 47-60.
17. Svoboda ME, Van Wyk JJ, Klapper DG, Fellows RE, Grissom FE & Schlueter RJ. Purification of somatomedin-C from human plasma: chemical and biological properties, partial sequence analysis, and relationship to other somatomedins. *Biochemistry* 1980 **19** 790-797.
18. Van Wyk JJ, Underwood LE, Hintz RL, Clemmons DR, Voina SJ & Weaver RP. The somatomedins: a family of insulinlike hormones under growth hormone control. *Recent Prog Horm Res* 1974 **30** 259-318.
19. Rinderknecht E & Humbel RE. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *J Biol Chem* 1978 **253** 2769-2776.
20. Rinderknecht E & Humbel RE. Primary structure of human insulin-like growth factor II. *FEBS Lett* 1978 **89** 283-286.

21. Enberg G, Carlquist M, Jornvall H & Hall K. The characterization of somatomedin A, isolated by microcomputer-controlled chromatography, reveals an apparent identity to insulin-like growth factor 1. *Eur J Biochem* 1984 **143** 117-124.
22. Klapper DG, Svoboda ME & Van Wyk JJ. Sequence analysis of somatomedin-C: confirmation of identity with insulin-like growth factor I. *Endocrinology* 1983 **112** 2215-2217.
23. Sadick MD, Intintoli A, Quarmby V, McCoy A, Canova-Davis E & Ling V. Kinase receptor activation (KIRA): a rapid and accurate alternative to end-point bioassays. *J Pharm Biomed Anal* 1999 **19** 883-891.
24. Furlanetto RW, Underwood LE, Van Wyk JJ & D'Ercole AJ. Estimation of somatomedin-C levels in normals and patients with pituitary disease by radioimmunoassay. *J Clin Invest* 1977 **60** 648-657.
25. Zapf J, Walter H & Froesch ER. Radioimmunological determination of insulinlike growth factors I and II in normal subjects and in patients with growth disorders and extrapancreatic tumor hypoglycemia. *J Clin Invest* 1981 **68** 1321-1330.
26. Chan SJ & Steiner DF. Insulin through the ages: Phylogeny of a growth promoting and metabolic regulatory hormone. *Amer. Zool.* 2000 **40** 213-222.
27. Humbel RE. Insulin-like growth factors I and II. *Eur J Biochem* 1990 **190** 445-462.
28. Sussenbach JS, Steenbergh PH & Holthuizen P. Structure and expression of the human insulin-like growth factor genes. *Growth Regul* 1992 **2** 1-9.
29. Howell SL & Tyhurst M. Microtubules, microfilaments and insulin-secretion. *Diabetologia* 1982 **22** 301-308.
30. Wollheim CB & Sharp GW. Regulation of insulin release by calcium. *Physiol Rev* 1981 **61** 914-973.
31. Froesch ER, Schmid C, Schwander J & Zapf J. Actions of insulin-like growth factors. *Annu Rev Physiol* 1985 **47** 443-467.
32. Froesch ER & Zapf J. Insulin-like growth factors and insulin: comparative aspects. *Diabetologia* 1985 **28** 485-493.
33. Holly JM & Perks CM. Insulin-like growth factor physiology: what we have learned from human studies. *Endocrinol Metab Clin North Am* 2012 **41** 249-263, v.
34. Murphy LJ, Bell GI & Friesen HG. Tissue distribution of insulin-like growth factor I and II messenger ribonucleic acid in the adult rat. *Endocrinology* 1987 **120** 1279-1282.
35. LeRoith D, Bondy C, Yakar S, Liu JL & Butler A. The somatomedin hypothesis: 2001. *Endocr Rev* 2001 **22** 53-74.
36. Baker J, Liu JP, Robertson EJ & Efstratiadis A. Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* 1993 **75** 73-82.
37. Walenkamp MJ & Wit JM. Genetic disorders in the GH IGF-I axis in mouse and man. *Eur J Endocrinol* 2007 **157 Suppl 1** S15-26.
38. Liu JP, Baker J, Perkins AS, Robertson EJ & Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* 1993 **75** 59-72.
39. DeChiara TM, Efstratiadis A & Robertson EJ. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* 1990 **345** 78-80.
40. Yakar S, Liu JL, Stannard B, Butler A, Accili D, Sauer B & LeRoith D. Normal growth and development in the absence of hepatic insulin-like growth factor I. *Proc Natl Acad Sci U S A* 1999 **96** 7324-7329.
41. Juul A. Serum levels of insulin-like growth factor I and its binding proteins in health and disease. *Growth Horm IGF Res* 2003 **13** 113-170.
42. Rietveld I, Janssen JA, Hofman A, Pols HA, van Duijn CM & Lamberts SW. A polymorphism in the IGF-I gene influences the age-related decline in circulating total IGF-I levels. *Eur J Endocrinol* 2003 **148** 171-175.

43. Deal C, Ma J, Wilkin F, Paquette J, Rozen F, Ge B, Hudson T, Stampfer M & Pollak M. Novel promoter polymorphism in insulin-like growth factor-binding protein-3: correlation with serum levels and interaction with known regulators. *J Clin Endocrinol Metab* 2001 **86** 1274-1280.
44. Bennett A, Wilson DM, Liu F, Nagashima R, Rosenfeld RG & Hintz RL. Levels of insulin-like growth factors I and II in human cord blood. *J Clin Endocrinol Metab* 1983 **57** 609-612.
45. Gluckman PD, Johnson-Barrett JJ, Butler JH, Edgar BW & Gunn TR. Studies of insulin-like growth factor -I and -II by specific radioligand assays in umbilical cord blood. *Clin Endocrinol (Oxf)* 1983 **19** 405-413.
46. Yu H, Mistry J, Nicar MJ, Khosravi MJ, Diamandis A, van Doorn J & Juul A. Insulin-like growth factors (IGF-I, free IGF-I and IGF-II) and insulin-like growth factor binding proteins (IGFBP-2, IGFBP-3, IGFBP-6, and ALS) in blood circulation. *J Clin Lab Anal* 1999 **13** 166-172.
47. Chao W & D'Amore PA. IGF2: epigenetic regulation and role in development and disease. *Cytokine Growth Factor Rev* 2008 **19** 111-120.
48. Brown J, Jones EY & Forbes BE. Keeping IGF-II under control: lessons from the IGF-II-IGF2R crystal structure. *Trends Biochem Sci* 2009 **34** 612-619.
49. Rajaram S, Baylink DJ & Mohan S. Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. *Endocr Rev* 1997 **18** 801-831.
50. Daughaday WH, Trivedi B & Mariz IK. Elevated insulin-like growth factor II (IGF II) in non-islet induced hypoglycemia detected by a new specific radioreceptor assay. *Diabetes* 1980 **29** Suppl. 2:5A (abstract) 19.
51. Hintz RL & Liu F. Insulin-like growth factor II radioimmunoassay based on antiserum against the synthetic C-peptide segment. *Clin Research* 1981 **29** 408A (Abstract).
52. Shimatsu A, Nakamura Y, Ihara C, Mizuta H & Murabe H. Serum Insulin-like growth factor-II (IGF-II) levels in patients with acromegaly and growth hormone deficiency: correlation with IGF-I and IGF binding protein-3. *Endocr J* 1996 **43 Suppl** S71-73.
53. Aguiar-Oliveira MH, Gill MS, de ABES, Alcantara MR, Miraki-Moud F, Menezes CA, Souza AH, Martinelli CE, Pereira FA, Salvatori R, Levine MA, Shalet SM, Camacho-Hubner C & Clayton PE. Effect of severe growth hormone (GH) deficiency due to a mutation in the GH-releasing hormone receptor on insulin-like growth factors (IGFs), IGF-binding proteins, and ternary complex formation throughout life. *J Clin Endocrinol Metab* 1999 **84** 4118-4126.
54. Ferrante E, Giavoli C, Porretti S, Vassallo E, Ronchi CL, Lania AG, Beck-Peccoz P & Spada A. Evaluation of the components of the insulin-like growth factors system in GH-deficient adults: effects of twelve-month rhGH treatment. *Horm Metab Res* 2006 **38** 352-355.
55. Fernholm R, Bramnert M, Hagg E, Hilding A, Baylink DJ, Mohan S & Thoren M. Growth hormone replacement therapy improves body composition and increases bone metabolism in elderly patients with pituitary disease. *J Clin Endocrinol Metab* 2000 **85** 4104-4112.
56. Ezzat S, Fear S, Gaillard RC, Gayle C, Landy H, Marcovitz S, Mattioni T, Nussey S, Rees A & Svanberg E. Gender-specific responses of lean body composition and non-gender-specific cardiac function improvement after GH replacement in GH-deficient adults. *J Clin Endocrinol Metab* 2002 **87** 2725-2733.
57. De Meyts, Sajid, Palsgaard, Theede, Gauguin, Aladdin & Whittaker. Mechanisims of insulin action. In *Insulin and IGF-I Receptor Structure and Binding Mechanism*. Ed ASJ Pessin, 2007.
58. Belfiore A, Frasca F, Pandini G, Sciacca L & Vigneri R. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. *Endocr Rev* 2009 **30** 586-623.
59. Moller DE, Yokota A, Caro JF & Flier JS. Tissue-specific expression of two alternatively spliced insulin receptor mRNAs in man. *Mol Endocrinol* 1989 **3** 1263-1269.

60. Mosthaf L, Grako K, Dull TJ, Coussens L, Ullrich A & McClain DA. Functionally distinct insulin receptors generated by tissue-specific alternative splicing. *Embo J* 1990 **9** 2409-2413.
61. Blakesley VA, Scrimgeour A, Esposito D & Le Roith D. Signaling via the insulin-like growth factor-I receptor: does it differ from insulin receptor signaling? *Cytokine Growth Factor Rev* 1996 **7** 153-159.
62. Harris LK & Westwood M. Biology and significance of signalling pathways activated by IGF-II. *Growth Factors* 2012 **30** 1-12.
63. El-Shewy HM & Luttrell LM. Insulin-like growth factor-2/mannose-6 phosphate receptors. *Vitam Horm* 2009 **80** 667-697.
64. Scott CD & Firth SM. The role of the M6P/IGF-II receptor in cancer: tumor suppression or garbage disposal? *Horm Metab Res* 2004 **36** 261-271.
65. Siddle K, Urso B, Niesler CA, Cope DL, Molina L, Surinya KH & Soos MA. Specificity in ligand binding and intracellular signalling by insulin and insulin-like growth factor receptors. *Biochem Soc Trans* 2001 **29** 513-525.
66. LeRoith D, Adamo D, Werner H & Roberts Jr CT. Molecular and cellular biology of the insulin-like growth factors. In *Molecular Endocrinology, Basic Concepts and Clinical Correlations*, ch. 13, pp 181-183. Ed W DE, 1995.
67. Dupont J & LeRoith D. Insulin and insulin-like growth factor I receptors: similarities and differences in signal transduction. *Horm Res* 2001 **55 Suppl 2** 22-26.
68. Bevan P. Insulin signalling. *J Cell Sci* 2001 **114** 1429-1430.
69. Boucher J, Tseng YH & Kahn CR. Insulin and insulin-like growth factor-1 receptors act as ligand-specific amplitude modulators of a common pathway regulating gene transcription. *J Biol Chem* 2010 **285** 17235-17245.
70. Froesh ER, Zenobi PD & Zapf J. Metabolic effects of insulin-like growth factor-I and possible therapeutic aspects in Diabetes. In *Growth hormone and Insulin-like Growth Factor*, pp 109-129. Ed O Flyvbjerg, Alberti 1993.
71. Vigneri P, Frasca F, Sciacca L, Pandini G & Vigneri R. Diabetes and cancer. *Endocr Relat Cancer* 2009 **16** 1103-1123.
72. Pandini G, Frasca F, Mineo R, Sciacca L, Vigneri R & Belfiore A. Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. *J Biol Chem* 2002 **277** 39684-39695.
73. Jones JI & Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev* 1995 **16** 3-34.
74. Firth SM & Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. *Endocr Rev* 2002 **23** 824-854.
75. Hwa V, Oh Y & Rosenfeld RG. The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocr Rev* 1999 **20** 761-787.
76. Wheatcroft SB & Kearney MT. IGF-dependent and IGF-independent actions of IGF-binding protein-1 and -2: implications for metabolic homeostasis. *Trends Endocrinol Metab* 2009 **20** 153-162.
77. Frystyk J, Skjaerbaek C, Dinesen B & Orskov H. Free insulin-like growth factors (IGF-I and IGF-II) in human serum. *FEBS Lett* 1994 **348** 185-191.
78. Chen JW, Ledet T, Orskov H, Jessen N, Lund S, Whittaker J, De Meyts P, Larsen MB, Christiansen JS & Frystyk J. A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum. *Am J Physiol Endocrinol Metab* 2003 **284** E1149-1155.
79. Frystyk J. Free insulin-like growth factors -- measurements and relationships to growth hormone secretion and glucose homeostasis. *Growth Horm IGF Res* 2004 **14** 337-375.

80. Clemmons DR. IGF-I assays: current assay methodologies and their limitations. *Pituitary* 2007 **10** 121-128.
81. Strasburger CJ. Methods in determining growth hormone concentrations: an immunofunctional assay. *Pediatrics* 1999 **104** 1024-1028.
82. Brugts MP, Ranke MB, Hofland LJ, van der Wansem K, Weber K, Frystyk J, Lamberts SW & Janssen JA. Normal values of circulating insulin-like growth factor-I bioactivity in the healthy population: comparison with five widely used IGF-I immunoassays. *J Clin Endocrinol Metab* 2008 **93** 2539-2545.
83. Tattersall RB. The history of Diabetes In *Textbook of Diabetes* ch. 1, pp 1.1-1.22. Ed PJW G: Blackwell Science, 2003.
84. Emdin SO, Dodson GG, Cutfield JM & Cutfield SM. Role of zinc in insulin biosynthesis. Some possible zinc-insulin interactions in the pancreatic B-cell. *Diabetologia* 1980 **19** 174-182.
85. FDA. Guidance for Industry Diabetes Mellitus: Developing Drugs and Therapeutic Biologics for Treatment and Prevention. 2008.
86. Slieker LJ, Brooke GS, DiMarchi RD, Flora DB, Green LK, Hoffmann JA, Long HB, Fan L, Shields JE, Sundell KL, Surface PL & Chance RE. Modifications in the B10 and B26-30 regions of the B chain of human insulin alter affinity for the human IGF-I receptor more than for the insulin receptor. *Diabetologia* 1997 **40 Suppl 2** S54-61.
87. Kurtzhals P, Schaffer L, Sorensen A, Kristensen C, Jonassen I, Schmid C & Trub T. Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. *Diabetes* 2000 **49** 999-1005.
88. Nakagawa SH & Tager HS. Role of the COOH-terminal B-chain domain in insulin-receptor interactions. Identification of perturbations involving the insulin mainchain. *J Biol Chem* 1987 **262** 12054-12058.
89. Mayer D, Shukla A & Enzmann H. Proliferative effects of insulin analogues on mammary epithelial cells. *Arch Physiol Biochem* 2008 **114** 38-44.
90. Bi RC, Dauter Z, Dodson E, Dodson G, Giordano F & Reynolds C. Insulin's structure as a modified and monomeric molecule. *Biopolymers* 1984 391 – 395.
91. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer* 2008 **8** 915-928.
92. Hansen BF, Danielsen GM, Drejer K, Sorensen AR, Wiberg FC, Klein HH & Lundemose AG. Sustained signalling from the insulin receptor after stimulation with insulin analogues exhibiting increased mitogenic potency. *Biochem J* 1996 **315 (Pt 1)** 271-279.
93. Hansen BF, Kurtzhals P, Jensen AB, Dejgaard A & Russell-Jones D. Insulin X10 revisited: a super-mitogenic insulin analogue. *Diabetologia* 2011 **54** 2226-2231.
94. Nielsen FS, Jorgensen LN, Ipsen M, Voldsgaard AI & Parving HH. Long-term comparison of human insulin analogue B10Asp and soluble human insulin in IDDM patients on a basal/bolus insulin regimen. *Diabetologia* 1995 **38** 592-598.
95. Drejer K. The bioactivity of insulin analogues from in vitro receptor binding to in vivo glucose uptake. *Diabetes Metab Rev* 1992 **8** 259-285.
96. Milazzo G, Sciacca L, Papa V, Goldfine ID & Vigneri R. ASPB10 insulin induction of increased mitogenic responses and phenotypic changes in human breast epithelial cells: evidence for enhanced interactions with the insulin-like growth factor-I receptor. *Mol Carcinog* 1997 **18** 19-25.
97. Drejer K, Kruse V, Larsen UD, Hougaard P, Bjorn S & Gammeltoft S. Receptor binding and tyrosine kinase activation by insulin analogues with extreme affinities studied in human hepatoma HepG2 cells. *Diabetes* 1991 **40** 1488-1495.

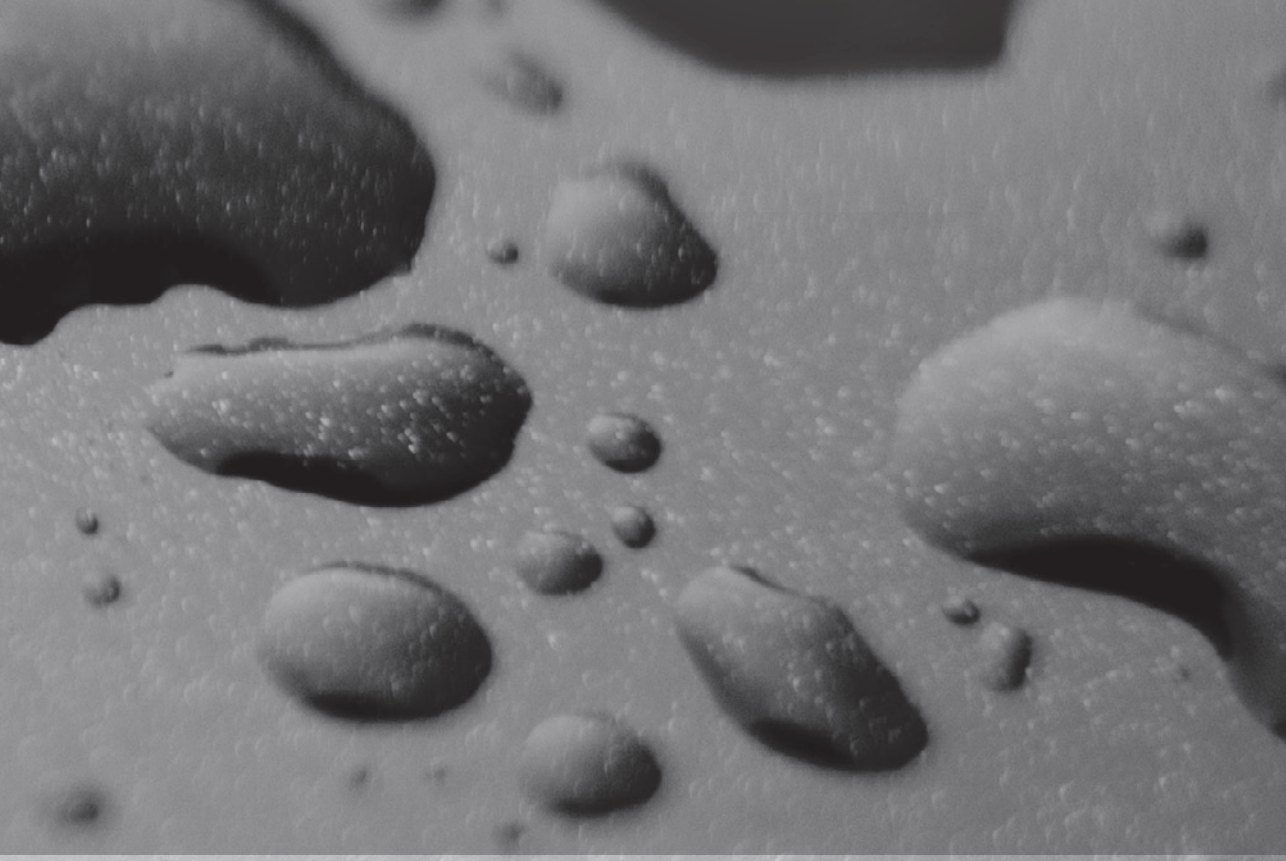
98. Owens D & Barnett A. Designer insulins: have they revolutionized insulin therapy? . In *Diabetes, current perspectives*, ch. 14, pp 223-265. Ed DJ Betteridge, 2000.
99. Vigneri R, Squatrito S & Sciacca L. Insulin and its analogs: actions via insulin and IGF receptors. *Acta Diabetol* 2010 **47** 271-278.
100. Stammberger I, Seipke G & Bartels T. Insulin glulisine--a comprehensive preclinical evaluation. *Int J Toxicol* 2006 **25** 25-33.
101. Havelund S, Plum A, Ribel U, Jonassen I, Volund A, Markussen J & Kurtzhals P. The mechanism of protraction of insulin detemir, a long-acting, acylated analog of human insulin. *Pharm Res* 2004 **21** 1498-1504.
102. Kuerzel GU, Shukla U, Scholtz HE, Pretorius SG, Wessels DH, Venter C, Potgieter MA, Lang AM, Koose T & Bernhardt E. Biotransformation of insulin glargine after subcutaneous injection in healthy subjects. *Curr Med Res Opin* 2003 **19** 34-40.
103. Heise T, Nosek L, Ronn BB, Endahl L, Heinemann L, Kapitza C & Draeger E. Lower within-subject variability of insulin detemir in comparison to NPH insulin and insulin glargine in people with type 1 diabetes. *Diabetes* 2004 **53** 1614-1620.
104. Markussen J, Havelund S, Kurtzhals P, Andersen AS, Halstrom J, Hasselager E, Larsen UD, Ribel U, Schaffer L, Vad K & Jonassen I. Soluble, fatty acid acylated insulins bind to albumin and show protracted action in pigs. *Diabetologia* 1996 **39** 281-288.
105. Vague P, Selam JL, Skeie S, De Leeuw I, Elte JW, Haahr H, Kristensen A & Draeger E. Insulin detemir is associated with more predictable glycemic control and reduced risk of hypoglycemia than NPH insulin in patients with type 1 diabetes on a basal-bolus regimen with premeal insulin aspart. *Diabetes Care* 2003 **26** 590-596.
106. Owens DR & Bolli GB. Beyond the era of NPH insulin--long-acting insulin analogs: chemistry, comparative pharmacology, and clinical application. *Diabetes Technol Ther* 2008 **10** 333-349.
107. Hastings KL. Pharmacology and toxicology review of insulin detemir. 2005.
108. Sciacca L, Cassarino MF, Genua M, Pandini G, Le Moli R, Squatrito S & Vigneri R. Insulin analogues differently activate insulin receptor isoforms and post-receptor signalling. *Diabetologia* 2010 **53** 1743-1753.
109. Zelobowska K, Gumprecht J & Grzeszczak W. Mitogenic potency of insulin glargine. *Endokrynol Pol* 2009 **60** 34-39.
110. Hansen BF. Insulin analogues with increased mitogenic potency--are they safe? *Horm Metab Res* 2008 **40** 431-433.
111. Staiger K, Hennige AM, Staiger H, Haring HU & Kellerer M. Comparison of the mitogenic potency of regular human insulin and its analogue glargine in normal and transformed human breast epithelial cells. *Horm Metab Res* 2007 **39** 65-67.
112. Eckardt K, May C, Koenen M & Eckel J. IGF-1 receptor signalling determines the mitogenic potency of insulin analogues in human smooth muscle cells and fibroblasts. *Diabetologia* 2007 **50** 2534-2543.
113. Sandow J. Growth effects of insulin and insulin analogues. *Arch Physiol Biochem* 2009 **115** 72-85.
114. Giorgino F, Belfiore A, Milazzo G, Costantino A, Maddux B, Whittaker J, Goldfine ID & Vigneri R. Overexpression of insulin receptors in fibroblast and ovary cells induces a ligand-mediated transformed phenotype. *Mol Endocrinol* 1991 **5** 452-459.
115. Parkin JM. Incidence of growth hormone deficiency. *Arch Dis Child* 1974 **49** 904-905.
116. Sassolas G, Chazot FB, Jaquet P, Bachelot I, Chanson P, Rudelli CC, Tauber JP, Allannic H, Bringer J, Roudaut N, Rohmer V, Roger P, Latapie JL, Reville P & Leutenegger M. GH deficiency in adults: an epidemiological approach. *Eur J Endocrinol* 1999 **141** 595-600.

117. Stochholm K, Gravholt CH, Laursen T, Jorgensen JO, Laurberg P, Andersen M, Kristensen LO, Feldt-Rasmussen U, Christiansen JS, Frydenberg M & Green A. Incidence of GH deficiency - a nationwide study. *Eur J Endocrinol* 2006 **155** 61-71.
118. Hwa V, Nadeau K, Wit JM & Rosenfeld RG. STAT5b deficiency: lessons from STAT5b gene mutations. *Best Pract Res Clin Endocrinol Metab* 2011 **25** 61-75.
119. Kelberman D, Rizzoti K, Lovell-Badge R, Robinson IC & Dattani MT. Genetic regulation of pituitary gland development in human and mouse. *Endocr Rev* 2009 **30** 790-829.
120. Mullis PE. Genetics of GHRH, GHRH-receptor, GH and GH-receptor: its impact on pharmacogenetics. *Best Pract Res Clin Endocrinol Metab* 2011 **25** 25-41.
121. Tauber M, Moulin P, Pienkowski C, Jouret B & Rochiccioli P. Growth hormone (GH) retesting and auxological data in 131 GH-deficient patients after completion of treatment. *J Clin Endocrinol Metab* 1997 **82** 352-356.
122. Abs R, Bengtsson BA, Hernberg-Stahl E, Monson JP, Tauber JP, Wilton P & Wuster C. GH replacement in 1034 growth hormone deficient hypopituitary adults: demographic and clinical characteristics, dosing and safety. *Clin Endocrinol (Oxf)* 1999 **50** 703-713.
123. de Boer H, Blok GJ & Van der Veen EA. Clinical aspects of growth hormone deficiency in adults. *Endocr Rev* 1995 **16** 63-86.
124. Molitch ME, Clemmons DR, Malozowski S, Merriam GR & Vance ML. Evaluation and treatment of adult growth hormone deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab* 2011 **96** 1587-1609.
125. Carroll PV, Christ ER, Bengtsson BA, Carlsson L, Christiansen JS, Clemmons D, Hintz R, Ho K, Laron Z, Sizonenko P, Sonksen PH, Tanaka T & Thorne M. Growth hormone deficiency in adulthood and the effects of growth hormone replacement: a review. Growth Hormone Research Society Scientific Committee. *J Clin Endocrinol Metab* 1998 **83** 382-395.
126. Shalet SM, Toogood A, Rahim A & Brennan BM. The diagnosis of growth hormone deficiency in children and adults. *Endocr Rev* 1998 **19** 203-223.
127. Strasburger CJ, Bidlingmaier M, Wu Z & Morrison KM. Normal values of insulin-like growth factor I and their clinical utility in adults. *Horm Res* 2001 **55 Suppl 2** 100-105.
128. Toogood AA, Jones J, O'Neill PA, Thorner MO & Shalet SM. The diagnosis of severe growth hormone deficiency in elderly patients with hypothalamic-pituitary disease. *Clin Endocrinol (Oxf)* 1998 **48** 569-576.
129. Ghigo E, Aimaretti G, Gianotti L, Bellone J, Arvat E & Camanni F. New approach to the diagnosis of growth hormone deficiency in adults. *Eur J Endocrinol* 1996 **134** 352-356.
130. Ho KK. Consensus guidelines for the diagnosis and treatment of adults with GH deficiency II: a statement of the GH Research Society in association with the European Society for Pediatric Endocrinology, Lawson Wilkins Society, European Society of Endocrinology, Japan Endocrine Society, and Endocrine Society of Australia. *Eur J Endocrinol* 2007 **157** 695-700.
131. Toogood AA, Beardwell CG & Shalet SM. The severity of growth hormone deficiency in adults with pituitary disease is related to the degree of hypopituitarism. *Clin Endocrinol (Oxf)* 1994 **41** 511-516.
132. Molitch ME. Diagnosis of GH deficiency in adults--how good do the criteria need to be? *J Clin Endocrinol Metab* 2002 **87** 473-476.
133. Ribeiro-Oliveira A, Jr. & Barkan A. The changing face of acromegaly--advances in diagnosis and treatment. *Nat Rev Endocrinol* 2012 **8** 605-611.
134. Neggers SJ, Biermasz NR & van der Lely AJ. What is active acromegaly and which parameters do we have? *Clin Endocrinol (Oxf)* 2012 **76** 609-614.

135. Jaffe CA, Pan W, Brown MB, DeMott-Friberg R & Barkan AL. Regulation of GH secretion in acromegaly: reproducibility of daily GH profiles and attenuated negative feedback by IGF-I. *J Clin Endocrinol Metab* 2001 **86** 4364-4370.
136. Melmed S, Colao A, Barkan A, Molitch M, Grossman AB, Kleinberg D, Clemmons D, Chanson P, Laws E, Schlechte J, Vance ML, Ho K & Giustina A. Guidelines for acromegaly management: an update. *J Clin Endocrinol Metab* 2009 **94** 1509-1517.
137. Larsen PR, Davies TF & Schlumberger MJ. Thyrotoxicosis. In *Williams Textbook of Endocrinology*, pp 374-421. Eds PR Larsen, K H.M. & S Melmed: Saunders Co, W.B., 2003.
138. Jameson JL & Weightman AP. Disorders of the thyroid gland. In *Harrison's Principles of Internal Medicine*, pp 2060-2071. Eds AS Fauci, E Braunwald & DL Kasper: McGraw-Hill, 2001.
139. Eckstein AK, Plicht M, Lax H, Neuhauser M, Mann K, Lederbogen S, Heckmann C, Esser J & Morgenthaler NG. Thyrotropin receptor autoantibodies are independent risk factors for Graves' ophthalmopathy and help to predict severity and outcome of the disease. *J Clin Endocrinol Metab* 2006 **91** 3464-3470.
140. Gerding MN, van der Meer JW, Broenink M, Bakker O, Wiersinga WM & Prummel MF. Association of thyrotrophin receptor antibodies with the clinical features of Graves' ophthalmopathy. *Clin Endocrinol (Oxf)* 2000 **52** 267-271.
141. Khoo DH, Eng PH, Ho SC, Tai ES, Morgenthaler NG, Seah LL, Fong KS, Chee SP, Choo CT & Aw SE. Graves' ophthalmopathy in the absence of elevated free thyroxine and triiodothyronine levels: prevalence, natural history, and thyrotropin receptor antibody levels. *Thyroid* 2000 **10** 1093-1100.
142. Perros P & Kendall-Taylor P. Demonstration of thyrotropin binding sites in orbital connective tissue: possible role in the pathogenesis of thyroid-associated ophthalmopathy. *J Endocrinol Invest* 1994 **17** 163-170.
143. Valyasevi RW, Erickson DZ, Harteneck DA, Dutton CM, Heufelder AE, Jyonouchi SC & Bahn RS. Differentiation of human orbital preadipocyte fibroblasts induces expression of functional thyrotropin receptor. *J Clin Endocrinol Metab* 1999 **84** 2557-2562.
144. Wakelkamp IM, Bakker O, Baldeschi L, Wiersinga WM & Prummel MF. TSH-R expression and cytokine profile in orbital tissue of active vs. inactive Graves' ophthalmopathy patients. *Clin Endocrinol (Oxf)* 2003 **58** 280-287.
145. Bahn RS, Dutton CM, Natt N, Joba W, Spitzweg C & Heufelder AE. Thyrotropin receptor expression in Graves' orbital adipose/connective tissues: potential autoantigen in Graves' ophthalmopathy. *J Clin Endocrinol Metab* 1998 **83** 998-1002.
146. Starkey KJ, Janezic A, Jones G, Jordan N, Baker G & Ludgate M. Adipose thyrotrophin receptor expression is elevated in Graves' and thyroid eye diseases ex vivo and indicates adipogenesis in progress in vivo. *J Mol Endocrinol* 2003 **30** 369-380.
147. Iranmanesh A, Lizarralde G, Johnson ML & Veldhuis JD. Nature of altered growth hormone secretion in hyperthyroidism. *J Clin Endocrinol Metab* 1991 **72** 108-115.
148. Miell JP, Taylor AM, Zini M, Maheshwari HG, Ross RJ & Valcavi R. Effects of hypothyroidism and hyperthyroidism on insulin-like growth factors (IGFs) and growth hormone- and IGF-binding proteins. *J Clin Endocrinol Metab* 1993 **76** 950-955.
149. Valimaki M, Liewendahl, Karonen SL, Helenius T & Suikkari AM. Concentrations of somatomedin-C and triiodothyronine in patients with thyroid dysfunction and nonthyroidal illnesses. *J Endocrinol Invest* 1990 **13** 155-159.
150. Frystyk J, Gronbaek H, Skjaerbaek C & Flyvbjerg A. Effect of hyperthyroidism on circulating levels of free and total IGF-I and IGFBPs in rats. *Am J Physiol* 1995 **269** E840-845.

151. Giustina A, Bussi AR, Legati F, Bossoni S, Licini M, Schettino M, Zuccato F & Wehrenberg WB. Galanin does not affect the growth hormone-releasing hormone-stimulated growth hormone secretion in patients with hyperthyroidism. *Acta Endocrinol (Copenh)* 1992 **127** 504-508.
152. Rodriguez-Arnao J, Miell JP & Ross RJ. Influence of thyroid hormones on the GH-IGF-I axis. *Trends Endocrinol Metab* 1993 **4** 169-173.
153. Valcavi R, Dieguez C, Zini M, Muruais C, Casanueva F & Portioli I. Influence of hyperthyroidism on growth hormone secretion. *Clin Endocrinol (Oxf)* 1993 **38** 515-522.
154. Lakatos P, Foldes J, Nagy Z, Takacs I, Speer G, Horvath C, Mohan S, Baylink DJ & Stern PH. Serum insulin-like growth factor-I, insulin-like growth factor binding proteins, and bone mineral content in hyperthyroidism. *Thyroid* 2000 **10** 417-423.
155. Krassas GE, Pontikides N, Kaltsas T, Dumas A, Frystyk J, Chen JW & Flyvbjerg A. Free and total insulin-like growth factor (IGF)-I, -II, and IGF binding protein-1, -2, and -3 serum levels in patients with active thyroid eye disease. *J Clin Endocrinol Metab* 2003 **88** 132-135.
156. Pritchard J, Han R, Horst N, Cruikshank WW & Smith TJ. Immunoglobulin activation of T cell chemoattractant expression in fibroblasts from patients with Graves' disease is mediated through the insulin-like growth factor I receptor pathway. *J Immunol* 2003 **170** 6348-6354.
157. Pritchard J, Horst N, Cruikshank W & Smith TJ. Igs from patients with Graves' disease induce the expression of T cell chemoattractants in their fibroblasts. *J Immunol* 2002 **168** 942-950.
158. Pritchard J, Tsui S, Horst N, Cruikshank WW & Smith TJ. Synovial fibroblasts from patients with rheumatoid arthritis, like fibroblasts from Graves' disease, express high levels of IL-16 when treated with Igs against insulin-like growth factor-1 receptor. *J Immunol* 2004 **173** 3564-3569.
159. Hansson HA, Petruson B & Skottner A. Somatomedin C in pathogenesis of malignant exophthalmos of endocrine origin. *Lancet* 1986 **1** 218-219.
160. Tramontano D, Cushing GW, Moses AC & Ingbar SH. Insulin-like growth factor-I stimulates the growth of rat thyroid cells in culture and synergizes the stimulation of DNA synthesis induced by TSH and Graves'-IgG. *Endocrinology* 1986 **119** 940-942.
161. Weightman DR, Perros P, Sherif IH & Kendall-Taylor P. Autoantibodies to IGF-1 binding sites in thyroid associated ophthalmopathy. *Autoimmunity* 1993 **16** 251-257.
162. Smith TJ & Hoa N. Immunoglobulins from patients with Graves' disease induce hyaluronan synthesis in their orbital fibroblasts through the self-antigen, insulin-like growth factor-I receptor. *J Clin Endocrinol Metab* 2004 **89** 5076-5080.
163. Wiersinga WM, Smit T, van der Gaag R & Koornneef L. Temporal relationship between onset of Graves' ophthalmopathy and onset of thyroidal Graves' disease. *J Endocrinol Invest* 1988 **11** 615-619.
164. Bahn RS & Heufelder AE. Pathogenesis of Graves' ophthalmopathy. *N Engl J Med* 1993 **329** 1468-1475.
165. Mourits MP, Prummel MF, Wiersinga WM & Koornneef L. Clinical activity score as a guide in the management of patients with Graves' ophthalmopathy. *Clin Endocrinol (Oxf)* 1997 **47** 9-14.
166. Brugts MP. IGF-I Bioactivity in Aging, Health and Disease. Thesis, ISBN 9789085599159, Erasmus University, 2009
167. Sommerfeld MR, Muller G, Tschank G, Seipke G, Habermann P, Kurrle R & Tennagels N. In vitro metabolic and mitogenic signaling of insulin glargine and its metabolites. *PLoS One* 2010 **5** e9540.
168. Hennige AM, Lehmann R, Weigert C, Moeschel K, Schauble M, Metzinger E, Lammers R & Haring HU. Insulin glulisine: insulin receptor signaling characteristics in vivo. *Diabetes* 2005 **54** 361-366.
169. Kohn WD, Micanovic R, Myers SL, Vick AM, Kahl SD, Zhang L, Strifler BA, Li S, Shang J, Beals JM, Mayer JP & DiMarchi RD. pI-shifted insulin analogs with extended in vivo time action and favorable receptor selectivity. *Peptides* 2007 **28** 935-948.

170. Berti L, Kellerer M, Bossenmaier B, Seffer E, Seipke G & Haring HU. The long acting human insulin analog HOE 901: characteristics of insulin signalling in comparison to Asp(B10) and regular insulin. *Horm Metab Res* 1998 **30** 123-129.
171. Liefvendahl E & Arnqvist HJ. Mitogenic effect of the insulin analogue glargine in malignant cells in comparison with insulin and IGF-I. *Horm Metab Res* 2008 **40** 369-374.



Circulating insulin-like growth factors may contribute substantially to insulin receptor isoform A and insulin receptor isoform B signalling

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ABSTRACT

Aims: Only a fraction of circulating insulin-like activity is due to insulin itself. The aim of this study was to determine total serum insulin-like activity mediated via the insulin receptor isoform A (IR-A) and isoform B (IR-B) by using Kinase Receptor Activation (KIRA) assays specific for the IR-A and IR-B.

Methods: The IR-A and IR-B KIRA assays use human embryonic kidney cells which have been transfected with the human IR-A or IR-B gene and quantify serum-mediated phosphorylation of the IR.

Results: Both IR KIRA assays were sensitive (detection limit 32 pmol/L) and precise (intra- and inter assay CV: <12% and <15%). The EC_{50}s of insulin, IGF-I and IGF-II were 1.4, 11.2 and 6.7 nmol/L for the IR-A KIRA assay, and 1.3, 31.0 and 15.7 nmol/L for the IR-B KIRA assay.

The operational range of both assays allowed for determination of total insulin-like activity in human serum. Analysis of serum samples showed that there was a significant positive correlation between serum insulin-like and immunoreactive insulin concentrations (IR-A: r=0.56, p= 0.01, IR-B: r=0.68, p=0.001). Importantly, addition of IGF-I or IGF-II antibodies to human serum samples could substantially decrease the endpoint signal in both KIRA assays.

Conclusions: We showed that serum IGF-I and IGF-II may substantially contribute to IR signalling. Since IR isoform specific KIRA assays also take into account the contribution of IGFs present in serum on IR signalling, they may help to gain more insight into the roles of IGF mediated IR-A and IR-B activation in health and disease.

INTRODUCTION

Early studies have shown that insulin-like mediated effects of serum on target tissues are much greater *in vitro* and *in vivo* than what would be expected on the basis of the measured immunoreactive insulin concentrations[1].

Insulin elicits its various biological responses by binding to the insulin receptor (IR), which is then followed by activation of its intrinsic tyrosine kinase[2]. In the human body, due to alternative splicing of exon 11 of the IR gene, two IR transcripts are generated, resulting in IR isoform A (IR-A) (lacking exon 11) and in IR isoform B (IR-B) (full length)[3]. The relative abundance of mRNAs encoding the IR-A and IR-B isoforms is regulated in a tissue-specific manner[4] and also differs by stage of cell development and differentiation. IR-A is the predominant isoform in foetal tissues and cancer cells, while the IR-B is the classical receptor for insulin with metabolic effects in muscle, liver and adipose tissues[4-5]. Although studies have suggested that differences between IR-A and IR-B in terms of receptor activation and signalling may result in different functions of each IR isoform[3], it appears that most cells have both IR isoforms and that the ratio of the two seems to be very important[3].

IGF-I and IGF-II primarily activate the IGF-I receptor (IGF-IR), but they can also activate the IRs[6]. According to the literature, the IR-B binds insulin with high affinity but the IGFs poorly, while the IR-A binds insulin and IGF-II with high affinity and IGF-I with low affinity[7]. In this respect, it is important to underline that although the IGFs have a lower affinity for the IR than insulin, in vivo circulating total concentrations of IGFs are much higher than of insulin (picomolar vs. nanomolar range) and that IGF-IR bioactivity (1-2% of the total IGF concentration) is modulated by the presence of IGF binding proteins (IGFBPs) and IGFBP proteases[8]. Nevertheless, insulin immunoassays measure only immunoreactive insulin and by doing so they ignore at least the potential insulin-like effects of the IGFs in blood. Moreover, insulin immunoassays do not assess potential biological effects of circulating insulin-like factors on the IR-A and the IR-B.

The aim of this study was to determine the potential biologic actions of serum on the IR-A and the IR-B and to assess the relative contribution of circulating IGFs in this respect. For this purpose we used cell-based kinase receptor activation (KIRA) assays, one specific for the human IR-A and one specific for the human IR-B. Since there are no specific antibodies for the two isoforms available, specificity was determined not by isoform-specific antibodies but by transfecting HEK cells with either the IR-A or the IR-B.

The principle of these two assays is based on quantification of phosphorylated tyrosine residues within the IR after *in vitro* stimulation with serum. The same principle has been used for IGF-IR KIRA assay specific for the IGF-IR[9-10].

MATERIALS AND METHODS

Peptides

MAI1, a monoclonal antibody directed against the extracellular domain of both human IRs, was used as capture antibody (Novozymes-Gropep (Aidelade, Australia)). Europium-labelled PY20 (Eu-PY20); a monoclonal anti-phosphotyrosine antibody was used as detection antibody (PerkinElmer life sciences (Groningen, Netherlands)). Human insulin (Actrapid®) was obtained from Novo Nordisk (Bagsvaerd, Denmark), human recombinant IGF-I from Invitrogen (Breda, Netherlands) and human recombinant IGF-II was obtained from Sigma-Aldrich (Zwijndrecht, Netherlands). An IGF-I neutralizing antibody was obtained from R&D Systems Europe Ltd. (Abingdon, UK). Monoclonal IGF-II antibody IgG1 m610 was developed in our laboratories[11].

Cell line and media

The human embryonic kidney (HEK) cell-line Flip-in™-293 from Invitrogen was transfected with plasmids (pNTK-2) containing a cDNA insert of the human IR-A (pNTK2-IR-A) or IR-B (pNTK2-IR-B) using Fugene® transfection reagens according to manufacturer's protocol. The plasmids were kindly provided by Axel Ullrich (Martinsried, Germany). After 48 hours, cells were trypsinized and cultured in Dulbecco's Modified Eagles Medium (DMEM: gluc+, L-Glutamin +, Pyr+) from Invitrogen supplemented with 10% foetal bovine serum (FBS) from Invitrogen and geneticin 1000 µg/mL from Invitrogen. Separate colonies were isolated, expanded and tested for IR expression defined by quantitative RT-PCR as relative copy number of mRNA (see below) and by immunocytochemistry (see below). The transfected cells were cultured in 75cm^3 culture flasks from Corning (Amsterdam, Netherlands) using DMEM containing 10% FBS, 100 U/mL penicillin, 100 µg/L streptomycin from Invitrogen and 500 µg/mL geneticin.

Quantitative RT-PC

Total RNA was isolated from 10^6 cells, using a commercially available kit (High pure RNA isolation kit) from Roche (Almere, Netherlands), according to the recommendation by the manufacturer. Complementary DNA was synthesized using 500 ng of total RNA in a Super Reverse Transcriptase (RT) buffer from HT Biotechnology Ltd. (Cambridge, UK), together with 40 nmol of each deoxynucleotide triphosphate, 15 ng oligo-dT primer, 20 U RNAse inhibitor, and 4 U AMV Super RT also from HT Biotechnology, in

a final volume of 40 µl. This mixture was incubated for 1 hour at 40^0C and thereafter diluted 5 times in bidest. A quantitative PCR was performed using the TaqMan Gold nuclease assay from Roche, according to the manufacturer's protocol. The primer and probe sequences (Sigma Aldrich (Zwijndrecht, The Nederlands)) were: IR-A forward, 5'-CGTTTGAGGATTACCTGCACAA-3'; IR-A reverse, 5'-GCCAAGGGACCTGCGTTT-3'; and IR-A probe, 5'-FAM-TGGTTTTCGTCCCCAGGCCATC-TAMRA-3'. IR-B forward, 5'-CCCAGAAAAACCTCTTCAGGC-3'; IR-B reverse, 5'-GGACCTGCGTTTCCGAGA-3'; and IR-B probe, 5'-FAM-CTGGTGCCGAGGACCCTAGGCC-TAMRA-3'. IGF-II Receptor forward, 5'-ACCGACCCCTCCACGC-3'; IGF-II Receptor reverse, 5'-CCTCCAAGGCCACCTTCAG-3'; and IGF-II Receptor probe, 5'-FAM-AGCAGTACGACCTCTCCAGTCTGGCAAA-TAMRA-3'. Samples were normalized against the expression of the housekeeping gene hypoxanthine-phospho-ribosyl-transferase (HPRT). Dilution curves were constructed for calculating the PCR efficiency for every primer set[12]. PCR efficiencies were: HPRT= 1.98, IR-A= 1.89, IR-B= 1.92, IGF-II Receptor= 1.87. The primer and probe sequences for IGF-I, IGF-II and for the IGF-IR have been previously described[13-14]. The relative expression of genes were calculated using the comparative threshold method, 2-ΔCt[15], after efficiency correction[16] of target and reference transcripts.

Total human IR sandwich ELISA

HEK IR-A and HEK IR-B cells were plated into a 6 well culture plate (Corning, NY, USA), 300000 cells/well in 2mL of culture medium at 37°C and 5% CO_2. After 72 hours medium was removed and replaced with 2mL DMEM containing 0.1% HSA, 100 U/mL penicillin and 100 µg/L streptomycin and 500 µg/mL geneticin. The next day, a total IR sandwich ELISA was performed according to manufactures protocol (Human total Insulin R, R&D Systems Europe Ltd., Abingdon, UK). Quantification of protein concentration was performed by using

Nanodrop Nanodrop ND-1000 (Thermo Scientific). For the HEK IR-A 200 µg of lysate was used to perform the ELISA, compared to 100µg for the HEK IR-B.

Immunocytochemistry

HEK IR-A and HEK IR-B cells were cultured on chamber slides from Invitrogen (Breda, Netherlands) for two days (20.000 cells/ chamber) in DMEM containing 10% FBS, 100 U/mL penicillin, 100 µg/L streptomycin and 500 µg/mL geneticin. Prior to immunostaining, cells were fixed with 4% paraformaldehyde and 0.2% picric acid in phosphate buffer, pH 6.9 for 40 min at room temperature. After washing with Tris/HCl/ Tween 0.5%, fixation of cells was finalized by incubating them with 50% methanol (3 min at room temperature) and 100% methanol (3 min at room temperature). Fixed cells were washed again with Tris/HCl/ Tween 0.5% followed by washing with PBS. Subsequently they were incubated with H_2O_2 (30%) (15 min at room temperature) to quench endogenous peroxidase and

were washed with Tris/HCl/ Tween 0.5% thereafter. Fixed cells were then incubated with the following primary antibodies for 1 hr at room temperature: anti-IR (Enzo Life Sciences, Antwerp, Belgium; mouse monoclonal, 1:25) and anti-IGF-IR (Novus Biologicals, Cambridge, United Kingdom; mouse monoclonal, 1:500). After incubation, cells were washed and two drops of HRP-Rabbit/Mouse from Dako (Heverlee, Belgium) were added to chamber slides and incubated for 30 min. Bound antibodies were visualized with freshly prepared 100 µl of DAB from Dako (Heverlee, Belgium) twice for 5 min at room temperature, in the dark. Staining was then stopped by rinsing with water. Slides were counterstained with haematoxylin and eosin and coverslipped. For negative controls, the primary antibody was omitted.

Buffers

Preparation of the antibody coating buffer (ACB), blocking buffer and lysis buffer has been previously described[10]. Standards and serum samples were diluted in Krebs Ringer bicarbonate (KRB) buffer that was adjusted to pH 7.4 by CO_2 and supplemented with 0.5% and 0.1% respectively (wt/vol), Human Serum Albumin (HSA), (Octalbine®) (Octopharma (Lachen, Switzerland)). EU-PY20 was diluted in a commercial assay buffer obtained from PerkinElmer Life Sciences as was the washing solution and the enhancement solution.

IR KIRA assay procedure

On day 1, cells were plated into a 48 well culture plate (Corning, NY, USA), 200000 cells/ well in 500µl culture medium for 24 hours at 37°C and 5% CO_2.

On day 2, medium was removed and replaced with 500 µl DMEM containing 0.1% HSA, 100 U/mL penicillin and 100 µg/L streptomycin and 500 µg/mL geneticin. In addition, a 96-well microtiter test plate from Biozym (Landgraaf, Netherlands) was coated with MAI-1 diluted in ACB in the indicated concentrations and incubated overnight at 4°C. On day 3 the MAI-1 solution was replaced with 300 µl blocking buffer containing 1% HSA. The plate was incubated for 3 hours at RT.

Transfected HEK IR cells were stimulated for the times indicated with either serial dilutions of insulin, IGF-I or IGF-II or human serum diluted in KRB containing 0.5% or 0.1% HSA at 37° C as indicated. After stimulation, supernatant was removed and 250 µl lysis buffer was added to each well. The plate was incubated for 1 hour on a plate shaker at 4 ° C. Crude cell lysate (150 µl per well) was transferred to the blocked antibody plate and incubated overnight at 4 ° C on a plate shaker.

On day 4, wells were washed 3 times with 250 µl wash buffer and 150 µl of EU-PY20 diluted in assay buffer was added in the indicated concentrations. Samples were incubated for 2 hours at RT on a plate shaker. Wells were washed 6 times with 250 µl of wash buffer after which 150 µl of enhancement solution was added. The samples were incubated for

20 minutes at room temperature and were read in a time-resolved fluorometer (Victor^2 multilabel counter) from PerkinElmer Life Sciences.

By performing the IR KIRA assay procedure as described above, optimal assay conditions were found. MAI1 dilutions were tested at 0.5, 2.5 and 5.0 μg/mL in combination with EU-PY20 dilutions at 1.25μg/mL and 2.5 μg/mL. In both assays maximal S/N was reached at a concentration of 2.5 μg/mL of MAI-1 and at 1.25 μg/mL of EU-PY20 (data not shown); remaining experiments were performed at these concentrations. Sample incubation times tested were 5, 10, 15, 20 and 30 minutes. Maximal S/N was reached after 10 minutes of stimulation with insulin (data not shown) and so 10 minutes was chosen as stimulation time for the remaining experiments. Sensitivity and specificity were tested using dose titration curves of insulin, IGF-I and IGF-II, respectively. For both IR KIRA assays the detection limit for insulin was found to be 32 pmol/L (determined by repeated measurements of a blank sample and reported as the mean plus 3 SD of the blank). The intra-assay CVs of both IR KIRA assays were <12% and the inter-assay CVs were <15%, respectively, for the samples analyzed in duplicates (5 repetitions on 5 different days).

Previously it has been described that serum needs to be diluted to perform a KIRA assay, since cells become repressed at high serum concentrations[10]. In addition, by using the KIRA assay specific for the IGF-IR, it has previously been shown that the endpoint signal was relatively independent of serial dilutions of serum samples up to 20-fold[10]. This phenomenon has been explained by the buffering capacity of IGFBPs, which liberate IGFs during serum dilution to maintain equilibrium. Due to this phenomenon, for the IGF-IR specific KIRA assay, bioactivity of serum samples is measured in samples that have been diluted 10-fold.

Therefore, to test whether a similar phenomenon of liberation of IGFs could be detected in the IR KIRA assays, we compared the endpoint signals induced by 4-, 8-, 16-, and 32 fold diluted serum. Subsequently we determined the relative contribution of the IGFs to the endpoint signal by coincubating 2- and 20-fold diluted serum samples from 2 healthy subjects with IGF-I and IGF-II neutralizing antibodies. First we tested at known concentrations of IGF-I or IGF-II in KRB 0.5% HSA, which concentrations of specific IGF-I- and IGF-II neutralizing antibodies reduced the IR-A KIRA assay signal >90% (neutralization dose 90 (ND90)). The neutralizing antibodies were then pre-incubated (at these ND90 concentrations) with 2- or 20- fold diluted sera at 37°C for 1 hour (ND90; 65 nmol/L neutralizing IGF-I antibody; 40 nmol/L IGF-II neutralizing antibody). After incubation, the protocol was followed as described above.

Serum IR bioactivity (10-fold diluted) was compared to immunoreactive insulin concentrations as measured by a solid-phase, enzyme-labelled chemiluminescent immunometric assay (Immulite 2000, Siemens Medical Solutions).

All serum samples were collected in accordance with the Declaration of Helsinki. All subjects provided written informed consent.

Statistical analysis

Data were analysed using GraphPad software (Prism 5, London, United Kingdom). Statistical analysis was performed using the One-Way ANOVA or Two-way ANOVA repeated measures followed by Newman-Keuls Multiple Comparison Test or Bonferroni post tests, respectively. Means + SEM or 95% CI and signal-to-noise ratios of bioactivity measurements are presented. Noise was defined as the signal after stimulation with vehicle. Accordingly, the Signal-to-Noise ratios (S/N) were calculated by using the following formula: absolute counts after stimulation with a ligand/ absolute vehicle counts. The CVs were calculated by using the formula: (SD/mean) x 100%. A p-value of ($P<0.05$) was considered statistically significant.

RESULTS

Cell-lines

Before transfection, endogenous relative mRNA expression levels of IR-A, IR-B and IGF-IR (normalized against HPRT) in the non-transfected HEK cells were 0.056, 0.005 and 0.046, respectively. After transfection, the IR-A mRNA expression level in HEK IR-A cells was 2.82, while IR-B mRNA expression level in the HEK IR-B cells was 6.96. IR protein content determined by ELISA was 90 pg/100µg protein in the HEK IR-A cells compared to 450 pg/100 µg protein in the HEK IR-B cells. Immunocytochemical staining showed that both the HEK IR-A and HEK IR-B cells contained IRs and IGF-IRs (Figure 1). The mRNA expression levels of IGF-IIR, IGF-I, and IGF-II after transfection were respectively 0.044, undetectable, and 0.003 in the HEK IR-A cells and 0.049, undetectable, and 0.014 in the HEK IR-B cells. These expression ratios remained stable over at least 20 cell passages (data not shown).

Specificity

In the IR-A KIRA assay the EC_{50} for insulin was significantly lower than for IGF-I and IGF-II (1.4 nmol/L [1.3-1.7] (mean [95% CI]) vs. 11.2 nmol/L [8.6-14.8] and 6.7 [3.5-12.5], $p<0.001$, respectively) (Figure 2). The EC_{50} for IGF-II was significantly lower than for IGF-I ($p=0.02$) (Figure 2A). Also in the IR-B KIRA assay the EC_{50} for insulin was significantly

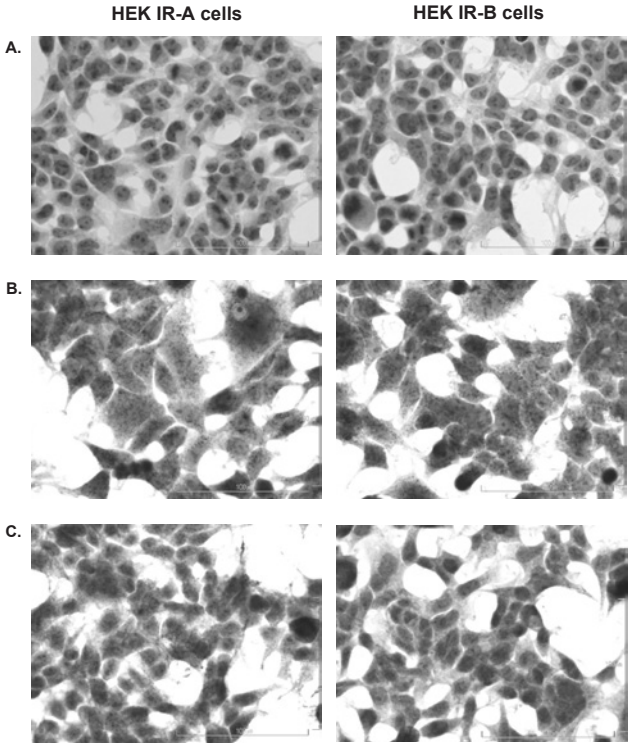

Figure 1.
Immunocytochemical staining of the IR and IGF-IR on HEK IR-A (left) and HEK IR-B (right) cells (400x). Cells were cultured on chamber slides for two days after which the immunostaining was performed. (A) negative control, (B) IR, (C) IGF-IR.

lower than for IGF-I and IGF-II (1.3 nmol/L [1.1-1.6] vs. 31.0 nmol/L [29.2-32.8] and 15.7 [12.9-19.1], $p<0.001$, respectively) and the EC_{50} for IGF-II was significantly lower than for IGF-I ($p<0.001$) (Figure 2B).

Contribution of IGFs to IR signalling

To test whether the endpoint signal was relatively independent of serial dilutions of serum samples, serum samples from two healthy subjects were serially diluted (1:4, 1:8, 1:16 and 1:32). Analysis of these serial dilutions showed that, as in the IGF-IR specific KIRA assay, the signal in both IR KIRA assays was relatively independent of serum dilution. For the IR-A KIRA assay, when a serum sample was diluted 16 fold it induced a significant lower signal than when it was diluted 4 fold (Figure 3 left panel). For the IR-B KIRA assay, when a serum sample was diluted 32 fold it induced a significant lower signal than when it was diluted 4 fold (Figure 3 right panel). To test whether indeed IGFs contribute substantially to the endpoint signal of both IR KIRA assays, we coincubated 2-fold and 20-fold diluted serum samples from two healthy subjects with IGF-I and IGF-II

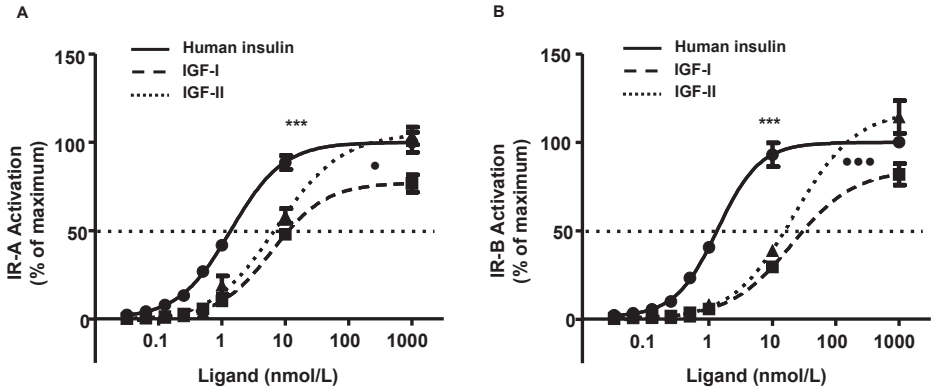

Figure 2.

Specificity of the IR-A (A) and IR-B (B) KIRA assays. Protocol was followed as described in Methods. Plates were coated with 2.5ug/mL of capture antibody (MAI1) and 1.25ug/mL of detection antibody (EU-PY20) was used. Cells were stimulated with a dose titration curve of insulin (solid line), IGF-I (dashed line, squares) or IGF-II (dashed line, triangles) for 10 minutes. Data are presented as mean (+SEM) KIRA assay signal of three independent experiments. Stimulation with 100 nmol/L of insulin was arbitrarily set at 100%.

*** P<0.001 for significant difference between insulin vs. IGF-I and IGF-II. • P<0.05 for significant difference between IGF-I vs. IGF-II. ••• P<0.001 for significant difference between IGF-I and IGF-II.

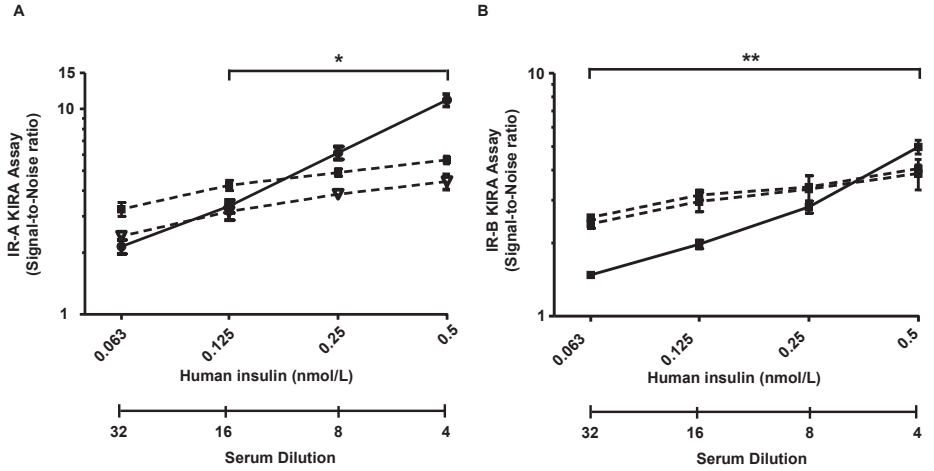

Figure 3.

Effects of serum dilution on the IR-A (A) and IR-B (B) KIRA assay signal. Protocol was followed as described in Methods. Cells were stimulated with insulin (range 63-500 pmol/L) (black line) or with serum samples from two healthy subjects (control 1 triangles, control 2 squares) that were diluted 1:4, 1:8, 1:16 and 1:32. Data are shown as mean (+SEM) IR bioactivity (expressed as Signal-to-Noise ratio) from three independent experiments. * P<0.05, ** P<0.01 for significant decrease in Signal-to-Noise ratio.

neutralizing antibodies. First the IR-A KIRA assay was used to test the specificities of the blocking antibodies that were used in the neutralizing experiments. Adding a constant amount of an IGF-I antibody (65 nmol/L) to a dilution of IGF-I blocked the endpoint signal with >90% (Figure 4A) whereas the endpoint signal produced by IGF-II or insulin was not influenced (data not shown). Adding a constant amount of an IGF-II neutralizing antibody (40 nmol/L) to a dilution of IGF-II blocked the endpoint signal with >90% (Figure 4B) whereas the endpoint signal produced by IGF-I or insulin was not influenced by co-incubation with an IGF-II antibody (data not shown).

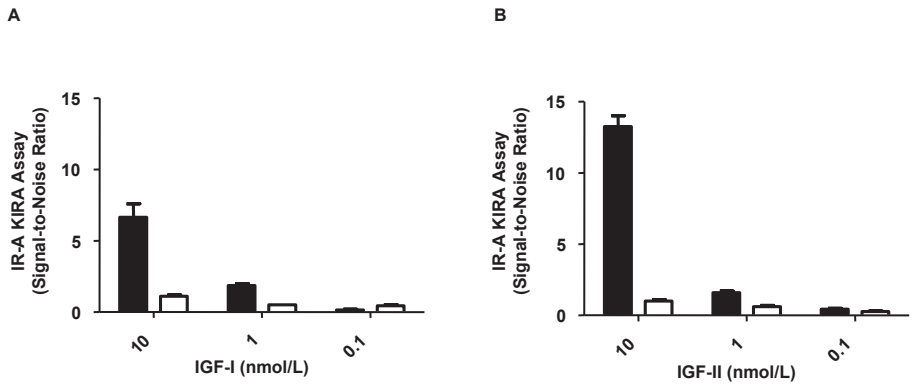

Figure 4.
Testing ND90 (dose at which the endpoint KIRA assay signal is 90% reduced) of IGF-I- and IGF-II neutralizing antibodies in the IR-A KIRA assay. Samples with IGF-I or IGF-II (range from 0.1-10nM) were preincubated with a constant concentration of neutralizing antibodies at 37° for 1 hr. After incubation, protocol was followed as described in Methods.
(A.) IR-A KIRA assay signal without (black bars) and with (white bars) addition of 65 nmol/L of an IGF-I neutralizing antibody to a standard curve of IGF-I. (B.*)* IR-A KIRA assay signal without (black bars) and with (white bars) addition of 40nmol/L of an IGF-II neutralizing antibody to a standard curve of IGF-II. Data are presented as mean (+SD) IR bioactivity (expressed as Signal-to-Noise-Ratio) of two experiments.

Signal reduction by the neutralizing antibodies directed to IGF-I or IGF-II in serum varied between the two subjects (Figure 5). In control 1, coincubation with an IGF-I or IGF-II antibody in 2-fold diluted serum did not reduce the endpoint signal in both assays. However, in 20-fold diluted serum addition of the IGF-I antibody reduced the endpoint signal with 38 % in the IR-A KIRA assay and with 26% in the IR-B KIRA assay. Also addition of the IGF-II neutralizing antibody reduced the signal in both assays with 41% and with 40% respectively. In control 2, coincubation of 2-fold diluted serum with an IGF-I or IGF-II antibody also did not reduce the endpoint signal in both assays. However, coincubation of 20-fold diluted serum with an IGF-I antibody reduced the signal with 73 % in the IR-A KIRA assay compared to 20% in the IR-B KIRA assay. Coincubation of an IGF-II antibody reduced the signal in both assays with 27% and 22 % respectively.

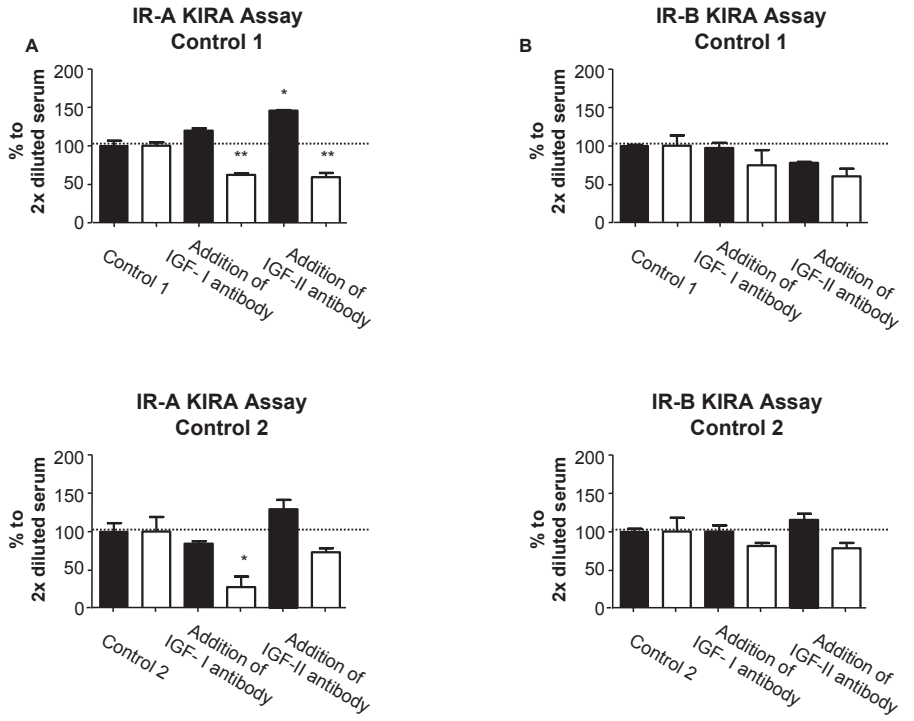

Figure 5.

Contribution of IGF-I and IGF-II in serum samples to the endpoint signal of the IR-A (A) and IR-B (B) KIRA assay. Serum samples from two healthy subjects were diluted 2 fold (black bars) and 20 fold (white bars) and preincubated at 37°C for 1 hr with an IGF-I neutralizing antibody (65 nmol/L) or with an IGF-II (40 nmol/L) neutralizing antibody. After incubation, protocol was followed as described in Methods. For both controls immunoreactive insulin, IGF-I, IGF-II, IGFBP-1 and IGFBP-3 concentrations were measured and were 14 pmol/L, 37 nmol/L, 69 nmol/L, 3.6 nmol/L and 174 nmol/L, respectively for control 1 and 46 pmol/L, 24 nmol, 73 nmol/L, 1.1 nmol/L and 205 nmol/L, respectively for control 2. Data are shown as mean (+SD) percentage reduction in endpoint signal. 100% is the endpoint signal measured in serum without adding neutralizing antibodies. * significant change compared to control ($P<0.05$). ** significant change compared to control ($P<0.01$)

Serum IR bioactivity was higher than its immunoreactive insulin content. Analysis of 20 serum samples (10-fold diluted) showed that there was a clear correlation between serum IR bioactivity and immunoreactive insulin concentrations (Figure 6).

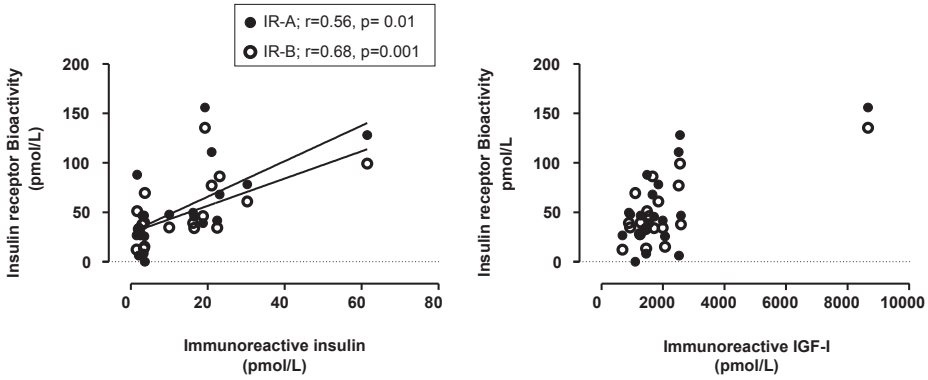

Figure 6.
A. Relationships between immunoreactive insulin concentrations, IR-A bioactivity (black dots) and IR-B bioactivity (open dots) measured in 20 serum samples collected in fasting state.
B. Relationships between immunoreactive IGF-I concentrations, IR-A bioactivity (black dots) and IR-B bioactivity (open dots) measured in 20 serum samples collected in fasting state.
Serum samples were diluted 10 fold before IR bioactivity was measured.

DISCUSSION

Here we show that IGF-I and IGF-II present in human serum may contribute substantially to IR-A and IR-B signalling in vitro. Sample dilution in a KIRA assay has been described to be necessary because cells become repressed at high serum concentrations[10]. Previously it has been shown that in the KIRA assay specific for the IGF-IR the endpoint signal is relatively independent of serum dilution[10]. This phenomenon has not only been described for free IGF-I but also for free thyroid hormones[17]. In the case of IGFs this has been attributed to the liberation of IGFs from IGFBPs during dilution to maintain equilibrium. We determined whether the same phenomenon was present in the IR KIRA assays. Also in the IR KIRA assays the endpoint signal was relatively independent of serum dilution as long as the serum dilutions were kept below 1:16. Although there may be several reasons for different kinetics by either insulin or serum (e.g. including different ligand-receptor affinity, binding proteins, non-specific serum protein interference), the non-parallelism could also indicate that circulating IGFs substantially contribute to the endpoint signal in both IR KIRA assays. The latter was supported by the finding that, in both IR KIRA assays, IGF-I and IGF-II antibodies could partially reduce the endpoint signal.

When antibodies directed against IGF-I and IGF-II were added to 20-fold diluted serum samples, as discussed above, only part of the IR-A or IR-B bioactivity was neutralized. However, when these specific antibodies were added to "pure" samples containing IGF-I or IGF-II, IR-A or IR-B activation could almost completely neutralized by these antibodies.

The only partial effect of the neutralizing antibodies in 20-fold diluted serum may be explained as follows; Since up to 90% of the circulating IGFs are normally contained in heterotrimeric complexes with the acid-labile subunit and IGFBP-3 or IGFBP-5[18], the effect of these neutralizing antibodies in serum could be decreased (or even absent) if the IGFs are sequestered by the IGFBPs in these complexes. This suggests that, in contrast to "pure" samples of the IGFs, the interactions and competition between insulins, IGFs, IGFBPs in serum at the level of the IRs are very intricate. In order to explain the substantial contributions of serum IGFs to IR signalling we would like to provide the reader a mathematical example. Under healthy circumstances, fasting insulin levels are around 50 pmol/L. Giving an individual with a concentration of total IGF-I of around 20 nmol/L and an IGF-II concentration of around 80 nmol/L, there will be around 2000 pmol/L of free IGFs (free IGFs are around 2% of the total IGF concentration[9]. Although the affinity of insulin for the IRs is 10-100x fold higher than of IGF-I and IGF-II, the free concentration of IGFs, in this example, is about 40-fold compared to insulin. Since the biological response of a target cell is not only determined by the affinity for the IR, but also by the concentrations of insulin and IGFs, this example illustrates that it may not be surprising that IGFs may substantially contribute to the total IR receptor bioactivity. More than fifty years ago it was already found that serum contained a higher amount of total insulin-like bioactivity than immunoreactive insulin[19] and that anti-insulin antibodies could only block a small portion of the total insulin bioactivity of serum[20]. However, due to the highly variable results of bioassays, they were replaced by insulin radioimmunoassays (RAI) in the sixties[19]. Although the RIA for insulin has greatly increased knowledge of the physiology of glucose homeostatasis and of the diverse causes of diabetes mellitus it neglects the potential contributions of IGFs to insulin-like signalling[21].

Over time several attempts have been made to introduce new bioassays to measure insulin-like activity but so far they have not been able to determine human serum bioactivity on human cells with high sensitivity and low intra- and interassay variability. The two IR KIRA assays that were used in this study both show a reasonably sensitivity and for bioassays an exceptionally low intra- and inter-assay CVs. In line with previous studies, the EC_{50} of IGF-I was higher than IGF-II in both the IR-A and IR-B KIRA assay[7, 22]. In addition, since they are specific for either the human IR-A or human IR-B, it is possible to distinguish the potential insulin-like mediated effects of serum on both IR isoforms. The latter may help to gain more insight into the functional roles of the two IR isoforms that, in recent years, have attracted renewed interest due to the finding that the IR isoform A besides binding to insulin also binds IGF-II with high affinity[23]. So, the two IR KIRA assays may be useful tools to disentangle the relative contribution of the IGFs present in serum on IR specific signalling. Nevertheless the authors would like to emphasize that also with the IR KIRA assays true quantification of the specific and relative contribution of IGFs to

overall IR activity remains difficult and one should be cautious with potential clinical applications and results should be interpreted with care.

Although the operational range of the assays allows to quantify the overall effect of total insulin-like factors in serum, it should be acknowledged that KIRA assays (as for all in-vitro systems) do not mimic the exact *in vivo* situation. KIRA assays only provide a crude, albeit convenient, measure of IR activation. The contribution of various circulating peptides in activating the IRs *in vivo* depends not only on their concentrations and bioavailability, but also on the relative and absolute concentrations of the two IR present on various target tissues. In most tissues and cells where significant levels of both IRs and IGF-IRs are present, hybrids may be formed. These hybrids are heterodimeric receptors consisting of an IR alpha/beta monomer and an IGF-IR alpha/beta monomer linked by disulfide bonds. Such hybrids are probably formed during normal post- translational processing of both receptors[24] and are widely expressed in normal tissues and often aberrantly expressed in cancer cells[25]. Since both IR isoforms and IGF-IRs are endogenously expressed in our cell lines, at least three hybrids may be formed: IR-A/IR-B, IR-A/IGF-IR and IR-B/IGF-IR which could influence our findings. However, our cell lines have very low endogenous levels of both IR-isoforms and the IGF-IRs compared to the transfected IR-isoform. The endogenous IRs and IGF-IRs are most probably out-competed in our IR KIRA assays following the high expression levels of the exogenous IRs achieved by transfection. In addition, although the precise biological role of the hybrids is still unclear, functional studies have demonstrated that IR/IGF-IR hybrids behave more like IGF-IRs than IRs[3]. So, although hybrid receptors may well play an important role in vivo, their relative contribution to the endpoint signal of both KIRA assays is assumed to be low but cannot be completely excluded.

Moreover, the IRs have up to 6 key tyrosine residues and the antibody used in our study to detect the tyrosine residues may not necessarily recognize all residues with the same affinity. As the distinct roles of the different tyrosine residues remain to be clarified, this aspect may be of importance.

In conclusion, serum IGF-I and IGF-II may substantially contribute to IR signalling. Since IR isoform specific KIRA assays, in sharp contrast to insulin immunoassays, take into account the contribution of the IGFs on IR signalling they may help to gain more insight into the roles of IGF mediated IR-A and IR-B activation in health and disease.

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DISCLOSURES

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All authors have nothing to disclose.

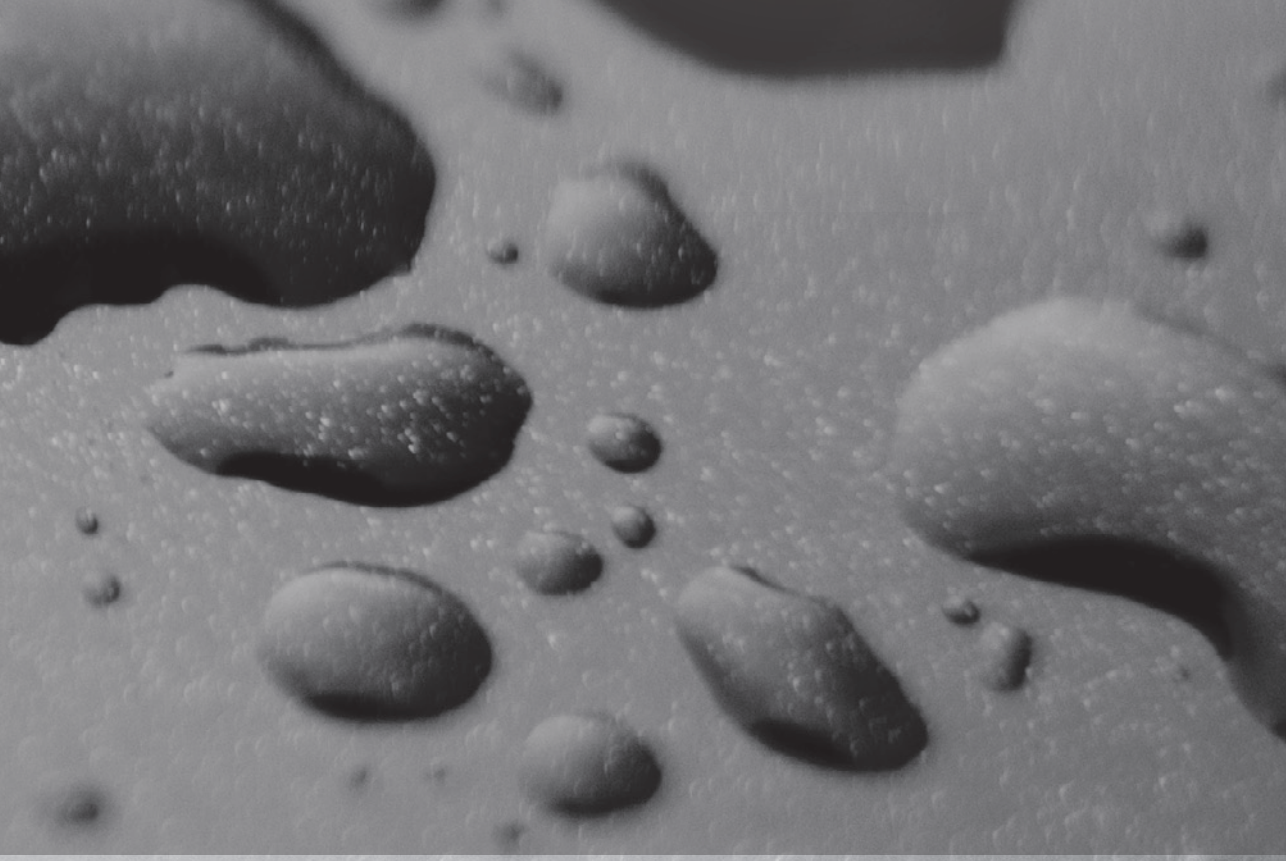
AUTHOR CONTRIBUTIONS

A.J. and M.P. researched data and wrote the manuscript, J. reviewed/edited the manuscript and contributed to the discussion, J.A. researched data, P. researched data, A.M. researched data, Y. reviewed/edited the manuscript, D.S. reviewed/edited the manuscript, S.W.J. reviewed/edited the manuscript and contributed to the discussion, L.J. reviewed/edited the manuscript and contributed to the discussion, J.A.M.J.L. researched data, wrote manuscript, reviewed/edited the manuscript.

REFERENCES

1. Froesch ER, Schmid C, Schwander J & Zapf J. Actions of insulin-like growth factors. *Annu Rev Physiol* 1985 **47** 443-467.
2. Okada Y, Yokono K, Katsuta A, Yoshida M, Morita S, Irino H, Goto T, Baba S, Roth RA & Shii K. Development of an assay for bioactive insulin. *Anal Biochem* 1998 **257** 134-138.
3. Belfiore A, Frasca F, Pandini G, Sciacca L & Vigneri R. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. *Endocr Rev* 2009 **30** 586-623.
4. Moller DE, Yokota A, Caro JF & Flier JS. Tissue-specific expression of two alternatively spliced insulin receptor mRNAs in man. *Mol Endocrinol* 1989 **3** 1263-1269.
5. Mosthaf L, Grako K, Dull TJ, Coussens L, Ullrich A & McClain DA. Functionally distinct insulin receptors generated by tissue-specific alternative splicing. *Embo J* 1990 **9** 2409-2413.
6. Boucher J, Tseng YH & Kahn CR. Insulin and insulin-like growth factor-1 receptors act as ligand-specific amplitude modulators of a common pathway regulating gene transcription. *J Biol Chem* **285** 17235-17245.
7. Denley A, Bonython ER, Booker GW, Cosgrove LJ, Forbes BE, Ward CW & Wallace JC. Structural determinants for high-affinity binding of insulin-like growth factor II to insulin receptor (IR)-A, the exon 11 minus isoform of the IR. *Mol Endocrinol* 2004 **18** 2502-2512.
8. Blakesley VA, Scrimgeour A, Esposito D & Le Roith D. Signaling via the insulin-like growth factor-I receptor: does it differ from insulin receptor signaling? *Cytokine Growth Factor Rev* 1996 **7** 153-159.
9. Brugts MP, Ranke MB, Hofland LJ, van der Wansem K, Weber K, Frystyk J, Lamberts SW & Janssen JA. Normal values of circulating insulin-like growth factor-I bioactivity in the healthy population: comparison with five widely used IGF-I immunoassays. *J Clin Endocrinol Metab* 2008 **93** 2539-2545.
10. Chen JW, Ledet T, Orskov H, Jessen N, Lund S, Whittaker J, De Meyts P, Larsen MB, Christiansen JS & Frystyk J. A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum. *Am J Physiol Endocrinol Metab* 2003 **284** E1149-1155.
11. Feng Y, Zhu Z, Xiao X, Choudhry V, Barrett JC & Dimitrov DS. Novel human monoclonal antibodies to insulin-like growth factor (IGF)-II that potently inhibit the IGF receptor type I signal transduction function. *Mol Cancer Ther* 2006 **5** 114-120.
12. Rasmussen R. Quantification on the LightCycler. In *Rapid Cycle real-time PCR, methods and application*, pp 21-34. Ed WC Meuer S, Nagakawara K. Heidelberg: Springer Press, 2001.
13. van Koetsveld PM, Vitale G, de Herder WW, Feelders RA, van der Wansem K, Waaijers M, van Eijck CH, Speel EJ, Croze E, van der Lely AJ, Lamberts SW & Hofland LJ. Potent inhibitory effects of type I interferons on human adrenocortical carcinoma cell growth. *J Clin Endocrinol Metab* 2006 **91** 4537-4543.
14. Vitale G, van Koetsveld PM, de Herder WW, van der Wansem K, Janssen JA, Colao A, Lombardi G, Lamberts SW & Hofland LJ. Effects of type I interferons on IGF-mediated autocrine/paracrine growth of human neuroendocrine tumor cells. *Am J Physiol Endocrinol Metab* 2009 **296** E559-566.
15. Schmittgen TD & Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008 **3** 1101-1108.
16. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001 **29** e45.
17. Weeke J & Orskov H. Ultrasensitive radioimmunoassay for direct determination of free triiodothyronine concentration in serum. *Scand J Clin Lab Invest* 1975 **35** 237-244.
18. Twigg SM & Baxter RC. Insulin-like growth factor (IGF)-binding protein 5 forms an alternative ternary complex with IGFs and the acid-labile subunit. *J Biol Chem* 1998 **273** 6074-6079.

19. Yalow RS & Berson SA. Immunoassay of endogenous plasma insulin in man. *J Clin Invest* 1960 **39** 1157-1175.
20. Froesch ER, Buergi H, Ramseier EB, Bally P & Labhart A. Antibody-Suppressible and Nonsuppressible Insulin-Like Activities in Human Serum and Their Physiologic Significance. An Insulin Assay with Adipose Tissue of Increased Precision and Specificity. *J Clin Invest* 1963 **42** 1816-1834.
21. Blumenthal S. The insulin immunoassay after 50 years: a reassessment. *Perspect Biol Med* 2009 **52** 343-354.
22. Frasca F, Pandini G, Scalia P, Sciacca L, Mineo R, Costantino A, Goldfine ID, Belfiore A & Vigneri R. Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. *Mol Cell Biol* 1999 **19** 3278-3288.
23. Morcavallo A, Gaspari M, Pandini G, Palummo A, Cuda G, Larsen MR, Vigneri R & Belfiore A. Research resource: New and diverse substrates for the insulin receptor isoform A revealed by quantitative proteomics after stimulation with IGF-II or insulin. *Mol Endocrinol* 2011 **25** 1456-1468.
24. LeRoith D, Adamo D, Werner H & Roberts Jr CT. Molecular and cellular biology of the insulin-like growth factors. In *Molecular Endocrinology, Basic Concepts and Clinical Correlations*, ch. 13, pp 181-183. Ed W DE, 1995.
25. Pandini G, Frasca F, Mineo R, Sciacca L, Vigneri R & Belfiore A. Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. *J Biol Chem* 2002 **277** 39684-39695.



Insulin Glargine is More Potent in Activating the Human IGF-I Receptor than Human Insulin and Insulin Detemir

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ABSTRACT

Aims: To investigate whether human insulin and insulin analogues differ in their ability to activate the human IGF-I receptor (IGF-IR), the human insulin receptor A (IR-A) and the human insulin receptor B (IR-B) in vitro.

Methods: Human insulin, short-acting insulin analogues (insulin aspart; insulin lispro) and long-acting insulin analogues (insulin glargine; insulin detemir) were compared by using kinase receptor activation (KIRA) bioassays specific for IGF-IR, IR-A or IR-B, respectively. These assays quantify ligand activity by measuring receptor auto-phosphorylation upon ligand binding. Human and insulin analogues were tested in a range from 0.1-100nM.

Results: Short-acting analogues: Overall, short-acting insulin analogues did not differ substantially from human insulin, nor from each other. Insulin lispro was slightly more potent than human insulin and insulin aspart in activating the IGF-IR, only reaching statistical significance at 100nM ($p<0.01$).

Long-acting analogues: At <10nM insulin glargine was as potent as human insulin in activating the IRs and IGF-IR. At 10-100nM insulin glargine was significantly more potent than human insulin in activating the IR-B ($p<0.05$) and IGF-IR ($p<0.001$). Insulin glargine was more potent than insulin detemir in activating all three receptors ($p<0.001$). Insulin detemir was less potent than human insulin in activating the IRs at 1-10nM ($p<0.01$) and IGF-IR at >1nM ($p<0.05$).

Conclusions: Insulin glargine was more potent in activating the IGF-IR than human insulin and insulin detemir. Since KIRA bioassays do not mimic the exact in vivo situation, further research is needed to find out whether our data have implications for clinical use of insulin glargine.

INTRODUCTION

The insulin receptor (IR) is a transmembrane receptor tyrosine kinase and exists in two isoforms: isoform A (IR-A) and isoform B (IR-B). The IR-A is expressed ubiquitously but is predominantly expressed in central nervous system, haematopoietic cells and in cancer tissues[1-4]. The IR-B is expressed predominantly in the liver, but is also substantially expressed in muscle and adipose tissue, the major target tissues for the metabolic effects of insulin[1-4]. Both IR isoforms show great homology with the IGF- I Receptor (IGF-IR)[4-6]. The IGF-IR is found in most tissues and brings about mitogenic, pro-invasive and anti-apoptotic effects[7].

Insulin can not only stimulate the IR-B, but also the IR-A and IGF-IR[4]. Subtle modifications of the insulin structure made to engineer insulin analogues, may affect receptor specificity signalling and thereby result in abnormal metabolic:mitogenic ratios, e.g. an increased activation of the IGF-IR may inhibit apoptosis and promote cancer by increasing cell proliferation[4]. Recently a possible relationship between insulin analogues, in particular insulin glargine, and risk of cancer was raised by epidemiological studies[8-10]. However, the results of these studies were found to be inconsistent[11-12]. Although, based on the current evidence, short-acting insulin analogues do not seem to bring additive risks in this respect, further evaluation of especially insulin glargine is required[12].

Kinase Receptor Activation (KIRA) bioassays make use of cell lines stably transfected with receptors and quantify ligand bioactivity by measuring ligand-induced receptor tyrosine kinase activation in terms of receptor-phosphorylation[13]. In our laboratory we have running KIRA bioassays specific for the human IGF-IR[14], human IR-A or human IR-B, respectively. This gives us the possibility to compare bioactivity of insulin analogues with respect to their ability to activate the IGF-IR, IR-A and IR-B.

In the present study we investigated whether (short- and long-acting) insulin analogues differ from human insulin in their bioactivity.

MATERIALS AND METHODS

Materials

The human embryonic kidney cell-line (HEK) Flip-in™-293 was obtained from Invitrogen life technologies (Breda, The Netherlands). Plasmids (pNTK-2) containing a cDNA insert of the human IR-B (pNTK2-IR-B) or IR-A (pNTK2-IR-A) were kindly provided by Axel Ullrich (Martinsried, Germany). The HEK IGF-IR cell-line was a kind gift from P. de Meyts (Gentofte, Denmark).

Dulbecco's Modified Eagles Medium (DMEM: gluc+, L-Glutamin +, Pyr+), Penicillin/Streptovidin, Hygromycine, Geneticin, Fugene® transfection reagens and FBS were

obtained from Invitrogen life technologies (Breda, The Netherlands). Human Serum Albumin (HSA), (Octalbine®) was obtained from Octopharma (Lachen, Switzerland). Culture plates (flat-bottomed 48 wells) were obtained from Corning Costar (Schiphol, The Netherlands). Microtiter 96-wells plates were purchased from Biozym (Landgraaf, The Netherlands).

Antibody coating buffer (ACB) contained 15 mM sodiumcarbonate and 35 mM sodium hydrogen carbonate (pH 9.6). Blocking solution contained 40 mM phosphate, 0.05% (wt/vol) NaN_3, 0.6% (wt/vol) NaCl, 0.2% (wt/vol) Titriplex V (EDTA), and 1% (wt/vol) HSA (pH 8.0). Krebs Ringer bicarbonate (KRB) buffer was adjusted to pH 7.4 by CO_2 and supplemented with 0.1% (wt/vol) HSA. Lysis buffer contained 50mM HEPES, 137mM NaCl, 10mM NaP_2O_7, 10mM NaF, 0.1mM $MgCl_2.6H_20$, 0.1mM $CaCl_2.2H_20$, 1% NP-40, 10% (vol/vol) Glycerol (pH 7.4) (before use, 1 tablet of EDTA free protease inhibitor and 0.5ml 200mM sodium orthovanadate $14H_20$ was added to 50mL of lysis buffer).

MAI1 and MAD1, monoclonal antibodies directed against the extracellular domain of the human IR and human IGF-IR respectively, were obtained from Novozymes-Gropep (Adelaide, Australia). Europium-labelled PY20 (Eu-PY20); a monoclonal anti- phosphotyrosine antibody was obtained from Perkin Elmer- life sciences (Groningen, The Netherlands). DELFIA assay reagents (assay buffer, wash buffer and enhancement solution) and a time-resolved fluorometer (Victor^2 multilabel counter) were also purchased from Perkin Elmer- life sciences.

Human insulin (Actrapid®), insulin aspart (Novorapid®) and insulin detemir (Levemir®) were obtained from Novo Nordisk (Bagsvaerd, Denmark). Insulin lispro (Humalog®) was obtained from Eli Lilly (Houten, The Netherlands). Insulin glargine (Lantus ®) was obtained from Sanofi- aventis (Frankfurt am Main, Germany). Human recombinant IGF-I was obtained from Invitrogen (Breda, The Netherlands).

Methods

Insulin analogues were compared to human insulin by using in-house KIRA bioassays specific for IGF-IR, IR-A or the IR-B. The IGF-IR KIRA assays has been previously described[15] and the IR-A and IR-B specific bioassays have recently been developed based on this same principle (article in preparation). All three assays use human embryonic renal cells stably transfected with either cDNA of the human IR-A or human IR-B gene (HEK IR-A or IR-B) or with cDNA of the human IGF-IR gene (HEK IGF-IR).

After 48 hours of culture, HEK IR-A and HEK IR-B cells were stimulated for 10 minutes at 37°C with an equimolar dose titration ranging from 0.1-100nM of insulin analogues or human insulin, respectively. HEK IGF-IR cells were stimulated for 15 minutes at 37°C with an equimolar dose titration ranging from 0.1-100nM of insulin analogues, human insulin or human recombinant IGF-I.

After stimulation cells were lysed. Crude lysates were transferred to a sandwich assay. Capture antibodies MAI1 and MAD1 were used in a concentration of 2.5µg/mL and 5.0µg/mL respectively. Eu-PY20 was used as tracer antibody in a concentration of 1.25µg/mL. Contents were read in a time-resolved fluorometer. Assays were performed in 48 well plates.

A control dose-titration-curve of human insulin or human recombinant IGF-I was implemented on all plates to ensure assay performance. The maximal inter-assay CVs for the IGF-IR, IR-A and IR-B KIRA were 11.2%, 14.7% and 11.3% respectively. The maximal intra-assay CVs were 7.0%, 9.7% and 11.1% respectively**.**

Statistical analysis

For bioactivity measurements means ± SD and signal-to-noise ratios are presented. Noise was defined as signal after stimulation with vehicle and so signal-to-noise ratios were calculated by using the formula: absolute counts after stimulation with a ligand, divided by absolute vehicle counts. Statistical analysis was performed using repeated measures Two Way ANOVA followed by Bonferroni post-tests. Data were analysed using GraphPad software (Prism 5, London, United Kingdom). A p-value of <0.05 was considered statistically significant.

RESULTS

IR-B activation

I. Comparing short-acting insulin analogues vs. human insulin

Overall there were no great differences between short-acting insulin analogues and human in IR-B activation. Only very small differences were observed at concentrations of 10nM and higher, where insulin aspart was slightly more potent in IR-B activation that human insulin (Fig. 1A).

II. Comparing long-acting insulin analogues vs. human insulin

Insulin glargine was as least as potent as human insulin. At 10-100nM insulin glargine was slightly more potent than human insulin in activating IR-B ($p<0.05$; Fig. 1B). Insulin detemir was significantly less potent than human insulin at 1-10nM ($p<0.001$; Fig. 1B). Insulin glargine was significantly more potent than insulin detemir in activating IR-B at concentrations >0.1nM ($p<0.001$; Fig. 1B).

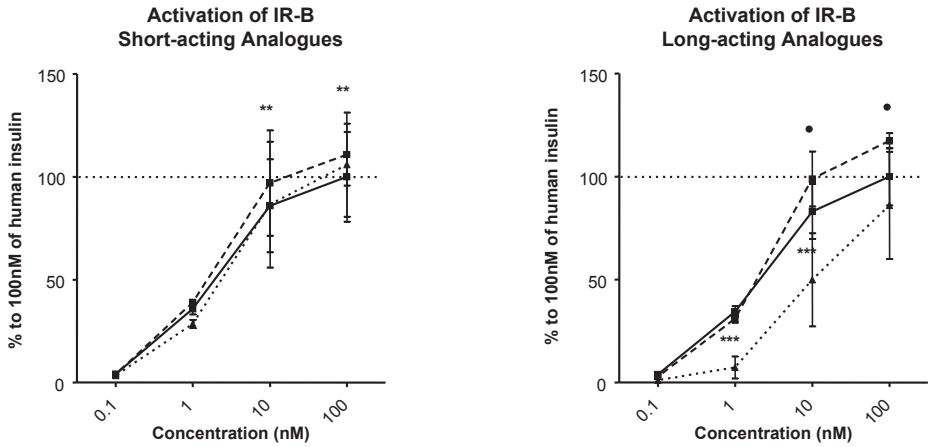

Figure 1.
Activation of insulin receptor isoform B (IR-B). **Figure 1A**: Comparing human insulin (black solid line) and short-acting insulin analogues insulin aspart (dashed line with squares) and insulin lispro (dashed line with triangles). **Figure 1B**: Comparing human insulin (black solid line) and long-acting insulin analogues insulin glargine (dashed line with squares) and insulin detemir (dashed line with triangles). Dose-response profiles ranged from 0.1-100nM. Stimulation with 100nM of human insulin was arbitrarily set at 100% (thin horizontal dashed line). Each point represents the mean value ± SD of four independent experiments. •• $p<0.001$ insulin aspart vs. human insulin • $p<0.05$ insulin glargine vs. human insulin *** $p<0.001$ insulin detemir vs. human insulin

IR-A activation

I. Comparing short-acting insulin analogues vs. human insulin

At all concentrations tested insulin aspart, insulin lispro and human insulin did not differ in their ability to activate the IR-A (Fig. 2A).

II. Comparing long-acting insulin analogues vs. human insulin

Insulin glargine was as potent as human insulin in activating IR-A (Fig. 2B). Insulin detemir was significantly less potent than human insulin at 1-10nM ($p<0.01$) (Fig. 2B). At >0.1nM, insulin glargine generated significant higher responses than insulin detemir ($p<0.05$; Fig. 2B).

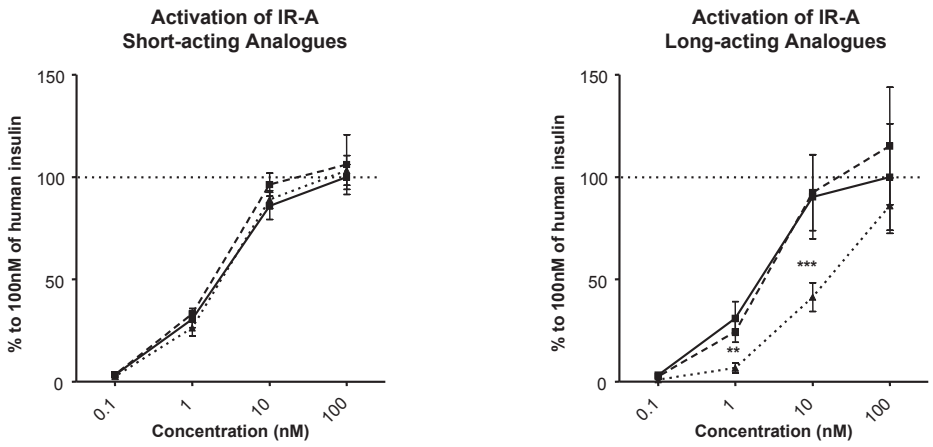

Figure 2.

Activation of insulin receptor isoform A (IR-A). **Figure 2A**: Comparing human insulin (black solid line) and short-acting insulin analogues insulin aspart (dashed line with squares) and insulin lispro (dashed line with triangles). **Figure 2B**: Comparing human insulin (black solid line) and long-acting insulin analogues insulin glargine (dashed line with squares) and insulin detemir (dashed line with triangles). Dose-response profiles ranged from 0.1-100nM. Stimulation with 100nM of human insulin was arbitrarily set at 100% (thin horizontal dashed line). Each point represents the mean value ± SD of four independent experiments.

** p<0.01 *** p<0.001 insulin detemir vs. human insulin

IGF-IR activation

I. Comparing short-acting insulin analogues vs. human insulin

Overall there was no difference in potencies between the short-acting insulin analogues and human insulin in activating the IGF-IR. Only at 100nM insulin lispro was slightly more potent than human insulin and insulin aspart (Fig. 3A).

Human recombinant IGF-I generated >2 fold higher responses than human insulin, insulin aspart and insulin lispro at 1-100nM (p<0.001) (Fig. 3A).

II. Comparing long-acting insulin analogues vs. human insulin

At 0.1-1nM, insulin glargine was as potent as human insulin. At 10-100nM, insulin glargine was more potent than human insulin (p<0.001), whereas insulin detemir generated significantly lower dose-respons effects than human insulin (p<0.05; Fig. 3B). Insulin glargine was significantly more potent than insulin detemir at concentrations >1nM (Fig. 3B).

Human recombinant IGF-I generated >2 fold higher responses than human insulin and insulin detemir at 1-100nM (p<0.001) (Fig. 3B). Only insulin glargine, at 100nM, was equally potent to IGF-I (Fig. 3B).

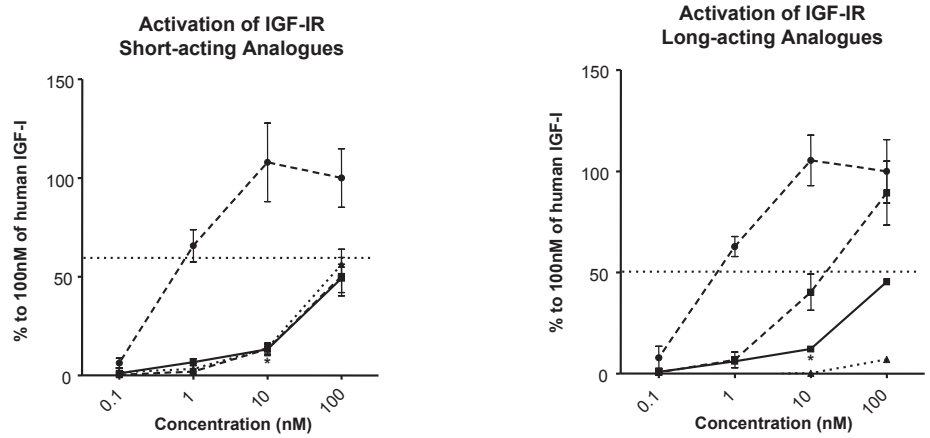

Figure 3.

Activation of IGF-I receptor (IGF-IR): **Figure 3A**: Comparing human insulin (black solid line) and short-acting insulin analogues insulin aspart (dashed line with squares) and insulin lispro (dashed line with triangles). **Figure 3B**: Comparing human insulin (black solid line) and long-acting insulin analogues insulin glargine (dashed line with squares) and insulin detemir (dashed line with triangles). Dose-response profiles ranged from 0.1-100nM. Stimulation with 100nM of human IGF-I was arbitrarily set at 100% (thin horizontal dashed line). Each point represents the mean value ± SD of three independent experiments. ** p< 0.01 insulin lispro vs. human insulin ••• <0.001 insulin glargine vs. human insulin • p<0.05, *** p<0.001 insulin detemir vs. human insulin.

EC50 values

EC50 values of human insulin and insulin analogues for all three receptors are presented in Table 1. The EC50 value of human insulin was similar for both IR isoforms and was substantially lower for the IGF-IR. Both short-acting insulin analogues had EC50 values similar to human insulin for all three receptors. The EC50 values of insulin glargine for the IR isoforms were equal to human insulin, however, the EC50 value for the IGF-IR was 41% of human insulin. The EC50 values of insulin detemir for both IR isoforms and for the

Table 1 EC50 values of human insulin, human IGF-I and insulin analogues

Receptor types	EC50 (nmol/L)					
	Human Insulin	Human IGF-I	Insulin aspart	Insulin lispro	Insulin detemir	Insulin glargine
IR-B	2.0 ± 1.5	N.D.	1.8 ± 1.5	2.7 ± 1.8	8.8 ± 1.7	2.5 ± 1.2
IR-A	2.0 ± 1.4	N.D.	1.9 ± 1.3	2.5 ± 1.2	13.8 ± 1.3	3.2 ± 1.6
IGF-IR	33.0 ± 1.5	0.45 ± 1.6	29.2 ± 1.6	33.8 ± 1.5	55.3 ± 1.9	13.9 ± 1.4

EC50 of human insulin, human IGF-I and insulin analogues for the human IR-B, human IR-A and the human IGF-IR. Data represent the mean ± SE of minimally three independent experiments.
N.D. = not determined.

IGF-IR were higher relative to human insulin (440% for IR-A and 690% for IR-B and 170% for the IGF-IR, respectively).

DISCUSSION

Our main finding was that insulin glargine was significantly more potent than human insulin in activating the IGF-IR. This result is in agreement with a study of Kurtzhals et al., where a 3-8 fold increased mitogenicity of insulin glargine compared to human insulin was found[16]. They used downstream events, such as glucose uptake (of mouse adipocytes) and proliferation (of human osteosarcoma cells), to compare metabolic and mitogenic potencies of insulin analogues with human insulin and correlated these effects to IGF-IR and IR-A affinity. However, there is a considerable crosstalk between IRA, IR-B and IGF-IR-mediated functions at receptor- and at post-receptor level and the final effects are due to a combination of IGF-IR- and IRs-mediated processes. Recently it was even suggested that the IR and IGF-IR act as identical portals to the regulation of gene expression, with differences between insulin and IGF-I effects due to a modulation of the signal created by the specific ligand-receptor interactions[17]. As a consequence it is almost impossible in most in vitro cell lines to disentangle the individual contribution of each type receptor to the final downstream event.

Recently, Sciacca et al. found an increased mitogenic potency of insulin glargine and insulin detemir compared to human insulin[18]. However, in contrast to our findings and previous reports[16, 19], they found that insulin detemir induced significantly more IGF-IR phosphorylation than human insulin, while IGF-IR phosphorylation by insulin glargine was similar to human insulin. Sciacca et al. suggested that different findings on the effects of insulin detemir in vitro may be attributed to different albumin concentrations used in different studies.

In our study we used three KIRA assays; one developed for the human IGF-IR, one for the human IR-A and one for the human IR-B. These bioassays are very specific in that only the initial activation step of a particular receptor (i.e. tyrosine-phosphorylation) after stimulation with an insulin analogue is used to quantify receptor-mediated signalling. By using these three KIRA bioassays, we were thus able to assess the specific activation of each type of receptor after stimulation with an insulin analogue.

As for all in vitro systems, KIRA bioassays do not mimic the exact in vivo situation. KIRA bioassays only provide a crude, albeit convenient, measure of kinase activation. Although the endpoint signal is readily direct and specific, the IR and IGF-IR have up to 6 key tyrosine residues of which of some the role in vivo is not yet fully clear. In addition, the antibody used to capture the tyrosine residues may well not recognize all residues equally because of dependence of affinity on flanking sequence and proximity of other

sites. Furthermore, IR and IGF-IR expression may vary in a tissue-specific manner and inter-individual differences in the levels of proteins of the IGF-IR system may function as a critical determinant of the mitogenic potency of insulin analogues[20]. Moreover, IR/IGF-IR hybrids (Hybrid –Rs) are formed in many tissues and have been shown to be the most represented subtype[21]. However, although the precise biological role of these Hybrid –Rs is still unclear, functional studies have demonstrated that Hybrid –Rs behave more like IGF-IRs than IRs[21]. So, in this context the effects we found on IGF-IR phosphorylation could be important in vivo.

Increased IGF-IR activation by insulin glargine may result in an abnormal metabolic:mitogenic ratio in vivo. However, differences in IGF-IR activation between insulin glargine and human insulin only reached statistical significance when stimulating cells with relatively high concentrations. On the other hand, in contrast to in vitro conditions, cells in vivo are continuously exposed to insulin glargine 24 hours a day during many years. In vivo, insulin glargine is partially degraded into two bioactive products (M1 and M2, respectively) after subcutaneous injection[22]. It has been found that one of these degradation products (M1) is less mitogenic than insulin glargine[16], which could implicate that insulin glargine is less mitogenic in vivo than it is in vitro. To make relationships even more complex, markedly increased circulating IGF-I levels have been reported during treatment with insulin glargine[23-25].

Comparing human insulin and long-acting insulin analogues in their ability to activate the IR-B, we found that insulin glargine was at least as potent as human insulin. Insulin detemir was less potent than human insulin in IR-B activation; only at 100nM insulin detemir induced similar IR-B activation levels compared to human insulin. This could be attributed to lower receptor affinity and to binding of insulin detemir to HSA[16] although in our experiments the HSA concentration was only 0.1%. In vivo, a reduced potency to activate IR-B will result in decreased glucose lowering effects. Indeed, insulin detemir has a 4-fold concentrated formulation in order to match biological potency of human insulin[26]. Therefore, administration of insulin detemir has been shown to result in higher plasma insulin levels than human insulin and insulin glargine[27-28]. Sciacca et al. tested insulin at bioequivalent (i.e. glucose lowering) concentrations[18]. They found that insulin glargine, insulin detemir and human insulin had a similar effect on IR-A and IR-B phosphorylation but that insulin detemir was more potent than human insulin in activating the IGF-IR. In accordance with these latter results we found that insulin detemir at highest concentrations (100nM) was as potent as human insulin in phosphorylating both IR isoforms. However, we found that, even at 100nM, insulin detemir was not able to activate the IGF-IR to any extent.

Belfiore et al. have shown that equivalent IR-A activation can elicit different downstream signalling pathways and biological effects depending on the ligand activating the receptor[29]. So, even at equivalent IR-A activation, human insulin, insulin detemir and

insulin glargine may result in different downstream signalling effects, being either more metabolic or more mitogenic.

Overall, short-acting insulin analogues did not differ substantially from human insulin, nor from each other. In agreement with previous studies[16], insulin lispro was slightly more potent than human insulin and insulin aspart in activating the IGF-IR, only reaching statistical significance at 100nM.

In conclusion, insulin glargine was more potent than human insulin especially in IGF-IR activation, whereas insulin detemir did not activate the IGF-IR to any extent. This result clearly underlines the importance of further research to elucidate whether increased IGF-IR activation by insulin glargine in vitro translates into clinical effects on cancer risk.

ACKNOWLEDGEMENTS

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DISCLOSURES

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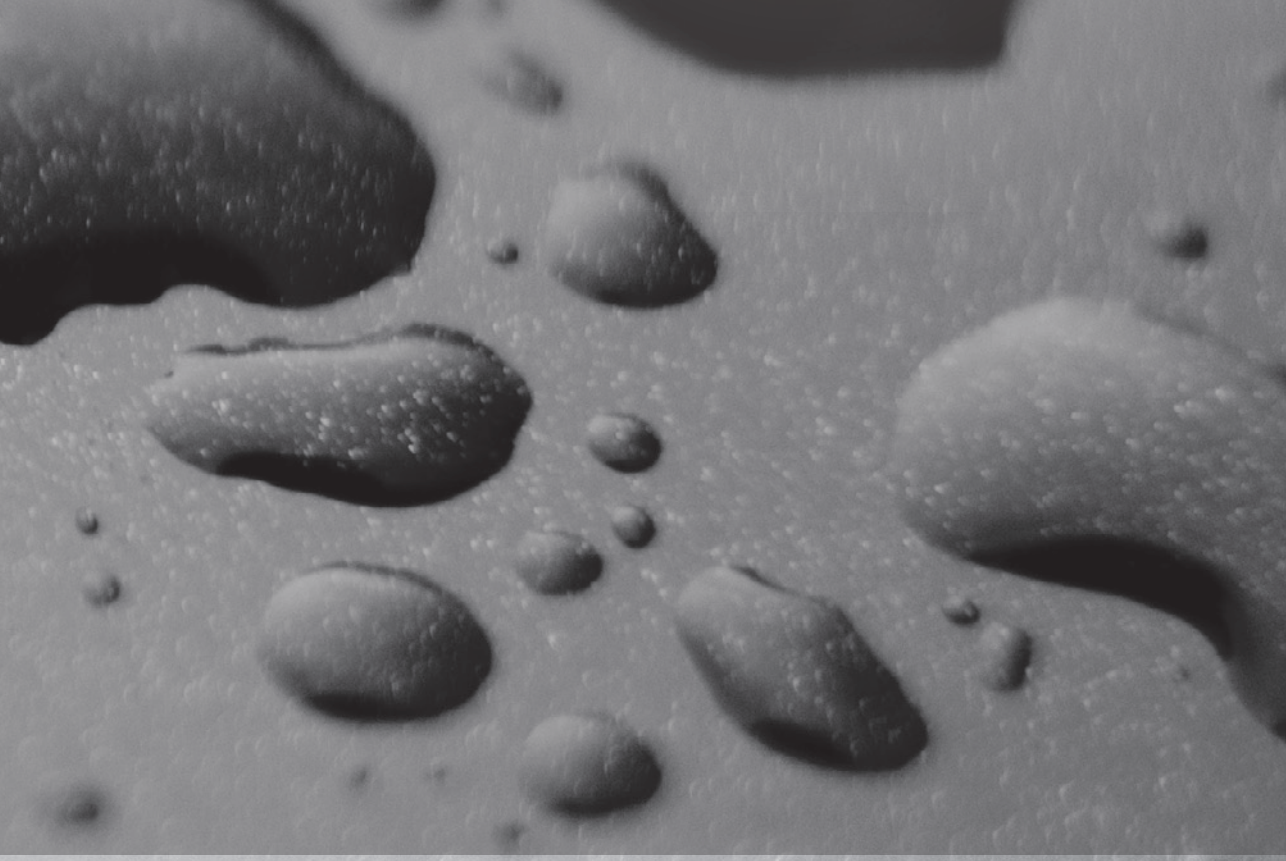
REFERENCES

1. Seino S, Seino M, Nishi S & Bell GI. Structure of the human insulin receptor gene and characterization of its promoter. *Proc Natl Acad Sci U S A* 1989 **86** 114-118.
2. Mosthaf L, Grako K, Dull TJ, Coussens L, Ullrich A & McClain DA. Functionally distinct insulin receptors generated by tissue-specific alternative splicing. *Embo J* 1990 **9** 2409-2413.
3. Benecke H, Flier JS & Moller DE. Alternatively spliced variants of the insulin receptor protein. Expression in normal and diabetic human tissues. *J Clin Invest* 1992 **89** 2066-2070.
4. Belfiore A, Frasca F, Pandini G, Sciacca L & Vigneri R. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. *Endocr Rev* 2009 **30** 586-623.
5. LeRoith D, Werner H, Beitner-Johnson D & Roberts CT, Jr. Molecular and cellular aspects of the insulin-like growth factor I receptor. *Endocr Rev* 1995 **16** 143-163.
6. Yarden Y & Ullrich A. Growth factor receptor tyrosine kinases. *Annu Rev Biochem* 1988 **57** 443-478.
7. De Meyts P & Whittaker J. Structural biology of insulin and IGF1 receptors: implications for drug design. *Nat Rev Drug Discov* 2002 **1** 769-783.
8. Hemkens LG, Grouven U, Bender R, Gunster C, Gutschmidt S, Selke GW & Sawicki PT. Risk of malignancies in patients with diabetes treated with human insulin or insulin analogues: a cohort study. *Diabetologia* 2009 **52** 1732-1744.
9. Jonasson JM, Ljung R, Talback M, Haglund B, Gudbjornsdottir S & Steineck G. Insulin glargine use and short-term incidence of malignancies-a population-based follow-up study in Sweden. *Diabetologia* 2009 **52** 1745-1754.
10. Colhoun HM. Use of insulin glargine and cancer incidence in Scotland: a study from the Scottish Diabetes Research Network Epidemiology Group. *Diabetologia* 2009 **52** 1755-1765.
11. Pocock SJ & Smeeth L. Insulin glargine and malignancy: an unwarranted alarm. *Lancet* 2009 **374** 511-513.
12. Smith U & Gale EA. Does diabetes therapy influence the risk of cancer? *Diabetologia* 2009 **52** 1699-1708.
13. Sadick MD, Intintoli A, Quarmby V, McCoy A, Canova-Davis E & Ling V. Kinase receptor activation (KIRA): a rapid and accurate alternative to end-point bioassays. *J Pharm Biomed Anal* 1999 **19** 883-891.
14. Brugts MP, van den Beld AW, Hofland LJ, van der Wansem K, van Koetsveld PM, Frystyk J, Lamberts SW & Janssen JA. Low circulating insulin-like growth factor I bioactivity in elderly men is associated with increased mortality. *J Clin Endocrinol Metab* 2008 **93** 2515-2522.
15. Chen JW, Ledet T, Orskov H, Jessen N, Lund S, Whittaker J, De Meyts P, Larsen MB, Christiansen JS & Frystyk J. A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum. *Am J Physiol Endocrinol Metab* 2003 **284** E1149-1155.
16. Kurtzhals P, Schaffer L, Sorensen A, Kristensen C, Jonassen I, Schmid C & Trub T. Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. *Diabetes* 2000 **49** 999-1005.
17. Boucher J, Tseng YH & Kahn CR. Insulin and insulin-like growth factor-1 receptors act as ligand-specific amplitude modulators of a common pathway regulating gene transcription. *J Biol Chem* 2010 **285** 17235-17245.
18. Sciacca L, Cassarino MF, Genua M, Pandini G, Le Moli R, Squatrito S & Vigneri R. Insulin analogues differently activate insulin receptor isoforms and post-receptor signalling. *Diabetologia* 2010 **53** 1743-1753.

19. Shukla A, Grisouard J, Ehemann V, Hermani A, Enzmann H & Mayer D. Analysis of signaling pathways related to cell proliferation stimulated by insulin analogs in human mammary epithelial cell lines. *Endocr Relat Cancer* 2009 **16** 429-441.
20. Eckardt K, May C, Koenen M & Eckel J. IGF-1 receptor signalling determines the mitogenic potency of insulin analogues in human smooth muscle cells and fibroblasts. *Diabetologia* 2007 **50** 2534-2543.
21. Pandini G, Frasca F, Mineo R, Sciacca L, Vigneri R & Belfiore A. Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. *J Biol Chem* 2002 **277** 39684-39695.
22. Kuerzel GU, Shukla U, Scholtz HE, Pretorius SG, Wessels DH, Venter C, Potgieter MA, Lang AM, Koose T & Bernhardt E. Biotransformation of insulin glargine after subcutaneous injection in healthy subjects. *Curr Med Res Opin* 2003 **19** 34-40.
23. Ekstrom K, Salemyr J, Zachrisson I, Carlsson-Skwirut C, Ortqvist E & Bang P. Normalization of the IGF-IGFBP axis by sustained nightly insulinization in type 1 diabetes. *Diabetes Care* 2007 **30** 1357-1363.
24. Murthy SN, Sukhanov S, McGee J, Greco JA, Chandra S, Delafontaine P, Kadowitz PJ, McNamara DB & Fonseca VA. Insulin glargine reduces carotid intimal hyperplasia after balloon catheter injury in Zucker fatty rats possibly by reduction in oxidative stress. *Mol Cell Biochem* 2009 **330** 1-8.
25. Slawik M, Schories M, Busse Grawitz A, Reincke M & Petersen KG. Treatment with insulin glargine does not suppress serum IGF-1. *Diabet Med* 2006 **23** 814-817.
26. Owens DR & Bolli GB. Beyond the era of NPH insulin--long-acting insulin analogs: chemistry, comparative pharmacology, and clinical application. *Diabetes Technol Ther* 2008 **10** 333-349.
27. Porcellati F, Rossetti P, Busciantella NR, Marzotti S, Lucidi P, Luzio S, Owens DR, Bolli GB & Fanelli CG. Comparison of pharmacokinetics and dynamics of the long-acting insulin analogs glargine and detemir at steady state in type 1 diabetes: a double-blind, randomized, crossover study. *Diabetes Care* 2007 **30** 2447-2452.
28. Porcellati F, Rossetti P, Candeloro P, Lucidi P, Cioli P, Andreoli AM, Ghigo E, Bolli GB & Fanelli CG. Short-term effects of the long-acting insulin analog detemir and human insulin on plasma levels of insulin-like growth factor-I and its binding proteins in humans. *J Clin Endocrinol Metab* 2009 **94** 3017-3024.
29. Sacco A, Morcavallo A, Pandini G, Vigneri R & Belfiore A. Differential signaling activation by insulin and insulin-like growth factors I and II upon binding to insulin receptor isoform A. *Endocrinology* 2009 **150** 3594-3602.



Addition of insulin glargine or NPH insulin to metformin monotherapy in poorly controlled type 2 diabetic patients decreases IGF-IR bioactivity similarly

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ABSTRACT

Aim: To compare IGF-IR bioactivity 36 weeks after addition of insulin glargine or NPH insulin to metformin therapy in type 2 diabetic patients who were poorly controlled under metformin monotherapy.

Methods: In the LANMET Study, 110 poorly controlled insulin-naive type 2 diabetic patients were randomized to receive metformin with either insulin glargine (G+MET) or NPH insulin (NPH+MET). In the present study IGF-IR bioactivity was measured, retrospectively, in 104 out of the 110 initially included LANMET participants before and after 36 weeks of insulin therapy. IGF-IR bioactivity was measured by an IGF-I kinase receptor activation assay.

Results: After 36 weeks of insulin therapy, insulin doses were comparable between the G+MET (68±5.7 U/day) and NPH+MET (71±6.2 U/day) groups (p=0.68). Before insulin therapy, circulating IGF-IR bioactivity was similar between the G+MET (134±9 pmol/l) and NPH+MET (135±10 pmol/l) groups (p=0.83). After 36 weeks, IGF-IR bioactivity had decreased significantly (p=0.001) and did not differ between the G+MET (116±9 pmol/l) and NPH+MET (117±10 pmol/l) groups (p=0.91). At baseline and after insulin therapy, total IGF-I concentrations were comparable in both groups (baseline; G+MET 13.3±1.0 vs. NPH+MET 13.3±1.0 nmol/l, p=0.97 and 36 weeks; 13.4±1.0 vs. 13.1±0.9 nmol/l, p=0.71). Total IGF-I concentrations did not change during insulin therapy (13.3±0.7 vs. 13.3±0.7 nmol/l, baseline vs. 36 weeks, p=0.86).

Conclusion: Addition of insulin glargine or NPH insulin to metformin monotherapy in poorly controlled type 2 diabetic patients decreases serum IGF-IR bioactivity similarly.

INTRODUCTION

In 2009 four observational studies were published of which three suggested that use of insulin glargine was associated with an increased risk of cancer[1-4]. The FDA reviewed these four studies and concluded that *'the evidence presented was inconclusive, due to limitations in how the studies were designed, carried out and in the data available for analysis'*[5].

Nevertheless these findings have raised concerns as to whether or not insulin glargine promotes cell proliferation and growth of neoplasms. *In vitro*, at high concentrations, insulin glargine has been found to be more potent than human insulin in stimulating the insulin-like growth factor-I (IGF-I) receptor (IGF-IR)[6-10]. By using an in-house Kinase Receptor Activation (KIRA) assay specific for the IGF-IR, we recently confirmed that insulin glargine, at high concentrations, is more potent than either human insulin or insulin detemir in activating the human IGF-IR *in vitro*[7]. This assay uses human embryonic kidney cells overexpressing the human IGF-IR to quantify IGF-IR phosphorylation in response to stimulation by specific ligands or human serum[11-12]. The dose-dependent effect of insulin glargine supports the finding of a potential link between higher doses of insulin glargine and incidence of cancer reported in a case control study by Mannucci et al.[13].

Previously, Mayer et al. have published a pilot study in which serum of type 1 diabetic patients treated with insulin glargine had a greater proliferative effect on MCF-7 breast cancer cells than serum containing human insulin[14]. There are however, as to yet, no studies comparing IGF-IR bioactivity in type 2 diabetic patients during treatment with insulin glargine or NPH insulin. However, such studies would help to clarify whether insulin-mediated differences in IGF-IR activation *in vitro* translate into differences in IGF-IR bioactivity *in vivo*.

In the LANMET Study, the efficacy and safety of bedtime insulin glargine was compared to NPH insulin in poorly controlled insulin-naive type 2 diabetic patients treated with metformin[15].

In the present study, we examined whether addition of insulin glargine or NPH insulin to metformin monotherapy in poorly controlled type 2 diabetic patients induced changes in circulating IGF-IR bioactivity as measured by the IGF-IR KIRA assay and whether this response differed between the two insulins.

STUDY DESIGN AND STUDY POPULATION

The LANMET Study was a multicentre, open, randomized, parallel-group study[15]. Briefly, in the LANMET Study the efficacy and safety of bedtime insulin glargine and metformin (G+MET) was compared to NPH insulin and metformin (NPH+MET) treatment in insulin-naive poorly controlled type 2 diabetic patients (HbA_{1c} 8.0% (64 mmol/mol) or higher). The study consisted of a 4-week run-in phase and a 36–week treatment phase. It was performed at six sites in Finland and one in the United Kingdom. The study was performed in accordance with the Declaration of Helsinki and good clinical practice as described by Note for Guidance CPMP/ICH/135/95. Approval by institutional ethics committees was obtained for each participating site. All patients provided written informed consent before entry into the study.

In the present study IGF-IR bioactivity was measured, retrospectively, in participants of the LANMET study. All LANMET participants were included from whom extra serum samples had been collected as part of the original protocol (104 out of 110 LANMET participants (95% of the total number)). In addition, 41 serum samples from non-diabetic subjects (mostly spouses) were collected at the time of the baseline visit. The latter samples were analyzed to compare IGF-IR bioactivity and total IGF-I between type 2 diabetic patients and non-diabetic subjects.

Since, previously, a dose-dependent increase in cancer risk was observed for treatment with insulin glargine compared to human insulin[1, 13] and differences between insulin analogues and human insulin in IGF-IR activation *in vitro* have only been reported at relatively high concentrations[6-10], we also performed a sub-analysis in 40 patients, who were treated with a mean daily insulin dose above 70 U/day (20 patients in each treatment group).

MATERIALS AND METHODS

Serum IGF-IR bioactivity and total serum IGF-I were measured in fasting blood samples before and after 36 weeks of insulin treatment from type 2 diabetic patients and from non-diabetic subjects (mostly spouses).

IGF-IR KIRA bioassay

The IGF-IR KIRA assay has been previously described[11-12]. Briefly, autophosphorylation of tyrosine residues located within the intracellular kinase domain of the IGF-IR is the first step in the intracellular signalling cascade. The IGF-IR KIRA assay uses a human embryonic kidney (HEK) cell line that is stably transfected with the human IGF-IR gene

(HEK IGF-IR) and quantifies phosphorylation of tyrosine residues of the transfected IGF-IR to assess IGF-IR bioactivity. After 48 h of culture, HEK IGF-IR cells were stimulated for 15 min at 37°C with recombinant IGF-I standards (Austral Biologicals, San Ramon, CA), insulin glargine (Lantus®, Sanofi-aventis, Frankfurt am Main, Germany) and NPH insulin (Insulatard®, Novo Nordisk, Bagsvaerd, Denmark). Equimolar concentrations of IGF-I, insulin glargine and NPH insulin were tested in a range of 100-100 000 pmol/l. Standards were diluted in Krebs-Ringer bicarbonate buffer adjusted to pH 7.4 by CO_2 and supplemented with 0.1% human serum albumin (Octalbine) (Octopharma, Lachen, Switzerland). After stimulation, cells were lysed. Crude lysates were transferred to a sandwich assay. Wells were coated with a monoclonal antibody (MAD-1) directed against the IGF-IR (Novozymes-Gropep, Adelaide, Australia) that was used as capture antibody in a concentration of 5.0 µg/ml. A biotinylated anti-phosphotyrosine monoclonal antibody (BAM 1676) (R&D Systems Europe Ltd (Abingdon, UK)) was used in a concentration of 0.2 µg/ml together with streptavidin labelled europium (DELFIA Eu-N1) (PerkinElmer Life Sciences, Groningen, The Netherlands) in a concentration of 50 pmol/l as detection antibody. Contents were read in a time-resolved fluorometer (Victor2 multilabel counter; PerkinElmer Life Sciences). Assays were performed in 48-well plates (Corning, Corning, NY).

For measurements of serum IGF-IR bioactivity, an IGF-I standard, two internal control samples and two study control samples were included on each culture plate. Serum samples were diluted 1:10. All measurements were done in duplicate. The intra and interassay CVs were below 15%.

Statistical analysis

Baseline characteristics are shown as means (or geometric means) ± SE. The Kolmogorov-Smirnov-test was used to test normality of variables (data were considered to be normally distributed when $p>0.05$). For data that did not meet the criteria for normality, logarithmic transformations were applied. Pearson's correlation coefficients were calculated to assess the associations between variables. Pearson's correlation coefficients for IGF-I were calculated after adjustment for age. A univariate general linear model was used to test differences in continuous variables between the insulin treatment groups and non-diabetic subjects; differences for total IGF-I and IGF-IR bioactivity were calculated after adjustment for age and sex. Differences in categorical variables were tested by using the Chi-Squared test. A paired t-test was used to test differences before and after starting insulin treatment. A P-value of 0.05 or less was considered statistically significant. Data were analysed using SPSS 17 for Windows (SPSS Inc., Chicago, IL).

RESULTS

A. *In vitro measurements*

Effects of insulin glargine and NPH insulin on IGF-IR activation

Figure 1 shows that insulin glargine and NPH insulin were equally effective in activating the IGF-IR at 1 00 and 1 000 pmol/l (p= 0.26 and p= 0.34, respectively). At 10 000 and 100 000 pmol/l, insulin glargine was more potent than NPH insulin (p= 0.02 and p= 0.04, respectively) (Figure 1). Human recombinant IGF-I was more potent than human insulin and insulin glargine over the whole range tested (Figure 1).

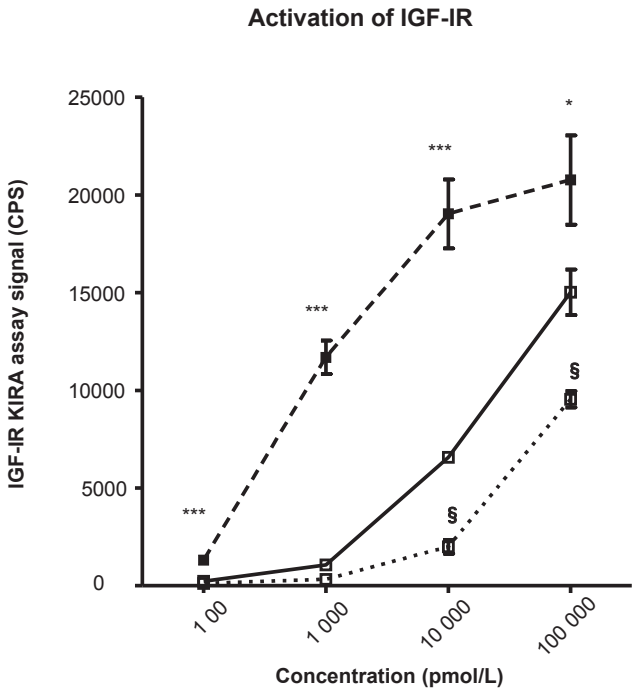

Figure 1.

Activation of IGF-I receptor (IGF-IR): Comparing human IGF-I (dashed line, solid squares), insulin glargine (solid line, open squares) and NPH insulin (dashed line, open quares). Dose-response profiles ranged from 100-100 000 pmol/l. Points represent the mean value (+ SEM) of three independent experiments.
* P<0.05, for human IGF-I vs. insulin glargine and NPH insulin. *** P<0.001, for human IGF-I vs. insulin glargine and NPH insulin. **§** P<0.05, for insulin glargine vs. NPH insulin.

B. *In vivo measurements*

Baseline characteristics

Baseline characteristics of the two patient groups did not significantly differ from each other (Table 1). The non-diabetic subjects (matched with respect to age and sex) were leaner and metabolically healthier compared to the type 2 diabetic subjects (Table 1).

Glycaemic control and insulin doses

Mean HbA_{1c} concentrations were comparable in the G+MET and NPH+MET groups before insulin therapy (9.0±0.1% (75±1.1 mmol/mol) vs. 9.2±0.2% (77±2.2mmol/mol), p=0.33) and decreased similarly in both groups after insulin therapy (7.0±0.1% (53±1.1mmol/mol) vs. 7.1±0.1%, (54±1.1mmol/mol) G+MET vs. NPH+MET, p=0.93).

In the G+MET group the mean insulin dose was 68±5.7 U/day compared to 71±6.2 U/day in the NPH+MET group (p=0.68) and the insulin dose per kilogram was 0.69±0.05 U/kg in the G+MET group compared to 0.69±0.05 U/ kg in the NPH+MET group (p= 0.98).

Circulating IGF-IR bioactivity and total IGF-I at baseline

Mean serum IGF-IR bioactivity and total IGF-I did not differ between the two treatment groups at baseline (IGF-IR bioactivity: 134±9 vs. 135±10 pmol/l, G+MET vs. NPH+MET, p=0.83 and total IGF-I: 13.3±1.0 vs. 13.3±1.0 nmol/l, G+MET vs. NPH+MET, p=0.97).

There were also no differences in IGF-IR bioactivity and total IGF-I between the two treatment groups when comparing individuals using >70 U/day (G+MET: 118±16 pmol/l vs. NPH+MET: 129±14 pmol/l, p=0.67; and G+MET: 10.1±1.0 nmol/l vs. NPH+MET: 10.4±1.0 nmol/l, p=0.77).

IGF-IR bioactivity was borderline significantly lower in patients with type 2 diabetes than in non-diabetic controls (135±7 vs. 161±11 pmol/l, p=0.09) (Figure 2A). Total IGF-I concentrations were significantly lower in patients with type 2 diabetes than in non–diabetic subjects (13.3±0.7 vs. 16.3±1.0 nmol/l, p=0.03) (Figure 2B).

Changes in circulating IGF-IR bioactivity and total IGF-I in response to insulin therapy

After 36 weeks of insulin treatment there were no differences in mean IGF-IR bioactivity between the G+MET and the NPH+MET groups (116±9 vs. 117±10 pmol/l, p=0.91) (Figure 2A). Also mean total IGF-I concentrations were not different between the treatment groups (13.4±1.0 vs. 13.1±0.9 nmol/l, G+MET vs. NPH+MET, p=0.71) (Figure 2B).

In addition, mean IGF-IR bioactivity and total IGF-I did not differ between the G+MET and the NPH+MET groups when comparing individuals using > 70 U insulin/day (IGF-IR bioactivity: 102±15 pmol/l vs. 114±8 pmol/l, p=0.63; total IGF-I: 10.8±1.1 nmol/l vs. 10.5±0.9 nmol/l, p= 0.23).

Table 1. Baseline characteristics of diabetic patients randomized to the G+MET group or NPH+MET group within the original LANMET Study and of diabetic patients (in the G+MET or NPH group) and non-diabetic subjects analyzed in the present study

Characteristic	LANMET Study G+MET[b]	LANMET Study NPH+MET[b]	G+MET group[c]	NPH+MET group[c]	Non-diabetic subjects[c]	P-value[d]	P-value[e]
Patients (n)	61	49	57	47	41	-	-
Sex (m/f)	38/23	32/17	35/22	31/16	19/22	0.63	0.06
Age (yrs)[a]	56±1	57±1	56.0±1.2	56.7±1.2	56.6±1.3	0.71	0.88
Metformin dose (g/day)	2.28±0.06	2.19±0.05	2.16±0.05	2.23±0.06	-	0.38	-
Previous sulfonylurea (%)	79	86	81	89	-	0.28	
Previous ACE inhibitor (%)	57	55	53	49	-	0.84	
Previous beta-blocker or Thiazide(%)	71	63	67	59	-	0.54	
Weight (kg)	92.0±2.4	94.4±1.2	92.3±2.4	95.5±2.6	82.1±3.1	0.36	0.001
BMI (kg/m^2)[a]	31.3±0.7	32.0±0.8	31.0±0.7	31.9±0.8	27.8±0.7	0.35	<0.001
HbA_{1C} (%)[a] HbA_{1C} [a] (mmol/mol)	9.5±0.1 80±1.1	9.6±0.1 81±1.1	9.0±0.1 75±1.1	9.2±0.2 77±2.2	5.4±0.1 36±1.1	0.33	<0.001
FPG (mmol/l)[a]	13.0±0.3	12.9±0.3	12.3±0.3	11.9±0.4	5.5±0.1	0.43	<0.001
C-peptide (nmol/l)	0.98±0.04	1.00±0.07	1.0±0.04	1.0±0.07	0.7±0.06	0.86	<0.001
Serum TG (mmol/l)[a]	2.3±0.2	2.5±0.2	2.0±0.2	2.2±0.2	1.2±0.1	0.17	<0.001
HDL- cholesterol (mmol/l)[a]	1.18±0.04	1.18±0.04	1.2±0.04	1.1±0.03	1.5±0.07	0.25	<0.001
LDL- cholesterol (mmol/l)[a]	2.8±0.1	2.9±0.1	2.7±0.1	2.8±0.1	3.4±0.1	0.40	<0.001

Data are presented as mean ± SE.[a.] geometric mean.[b.] Values from the original LANMET Study.[c.] Values from the present study.
[d.] P-values are shown for differences between the G+MET group vs. NPH+MET group in the present study.[e.] P-values are shown for differences between the diabetic subjects vs. non-diabetic subjects in the present study.

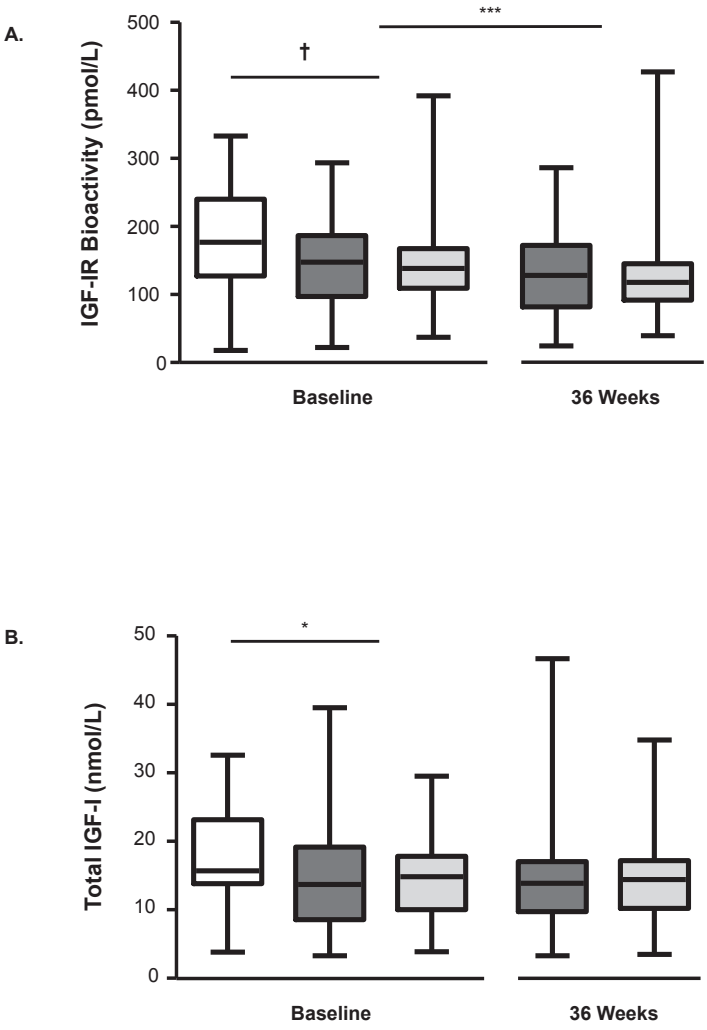

Figure 2.

A. IGF-IR bioactivity (pmol/l) in non-diabetic subjects (white box) at baseline and in diabetic subjects in the G+MET group (dark grey boxes) and in the NPH+MET group (light grey boxes) at baseline and after 36 weeks of insulin treatment. The line in the boxes represents the mean of the data. The boxes extend from the 25th percentile to the 75th percentile values, the whiskers show the minimum and maximum values of each group.

† P= 0.09, for IGF-IR bioactivity in non-diabetic subjects at baseline vs. IGF-IR bioactivity in patients at baseline. *** P<0.001, for IGF-IR bioactivity at baseline vs. IGF-IR bioactivity at 36 weeks in all patients.

B. Total IGF-I (nmol/l) in non-diabetic subjects in non-diabetic subjects (white box) at baseline and in diabetic subjects in the G+MET group (dark grey boxes) and in the NPH+MET group (light grey boxes) at baseline and after 36 weeks of insulin treatment. The line in the boxes represents the mean of the data. The boxes extend from the 25th percentile to the 75th percentile values, the whiskers show the minimum and maximum values of each group. * P= 0.03, for total IGF-I in non-diabetic subjects at baseline vs. total IGF-I in patients at baseline.

Mean serum IGF-IR bioactivity decreased significantly from 135±7 pmol/l at baseline to 117±6 pmol/l (p=0.001) at 36 weeks in all patients (Figure 2A). A significant decline in IGF-IR bioactivity was also observed in individuals using >70 U insulin/day: 123±11 pmol/l at baseline vs. 108±9 pmol/l after 36 weeks of insulin therapy (p=0.02).

Serum total IGF-I concentrations remained unchanged during insulin therapy (baseline: 13.3±0.7 vs. 13.3±0.7 nmol/l after 36 weeks of insulin therapy; p=0.86) (Figure 2B). Also in patients using >70 U insulin/day total IGF-I did not change during treatment (10.3±0.7 nmol/l at baseline vs. 10.6±0.7 nmol/l at 36 weeks, p=0.24).

Interrelationships between insulin dose at 36 weeks, IGF-IR bioactivity and total IGF-I

At 36 weeks, there was a highly significant inverse relationship between insulin dose and serum total IGF-I in both groups (total insulin dose: G+MET group r = -0.36, p=0.007; NPH+MET group r= -0.41, p= 0.005, insulin dose/kg: G+MET group r= -0.33, p= 0.01; NPH+MET group r= -0.36, p= 0.02), (Figure 3). There was no significant correlation between insulin dose and IGF-IR bioactivity (total insulin dose: G+MET group r = -0.23, p=0.10; NPH+MET group r= -0.08, p= 0.58, insulin dose/kg: G+MET group r= -0.17, p= 0.22; NPH+MET group r= -0.08, p= 0.58), at 36 weeks.

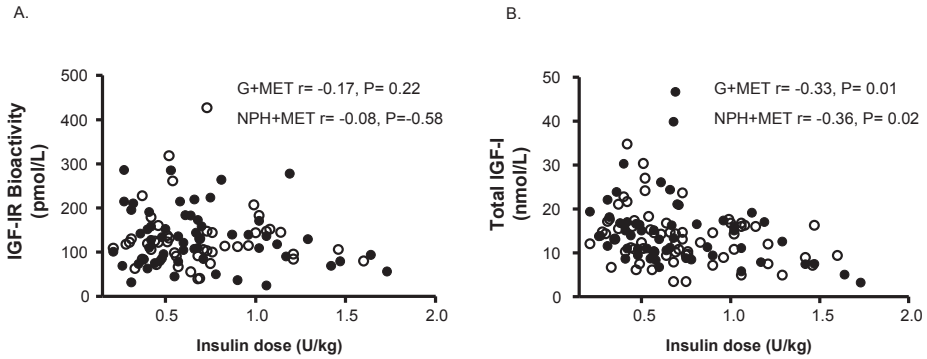

Figure 3.
Correlations between daily insulin dose and IGF-IR bioactivity (A) and total IGF-I (B) in the G+MET (black dots) and NPH+MET (open dots) group.

DISCUSSION

Insulin glargine has previously been found to be more potent than human insulin in activating the IGF-IR *in vitro*. However, this effect has only been observed at high (supra-physiological) insulin concentrations[6-10]. Indeed, in the present study, by using our in house IGF-IR KIRA assay, we confirmed that at high concentrations insulin glargine was more potent than NPH insulin in activating the IGF-IR *in vitro*. However, in contrast to

these *in vitro* findings, serum from type 2 diabetic patients who were treated for 36 weeks with metformin and insulin glargine induced similar IGF-IR activation compared to that of patients treated with metformin and NPH insulin for 36 weeks. Moreover, after 36 weeks of insulin treatment, IGF-IR bioactivity decreased significantly in both groups. Total IGF-I concentrations were similar in both groups at baseline and remained unchanged during insulin therapy.

A sub-analysis was performed in patients who had used high mean daily insulin doses (above 70 U/day) in order to address a previous observation of a dose-dependent increase in cancer risk for insulin glargine treatment. However, also in this sub-group, IGF-IR bioactivity was similar in both groups after 36 weeks of therapy and had also decreased significantly, whereas total IGF-I remained unchanged. In addition, we found a significant inverse relationship between insulin dose and total IGF-I, but not for insulin dose and IGF-IR bioactivity. So, although we did not study effects of insulin therapy on cancer incidence, our findings do not support the idea that insulin therapy raises cancer risk through an increase in IGF-IR bioactivity.

In the present study insulin concentrations were not measured due to the inability of commercially available serum insulin assays to accurately measure insulin glargine. However, in a previous study, in which type 2 diabetic patients were treated with insulin glargine, a mean dose of 0.43 U/kg/day resulted in mean free plasma insulin concentrations of 126 pmol/l[16]. In another study, the maximal concentration of serum insulin at an insulin glargine dose of 1.4 U/kg/day was around 700 pmol/l[17]. Extrapolating these results to the maximal insulin doses that were used in our study (1.7 U/kg/day), it seems unlikely that free plasma insulin concentrations exceeded 500-850 pmol/l during treatment with insulin glargine. Moreover, it has been reported that insulin glargine concentrations measured by regular insulin immunoassays are usually overestimated by ~30%[18]. Taken together this suggests that *in vivo* concentrations of insulin glargine do not likely reach concentrations at which we and others have observed differences in IGF-IR activation *in vitro*. The latter could be an explanation why we did not observe differences in IGF-IR bioactivity between the two treatment arms. In addition, after subcutaneous injection, insulin glargine is partially degraded into two bioactive products (M1 and M2)[19]. The M1 degradation product has been shown to have less mitogenic potency than insulin glargine itself and even less potency than human insulin[6]. These findings raise the possibility that insulin glargine would be less mitogenic *in vivo* than it is *in vitro* and could be another explanation for the absence of differences in IGF-IR bioactivity between the two treatment arms. Direct measurement of circulating concentrations of insulin glargine and its metabolites during insulin therapy would be helpful in clarifying this latter possibility[20].

The observed decline in IGF-IR bioactivity during insulin therapy in both treatment arms is in line with a recent study, which showed that acute hyperinsulinemia suppressed IGF-IR bioactivity, whereas total serum IGF-I did not change[21]. The decrease in circulating IGF-IR bioactivity in the latter study was attributed to insulin-mediated effects on circulating IGFBPs: insulin suppressed IGFBP-4 and IGFBP-1 and increased IGFBP-2 concentrations[21]. In addition, it has been reported that IGFBP-3 protease activity is increased in untreated diabetic patients and decreases after several days of insulin treatment[22-23]. The latter effect may also have attributed to the observed decrease in IGF-IR bioactivity during insulin treatment in our study.

At baseline, IGF-IR bioactivity was slightly lower in the type 2 diabetic patients than in the leaner non-diabetic subjects. Although this comparison was not the primary aim of our study, this finding is in line with previous data comparing patients with type 2 diabetes to those with impaired or normal glucose tolerance[24]. One mechanism responsible for lower IGF-IR bioactivity in the diabetic patients could be chronic hyperinsulinemia (C-peptide levels were 50% higher in the type 2 diabetic patients than in non-diabetic subjects), since it has been suggested that prolonged insulin exposure inhibits GH-induced signalling at both receptor and postreceptor level in the liver[25].

Total IGF-I concentrations were significantly lower in the diabetic patients than in the non-diabetic subjects. In previous studies total IGF-I concentrations in type 2 diabetic patients have been reported to be lower, similar or higher than in non-diabetic control subjects[26]. Different results in these studies could be due to variability in insulin concentrations because of different treatment regimens and/or variation in insulin sensitivity in type 2 diabetes[26]. Another factor may be that in many previous studies immunoassays have been used that may have not accurately measured IGF-I levels due to problems with assay standardization and/or with assay methodology[27].

It has been suggested that the IGF-IR KIRA assay is more sensitive than the common total IGF-I immunoassays to detect differences in clinical state[28]. Moreover, the IGF-IR KIRA assay seems to be superior to common IGF-I immunoassays to monitor therapeutic interventions[28]. Thus the measurement of IGF-IR bioactivity could be an important tool to clarify controversies that exist about the precise role of the IGF-I system in diabetes.

Our data do not support the idea that use of insulin glargine in type 2 diabetes leads to higher circulating IGF-IR bioactivity *in vivo* than NPH insulin. Our findings are in line with a recently published paper in which, in an animal model of type 2 diabetes, no differences were demonstrated in the degree of colonic epithelial proliferation between animals treated with insulin glargine or NPH insulin[29]. Nevertheless, in that study insulin treatment did result in a higher degree of colonic epithelial proliferation, thereby point-

ing towards the potential mitogenic properties of all insulins, irrespective of the type of insulin[29].

It is important to emphasize that with the IGF-IR KIRA phosphorylation of the tyrosine residues within the beta-subunits of the IGF-IR is quantified but it cannot be assessed whether activation of the IGF-IR by an insulin analogue results in a normal (i.e. balanced metabolic and mitogenic) activity at cellular level in vivo. The IGF-IR may differentially elicit biological effects and intracellular signalling upon binding of different insulin analogues. In this respect it has been suggested that intracellular signalling induced by insulin analogues differs from human insulin: insulin analogues preferentially activate the ERK pathway rather than the AKT pathway[30]. Thus there is still a chance that (subtle) differences in the molecular structure of insulin analogues may affect in vivo signalling at the postreceptor level and thereby induce an abnormal metabolic:mitogenic ratio (e.g. an increased activation of the IGF-IR may inhibit apoptosis and promote cancer by increasing cell proliferation[20]).

It should be stressed that in this study the decline in serum IGF-IR bioactivity during insulin treatment was found under co-medication with relatively high doses of metformin. Metformin is a widely prescribed anti-diabetic drug which is recommended as the initial pharmacological therapy together with lifestyle interventions for type 2 diabetes[31]. All patients in our study were already treated with metformin for at least three months before being randomly assigned to either insulin glargine or NPH insulin therapy. Several studies have shown that metformin treatment is associated with a lower cancer risk[4, 32-33]. Therefore, concomitant metformin use has been suggested to be a potential confounder when it comes to estimating the risks of insulin therapy for cancer[34]. However, in our study we merely addressed IGF-IR bioactivity and not cancer incidence. The potential mechanisms of a potential anticancer effect of metformin seem to be very complex[35]. Metformin reverses endogenous hyperinsulinemia through its effects on glucose homeostasis and therefore may directly have antiproliferative effects[36]. Indirectly, a reduction of endogenous insulin levels may also lower IGF-IR bioactivity by improving insulin sensitivity[37]. Most importantly, metformin activates the AMP-activated protein kinase (AMPK) signalling pathway[35]. One of the major growth regulatory pathways controlled by AMPK is the mammalian target of rapamycin (mTOR) pathway and its downstream substrates[38-39]. In addition, in NIH-3T3 cells, stimulation of AMPK inhibits the ability of IGF-I to activate ras and its downstream targets but phosphorylation of the activated IGF-IR appeared to be unaffected by this increase in AMPK activity[40]. So, metformin seems to interfere with IGF-I signalling at the postreceptor level and not at the receptor level. Nevertheless, we cannot exclude that concomitant metformin therapy has influenced our findings.

Finally, a few limitations of our study need to be addressed. First of all the LANMET study was not primarily designed and performed to study effects of insulin therapy on IGF-IR bioactivity and/or cancer incidence. We could only study IGF-IR bioactivity during a relatively short follow-up period and in subjects who were already treated with metformin. Therefore the post-hoc analysis in our study was inevitable. Nevertheless, again, we did not study cancer incidence. In addition, we would like to underline that, after 36 weeks of insulin treatment, there was no difference in IGF-IR bioactivity between subjects treated with insulin glargine or NPH insulin.

Secondly, as for all in vitro systems, the IGF-IR KIRA bioassay does not mimic the exact in vivo conditions. Responses to insulin and insulin analogues in vivo are far more complex and are mediated by insulin receptors next to IGF-IRs and possibly also by hybrid receptors, that are widely expressed on normal tissues and often aberrantly expressed in cancer cells[41]. However, although the precise biological role of these hybrids is still unclear, functional studies have demonstrated that hybrid receptors behave more like IGF-IRs than IRs[41]. So, in this context the effects we found on IGF-IR phosphorylation could be important in vivo.

In conclusion, circulating IGF-IR bioactivity was similar in poorly controlled type 2 diabetic patients who had been treated for 36 weeks with either insulin glargine or NPH insulin combined with metformin. Moreover, insulin treatment decreased IGF-IR bioactivity. In addition, there was a significant inverse relationship between insulin dose and total IGF-I. So, our data do not indicate increased IGF-IR signalling in type 2 diabetic patients treated with insulin glargine.

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DUALITY OF INTEREST STATEMENT

The authors declare that there is no duality of interest associated with this manuscript.

DISCLOSURES

The LANMET study was sponsored by sanofi-aventis but the present study was investigator-initiated and not supported by grants from the industry. Sanofi-aventis had no involvement in the study design nor in the collection, analysis, and interpretation of data. J.AM.J.L. Janssen has received an unrestricted grant from Novo Nordisk A/S (Alphen aan de Rijn, the Netherlands) and sanofi-aventis (Frankfurt am Main, Germany). All other authors have nothing to disclose.

AUTHOR CONTRIBUTION

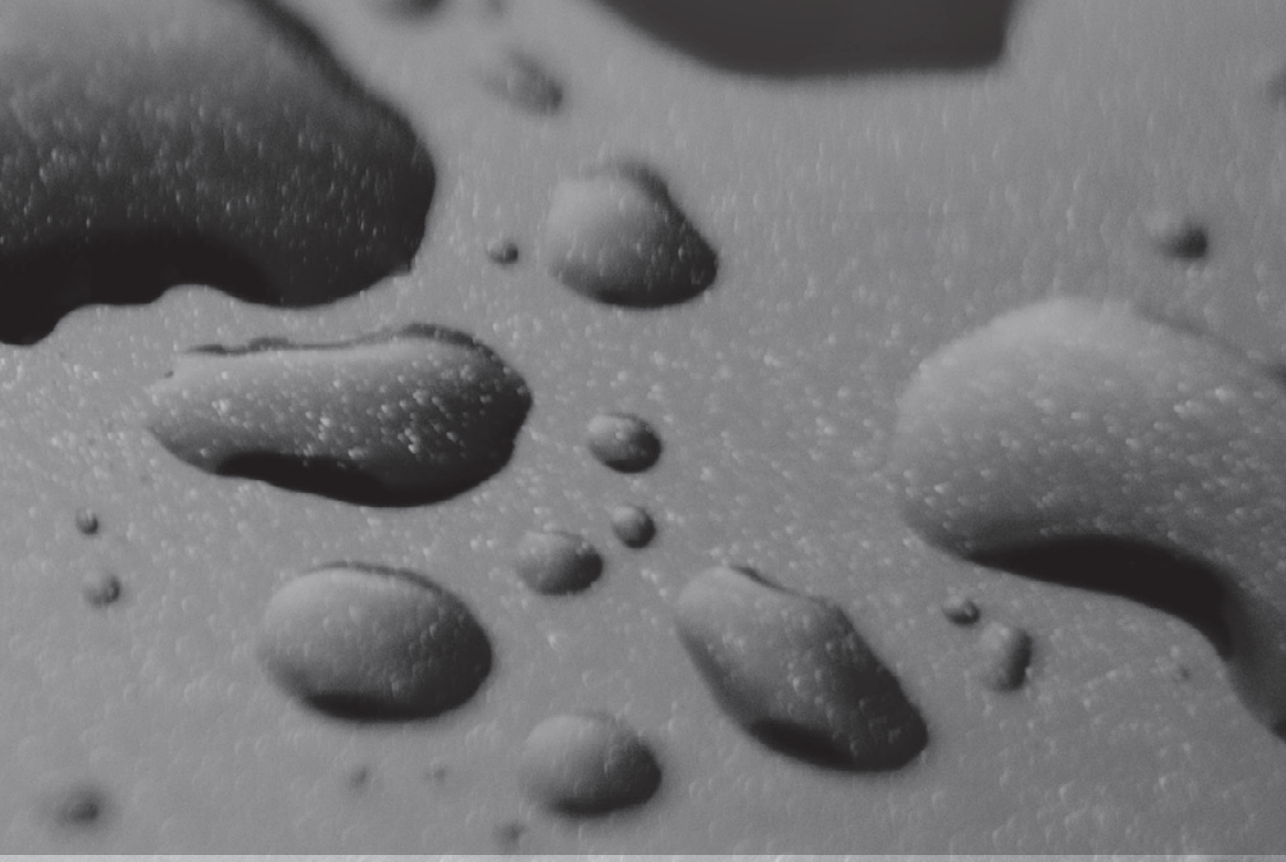
A.J. wrote the manuscript and researched data and edited the manuscript, J.A.M.J.L. contributed to conception and design, reviewed/edited the manuscript, M. contributed to conception and design and contributed to the discussion, L.J. contributed to conception and design and reviewed/edited the manuscript, S.W.J. contributed to conception and design and contributed to the discussion, H. contributed to conception and design researched data and reviewed/edited the manuscript.

REFERENCES

1. Hemkens LG, Grouven U, Bender R, Gunster C, Gutschmidt S, Selke GW & Sawicki PT. Risk of malignancies in patients with diabetes treated with human insulin or insulin analogues: a cohort study. *Diabetologia* 2009 **52** 1732-1744.
2. Jonasson JM, Ljung R, Talback M, Haglund B, Gudbjornsdottir S & Steineck G. Insulin glargine use and short-term incidence of malignancies-a population-based follow-up study in Sweden. *Diabetologia* 2009 **52** 1745-1754.
3. Colhoun HM. Use of insulin glargine and cancer incidence in Scotland: a study from the Scottish Diabetes Research Network Epidemiology Group. *Diabetologia* 2009 **52** 1755-1765.
4. Currie CJ, Poole CD & Gale EA. The influence of glucose-lowering therapies on cancer risk in type 2 diabetes. *Diabetologia* 2009 **52** 1766-1777.
5. FDA. FDA Drug Safety Communication: Update to ongoing safety review of Lantus (insulin glargine) and possible risk of cancer. http://www.fda.gov/Drugs/DrugSafety/ucm239376.htm, 2011.
6. Kurtzhals P, Schaffer L, Sorensen A, Kristensen C, Jonassen I, Schmid C & Trub T. Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. *Diabetes* 2000 **49** 999-1005.
7. Varewijck AJ, Goudzwaard JA, Brugts MP, Lamberts SW, Hofland LJ & Janssen JA. Insulin glargine is more potent in activating the human IGF-I receptor than human insulin and insulin detemir. *Growth Horm IGF Res* **20** 427-431.
8. Sciacca L, Cassarino MF, Genua M, Pandini G, Le Moli R, Squatrito S & Vigneri R. Insulin analogues differently activate insulin receptor isoforms and post-receptor signalling. *Diabetologia* 2010 **53** 1743-1753.
9. Eckardt K, May C, Koenen M & Eckel J. IGF-1 receptor signalling determines the mitogenic potency of insulin analogues in human smooth muscle cells and fibroblasts. *Diabetologia* 2007 **50** 2534-2543.
10. Weinstein D, Simon M, Yehezkel E, Laron Z & Werner H. Insulin analogues display IGF-I-like mitogenic and anti-apoptotic activities in cultured cancer cells. *Diabetes Metab Res Rev* 2009 **25** 41-49.
11. Chen JW, Ledet T, Orskov H, Jessen N, Lund S, Whittaker J, De Meyts P, Larsen MB, Christiansen JS & Frystyk J. A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum. *Am J Physiol Endocrinol Metab* 2003 **284** E1149-1155.
12. Brugts MP, Ranke MB, Hofland LJ, van der Wansem K, Weber K, Frystyk J, Lamberts SW & Janssen JA. Normal values of circulating insulin-like growth factor-I bioactivity in the healthy population: comparison with five widely used IGF-I immunoassays. *J Clin Endocrinol Metab* 2008 **93** 2539-2545.
13. Mannucci E, Monami M, Balzi D, Cresci B, Pala L, Melani C, Lamanna C, Bracali I, Bigiarini M, Barchielli A, Marchionni N & Rotella CM. Doses of insulin and its analogues and cancer occurrence in insulin-treated type 2 diabetic patients. *Diabetes Care* **33** 1997-2003.
14. Mayer D & Chantelau E. Treatment with insulin glargine (Lantus) increases the proliferative potency of the serum of patients with type-1 diabetes: a pilot study on MCF-7 breast cancer cells. *Arch Physiol Biochem* **116** 73-78.
15. Yki-Jarvinen H, Kauppinen-Makelin R, Tiikkainen M, Vahatalo M, Virtamo H, Nikkila K, Tulokas T, Hulme S, Hardy K, McNulty S, Hanninen J, Levanen H, Lahdenpera S, Lehtonen R & Ryysy L. Insulin glargine or NPH combined with metformin in type 2 diabetes: the LANMET study. *Diabetologia* 2006 **49** 442-451.
16. Vehkavaara S & Yki-Jarvinen H. 3.5 years of insulin therapy with insulin glargine improves in vivo endothelial function in type 2 diabetes. *Arterioscler Thromb Vasc Biol* 2004 **24** 325-330.

17. Owens DR & Bolli GB. Beyond the era of NPH insulin--long-acting insulin analogs: chemistry, comparative pharmacology, and clinical application. *Diabetes Technol Ther* 2008 **10** 333-349.
18. Westerbacka J, Bergholm R, Tiikkainen M & Yki-Jarvinen H. Glargine and regular human insulin similarly acutely enhance endothelium-dependent vasodilatation in normal subjects. *Arterioscler Thromb Vasc Biol* 2004 **24** 320-324.
19. Kuerzel GU, Shukla U, Scholtz HE, Pretorius SG, Wessels DH, Venter C, Potgieter MA, Lang AM, Koose T & Bernhardt E. Biotransformation of insulin glargine after subcutaneous injection in healthy subjects. *Curr Med Res Opin* 2003 **19** 34-40.
20. Belfiore A, Frasca F, Pandini G, Sciacca L & Vigneri R. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. *Endocr Rev* 2009 **30** 586-623.
21. Arafat AM, Weickert MO, Frystyk J, Spranger J, Schofl C, Mohlig M & Pfeiffer AF. The role of insulin-like growth factor (IGF) binding protein-2 in the insulin-mediated decrease in IGF-I bioactivity. *J Clin Endocrinol Metab* 2009 **94** 5093-5101.
22. Bereket A, Lang CH, Blethen SL, Fan J, Frost RA & Wilson TA. Insulin-like growth factor binding protein-3 proteolysis in children with insulin-dependent diabetes mellitus: a possible role for insulin in the regulation of IGFBP-3 protease activity. *J Clin Endocrinol Metab* 1995 **80** 2282-2288.
23. Bang P, Brismar K & Rosenfeld RG. Increased proteolysis of insulin-like growth factor-binding protein-3 (IGFBP-3) in noninsulin-dependent diabetes mellitus serum, with elevation of a 29-kilodalton (kDa) glycosylated IGFBP-3 fragment contained in the approximately 130- to 150-kDa ternary complex. *J Clin Endocrinol Metab* 1994 **78** 1119-1127.
24. Brugts MP, van Duijn CM, Hofland LJ, Witteman JC, Lamberts SW & Janssen JA. Igf-I bioactivity in an elderly population: relation to insulin sensitivity, insulin levels, and the metabolic syndrome. *Diabetes* 2010 **59** 505-508.
25. Ji S, Guan R, Frank SJ & Messina JL. Insulin inhibits growth hormone signaling via the growth hormone receptor/JAK2/STAT5B pathway. *J Biol Chem* 1999 **274** 13434-13442.
26. Clauson PG, Brismar K, Hall K, Linnarsson R & Grill V. Insulin-like growth factor-I and insulin-like growth factor binding protein-1 in a representative population of type 2 diabetic patients in Sweden. *Scand J Clin Lab Invest* 1998 **58** 353-360.
27. Chestnut RE & Quarmby V. Evaluation of total IGF-I assay methods using samples from Type I and Type II diabetic patients. *J Immunol Methods* 2002 **259** 11-24.
28. Janssen JAMJL. IGF-I: Pro 's and Con's of a Bioassay. *Horm Res Paediatr* 2011 **76(suppl 1)** 106-110.
29. Nagel JM, Staffa J, Renner-Müller I, Horst D, Vogeser M, Langkamp M, Hoeflich A, Göke B, Kolligs FT & Mantzoros CS. Insulin Glargine and NPH Insulin Increase to a Similar Degree Epithelial Cell Proliferation and Aberrant Crypt Foci Formation in Colons of Diabetic Mice *Hormones and Cancer* 2010 **1** 320-330.
30. Vigneri R, Squatrito S & Sciacca L. Insulin and its analogs: actions via insulin and IGF receptors. *Acta Diabetol* 2010 **47** 271-278.
31. Nathan DM, Buse JB, Davidson MB, Ferrannini E, Holman RR, Sherwin R & Zinman B. Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes Care* 2009 **32** 193-203.
32. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR & Morris AD. Metformin and reduced risk of cancer in diabetic patients. *Bmj* 2005 **330** 1304-1305.
33. Bowker SL, Majumdar SR, Veugelers P & Johnson JA. Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. *Diabetes Care* 2006 **29** 254-258.

34. Smith U & Gale EA. Does diabetes therapy influence the risk of cancer? *Diabetologia* 2009 **52** 1699-1708.
35. Jalving M, Gietema JA, Lefrandt JD, de Jong S, Reyners AK, Gans RO & de Vries EG. Metformin: taking away the candy for cancer? *Eur J Cancer* 2010 **46** 2369-2380.
36. Goodwin PJ, Pritchard KI, Ennis M, Clemons M, Graham M & Fantus IG. Insulin-lowering effects of metformin in women with early breast cancer. *Clin Breast Cancer* 2008 **8** 501-505.
37. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer* 2008 **8** 915-928.
38. Ning J & Clemmons DR. AMP-activated protein kinase inhibits IGF-I signaling and protein synthesis in vascular smooth muscle cells via stimulation of insulin receptor substrate 1 S794 and tuberous sclerosis 2 S1345 phosphorylation. *Mol Endocrinol* 2010 **24** 1218-1229.
39. Shaw RJ, Bardeesy N, Manning BD, Lopez L, Kosmatka M, DePinho RA & Cantley LC. The LKB1 tumor suppressor negatively regulates mTOR signaling. *Cancer Cell* 2004 **6** 91-99.
40. Kim J, Yoon MY, Choi SL, Kang I, Kim SS, Kim YS, Choi YK & Ha J. Effects of stimulation of AMP-activated protein kinase on insulin-like growth factor 1- and epidermal growth factor-dependent extracellular signal-regulated kinase pathway. *J Biol Chem* 2001 **276** 19102-19110.
41. Pandini G, Frasca F, Mineo R, Sciacca L, Vigneri R, Belfiore A . Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. *J Biol Chem* 2002 **277** 39684-39695



Concentrations of insulin glargine and its metabolites during long-term insulin therapy in type 2 diabetic patients and comparison of effects of insulin glargine, its metabolites, IGF-I and human insulin on insulin- and IGF-I receptor signalling

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ABSTRACT

Aims: We investigated 1) the ability of purified insulin glargine, M1, M2, IGF-I and NPH insulin to activate the IR-A and IR-B and IGF-IR *in vitro*, 2) plasma concentrations of insulin glargine and M1 and M2 during long-term insulin therapy in type 2 diabetic patients and 3) IR-A and IR-B activation *in vitro* induced by serum from patients treated with insulin glargine or NPH insulin.

Methods: 104 patients (age 56.3±0.8 yrs, BMI 31.4±0.5 kg/m^2, A1c 9.1±0.1% (mean±se)) were randomized to insulin glargine or NPH insulin therapy for 36 weeks. Plasma concentrations of insulin glargine, M1 and M2 were determined by LCMS. IR-A, IR-B and IGF-IR autophosphorylation induced by purified hormones or serum by kinase receptor activation assays.

Results: In vitro, M1 induced comparable IR-A, IR-B and IGF-IR autophosphorylation (activation) as NPH insulin. After 36 weeks, M1 increased from undetectable (<0.2 ng/mL) to 1.5 ng/mL [0.9-2.1] while insulin glargine and M2 remained undetectable. Insulin glargine dose correlated with M1 ($r=0.84$, $p<0.001$). Serum from patients treated with insulin glargine or NPH insulin induced similar IR-A and IR-B activation.

Conclusions: These data suggest that M1 rather than insulin glargine mediates insulin glargine effects and that compared to NPH insulin insulin glargine does not increase IGF-IR signalling during long term insulin therapy in type 2 diabetes.

INTRODUCTION

Besides its typical metabolic effects, insulin also has growth effects. In 2009 four observational studies were published of which three suggested that use of insulin glargine was associated with an increased risk of cancer[1-4]. Since it was shown that insulin glargine had an increased affinity for the IGF-I receptor (IGF-IR)[5-6], mitogenic effects of insulin glargine were believed to occur through increased stimulation of the IGF-IR. However, we recently observed that serum from type 2 diabetic patients, who were treated for 36 weeks with metformin and insulin glargine induced similar IGF-IR activation compared with that of patients treated with metformin and NPH insulin[7].

After subcutaneous injection, insulin glargine is metabolized into two metabolites M1 and M2[8-10]. Insulin glargine undergoes sequential cleavage of the carboxy terminus of the B-chain forming metabolites M1 and M2 that lack the di-arginine (M1 after removal of the two arginines, M2 with additional deamination of threonine at position B30). M1 and M2 have the same metabolic properties as human insulin and do not differ from human insulin in affinity for the IGF-IR[5]. Our previous observation of a lack of increase in IGF-IR activation by serum[7] could have been the result of insulin glargine not reaching in vivo concentrations needed to stimulate the IGF-IR[11]. However, it may also be the result of metabolism of insulin glargine to M1 or M2 before it enters the circulation. Pharmacodynamic studies have shown that in type 1 diabetes mellitus, after a subcutaneous injection, insulin glargine is rapidly and dose-independently metabolized almost completely into M1[10]. Moreover, in type 2 diabetic patients, after a subcutaneous injection of a therapeutic dose, insulin glargine was minimally detectable in blood and M2 was undetectable in blood, whereas M1 accounted for most (≈90%) of the plasma insulin concentration up to 31 hrs[8]. Yet, whether insulin glargine accumulates during long-term insulin glargine therapy is still unknown.

Mitogenic effects of insulin may also occur via increased stimulation of the insulin receptor (IR)[12]. IRs are usually expressed at high levels in cancer cells[13-14]. Moreover, cells can express two IR isoforms (IR-A and IR-B), generated by alternative splicing of the IR gene[15]. The two IR isoforms have slightly different biological properties[15]. IR-B is the classical form of the IR, which is primarily expressed in liver, muscle and adipose tissues and predominantly mediates metabolic effects[15-18]. IR-A is expressed ubiquitously but is predominantly expressed in central nervous system, haematopoietic cells and in cancer tissues and has predominantly mitogenic effects[15-18].

The aims of the present study were to measure 1) the ability of purified insulin glargine, M1, M2, IGF-I and NPH insulin to activate the IR-A and IR-B and IGF-IR *in vitro*, 2) plasma

concentrations of insulin glargine and M1 and M2 during long-term insulin therapy in type 2 diabetic patients and 3) IR-A and IR-B activation *in vitro* induced by serum from patients treated with insulin glargine or NPH insulin.

STUDY DESIGN AND SUBJECTS

In vitro effects of insulin glargine, M1, M2, NPH insulin and IGF-I on IR-A, IR-B and IGF-IR autophosphorylation (activation)

Equimolar concentrations of insulin glargine (Lantus®, sanofi, Frankfurt am Main, Germany), M1 (kindly provided by sanofi, Frankfurt Germany), M2 (kindly provided by sanofi, Frankfurt Germany), NPH insulin (Insulatard®, Novo Nordisk, Bagsvaerd, Denmark) and IGF-I (Austral Biologicals, San Ramon, CA) were tested in a range of 0.1-100 nmol/l. IR-A, IR-B and IGF-IR activation was measured by Kinase Receptor Activation (KIRA) assays as detailed below (Methods section).

Concentrations of metabolites in serum and effects of serum on IR-A and IR-B activation in vitro

Serum samples from type 2 diabetic patients treated with insulin glargine were used to measure concentrations of insulin glargine, M1 and M2 by LCMS (please see Methods section) (vide infra) at baseline and after 36 weeks of insulin glargine treatment. Serum-induced IR-A and IR-B activation was measured by KIRA assays as detailed below (Methods section) before and after 36 weeks of insulin treatment in the same type 2 diabetic patients and in type 2 diabetic patients treated with NPH insulin for 36 weeks. Serum samples tested were samples obtained from the previously reported LANMET study[19] in which extra serum samples had been collected as part of the original protocol (104 out of 110 LANMET participants). The LANMET study was a multicentre, open, randomized, parallel-group study. Briefly, in the LANMET study, the efficacy and safety of bedtime insulin glargine and metformin was compared to NPH insulin and metformin treatment in insulin-naive poorly controlled type 2 diabetic patients (HbA_{1c} 8.0% or higher). The study consisted of a 4-week run-in phase and a 36–week treatment phase. It was performed at six sites in Finland and one in the United Kingdom. The study was performed in accordance with the Declaration of Helsinki and good clinical practice as described by Note for Guidance CPMP/ICH/135/95. Approval by institutional ethics committees, including permission to obtain extra serum samples at 0 and 36 weeks, was obtained for each participating site. All patients provided written informed consent before entry into the study.

METHODS

IR-A, IR-B and IGF-IR KIRA assays using pure hormone, metabolites or serum

The IR-A, IR-B and IGF-IR KIRA assays have been previously described[20-21]. All three assays use human embryonic kidney cells stably transfected with either cDNA of the human IR-A or human IR-B gene (HEK IR-A or IR-B) or with cDNA of the human IGF-IR gene (HEK IGF-IR). The principle of all three KIRA bioassays is based on quantification of tyrosine residue phosphorylation of the IR or IGF-IR. For the in vitro experiments, after 48 h of culture, cells were stimulated for 10 min (for IR KIRA assays) or 15 min (for the IGF-IR KIRA assay) at 37 °C. Cells were stimulated with equimolar concentrations of insulin glargine, M1, M2, NPH insulin or human recombinant IGF-I diluted in 0.5% HSA (Octalbine®) (Octopharma, Lachen, Switzerland).

For serum experiments, after 48 h of culture, cells were stimulated for 10 min (for IR KIRA assays) at 37 °C. Cells were stimulated with in serum or increasing amounts of human recombinant insulin (Actrapid®, Novo Nordisk, Bagsvaerd, Denmark) in a range of 0.06-1.0 nmol/L. In addition, 2 control serum samples were tested on every plate to ensure optimal performance. Standards and serum samples were diluted in Krebs-Ringer bicarbonate (KRB) buffer adjusted to pH 7.4 by CO_2 and supplemented human serum albumin diluted to 0.5% and 0.1% respectively. Serum samples were diluted 1:10.

After stimulation, cells were lysed. Crude lysates were transferred to a sandwich assay. In the IR KIRA assays wells were coated with a monoclonal antibody (MAI-1) directed against the IR (Novozymes-Gropep, Adelaide, Australia) that was used as capture antibody in a concentration of 2.5 µg/ml. In the IGF-IR KIRA assay wells were coated with a monoclonal capture antibody (MAD-1) directed against the IGF-IR in a concentration of 5.0µg/ml. A biotinylated anti-phosphotyrosine monoclonal antibody (BAM 1676) (R&D Systems Europe Ltd (Abingdon, UK)) was used in a concentration of 0.2 µg/ml together with streptavidin labelled europium (DELFIA Eu-N1) (PerkinElmer Life Sciences, Groningen, The Netherlands) in a concentration of 50 pmol/l as detection antibody. Contents were read in a time-resolved fluorometer (Victor2 multilabel counter; PerkinElmer Life Sciences). Assays were performed in 48-well plates (Corning, Corning, NY). All measurements were done in duplicate. The inter- and intra-assay CVs were below 15%.

Insulin glargine, M1 and M2 metabolite assays

Plasma insulin glargine, M1 and M2 concentrations were determined in citrate plasma samples taken after an overnight fast after 36 weeks of insulin treatment as follows: insulin glargine, M1 and M2 were extracted in plasma samples by immunoaffinity columns and quantified by a specific liquid chromatography tandem mass spectrometry assay

(LCMS), without cross-reactivity to endogenous human or other insulins. The limit of quantitation (LOQ) was ≈33 pmol/l (0.2 ng/ml)[10].

Statistical analysis

Baseline characteristics are shown as means (or geometric means) ± SE. The Kolmogorov- Smirnov-test was used to test normality of variables (data were considered to be normally distributed when P> 0.05). For data that did not meet the criteria for normality, logarithmic transformations were applied and are presented as geometric mean with 95% CI or if logarithmic transformation did not normalize data as median with interquartile range Spearmans' correlation coefficients were calculated to assess the associations between variables. Differences in continuous variables were calculated by an unpaired t-test or Mann Whitney test. Differences in categorical variables were tested by using the Chi-Squared test. A P-value of 0.05 or less was considered statistically significant. Data were analyzed using SPSS 17 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Effects of purified insulin glargine, M1, M2 and NPH insulin on IGF-IR, IR-A and IR-B activation in vitro

IGF-IR activation. As depicted in Fig. 1A, at 10-100 nmol/l insulin glargine was significantly more potent than NPH insulin or M1 and M2 to activate the IGF-IR. IGF-I was the

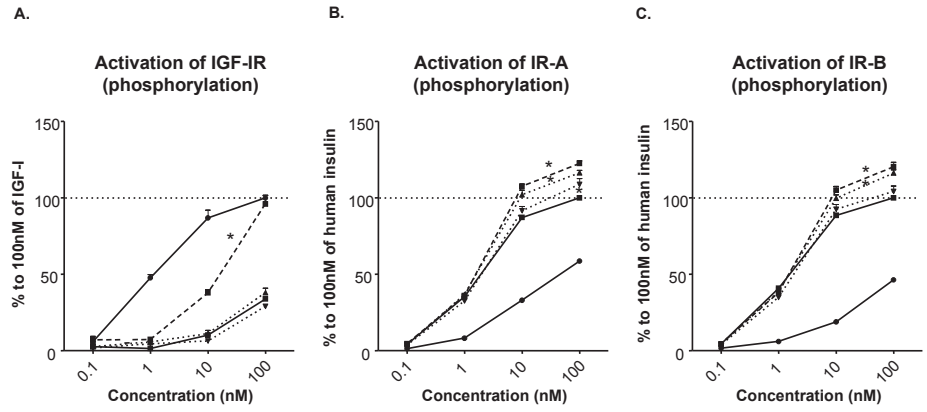

Figure 1.

Activation of the IR-A, IR-B and IGF-IR in vitro: comparing equimolar concentrations of NPH insulin (solid black line, squares), human IGF-I (solid black line, dots), insulin glargine (dashed line, squares) and M1 (dashed line, triangles down) and M2 (dashed line, triangles up). Dose–response profiles ranged from 0.1-100 nmol/L. Points represent the mean value (+SEM) of three independent experiments. *P-value < 0.05 compared to NPH insulin.

most potent activator of IGF-IR (Fig. 1A). M1 and M2 and NPH insulin activated the IGF-IR in a comparable manner (Fig. 1A).

IR-A activation. At 10-100 nmol/L, insulin glargine and M2 were more potent than NPH insulin in activating the IR-A ($p<0.001$) (Fig. 1B). M1 activates IR-A similarly to NPH insulin at concentrations up to 100 nmol/l (Fig 1B) and was only slightly more potent than NPH insulin at 100 nmol/L ($p=0.04$) (Fig 1B).

IR-B activation. At 10-100 nmol/L, insulin glargine and M2 were more potent than NPH insulin in activating the IR-B ($p<0.001$) (Fig. 1C). M1 was as potent as NPH insulin over the entire range tested (Fig. 1C).

Table 1 shows the EC50 values of IGF-I, NPH insulin, insulin glargine, M1 and M2 for the IGF-IR, IR-A and IR-B, respectively. For the IGF-IR, the EC50 of insulin glargine was substantially lower than NPH insulin for the IGF-IR, while EC50 values for M1 and M2 were higher compared to NPH insulin. For the IR-A and IR-B, the EC50 values were similar for NPH insulin, insulin glargine, M1 and M2.

Table 1. EC50 values of IGF-I, NPH insulin, insulin glargine, M1 and M2 for the human IR-A, human IR-B and the human IGF-IR.

Receptor types	EC50 (nmol/L)				
	IGF-I	NPH insulin	Insulin glargine	M1	M2
IR-A	9.6 ± 1.0	1.8 ± 1.0	2.2 ± 1.1	2.2 ± 1.1	2.0 ± 1.1
IR-B	23.0 ± 1.1	1.4 ± 1.1	2.0 ± 1.1	1.8 ± 1.2	2.0 ± 1.1
IGF-IR	1.1 ± 1.2	40.0 ± 1.4	23.4 ± 1.3	85.1 ± 1.9	49.1 ± 1.8

Data represent the mean±SE of minimally three independent experiments

Plasma concentrations of Insulin glargine and its metabolites after 36 weeks therapy

As reported previously[7, 19], baseline characteristics were comparable (Table 2). At 36 weeks, the mean doses of insulin glargine and NPH insulin to achieve HbA_{1c} of 7.0±0.1% and 7.1±0.1% ($p=0.93$) were 68±6 IU/day and 71±6 IU/day ($p=0.68$). The insulin doses (IU/kg) were also comparable (insulin glargine: 0.69±0.05 IU/kg vs. NPH insulin: 0.69±0.05 IU/kg, $p=0.98$).

At 36 weeks of insulin glargine treatment, plasma M1 concentrations increased from undetectable (<0.2 ng/mL) to 1.5 ng/mL [0.9-2.1] (median [interquartile range]) whereas Insulin glargine and M2 concentrations remained undetectable (<0.2 ng/mL) (Figure 2). The dose of insulin insulin glargine (IU/kg) correlated with M1 ($r=0.84$, $p<0.001$) (Figure 3).

Table 2. Baseline characteristics of diabetic patients randomized to the insulin glargine therapy or NPH insulin therapy

Characteristic	insulin glargine group	NPH insulin group	P-value[b]
Patients (n)	57	47	-
Sex (m/f)	35/22	31/16	0.63
Age (yrs)[a]	56.0±1.2	56.7±1.2	0.71
Metformin dose (g/day)	2.16±0.05	2.23±0.06	0.38
Previous sulfonylurea (%)	81	89	0.28
Weight (kg)	92.3±2.4	95.5±2.6	0.36
BMI (kg/m^2)[a]	31.0±0.7	31.9±0.8	0.35
HbA_{1C} (%)[a] HbA_{1C}[a] (mmol/mol)	9.0±0.1 75±1.1	9.2±0.2 77±2.2	0.33
FPG (mmol/l)[a]	12.3±0.3	11.9±0.4	0.43
C-peptide (nmol/l)	1.0±0.04	1.0±0.07	0.86
Serum TG (mmol/l)[a]	2.0±0.2	2.2±0.2	0.17
HDL- cholesterol (mmol/l)[a]	1.2±0.04	1.1±0.03	0.25
LDL- cholesterol (mmol/l)[a]	2.7±0.1	2.8±0.1	0.40

Data are presented as mean ± SE.

[a.] geometric mean.[b.] P-values are shown for differences between the insulin glargine group vs. NPH insulin group in the present study.

IR-A and IR-B activation in vitro, induced by serum from patients in the insulin glargine vs. NPH insulin treated groups

At baseline, serum-induced IR-A and IR-B activation did not differ between the two treatment groups (IR-A: insulin glargine vs. NPH insulin: 76 pmol/L [63-91] (median [interquartile range]) vs. 72 pmol/L [52-83]; p=0.09; IR-B: insulin glargine vs. NPH insulin: 134 pmol/L [106-166] vs. 123 pmol/L [102-159] p=0.33).

At 36 weeks of insulin therapy, serum-induced IR-A and IR-B activation did not differ between the two treatment groups (IR-A: insulin glargine vs. NPH insulin: 72 pmol/L [54-82] vs. 71 pmol/L [57-92]; p=0.96; IR-B: insulin glargine vs. NPH insulin: 116 pmol/L [93-148] vs. 121 pmol/L [102-133]; p=0.65).

At 36 weeks, the insulin dose (IU/kg) was positively correlated with serum-induced IR-A (r=0.28, p=0.004) but not to IR-B activation (r=0.16, p=0.12). In the NPH insulin treated group, the insulin dose (IU//kg) was positively correlated with serum-induced IR-A (r=0.39, p=0.008) but not to IR-B activation (r=0.24, p=0.11) (Figure 4A). In subjects treated with insulin glargine, the insulin dose did not correlate to serum-induced IR-A (r=0.17, p=0.22) or IR-B activation (r=0.08, p=0.55). However, M1 correlated with serum-induced IR-A (r=0.33, p=0.01) but not IR-B (r=0.17, p=0.20) activation (Figure 4B).

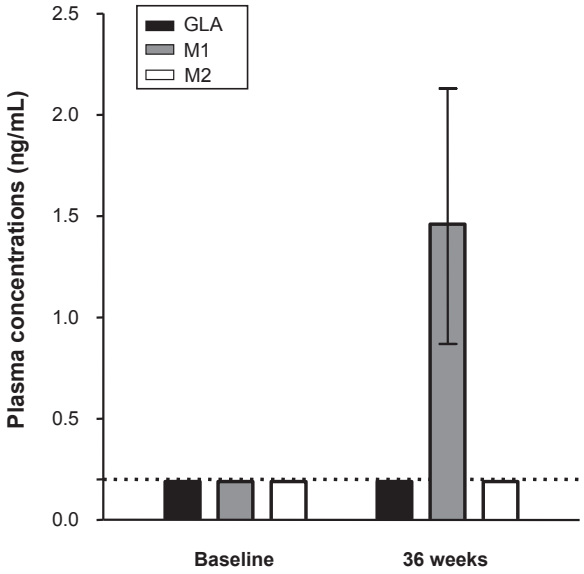

Figure 2.
Circulating plasma concentrations of insulin glargine (GLA) and its metabolites M1 and M2 at baseline and at 36 weeks of insulin glargine therapy determined by LCMS. Results are shown as median with interquartile ranges. The under broken line shows the detection limit for all 3 substances (0.20 ng/mL).

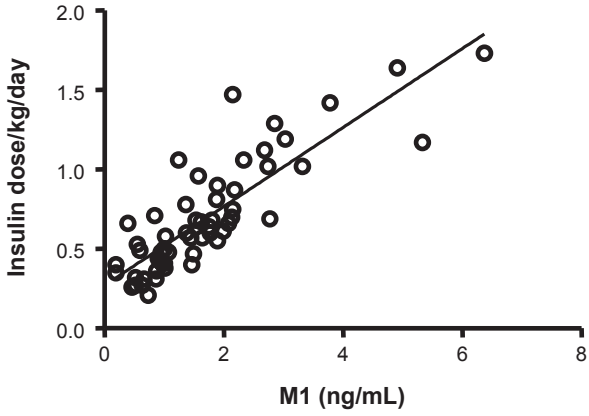

Figure 3.
Correlation between insulin glargine dose/kg/day at 36 weeks of insulin therapy and concentrations of its metabolite M1.

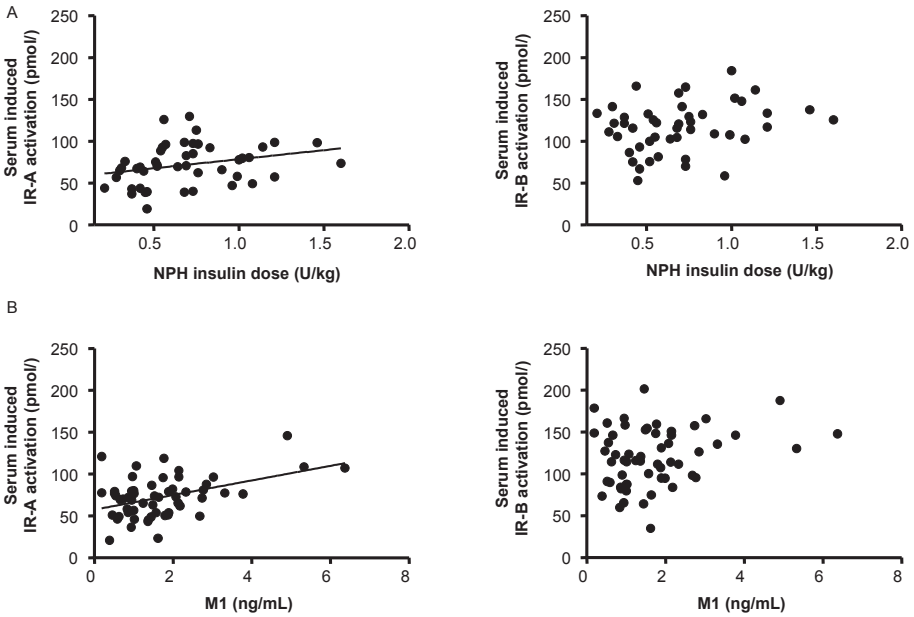

Figure 4.

A. Correlation between NPH insulin dose/kg/day and serum-induced IR-A and IR-B activation at 36 weeks of insulin therapy. NPH dose/kg/day was positively related to IR-A activation.

B. Correlation between concentrations of metabolite M1 and serum-induced IR-A and IR-B activation at 36 weeks of insulin glargine therapy. M1 concentrations were positively correlated to IR-A activation.

DISCUSSION

The most important finding of this study was that there was no detectable insulin glargine in the circulation of type 2 diabetic patients treated with high doses of insulin glargine for 36 weeks. Of the metabolites of insulin glargine, only M1 could be detected in the circulation of patients treated with insulin glargine. Circulating M1 concentrations correlated closely with the insulin glargine dose and with the ability of serum (from the same patients) to induce IR-A activation. In contrast to purified insulin glargine, purified M1 did not activate IGF-IR *in vitro*, even at high concentrations and activated IR-A and IR-B similar to NPH insulin.

Sommerfeld et al, found that insulin glargine had a higher affinity for the IGF-IR and was also more potent than human insulin to stimulate thymidine incorporation in Saos-2 cells[6]. In contrast, M1 and M2 were significantly less active than human insulin in binding to and activating the IGF-IR[6]. Their mitogenicity in Saos-2 cells was equal to that of

human insulin[6]. In another study, IGF-I and insulin glargine more efficiently stimulated phosphatidyl-inositol 3 phosphate PIP(3) production in breast cancer-derived MCF-7 cells as compared to human insulin[22]. In contrast, as compared to insulin, M1 and M2 showed lower potency in stimulating hybrid receptors (IR/IGF-IR), they induced less PIP(3) production, less Akt and Erk1/2 phosphorylation and less DNA synthesis in MCF-7 cells. These experimental results are in keeping with our findings of slightly greater IR-A, IR-B and IGF-IR activation at high (supraphysiologic) concentrations by insulin glargine compared to human insulin, and with data showing that insulin glargine metabolites M1 and M2 do not share these properties of insulin glargine (Fig. 1 en Table 1).

These findings may thus explain why the ability of serum from type 2 diabetic patients to induce IR-A and IR-B activation in the insulin glargine treated group was comparable to that of patients treated with NPH insulin. Moreover, they may also explain why we previously were not able to detect differences in serum-induced IGF-IR activation between patients treated with insulin glargine or NPH insulin[7].

In a recent study, circulating concentrations of insulin glargine, M1 and M2 were determined in type 1 diabetic subjects after a single subcutaneous dose of 0.3, 0.6, or 1.2 IU/kg insulin glargine respectively[10]. The authors concluded that insulin glargine was rapidly and nearly completely processed into M1. In another study, 9 type 2 diabetic subjects were investigated after 1 week of insulin glargine treatment[8]. Insulin glargine was detected at low concentrations in five of the nine subjects and at only a few time points. In contrast, M1 was detected in all subjects and accounted for most of the plasma insulin and metabolic action of the injected insulin glargine while M2 was undetectable. In contrast to our study, these studies did not include measurements of the ability of pure hormones or serum from the patients to activate the IR-A, IR-B and IGF-IR. Moreover, these studies did not address long term effects of high dose insulin glargine treatment on insulin glargine and its metabolites. Nevertheless, our findings are in line and consistent with conclusions of these latter studies.

The data in the present and our previous study[7] do not give support to the idea that treatment with insulin glargine in type 2 diabetes leads to a stronger stimulation of the IRs or IGF-IR than NPH insulin. In spite of that, we did find a positive relationship between insulin dose and serum-induced IR-A activation for both treatment groups. This suggests that, irrespective of insulin type, there may be an enhanced IR-A signalling in subjects who are treated with relatively high insulin doses. Whether these results have any clinical consequences is still uncertain. In a recent study, in which an animal model of type 2 diabetes was studied, no differences could be demonstrated in the degree of colonic epithelial proliferation between animals treated with insulin glargine or NPH insulin[23]. However, insulin treatment as such did result in a higher degree of colonic epithelial proliferation, suggesting that potential insulin-mediated mitogenic properties are irrespective of the type of insulin[23].

Finally, a few limitations of our study need to be addressed. The LANMET study was not primarily designed and performed to study the effects of insulin therapy on IR-A or IR-B or IGF-IR stimulating activities. Therefore the present study is inevitably a post-hoc analysis. Secondly, as for all in vitro systems, the IR-A and IR-B KIRA assays do not mimic the exact in vivo conditions. The autophosphorylation of the tyrosine residues at the beta-subunits of the IGF-IR and IRs does not necessarily give insights into whether activation of the IGF-IR or IR by an insulin analogue results in a normal (i.e. balanced metabolic and mitogenic) activity at cellular level *in vivo*. Thus, it would be important also to study downstream signalling pathways of the IGF-IR and IRs. However, lack of detectable insulin glargine in plasma after long-term insulin therapy precludes any direct role of insulin glargine in mediating metabolic or mitogenic effects during insulin therapy. Another limitation of the present study is that metabolite measurements were performed at 0 and 36 weeks, but not at other time points.

In conclusion, M1 induced similar IGF-IR, IR-A and IR-B activation as human insulin. After long-term, high dose, insulin glargine therapy, only M1 but not insulin glargine nor M2, could be detected in the circulation of type 2 diabetic patients. The concentration of plasma M1 correlated with insulin dose and also with IR-A stimulating activity. Serum from type 2 diabetic patients treated with insulin glargine or NPH insulin induced similar IR-A and IR-B activation. Taken together these data show that long-term high dose insulin glargine therapy does not increase IGF-IR signalling in type 2 diabetic patients.

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AUTHOR CONTRIBUTION

A.J. wrote the manuscript and researched data and edited the manuscript, H. contributed to conception and design researched data and reviewed/edited the manuscript, R. researched data and reviewed the manuscript, N. researched data and reviewed the manuscript, J.A.M.J.L. contributed to conception and design, reviewed/edited the manuscript, All authors gave their final approval of this version of the manuscript to be published.

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DISCLOSURES

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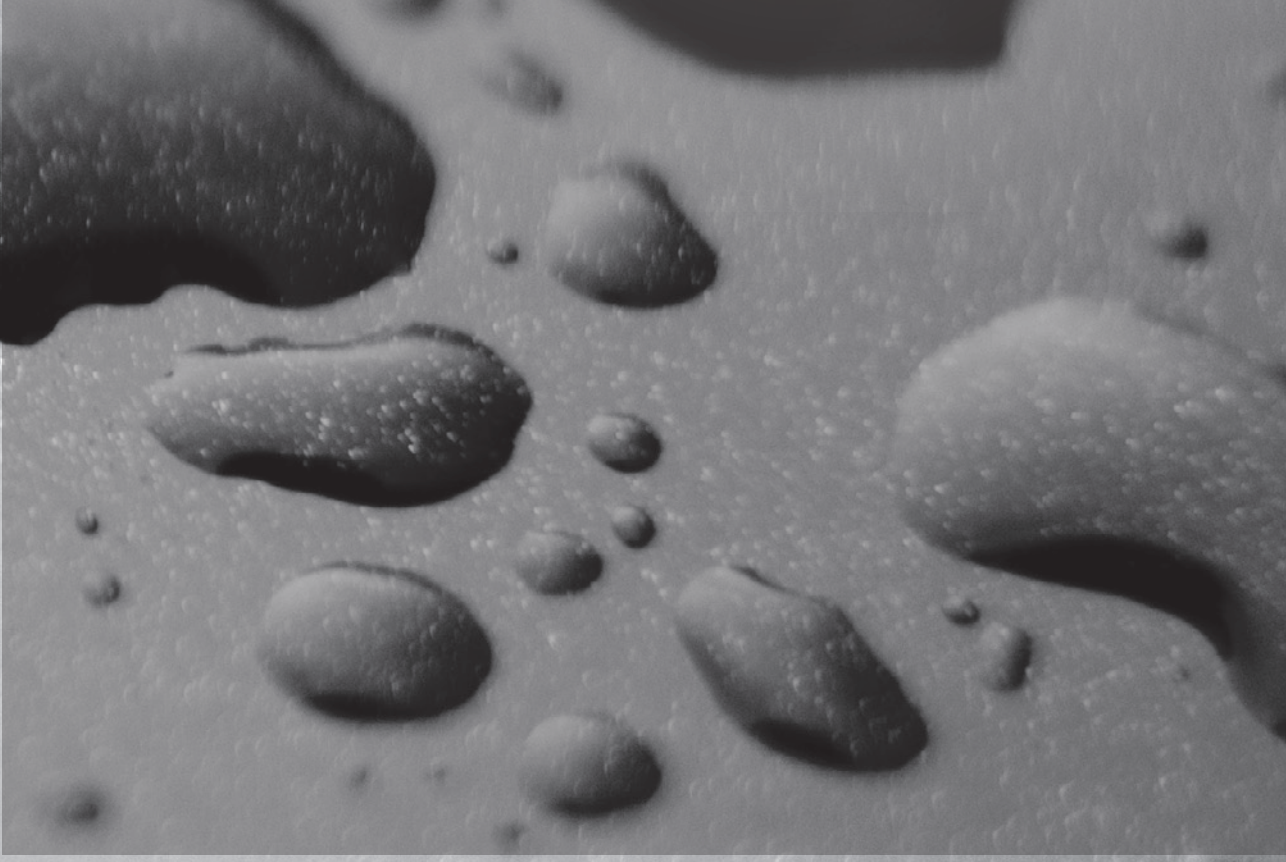
REFERENCES

1. Colhoun HM. Use of insulin glargine and cancer incidence in Scotland: a study from the Scottish Diabetes Research Network Epidemiology Group. *Diabetologia* 2009 **52** 1755-1765.
2. Currie CJ, Poole CD & Gale EA. The influence of glucose-lowering therapies on cancer risk in type 2 diabetes. *Diabetologia* 2009 **52** 1766-1777.
3. Hemkens LG, Grouven U, Bender R, Gunster C, Gutschmidt S, Selke GW & Sawicki PT. Risk of malignancies in patients with diabetes treated with human insulin or insulin analogues: a cohort study. *Diabetologia* 2009 **52** 1732-1744.
4. Jonasson JM, Ljung R, Talback M, Haglund B, Gudbjornsdottir S & Steineck G. Insulin glargine use and short-term incidence of malignancies-a population-based follow-up study in Sweden. *Diabetologia* 2009 **52** 1745-1754.
5. Kurtzhals P, Schaffer L, Sorensen A, Kristensen C, Jonassen I, Schmid C & Trub T. Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. *Diabetes* 2000 **49** 999-1005.
6. Sommerfeld MR, Muller G, Tschank G, Seipke G, Habermann P, Kurrle R & Tennagels N. In vitro metabolic and mitogenic signaling of insulin glargine and its metabolites. *PLoS One* 2010 **5** e9540.
7. Varewijck AJ, Janssen JA, Vahatalo M, Hofland LJ, Lamberts SW & Yki-Jarvinen H. Addition of insulin glargine or NPH insulin to metformin monotherapy in poorly controlled type 2 diabetic patients decreases IGF-I bioactivity similarly. *Diabetologia* 2012 **55** 1186-1194.
8. Lucidi P, Porcellati F, Rossetti P, Candeloro P, Andreoli AM, Cioli P, Hahn A, Schmidt R, Bolli GB & Fanelli CG. Metabolism of insulin glargine after repeated daily subcutaneous injections in subjects with type 2 diabetes. *Diabetes Care* 2012 **35** 2647-2649.
9. Kuerzel GU, Shukla U, Scholtz HE, Pretorius SG, Wessels DH, Venter C, Potgieter MA, Lang AM, Koose T & Bernhardt E. Biotransformation of insulin glargine after subcutaneous injection in healthy subjects. *Curr Med Res Opin* 2003 **19** 34-40.
10. Bolli GB, Hahn AD, Schmidt R, Eisenblaetter T, Dahmen R, Heise T & Becker RH. Plasma exposure to insulin glargine and its metabolites M1 and M2 after subcutaneous injection of therapeutic and supratherapeutic doses of glargine in subjects with type 1 diabetes. *Diabetes Care* 2012 **35** 2626-2630.
11. Sciacca L, Le Moli R & Vigneri R. Insulin analogs and cancer. *Front Endocrinol (Lausanne)* 2012 **3** 21.
12. Ish-Shalom D, Christoffersen CT, Vorwerk P, Sacerdoti-Sierra N, Shymko RM, Naor D & De Meyts P. Mitogenic properties of insulin and insulin analogues mediated by the insulin receptor. *Diabetologia* 1997 **40 Suppl 2** S25-31.
13. Frasca F, Pandini G, Scalia P, Sciacca L, Mineo R, Costantino A, Goldfine ID, Belfiore A & Vigneri R. Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. *Mol Cell Biol* 1999 **19** 3278-3288.
14. Papa V, Pezzino V, Costantino A, Belfiore A, Giuffrida D, Frittitta L, Vannelli GB, Brand R, Goldfine ID & Vigneri R. Elevated insulin receptor content in human breast cancer. *J Clin Invest* 1990 **86** 1503-1510.
15. Belfiore A, Frasca F, Pandini G, Sciacca L & Vigneri R. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. *Endocr Rev* 2009 **30** 586-623.
16. Benecke H, Flier JS & Moller DE. Alternatively spliced variants of the insulin receptor protein. Expression in normal and diabetic human tissues. *J Clin Invest* 1992 **89** 2066-2070.
17. Mosthaf L, Grako K, Dull TJ, Coussens L, Ullrich A & McClain DA. Functionally distinct insulin receptors generated by tissue-specific alternative splicing. *Embo J* 1990 **9** 2409-2413.

18. Seino S, Seino M, Nishi S & Bell GI. Structure of the human insulin receptor gene and characterization of its promoter. *Proc Natl Acad Sci U S A* 1989 **86** 114-118.
19. Yki-Jarvinen H, Kauppinen-Makelin R, Tiikkainen M, Vahatalo M, Virtamo H, Nikkila K, Tulokas T, Hulme S, Hardy K, McNulty S, Hanninen J, Levanen H, Lahdenpera S, Lehtonen R & Ryysy L. Insulin glargine or NPH combined with metformin in type 2 diabetes: the LANMET study. *Diabetologia* 2006 **49** 442-451.
20. Chen JW, Ledet T, Orskov H, Jessen N, Lund S, Whittaker J, De Meyts P, Larsen MB, Christiansen JS & Frystyk J. A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum. *Am J Physiol Endocrinol Metab* 2003 **284** E1149-1155.
21. Varewijck AJ, Brugts MP, Frystyk J, Goudzwaard JA, Uitterlinden P, Waaijers AM, Feng Y, Dimitrov DS, Lamberts SW, Hofland LJ & Janssen JA. Circulating insulin-like growth factors may contribute substantially to insulin receptor isoform A and insulin receptor isoform B signalling. *Mol Cell Endocrinol* 2013 **365** 17-24.
22. Pierre-Eugene C, Pagesy P, Nguyen TT, Neuille M, Tschank G, Tennagels N, Hampe C & Issad T. Effect of insulin analogues on insulin/IGF1 hybrid receptors: increased activation by glargine but not by its metabolites M1 and M2. *PLoS One* 2012 **7** e41992.
23. Nagel JM, Staffa J, Renner-Muller I, Horst D, Vogeser M, Langkamp M, Hoeflich A, Goke B, Kolligs FT & Mantzoros CS. Insulin glargine and NPH insulin increase to a similar degree epithelial cell proliferation and aberrant crypt foci formation in colons of diabetic mice. *Horm Cancer* 2010 **1** 320-330.



IGF-IR Bioactivity Better Reflects Growth Hormone Deficiency than Total-IGF-I

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ABSTRACT

Aim: Growth hormone (GH) is considered the main regulator of circulating insulin-like growth factor-I (IGF-I). Total (extractable) IGF-I is therefore routinely used for diagnosis of Growth Hormone Deficiency (GHD) and for monitoring treatment. Methods currently used for measurement of circulating total IGF-I may be hampered by interferences of IGF-binding proteins. Recently, a Kinase Receptor Activation (KIRA) assay was developed to determine IGF-IR bioactivity in human serum. The principle of this assay is based on quantification of IGF-I receptor (IGF-IR) activation after stimulation with serum in vitro. The aim of this study was to investigate the diagnostic potential of IGF-IR bioactivity in adults with GHD.

Methods: In a single centre observational study 94 GH-untreated patients diagnosed with GHD by GH-provocative-tests were included. IGF-IR bioactivity was determined by the IGF-IR KIRA assay, total IGF-I was determined by immunoassay in fasting blood samples.

Results: IGF-IR bioactivity was more frequently below the normal range (<-2SD) in untreated GH deficient patients than total IGF-I levels (81.9% vs. 61.7%, respectively), especially in patients >40 years of age. IGF-IR bioactivity decreased with the duration of GHD, whereas total IGF-I did not. With a decreasing number of additional pituitary deficits, total IGF-I levels more frequently remained within the normal range, whereas the percentage below the normal range was high for IGF-IR bioactivity, independent of additional deficits.

Conclusion: Determination of IGF-IR bioactivity may offer advantages in the evaluation of adult GHD compared to total IGF-I as bioactivity better reflects GHD as defined by GH stimulation tests, especially in subjects >40 years of age.

INTRODUCTION

Growth hormone (GH) is considered to be the main regulator of circulating insulin-like growth factor-I (IGF-I)[1]. Circulating total (extractable) IGF-I is therefore routinely used for diagnosing and monitoring treatment of adult Growth Hormone Deficiency (GHD)[2-3]. Nevertheless, it has been proven that the diagnosis of GH disorders cannot solely rely on determination of total IGF-I. In a substantial fraction of patients diagnosed as GH deficient, total IGF-I levels remain within the normal range, especially in patients above 40 years of age[4]. However, during GH replacement therapy, GH dose is titrated against total IGF-I levels and according to Consensus Guidelines total IGF-I values should be kept in the age-related normal range[5].

Many of the methods currently used for measurement of circulating total IGF-I are hampered by interferences of IGF-binding proteins (IGFBPs) remaining after extraction[6]. On the other hand, by extracting IGFBPs the modifying effects of these proteins on IGF-I action are ignored.

In 2003, a Kinase Receptor Activation (KIRA) bioassay was developed by Chen et al.[7] in order to measure IGF bioactivity at physiological conditions. The principle of this assay is based on quantification of IGF-I receptor (IGF-IR) activation after stimulation with serum in vitro[8]. In this way bioavailable IGF-I is quantified while taking into account the modifying effects of IGFBPs. Moreover, although it has been reported that cross-reactivity for IGF-II in this IGF-IR KIRA is only 12%[7], the contribution of IGF-II in GH deficient subjects may be relatively more significant considering the fact that IGF-II production is relatively GH independent.

The aim of this study was to investigate the diagnostic value of IGF-IR bioactivity in patients with proven GHD.

MATERIALS AND METHODS

Study Population

94 Patients diagnosed with GHD by GH-provocative tests were included in the present study. 81 Patients had already been treated with recombinant human GH, 13 patients were GH-naive.

For the diagnosis of GHD, patients with multiple pituitary hormone deficits had to have a serum GH peak below the cut-off value in one GH provocative test (arginine-GHRH test: GH peak< 16.5 µg/L, insulin tolerance test: GH peak< 5.0 µg/L) and patients with isolated GHD needed two GH provocative tests. Inclusion criteria were: 1. Male or female patients between 18-80 years, 2. Written voluntary informed consent, 3. Subjects using hormone replacement therapy for additional pituitary deficits had to be on an

optimized treatment regimen for at least three months prior to inclusion . Exclusion criteria were: 1. Patients who had received certain types of therapies for other reasons such as radiotherapies, surgeries, chemo-or immunotherapies in the three months prior to study start, 2. Female patients who were pregnant or lactating, or who wanted to become pregnant within one year, 3. Patients who were treated with insulin, 4. Subjects who, in the judgement of the investigators, were likely to be non-compliant or uncooperative during the study. From all subjects informed consent was obtained and the study was approved by the medical ethics committee of Erasmus MC.

Study Design

After inclusion, all 81 patients who had already been treated with recombinant human GH, were asked to discontinue GH treatment and were studied after four weeks. The 13 GH-naive patients were studied before starting GH therapy.

The following laboratory assessments were conducted in the fasting state: bioactive IGF-I, total IGF-I, insulin, glucose, IGFBP-1 and IGFBP-3. Moreover body weight, height, blood pressure. Body mass index (BMI) and waist-to-hip ratio (WHR) were calculated.

Duration of GHD was defined as time elapsed since GHD diagnosis (confirmed by a GH provocative test) and date of inclusion in this study.

Blood Measurements

The IGF-IR KIRA assay has been previously described[7-8]. Briefly, IGF-I binding to the IGF-IR results in autophosphorylation of tyrosine residues located within the intracellular kinase domain, being the first step in the intracellular signalling cascade. The IGF-IR KIRA assay uses a human embryonic kidney (HEK) cell line that is stably transfected with the human IGF-IR gene (HEK IGF-IR) and quantifies phosphorylation of tyrosine residues of the transfected IGF-IR to assess IGF-IR bioactivity. After 48 hours of culture HEK IGF-IR cells were stimulated for 15 minutes at 37°C with increasing known amounts of human recombinant IGF-I (Invitrogen, Breda, The Netherlands) in a range of 1-0.06 nmol/L and study serum samples. In addition, 2 control serum samples were tested on every plate to ensure optimal performance. Standards and serum samples were diluted in Krebs-Ringer bicarbonate (KRB) buffer adjusted to pH 7.4 by CO_2 and supplemented with 0.1% human serum albumin (HSA) (Octalbine®) (Octopharma, Lachen, Switserland). After stimulation cells were lysed. Crude lysates were transferred to a sandwich assay. Wells were coated with a monoclonal antibody directed against the IGF-IR (MAD1) (Novozymes-Gropep, Aidelade, Australia) that was used as capture antibody in a concentration of 5.0µg/mL. An europium labelled monoclonal anti-phosphotyrosine antibody (Eu-PY20) (Perkin-Elmer Life sciences, Groningen, the Netherlands) was used as a detection antibody in a concentration of 1.25µg/mL. Contents were read in a time-resolved fluorometer (Victor2 multilabel counter) (Perkin-Elmer life sciences, Groningen, The Netherlands). Assays were performed in 48 well plates (Corning, NY, USA). For measurements of IGF-IR bio-

activity, an IGF-I standard, two internal control samples were included on each culture plate. Serum samples were diluted 1/10. All measurements were done in duplicate. The intra-assay CV was 5.6%. The inter-assay CVs were 6.8% and 12.6%.

Serum total IGF-I, IGFBP-3 and insulin were measured by a solid-phase, enzyme-labelled chemiluminescent immunometric assays (intra-assay CVs were 3.9%, 4.4% and 3.3-5.5% and inter-assay CVs were 7.7%, 6.6% and 4.1-7.3% respectively) (Immulite 2000 supplied by Siemens Medical Solutions Diagnostics, Los Angeles, and USA). Since GH-levels after provocative tests were obtained between 1988 and 2010, different assays have been used to quantify GH levels. The inter- and intra-assay CVs were below 15%.

Statistics

The clinical characteristics of the study population are presented as mean with ranges or SE. The Kolmogorov- Smirnov-test was used to test normality of variables (data were considered to be normally distributed when P> 0.05). For data that did not meet the criteria for normality, logarithmic transformations were applied. Pearson's correlation coefficients were calculated (after adjustment for age) to assess associations between variables.

Both total IGF-I levels and IGF-IR bioactivity were compared with the age-specific normative range values for IGF-I that have been published before[8-9]. For total IGF-I normal values have been established in serum samples collected from 1584 healthy individuals, neonates, infants, children, adolescents and adults up to the age of 88 years by an enzyme-labelled chemiluminescent immunometric assays (Immulite 2000)[9]. In the present study, total IGF-I levels were also measured by an automated chemiluminescent assay system (Immulite 2000). The normal ranges for IGF-IR bioactivity have been previously published by our group and have been determined by using the same IGF-IR KIRA bioassay that was used in this study[8]. These normal values were established by measuring IGF-IR bioactivity in serum samples from healthy, non-fasting blood donors (total n = 427), whose ages ranged from 18 to 79 years (median: 44 years).

Both for total IGF-I and IGF-IR bioactivity individual Z-scores were calculated using the following formula: Z-score = (x–average x/S.D.) where x is the actual total IGF-I level or IGF-IR bioactivity, average x is the mean total IGF-I level or IGF-IR bioactivity at that age, and S.D. is standard deviation for the mean at that age.

The sensitivity of the parameters was defined as the percentage of GHD patients with a value below the lower bound of the normal range (<-2 SD) that is generally used in the diagnosis of GHD[10]. A P-value of 0.05 or less was considered statistically significant. Data were analysed using SPSS 15 for Windows (SPSS Inc., Chicago, IL).

Table 1. Baseline characteristics of the study population

	Mean	Range
Physical Measurements		
Age (yrs)	52.6	15.5-79.4
BMI (kg/m2)	29.2	17.9-49.8
	(m: 27.9±0.7; f: 30.4±1.1)*	
Systolic BP (mmHg)	130.6	96-164
Diastolic BP (mmHg)	80.7	58-102
Waist-to-Hip-Ratio (cm/cm)	1.0	0.8-1.2
	(m: 1.01±0.01; f: 0.96±0.01)***	
Laboratory tests		
Fasting Glucose level (mmol/L) +	4.9	3.0-11.2
Insulin (pmol/L) +	38.5	14-909
Total IGF-I (nmol/L) +	8.2	3.3-23.0
	(m: 9.6±0.7; f: 6.9±0.5)***	
IGF-IR bioactivity (pmol/L) +	112.3	20-500
	(m: 125.7±10.0; f: 99.5±10.8)*	
IGFBP-1 (ng/mL) +	23.5	0.6-131.2
IGFBP-3 (mg/L)	3.4	1.2-7.1
Duration of GHD (yrs)	8.9	0.0-22.0
GHD-categories		
Adult onset- GHD (N of subjects)	**78**	
· Functioning pituitary adenoma	22	
· Non-functioning pituitary adenoma	34	
· Craniopharyngeoma	10	
· Empty sella	2	
· Traumatic	3	
· Sheehan	2	
· Total Body Irradiation	4	
· Idiopathic	1	
Childhood onset- GHD (N of subjects)	**16**	
· Congenital	8	
· Empty sella	1	
· Functioning pituitary adenoma	1	
· Craniopharyngeoma	4	
· Traumatic	1	
· Total Body Irradiation	1	
Radiotherapy (N of subjects)	43	
Hormonal Deficits		
Isolated GH deficiency (N of subjects)	4	
Other pituitary deficits (N of subjects)		
· One	6	
· Two	17	
· Three or more	67	

Baseline characteristics are shown as mean (or geometric mean (+)) and range.

* $p<0.05$. ** $p<0.01$, *** $p<0.001$ significant differences between males and females.

RESULTS

Characteristics of the study population

Table 1 shows the characteristics of all GH deficient patients at baseline. 52.1% of the study population were male and had statistical significantly lower BMI (male: 27.9 ± 0.7 kg/m^2; female: 30.4 ± 1.1 kg/m^2; p= 0.04), and higher WHR than females (male: 1.01 ± 0.01; female: 0.96 ± 0.01; p <0.001).

In addition, compared to females, males had significantly higher total IGF-I levels (male: 9.6 ± 0.7 nmol/L; female 6.9 ± 0.5 nmol/L; p <0.001) and IGF-IR bioactivity (male: 125.7 ± 10.0 pmol/L; female 99.5 ± 10.8 pmol/L; p <0.03). Mean Z-scores for total IGF-I and IGF-IR bioactivity were significantly lower in women than in men (Figure 1). Mean Z-score for total IGF-I and IGF-IR bioactivity did not differ between women with (N=15) or without (N=30) oral oestrogen treatment (total IGF-I: p= 0.19; IGF-IR bioactivity: p= 0.75).

IGFBP-1 and IGFBP-3 were not different between males and females (IGFBP-1: male: 0.83 ± 0.15 nmol/L; female 1.05 ± 0.19 nmol/L; p=0.27 and IGFBP-3: male: 125.2 ± 3.5 nmol/L; female 114.8 ± 7.0 nmol/L; p= 0.31).

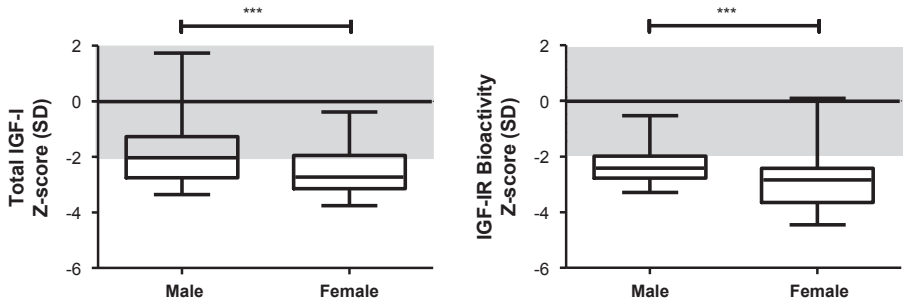

Figure 1.
Z-scores of total IGF-I (left) and IGF-IR bioactivity (right) are shown for men and women. The line in the boxes represents the mean of the data. The boxes extend from the 25th percentile to the 75th percentile values, the whiskers show the minimum and maximum values of each group. Grey area represents the normal range (-2 to +2 SD). *** P<0.001

IGF system parameters

Total IGF-I levels and IGF-IR bioactivity decreased borderline significantly with age (total IGF-I: r= -0.20, p= 0.06; IGF-IR bioactivity: r= -0.18, p= 0.08, respectively). IGFBP-3 decreased significantly with age (r= -0.25, p= 0.02) and IGFBP-1 did not decrease with age (r= -0.03, p=0.74). Insulin did not change with age (r= 0.07, p= 0.50), while the relationship between fasting glucose and age was borderline significant (r=0.18, p=0.08).

Total IGF-I and IGF-IR bioactivity were positively related to IGFBP-3, fasting insulin and glucose and negatively related to IGFBP-1 (Table 2). Total IGF-I and IGF-IR bioactivity were positively related to BMI, although not statistically significant (total IGF-I: r= 0.10, p= 0.34; IGF-IR bioactivity: r= 0.14, p= 0.19).

Table 2. Pearson 's correlations adjusted for age

	IGF-IR bioactivity	Total IGF-I	IGFBP-3	IGFBP-1	Insulin	Glucose
Total IGF-I	-0.39 (***)					
IGFBP-3	-0.41 (***)	-0.67 (***)				
IGFBP-1	-0.38 (***)	-0.39 (***)	-0.55 (***)			
Insulin	-0.41 (***)	-0.24 (*)	-0.42 (***)	-0.64 (***)		
Glucose	-0.18	-0.18	-0.32 (***)	-0.38 (***)	0.39 (***)	
BMI	-0.14	-0.10	-0.31 (***)	-0.46 (***)	0.45 (***)	0.28 (**)

*P< 0.05, **P< 0.01 and *** P < 0.001.

Identification of GHD by total IGF-I and IGF-IR bioactivity

The measurement of IGF-IR bioactivity better distinguished between GH deficient subjects and healthy subjects than total IGF-I (Figure 2). 60 out of 94 (63.8%) patients had total IGF-I levels below the lower bound of the 95% confidence interval (CI) while this was 81.9% for IGF-IR bioactivity (77 out of 94).

Z-scores for total IGF-I were positively related to age (r= 0.19, p= 0.06). For IGF-IR bioactivity Z-scores were negatively related to age (r= -0.28, p= 0.007). Sensitivity for GHD was 61.7% (58 out of 94 GHD patients) for total IGF-I and 81.9% (77 out of 94) for IGF-IR bioactivity. Below 40 years of age sensitivity of total IGF-I was 88.2% (15/19) and 73.7% (14/19) for IGF-IR bioactivity. Above 40 years of age sensitivity of total IGF-I was 57.3% (43 out of 75) and 84.0% (63 out of 75) for IGF-IR bioactivity.

Childhood onset (CO) vs. Adulthood onset (AO)

In total 16 patients were diagnosed as CO GHD. Of these patients, 14 were aged below 40 years and 2 above 40 years. Age-adjusted total IGF-I and IGF-IR bioactivity were not different between CO and AO GH deficient subjects (total IGF-I: 9.2 ± 1.5 vs. 8.0 ± 0.5 (p=0.78); IGF-IR bioactivity: 127.0 ± 28.6 pmol/L vs. 109.5 ± 6.7 pmol/L (p=0.82)).

Duration GHD

There was a significant inverse age-adjusted relationship between IGF-IR bioactivity and duration of GHD, but not between total IGF-I and duration of GHD (Figure 3).

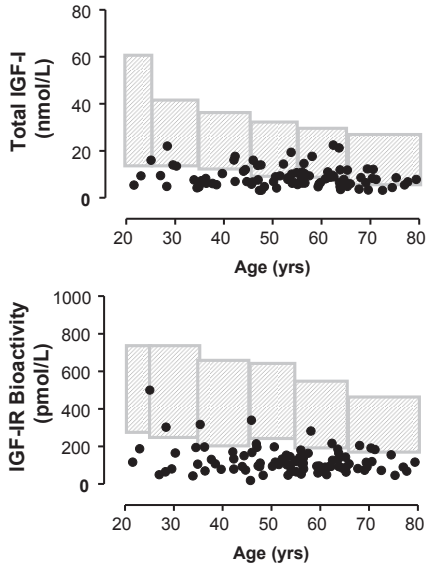

Figure 2.
Age distribution of serum total IGF-I levels (upper panel) and IGF-IR bioactivity (lower panel) in 94 patients diagnosed with GHD (black dots). The shaded area depicts the 95% confidence interval in normal subjects per decade of age.

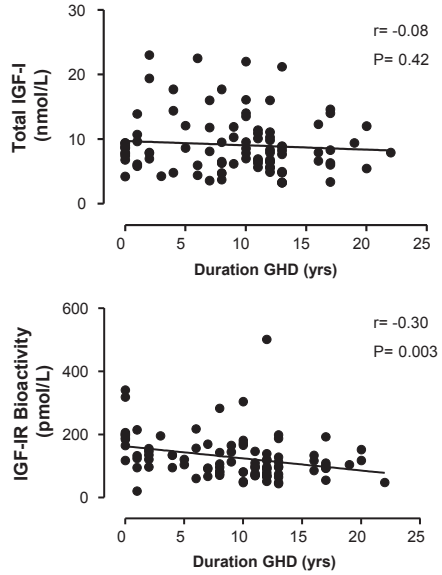

Figure 3.
Relation between duration of GHD and total IGF-I levels (upper panel) and IGF-IR bioactivity (lower panel)

IGF-I and number of additional pituitary deficits

Figure 4 shows baseline Z-scores of total IGF-I and IGF-IR bioactivity stratified by number of pituitary hormone deficits. Of the 94 patients, 4 patients had an isolated GHD, 6 patients had one other pituitary deficit, 17 patients had 2 other pituitary deficits and 67 had 3 or more pituitary deficits. With an increasing number of pituitary deficits, sensitivity of total IGF-I measurements increased (being 65% if ≥3 deficits present). For IGF-IR bioactivity the sensitivity was independent of the number of pituitary deficits and was >80% in all groups (Figure 4).

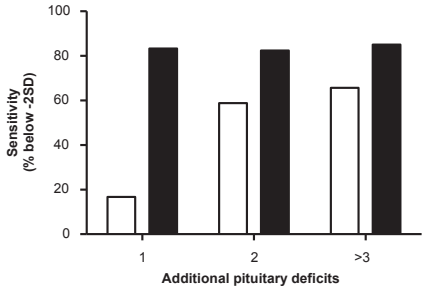

Figure 4
Sensitivity of total IGF-I measurement (open bars) and IGF-IR bioactivity (black bars) with increasing number of additional pituitary deficits.

GH treatment naive vs. GH treatment before

Mean IGF-IR bioactivity was significantly lower in patients who had been treated with GH therapy compared to GH-naive patients, also after adjustment for age and gender (103.3±7.4 pmol/L and 189.5 ± 18.4 pmol/L, p=<0.001) and also after further adjustment for duration of GHD and Body Mass Index. Total IGF-I levels did not differ between those who had been treated before compared to GH-naive patients, neither after adjustment for age and gender (8.2 ± 0.5 nmol/L and 8.1 ± 0.6 nmol/L, p=0.51) and neither after further adjustment for duration of GHD and Body Mass Index.

DISCUSSION

The most striking finding in our study was that in adults with GHD, IGF-IR bioactivity correlates better with GHD as defined by GH stimulation tests than total IGF-I measurements. This suggests that IGF-IR bioactivity can better distinguish GH deficient patients from healthy subjects and thus offers advantages over the measurement of total IGF-I in the diagnosis of GHD.

Our results are in contrast to a previous study by Chen et al, in which the same method was used to assess IGF-IR bioactivity[7]. In that study, there was no difference in IGF-IR bioactivity between GH deficient patients and healthy subjects. However, only a small number of patients (N=8) was included in that study. In addition, interpretation of circulating IGF-I measurements is only possible when age-adjusted normal ranges are available. As we have previously established age-specific normative values for the IGF-IR KIRA we were able to calculate Z-scores in our GH deficient study population[8].

IGF-I measurements were performed in GH deficient subjects that had either not been treated with GH before or had been withdrawn from GH treatment. In this latter group, blood was drawn four weeks after discontinuing GH therapy. IGF-IR bioactivity was significantly lower in patients who had been treated with GH before. Of the 81 patients who had already been treated with GH, 36 had a medical history of cranial radiotherapy (44%) compared to 7 out of 13 (54%) in the patients that were GH-naive. However, time elapsing after radiotherapy was significantly different between both groups (receiving GH therapy before: 19.6 ± 1.8 yrs; GH-naive: 8.1 ± 4.5 yrs, p=0.05). The latter may explain further deterioration of the GH-IGF-I axis during follow up. In addition, long-term GH-therapy may have influenced insulin sensitivity and thereby IGF-IR bioactivity. On the other hand, although an IGF-I value of <–2SD after withdrawing GH treatment for four weeks is generally considered as sufficient evidence of profound GHD, withdrawal from long-term GH therapy for 4 weeks may have not been enough to re-establish any residual function of the hypothalamic-pituitary-IGF-I axis[10].

In accordance with Ghigo et al.[4], total IGF-I levels were more often in the normal range in GH deficient subjects aged >40 yrs than <40 yrs. In contrast to total IGF-I, IGF-IR bioactivity more frequently remained below -2SD in subjects >40 yrs. This suggests that circulating IGF-IR bioactivity is a better marker of GH action throughout lifespan. In favour of this argument, IGF-IR bioactivity significantly decreased with longer duration of GHD, while such relationship was not found for total IGF-I.

With increasing age the contribution of GH to circulating total IGF-I levels appears to diminish, making total IGF-I a less useful diagnostic marker of GHD[11]. So, although total IGF-I levels are often reduced in adult GHD, a normal concentration does not exclude the diagnosis of GHD[5, 12]. Therefore, diagnosis of GHD remains based on a decreased GH response to a provocative test[5, 12-13]. Moreover, during therapy GH dose is titrated against total IGF-I levels and the Consensus guidelines advise that"...values should be kept in the age-related normal range"[12]. Thus the values of total IGF-I can be normal to begin with and yet that is the goal of therapy[14]. Our study suggests that measurement of IGF-IR bioactivity may help to solve this doublespeak, since IGF-IR bioactivity was lowered in a substantial number of GH-deficient subjects who had total IGF-I levels within the normal range.

Total IGF-I levels and IGF-IR bioactivity were significantly lower in females than in males. This is in agreement with previous findings[15-17]. However, although administration of oral estrogens is known to decrease IGF-I levels[18-19], we did not find differences in total IGF-I levels nor in IGF-IR bioactivity between untreated GH deficient women receiving oral estrogens and those who did not. This may be due to the small number of women on oral estrogens or just because the remaining IGF-I levels of these untreated GH deficient women could not become any lower.

Although it has been reported that the combination of ≥3 pituitary hormone deficits and a serum total IGF-I concentration <11nmol/L predicts adult GHD with 95% accuracy[20], many patients with GHD have <3 pituitary hormone deficits. Our study showed that the sensitivity of IGF-IR bioactivity was high and independent of the number pituitary deficits, whereas the sensitivity of total IGF-I decreased with decreasing number of pituitary deficits. Again, this suggests that circulating IGF-IR bioactivity is a better marker for diagnosing GHD than total IGF-I.

GH provocative testing is considered as the gold standard for diagnosing GHD. However, cut-off levels of GH levels currently used are arbitrary[2]. In addition, none of the currently used GH provocative tests satisfactorily mimic the normal secretory GH-pattern and there is poor reproducibility of the GH provocative tests[21]. So, which GH level is truly normal or abnormal with respect to total IGF-I levels or other aspects of GH action is not clear[14]. Yet, there are no studies in which clinical responses to GH therapy have been related to the level of total IGF-I before starting treatment[14]. Such studies might give some objective criteria as to whom might benefit the best from GH therapy[14]. It would be interesting to compare measurements of total IGF and IGF-IR bioactivity in this respect and to find out whether patients with low total IGF-I and/ or especially low IGF-IR bioactivity are those patients who respond best to GH therapy.

In conclusion, IGF-IR bioactivity correlated better with GHD as defined by GH stimulation tests than total IGF-I measurements, especially in subjects >40 years of age. In addition, for IGF-IR bioactivity, in contrast to total IGF-I, sensitivity was independent of the number of pituitary deficits. Moreover, IGF-IR bioactivity decreased with the duration of GHD, whereas total IGF-I did not. Taken together, this suggests that determination of IGF-IR bioactivity may offer advantages over total IGF-I in the evaluation of adult GHD. Further studies are needed to confirm our findings and to investigate whether it is possible to develop criteria for diagnosing GHD based on the measurement of IGF-IR bioactivity.

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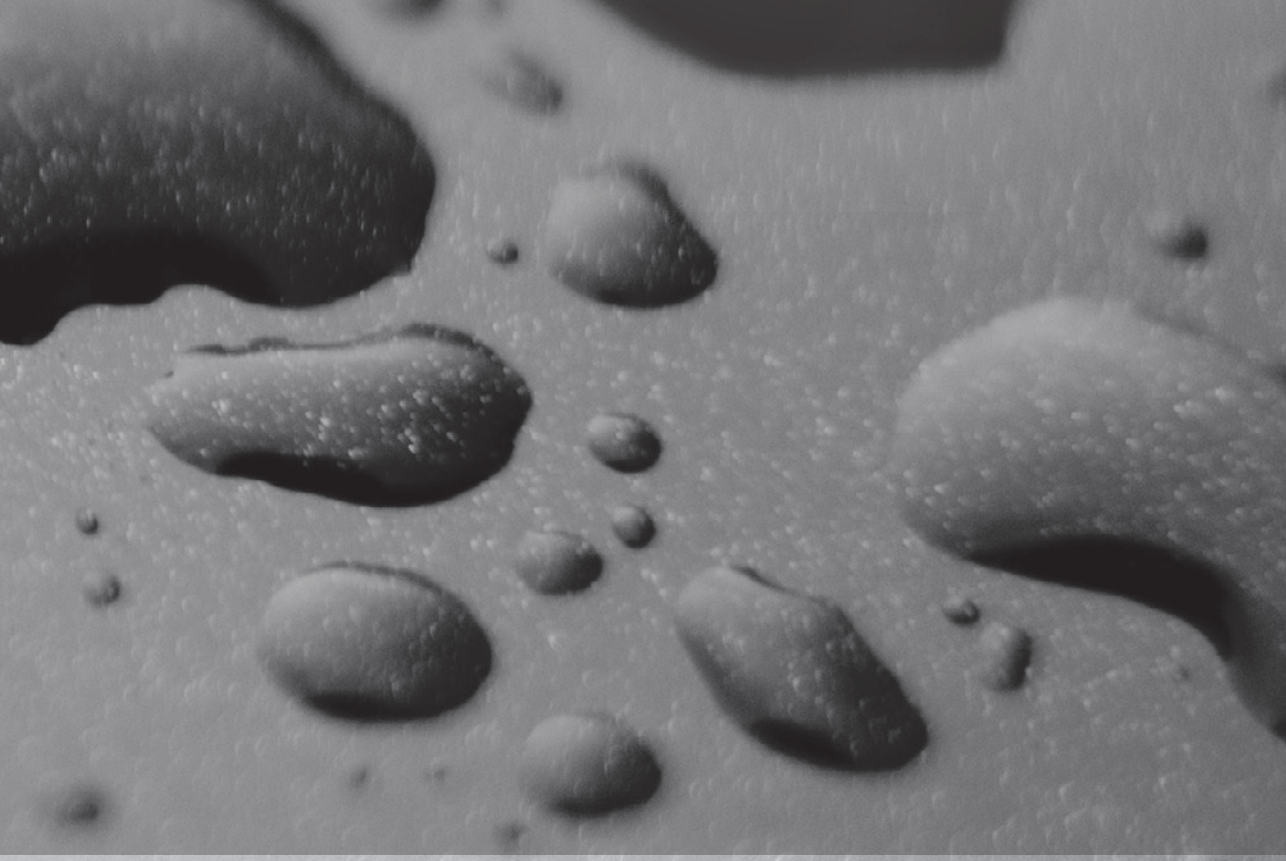
REFERENCES

1. Le Roith D, Bondy C, Yakar S, Liu JL & Butler A. The somatomedin hypothesis: 2001. *Endocr Rev* 2001 **22** 53-74.
2. Shalet SM, Toogood A, Rahim A & Brennan BM. The diagnosis of growth hormone deficiency in children and adults. *Endocr Rev* 1998 **19** 203-223.
3. Strasburger CJ, Bidlingmaier M, Wu Z & Morrison KM. Normal values of insulin-like growth factor I and their clinical utility in adults. *Horm Res* 2001 **55 Suppl 2** 100-105.
4. Ghigo E, Aimaretti G, Gianotti L, Bellone J, Arvat E & Camanni F. New approach to the diagnosis of growth hormone deficiency in adults. *Eur J Endocrinol* 1996 **134** 352-356.
5. Ho KK. Consensus guidelines for the diagnosis and treatment of adults with GH deficiency II: a statement of the GH Research Society in association with the European Society for Pediatric Endocrinology, Lawson Wilkins Society, European Society of Endocrinology, Japan Endocrine Society, and Endocrine Society of Australia. *Eur J Endocrinol* 2007 **157** 695-700.
6. Clemmons DR. IGF-I assays: current assay methodologies and their limitations. *Pituitary* 2007 **10** 121-128.
7. Chen JW, Ledet T, Orskov H, Jessen N, Lund S, Whittaker J, De Meyts P, Larsen MB, Christiansen JS & Frystyk J. A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum. *Am J Physiol Endocrinol Metab* 2003 **284** E1149-1155.
8. Brugts MP, Ranke MB, Hofland LJ, van der Wansem K, Weber K, Frystyk J, Lamberts SW & Janssen JA. Normal values of circulating insulin-like growth factor-I bioactivity in the healthy population: comparison with five widely used IGF-I immunoassays. *J Clin Endocrinol Metab* 2008 **93** 2539-2545.
9. Elmlinger MW, Kuhnel W, Weber MM & Ranke MB. Reference ranges for two automated chemiluminescent assays for serum insulin-like growth factor I (IGF-I) and IGF-binding protein 3 (IGFBP-3). *Clin Chem Lab Med* 2004 **42** 654-664.
10. Gasco V, Corneli G, Beccuti G, Prodam F, Rovere S, Bellone J, Grottoli S, Aimaretti G & Ghigo E. Retesting the childhood-onset GH-deficient patient. *Eur J Endocrinol* 2008 **159 Suppl 1** S45-52.
11. Toogood AA, Jones J, O'Neill PA, Thorner MO & Shalet SM. The diagnosis of severe growth hormone deficiency in elderly patients with hypothalamic-pituitary disease. *Clin Endocrinol (Oxf)* 1998 **48** 569-576.
12. Consensus guidelines for the diagnosis and treatment of adults with growth hormone deficiency: summary statement of the Growth Hormone Research Society Workshop on Adult Growth Hormone Deficiency. *J Clin Endocrinol Metab* 1998 **83** 379-381.
13. Aimaretti G, Corneli G, Baldelli R, Di Somma C, Gasco V, Durante C, Ausiello L, Rovere S, Grottoli S, Tamburrano G & Ghigo E. Diagnostic reliability of a single IGF-I measurement in 237 adults with total anterior hypopituitarism and severe GH deficiency. *Clin Endocrinol (Oxf)* 2003 **59** 56-61.
14. Molitch ME. Diagnosis of GH deficiency in adults--how good do the criteria need to be? *J Clin Endocrinol Metab* 2002 **87** 473-476.
15. Span JP, Pieters GF, Sweep CG, Hermus AR & Smals AG. Gender difference in insulin-like growth factor I response to growth hormone (GH) treatment in GH-deficient adults: role of sex hormone replacement. *J Clin Endocrinol Metab* 2000 **85** 1121-1125.
16. Leung KC, Johannsson G, Leong GM & Ho KK. Estrogen regulation of growth hormone action. *Endocr Rev* 2004 **25** 693-721.
17. Birzniece V, Sata A, Sutanto S & Ho KK. Paracrine regulation of growth hormone secretion by estrogen in women. *J Clin Endocrinol Metab* **95** 3771-3776.

18. Kam GY, Leung KC, Baxter RC & Ho KK. Estrogens exert route- and dose-dependent effects on insulin-like growth factor (IGF)-binding protein-3 and the acid-labile subunit of the IGF ternary complex. *J Clin Endocrinol Metab* 2000 **85** 1918-1922.
19. O'Sullivan AJ & Ho KK. Route-dependent endocrine and metabolic effects of estrogen replacement therapy. *J Pediatr Endocrinol Metab* 2000 **13 Suppl 6** 1457-1466.
20. Hartman ML, Crowe BJ, Biller BM, Ho KK, Clemmons DR & Chipman JJ. Which patients do not require a GH stimulation test for the diagnosis of adult GH deficiency? *J Clin Endocrinol Metab* 2002 **87** 477-485.
21. Hoeck HC, Jakobsen PE, Vestergaard P, Falhof J & Laurberg P. Differences in reproducibility and peak growth hormone responses to repeated testing with various stimulators in healthy adults. *Growth Horm IGF Res* 1999 **9** 18-24.



IGF-IR Bioactivity might reflect different aspects of Quality of Life than Total IGF-I in GH Deficient patients during GH treatment

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ABSTRACT

Aim: No relation has been found between improvement in quality of life (QoL) and total IGF-I during GH therapy. The aim of this study was to investigate the relationship between IGF-IR bioactivity and QoL in GH deficient patients receiving GH for 12 months.

Methods: 106 GH deficient patients; 84 on GH treatment discontinued therapy 4 weeks before establishing baseline values, 22 were GH-naive. IGF-IR bioactivity was determined by IGF-IR KIRA assay, total IGF-I by immunoassay (Immulite), QoL by disease-specific Question on Life Satisfaction Hypopituitarism (QLS-H) Module and by the general SF-36 questionnaire (SF-36Q).

Results: IGF-IR bioactivity increased after 6 months (-2.5SD vs. -1.9SD, $p<0.001$) and did not further increase after 12 months (-1.8SD, $p=0.23$); total IGF-I increased from -2.3SD to -0.9SD ($p<0.001$) and to -0.6SD ($p=0.005$), respectively.

QLS-H did not change over 12 months (-0.66±0.16SD to -0.56±0.17SD ($p=0.42$), to -0.68±0.17SD ($p=0.22$)).

The mental component summary (MCS) of the SF-36Q increased from 47.4 [38.7-52.8] to 50.2 [43.1-55.3] ($p=0.001$) and did not further improve (49.4 [42.1-54.1], $p=0.19$); the physical component summary (PCS) did not change (47.5 [42.0-54.2] vs. 47.0 [41.9-55.3], $p=0.91$, vs. 48.3 [39.9-55.4], $p=0.66$).

After 12 months, IGF-IR bioactivity was related to QLS-H ($r=0.28$, $p=0.01$); total IGF-I was not ($r=0.10$, $p=0.37$). IGF-IR bioactivity and total IGF-I were related to PCS ($r= 0.35$, $p=0.001$ and $r= 0.31$, $p=0.003$).

Conclusion: IGF-IR bioactivity remained subnormal after GH treatment and was positively related to QLS-H, while total IGF-I was not. This suggests that IGF-IR bioactivity reflects different aspects of QoL than total IGF-I in GH deficient patients during GH treatment.

INTRODUCTION

Growth hormone deficiency (GHD) is recognized to result in alterations in body composition, glucose and lipid metabolism, bone metabolism and physical performance[1]. The beneficial effects of long-term growth hormone (GH) replacement on body composition and metabolism in patients with GHD are well documented[1]. In addition to metabolic disturbances in GHD, quality of life (QoL) is impaired[2-5]. Evaluations of QoL, one of the key clinical endpoints, have shown a high degree of variability although it has been found to improve with GH replacement therapy when determined by disease specific questionnaires[5-10].

Until now, no correlations have been found between changes in QoL and total IGF-I concentrations during GH replacement[5].

During GH therapy, GH dose is titrated against total (extractable) IGF-I concentrations since GH is considered to be the main regulator of circulating IGF-I concentrations[11]. According to consensus guidelines, total IGF-I values should be kept in the age-related normal range[12]. In a substantial fraction of patients diagnosed as GH deficient, total IGF-I concentrations are within the normal range before starting GH therapy[13-14].

Many of the methods currently used for measurement of circulating total IGF-I are hampered by interferences of IGF-binding proteins (IGFBPs) remaining after extraction[15]. On the other hand, by extracting IGFBPs, the modifying effects of these proteins on IGF-I action are ignored. In 2003 a kinase receptor activation (KIRA) bioassay was developed by Chen et al.[16] to measure IGF-IR bioactivity at physiological conditions. The principle of this assay is based on quantification of IGF-I receptor (IGF-IR) activation after stimulation with serum in vitro[17]. In this way bioavailable IGF-I is quantified while taking into account the modifying effects of IGFBPs. It has been suggested that IGF-IR bioactivity is more sensitive than total IGF-I to detect differences in clinical state[18]. Previously we demonstrated that IGF-IR bioactivity offers advantages over total IGF-I in the diagnostic evaluation of adult GHD[14].

The aim of the present study was to investigate the value of IGF-IR bioactivity in monitoring QoL in patients with GHD treated with GH for 12 months.

PATIENTS AND METHODS

Study population

106 Patients diagnosed with GHD by GH-provocative tests were included in the present study. 84 patients were diagnosed as Adult Onset GH deficiency (AO-GHD) and 22 as Childhood onset GH deficiency (CO-GHD). 84 patients had already been treated with recombinant human GH, 22 patients were GH-naive.

For the diagnosis of GHD, patients with multiple pituitary hormone deficits had to have a serum GH peak below the cut-off value in one GH provocative test (arginine-GHRH test: GH peak< 16.5 µg/L, insulin tolerance test: GH peak< 5.0 µg/L) and patients with isolated GHD needed two GH provocative tests. Subjects using hormone replacement therapy for additional pituitary deficits had to be on an optimized treatment regimen for at least three months prior to inclusion. Inclusion and exclusion criteria have been previously described[14]. From all subjects informed consent was obtained and the study was approved by the medical ethics committee of Erasmus MC.

Study design

After inclusion, all 84 patients who had already been treated with recombinant human GH were asked to discontinue GH treatment for 4 weeks after which baseline values were established. Patients were studied at baseline and at 6 and 12 months after starting GH therapy. The 84 patients were using different brands of recombinant human GH (Genotropin (Pfizer) (n=4), Humatrope (Eli Lilly) (n=68). Norditropin (Novo Nordisk) (n=8), Nutropinaq (Ipsen) (n=1) and Zomacton (Ferring) (n=3). No patient was switched from one recombinant GH product to another. The 22 GH-naive patients were studied before starting GH therapy and at 6 and 12 months thereafter. In 14 of the GH-naive patients Norditropin was started, while in 8 patients Nutropinaq was started. GH dose was adjusted targeting serum total IGF-I concentrations to the middle (Z-score=0) of the normal gender- and age-related reference values for the healthy population[19]. The following laboratory assessments were conducted in the fasting state: bioactive IGF-I, total IGF-I, insulin, glucose, IGFBP-1, and IGFBP-3. Moreover, body weight, height, blood pressure, body mass index (BMI), and waist to hip ratio (WHR) were calculated. Quality of life (QoL) was measured by the Question on Life Satisfaction Hypopituitarism Module (QLS-H)[7-8] and the SF-36 questionnaire (SF-36Q)[20-22]. Duration of GHD was defined as the time that had elapsed since GHD diagnosis (confirmed by a GH provocative test) up to the date of inclusion in this study.

Blood measurements

The IGF-IR KIRA assay has been previously described[16-17]. Briefly, IGF-I binding to the IGF-IR results in autophosphorylation of tyrosine residues located within the intracellular kinase domain, being the first step in the intracellular signalling cascade. The IGF-IR KIRA assay uses a human embryonic kidney (HEK) cell line that is stably transfected with the human IGF-IR gene (HEK IGF-IR) and quantifies phosphorylation of tyrosine residues of the transfected IGF-IR to assess IGF-IR bioactivity. All measurements were done in duplicate. The intra-and interassay CVs were below 15%.

Serum total IGF-I, IGFBP-3 and insulin were measured by a solid-phase, enzyme-labelled chemiluminescent immunometric assays (intra-assay CVs were 3.9%, 4.4% and

3.3-5.5% and inter-assay CVs were 7.7%, 6.6% and 4.1-7.3% respectively) (Immulite 2000 supplied by Siemens Medical Solutions Diagnostics, Los Angeles, and USA).

Both total IGF-I concentrations and IGF-IR bioactivity were compared with the age-specific normative range values for IGF-I that have been published before[17, 23]. For total IGF-I normal values have been established in serum samples collected from 1584 healthy individuals, neonates, infants, children, adolescents and adults up to the age of 88 years by an enzyme-labelled chemiluminescent immunometric assays (Immulite 2000)[23]. In the present study, total IGF-I concentrations were also measured by Immulite 2000. In our laboratory we monitored the IGF-I batch-to-batch variation by internal controls. The coefficient of variation determined at concentrations of 6.5 to 34.9 nmol/L was 3.7-10.9%. Quality assessment by the SKML (Dutch Foundation for Quality Assessment in Clinical Laboratories) showed that the batch to batch variation of the total IGF-I Immulite assay between 2008-2012 was relatively small during these years.

The normal ranges for IGF-IR bioactivity have been previously published by our group and were established by measuring IGF-IR bioactivity in serum samples from healthy, non-fasting blood donors (total n = 427), whose ages ranged from 18 to 79 years (median: 44 years)[17].

Both for total IGF-I and IGF-IR bioactivity individual Z-scores were calculated using the following formula: Z-score = (x–average x/S.D.) where x is the actual total IGF-I level or IGF-IR bioactivity, average x is the mean total IGF-I level or IGF-IR bioactivity at that age, and S.D. is standard deviation for the mean at that age.

For the measurements of bioactive IGF-I, samples from the same patient at different time points were assayed in one assay run, while total IGF-I concentrations were separately determined after each visit since, total IGF-I was used to adjust GH dose.

Quality of Life (QoL) measurements

Questions on Life Satisfaction-Hypopituitarism (QLS-H) Module

The QLS-H module has been developed to assess the specific issues faced by adult patients with GHD[7]. It is self-administered and subjects must initially indicate how important a certain dimension of QoL is to them and then their degree of satisfaction with that dimension. The total QLS-H score is obtained by adding the individual item scores of nine dimensions and can range from -108 (representing very low satisfaction) to +180 (representing very high satisfaction)[7]. Reference ranges of total QLS-H scores have been constructed separately for each country by gender, using age as a continuous independent variable, as previously described. Results were expressed as Z-scores based on these reference ranges. Z-score= [QLS-H score - mean(age)]/sd(age) for the general population of the particular country[7].

Short form (SF) Health Survey-36 questionnaire (SF-36Q)

The SF-36 Health Survey is a widely used generic measure of health status[20]. The mental and physical component summeries (MCS and PCS, respectively) of the SF-36Q were calculated by standardizing the subscale scores using a linear Z-score transformation using national (Dutch) means and standard deviations. Then, Z-scores were multiplied by the US subscale factor score coefficients for PCS and MCS and summed over all eight subscales. Finally, T-scores were calculated by multiplying the obtained PCS en MCS sums by 10 and adding 50 to the product, to yield a mean of 50 and a standard deviation of 10 for the norm population[24].

Statistics

The clinical characteristics of the study population are presented as mean with ranges or SE. For data that did not meet the criteria for normality, logarithmic transformations were applied and are presented as geometric mean with ranges or 95% CI or if logarithmic transformation did not normalize data as median with interquartile range. The Kolmogorov- Smirnov-test was used to test normality of variables (data were considered to be normally distributed when P> 0.05). An unpaired t-test/ one-way ANOVA or Mann-Whitney/ Kruskal-Wallis test were used to test differences in continuous variables within a visit, differences in categorical variables were tested by using the Chi-Squared test. A paired t-test or a Wilcoxon Signed Ranks test were used to test differences between baseline and 12 months of GH therapy. Age adjusted Pearson's correlation coefficients were calculated to assess the associations between variables that were normally distributed. Spearman's correlation coefficient was used if they were not. A P-value of 0.05 or less was considered statistically significant. Data were analysed using SPSS 17 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Baseline characteristics

Baseline characteristics are shown in Table 1 and 2. 50.9% of the study population was male. 38% of the participants had total IGF-I levels >-2SD despite a proven GHD by a decreased GH response in provocative test (Figure 1 bottom right) while this was 22% for IGF-IR bioactivity (Figure 1 top).

Mean Z-scores for IGF-IR bioactivity and total IGF-I were significantly higher in males than in females (IGF-IR bioactivity: -2.3 ± 0.1 vs. -2.8 ± 0.1 SD, p=0.006; total IGF-I: -1.9 ± 0.2 vs. -2.6 ± 0.1 SD, p= 0.001). Differences for total IGF-I remained when only women not using estrogens (69% of all females) were compared to males.

Table 1. Baseline characteristics of the study population

	Mean	Range
Physical Measurements		
Age (yrs)	54.0#	18.0-79.4
BMI (kg/m2)	28.4	17.9-49.8
Systolic BP (mmHg)	129	96-164
Diastolic BP (mmHg)	80	58-102
WHR (cm/cm)	0.98	0.74-1.19
Laboratory tests		
Fasting Glucose level (mmol/L)	4.7#	3.0-11.2
Insulin (pmol/L)	35#	13-909
Total IGF-I (nmol/L)	8.1	2.7-23.0
IGF-IR bioactivity (pmol/L)	115	20-337
IGFBP1 (µg/L)	23.0	0.4-131.2
IGFBP3 (mg/L)	3.4	1.2-7.1
Duration of GHD (yrs)	9.5#	0-22
GHD-categories (N of subjects)		
AO- GHD	84	
CO- GHD	22	
GH-naive	22	
Previous GH treatment	84	
Aetiology (N of subjects)		
· Congenital	10	
· Functioning pituitary adenoma	24	
· Non-functioning pituitary adenoma	37	
· Craniopharyngeoma	15	
· Empty sella	4	
· Traumatic	4	
· Sheehan	3	
· Irradiation	8	
· Idiopathic	1	

Baseline characteristics are shown as mean (or median (#)) and range.
WHR: Waist-to-Hip Ratio; IGF: insulin-like growth factor; IGFBP: insulin-like growth factor binding protein; GHD: growth hormone deficiency; AO: adult-onset; CO: childhood-onset; GH: growth hormone

20.8% of the study population was diagnosed as CO-GHD. Mean Z-scores for IGF-IR bioactivity and total IGF-I were not different between CO-GH deficient patients vs. AO-GHD patients (IGF-IR bioactivity: -2.5 ± 0.1 vs. -2.6 ± 0.1 SD, p=0.78; total IGF-I: -2.4 ± 0.2 vs. -2.2 ± 0.1 SD, p= 0.48).

20.8% of the study population was GH-naive. Mean Z-scores for IGF-IR bioactivity and total IGF-I were not different between GH-naive patients vs. patients who had been

previously treated with GH (IGF-IR bioactivity: -2.3 ± 0.1 vs. -2.6 ± 0.1, p=0.08; total IGF-I: -2.5 ± 0.2 vs. -2.2 ± 0.1, p=0.23).

The overall mean QLS-H Z-score was -0.66 ± 0.17. The overall median MCS and PCS of the SF-36Q were 47.4 [38.7-52.8] and 47.5 [42.2-54.2], respectively.

QLS-H Z-scores were not different between patients within various subgroups (Table 2). Within the same subgroups, the MCS was not different (data not shown). In contrast, on the PCS, males scored significantly higher than females 50.8 [44.8-54.9] vs. 45.0 [35.4-50.5], p=0.01, respectively). In addition, patients with CO-GHD scored significantly higher on the PCS than AO-GHD patients (52.8 [45.8-55.8] vs. 46.4 [37.3-53.2], p= 0.01, respectively).

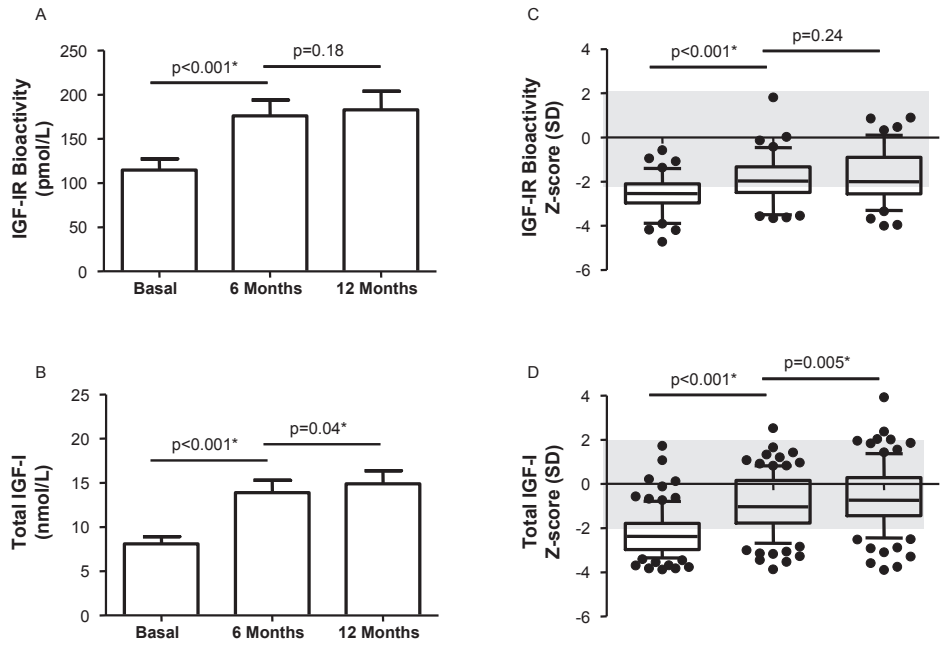

Figure 1.

A. Geometric mean (and 95% CI) of IGF-IR bioactivity (pmol/L) before and after 12 months of GH therapy.
B. Geometric mean (and 95% CI) of total IGF-I concentrations (nmol/L) before and after 12 months of GH therapy.
C. Z-scores of IGF-IR bioactivity before and after 12 months of GH therapy. The line in the boxes represents the mean of the data. The boxes extend from the 25th percentile to the 75th percentile; the whiskers show the 10 to 90% confidence interval. Grey area represents the normal range (-2 to +2 SD).
D. Z-scores of total IGF-I before and after 12 months of GH therapy. The line in the boxes represents the mean of the data. The boxes extend from the 25th percentile to the 75th percentile; the whiskers show the 10 to 90% confidence interval. Grey area represents the normal range (-2 to +2 SD).

Table 2. QLS-H Z-scores in GH deficient patients before and after 12 months of GH replacement therapy.

	QLS-H Z-score (SD)		p-value		
	Before	**After 12 months**	**Differences before**	**Differences after 12 months**	**Changes during treatment**
All patients	-0.66 ± 0.17	-0.68 ± 0.17			0.90
Gender					
M	-0.58 ± 0.21	-0.78 ± 0.22	0.59	0.50	0.22
F	-0.77 ± 0.28	-0.54 ± 0.29			0.13
Onset					
CO	-0.64 ± 0.26	-0.57 ± 0.33	0.94	0.74	0.79
AO	-0.67 ± 0.20	-0.71 ± 0.21			0.76
GH naive					
Yes	-0.46 ± 0.56	-1.13 ± 0.54	0.68	0.36	0.29
No	-0.69 ± 0.18	-0.62 ± 0.18			0.51
Duration GHD (yr)					
<5	-0.80± 0.36	-1.28 ± 0.33	ANOVA 0.89	ANOVA 0.14	0.11
5-10	-0.58 0.47	-0.40 ± 0.45			0.41
>10	-0.63 ± 0.19	-0.50 ± 0.22			0.32
Additional pituitary deficits					
1-3	-0.68 ± 0.33	-0.69 ± 0.29	0.93	0.95	0.97
≥3	-0.65 ± 0.19	-0.67 ± 0.22			0.89
Age group (yr)					
<25	-0.56 ± 0.73	-1.23 ± 0.88	ANOVA 0.32	ANOVA 0.04*	0.04*
25-35	-0.55 ± 0.37	-0.46 ± 0.27			0.76
35-45	-1.12 ± 0.33	-1.12 ± 0.45			0.96
45-55	-1.17 ± 0.45	-1.38 ± 0.44			0.59
55-65	-0.60 ± 0.31	-0.60 ± 0.31			0.98
>65	-0.01 ± 0.30	0.33 ± 0.27			0.05*

Data are shown as Mean±SE. *statistically significant. M; male; F; female; CO: childhood-onset; AO: adult-onset; GH: growth hormone; GHD: growth hormone deficiency

Relationships between IGF-I parameters and QoL measures at baseline

At baseline, there were no significant (age-adjusted) correlations between IGF-IR bioactivity or total IGF-I and QLS-H Z-scores (IGF-IR bioactivity: r=0.16, p=0.13; total IGF-I: r= 0.03, p=0.82).

In addition there were no correlations between IGF-IR bioactivity or total IGF-I and the MCS (IGF-IR bioactivity: r=-0.11, p=0.28; total IGF-I: r=0.05, p=0.62) or the PCS of the SF-36Q (IGF-IR bioactivity: r=0.16, p=0.11; total IGF-I: r=0.17, p=0.10).

GH doses and IGF-I concentrations after 12 months of GH therapy

At 12 months the median GH dose was 0.20 mg/day [0.10-0.30]. Males used significantly lower GH doses than females (0.20 [0.10-0.30] vs. 0.30 [0.15-0.40], p=0.02). IGF-IR bioactivity increased from 115 [95%CI 103-127] pmol/L (Z-score -2.5SD) to 176 [95%CI 160-196] pmol/L (Z-score -1.7SD) after 6 months (p<0.001) and did not further increase after 12 months (183 [95%CI 164-204], Z-score=1.8SD) (p=0.18) (Figure 1A). Total IGF-I increased from 8.1 [95%CI 7.3-8.9] nmol/L (Z-score -2.3SD) at baseline to 13.9 [95%CI 12.7-15.3] nmol/L (Z-score -0.6SD) at 6 months (p<0.001) and further increased to 14.9 [95% 13.5-16.4] (Z-score=-0.6SD) at 12 months (p=0.04) (Figure 1B). After 12 months of GH therapy, only 50% of patients had IGF-IR bioactivity values within the normal range, compared to 81% for total IGF-I concentrations (Figure 1C-D).

QoL measures after 12 months

QLS-H Z-score

QLS-H did not change over 12 months (from -0.66±0.16SD at baseline to -0.56±0.17SD after 6 months (p=0.42), to -0.68±0.17SD after 12 months (p=0.22)). Overall, the QLS-H Z-scores were not different between patients within the subgroups, although patients >65 years of age tended to have highest QLS-H Z-scores and reported significantly higher QLS-H Z-scores compared to patients aged 45-55 (p=0.02) (Table 2). In this subgroup, GH treatment had increased QLS-H Z-scores whereas in patients aged <25 yrs, QLS-H Z-scores had significantly decreased. In the latter subgroup total IGF-I and IGF-IR bioactivity had not increased significantly over time (p=0.14 and p=0.07 respectively), whereas in patients aged above 65 yrs both total IGF-I and IGF-IR bioactivity significantly increased (p=0.002 and p<0.05, respectively).

Interestingly, at 12 months, patients with IGF-IR bioactivity within the normal range had significantly higher QLS-H Z-scores compared to patients with IGF-IR bioactivity below the normal range (-0.19 ± 0.22 vs. -0.88 ± 0.21, p=0.02), see Figure 2. This difference was not found for total IGF-I (>-2SD vs. <-2SD: -0.46 ± 0.16 vs. -1.10 ± 0.45, p=0.12), Figure 2.

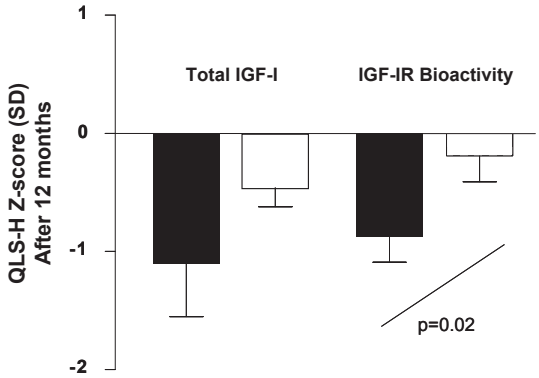

Figure 2.
Comparison of QLS-H Z-scores after 12 months of therapy in patients with IGF-IR bioactivity or total IGF-I below (black bars) or above (white bars) -2SD. QLS-H Z-scores are shown as mean+SEM.

SF-36 component summaries

The MCS of the SF-36Q increased from 47.4 [38.7-52.8] at baseline to 50.2 [43.1-55.3] at 6 months (p=0.001) and did not further improve after 12 months (49.4 [42.1-54.1], p=0.19). In addition, the MCS of the SF-36Q was not different at 12 months compared to baseline (p=0.12). At 6 and at 12 months the MCS was not different between patients within the various subgroups described above (data not shown).

Over 12 months, the physical component summary (PCS) did not change (47.5 [42.0-54.2] vs. 47.0 [41.9-55.3], p=0.91, vs. 48.3 [39.9-55.4], p=0.66). Males scored higher on the PCS than females throughout the treatment period (6 months: 49.1 [44.0-56.9] vs. 45.5 [40.0-51.8], p=0.01; 12 months: 49.5 [43.7-56.7] vs. 45.90 [36.0-53.4], p=0.03). In addition, throughout the treatment period, CO-GH deficient patients scored higher on the PCS than AO-GH deficient patients (6 months: 52.4 [45.2-58.2] vs. 45.9 [40.0-53.4], p=0.01; 12 months: 54.2 [48.2-58.2] vs. 46.5 [37.7-54.6], p=0.002).

Interestingly, at 12 months, patients with total IGF-I concentrations within in the normal range scored significantly higher on both the MCS and PCS than patients with subnormal IGF-I concentrations (MCS: 51.1 [44.6-55.0] vs. 42.1 [31.9-48.9], p=0.005; PCS: 49.0 [42.3-55.4] vs. 41.0 [33.7-49.9], p=0.02) (Figure 3). In contrast, such differences were not found for IGF-IR bioactivity (>-2SD vs. <-2SD; MCS: 50.7 [43.1-55.8] vs. 48.7 [40.1-53.1], p=0.37; PCS: 48.8 [43.0-56.7] vs. 45.1 [37.4-54.7], p=0.09) (Figure 3).

Relationships between IGF-I parameters and QoL measures after 12 months of GH therapy

At 12 months, there was a significant positive (age-adjusted) correlation between QLS-H Z-score and IGF-IR bioactivity (r=0.28, p=0.01), but not between QLS-H Z-score and total IGF-I (r=0.10, p=0.37) (Figure 4).

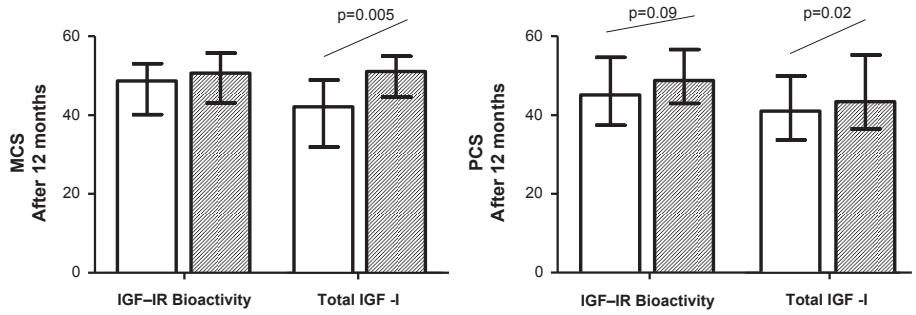

Figure 3.
MCS and PCS after 12 months of GH therapy.
A. Comparison of MCS of the SF-36Q in patients with IGF-IR bioactivity or total IGF-I below (white bars) or above (grey bars) -2SD. MCS is shown as median+interquartile range.
B. Comparison of PCS of the SF-36Q in patients with IGF-IR bioactivity or total IGF-I below (white bars) or above (grey bars) -2SD. PCS is shown as median+interquartile range.

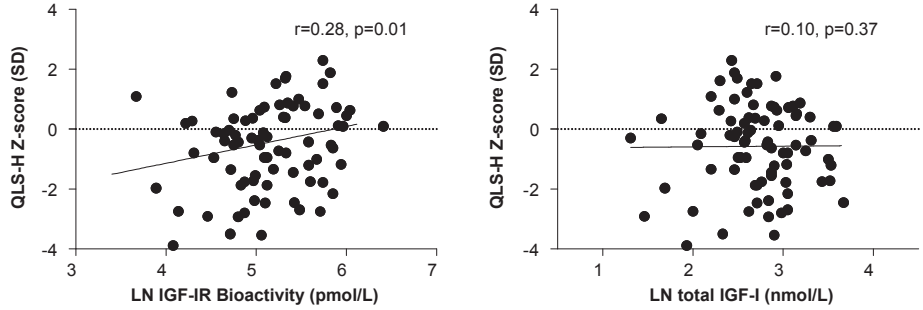

Figure 4.
Relationship between QLS-H Z-scores and the natural logarithm of IGF-IR bioactivity (left panel) and total IGF-I concentrations (right panel) after 12 months of GH therapy.

There was no correlation between the MCS and IGF-IR bioactivity (r= 0.21, p=0.06) or total IGF-I (r= 0.16, p=0.13). There was a significant positive correlation between the PCS and IGF-IR bioactivity (r= 0.35, p=0.001) and total IGF-I (r= 0.31, p=0.003).

DISCUSSION

The QLS-H module is more likely to identify impairments related to the GH deficiency and eventually benefits of GH treatment than the SF-36Q, which was designed to evaluate QoL in the general population[5, 25]. Twelve months of GH replacement therapy of GHD patients did not significantly improve QoL as measured by the disease-specific QLS-H module.

Our results are in contrast to previously published data from Rosilio et al., who reported a significant increase in QLS-H Z-scores after 1 yr of GH treatment in a much larger cohort of GHD patients[5]. The study by Rosalio was performed in 7 countries including the Netherlands. In contrast to that study, in our study the majority of patients was not GH-naive and had already been substituted for GH and other pituitary hormonal deficits for many years.

In our study baseline QLS-H Z-scores were established after 1 month off GH treatment. One month off GH treatment is generally considered to be long enough to restore baselines values for biochemical parameters such as IGF-IR bioactivity and total IGF-I, that existed before the start of the GH therapy[26]. However, our study suggests that this may not be long enough to obtain QoL values back to the level before GH therapy was first started. The fact that baseline QLS-H Z-scores were higher in our study than in the study of Rosalio et al. is in support of this latter possibility. As to yet there is no clear consensus how long a minimal holiday from GH-treatment should be to obtain QoL values back to the level before GH therapy was first started, although a recent study reported significant changes in QoL after 4 months of GH withdrawal[27].

The potential degree of improvement in QoL is generally proportional to the deviation from normality before start of GH therapy[28]. Poorer pretreatment QoL is usually associated with a greater improvement in QoL after administration of GH[29]. Thus the relatively high baseline QLS-H Z-scores in our study at baseline are probably an important factor for the lack of improvement in Qol after restarting GH therapy. However, QLS-H Z-scores also did not significantly increase after 12 months of GH replacement therapy in GH-naive patients. This may be due to the small sample size of GH-naive patients since GH replacement has been found to improve QoL only with a small effect size[30]. Moreover, we found that patients with a longer duration of GHD (and thus a longer exposure time to GH treatment) tended to have higher QLS-H Z-scores than patients with a shorter duration of GHD (Table 2). The latter suggests that GH therapy may further improve QoL even after many years of GH replacement therapy.

At baseline there was no correlation between IGF-IR bioactivity or total IGF-I and QLS-H Z-score. Both IGF-IR bioactivity and total IGF-I significantly increased after 12 months of GH therapy. Although the disease specific QLS-H Z-score had not improved after 12 months, it was significantly correlated to IGF-IR bioactivity but not to total IGF-I. Even more strikingly, we found that in patients in whom IGF-IR bioactivity had not normalized after 12 months, QLS-H Z-scores were significantly lower compared to patients in whom IGF-IR bioactivity had normalized. This discrepancy was not found for total IGF-I.

During GH replacement therapy GH dose was titrated against total IGF-I concentrations and according to recent guidelines, serum IGF-I concentrations were targeted to the middle (Z-score=0)[19]. Nevertheless, after 12 months, IGF-IR bioactivity was still below normal in more than 40% of patients in whom total IGF-I had normalized.

After twelve months of GH replacement therapy both the MCS and the PCS of the SF-36Q had not changed. Only the PCS was positively related to both total IGF-I and IGF-IR bioactivity. Patients with total IGF-I concentrations within the normal range scored significantly higher on the MCS and PCS than patients with IGF-I concentrations below the normal range, while for IGF-IR bioactivity such differences were not found. Our study shows that total IGF-I was more strongly related to changes in the SF-36Q than IGF-IR bioactivity. However, as above discussed, the SF-36Q is a generic instrument, that in contrast to the QLS-H, does not consider QoL parameters that are particular relevant for adult GHD patients[7, 31]

Previous studies have suggested that the IGF-IR KIRA assay is more sensitive than the common total IGF-I immunoassays to detect differences in clinical state[18]. Indeed, previously we have shown that IGF-IR bioactivity, compared to total IGF-I, better distinguished between untreated GH deficient patients and healthy subjects[14].

Our data suggest that IGF-IR bioactivity, compared to total IGF-I, reflects different aspects of QoL during GH replacement therapy of GH deficient patients. In addition, IGF-IR bioactivity was still below normal in more than 40% of patients, despite normalization of total IGF-I. In this respect, it would be interesting to study QLS-H scores in this latter subgroup using IGF-IR bioactivity as parameter to titrate GH dose.

Several limitations of our study should be addressed. Our study had not a randomized placebo-controlled design and GH was titrated on the results of total IGF-I concentrations, but IGF-IR bioactivity was not taken into account. Evaluation of effects of GH replacement is complex: IGF-I concentrations and IGF-IR bioactivity are regulated by many factors other than GH, such as nutrition, the immune system, insulin, cortisol, oestrogen, and last but not least genetic factors[32-33]. Many other factors other than IGF-I determine QoL. They include (abnormal) body composition, decreased exercise capacity, metabolic disturbances, possible neuroendocrine effects in the central nervous system and comorbidities[31]. There is evidence that the clinical manifestations and 'experience' of GHD in adulthood differs according to whether the patient acquired their disease in childhood or adulthood[34]. Unfortunately, the study was not powered to assess differences in QoL between subjects with adult-onset vs. childhood-onset GHD or to access differences between patients with different underlying disease or to study the effect of co-replaced hormones. In addition, the duration of follow-up was relatively short and some GH-mediated effects may only become manifest after long-term GH treatment.

In conclusion, twelve months GH replacement treatment of patients with adult GHD did not significantly improve QoL as measured by the disease-specific QLS-H module. After 12 months, despite normalization of total IGF-I, IGF-IR bioactivity remained subnormal in many subjects after GH treatment. Patients in whom IGF-IR bioactivity had normalized had a significantly higher QLS-H Z-score than patients in whom IGF-IR bioactivity had not normalized. Finally, there was a positive relationship between IGF-IR

bioactivity and the disease specific QLS-H module, whilst this relationship was absent for total IGF-I. In contrast, total IGF-I was more strongly related to changes in the general SF-36Q than IGF-IR bioactivity. This suggests that IGF-IR bioactivity may better reflect disease specific QoL in GH-deficient patients than total IGF-I and that both measurements reflect different aspects of QoL.

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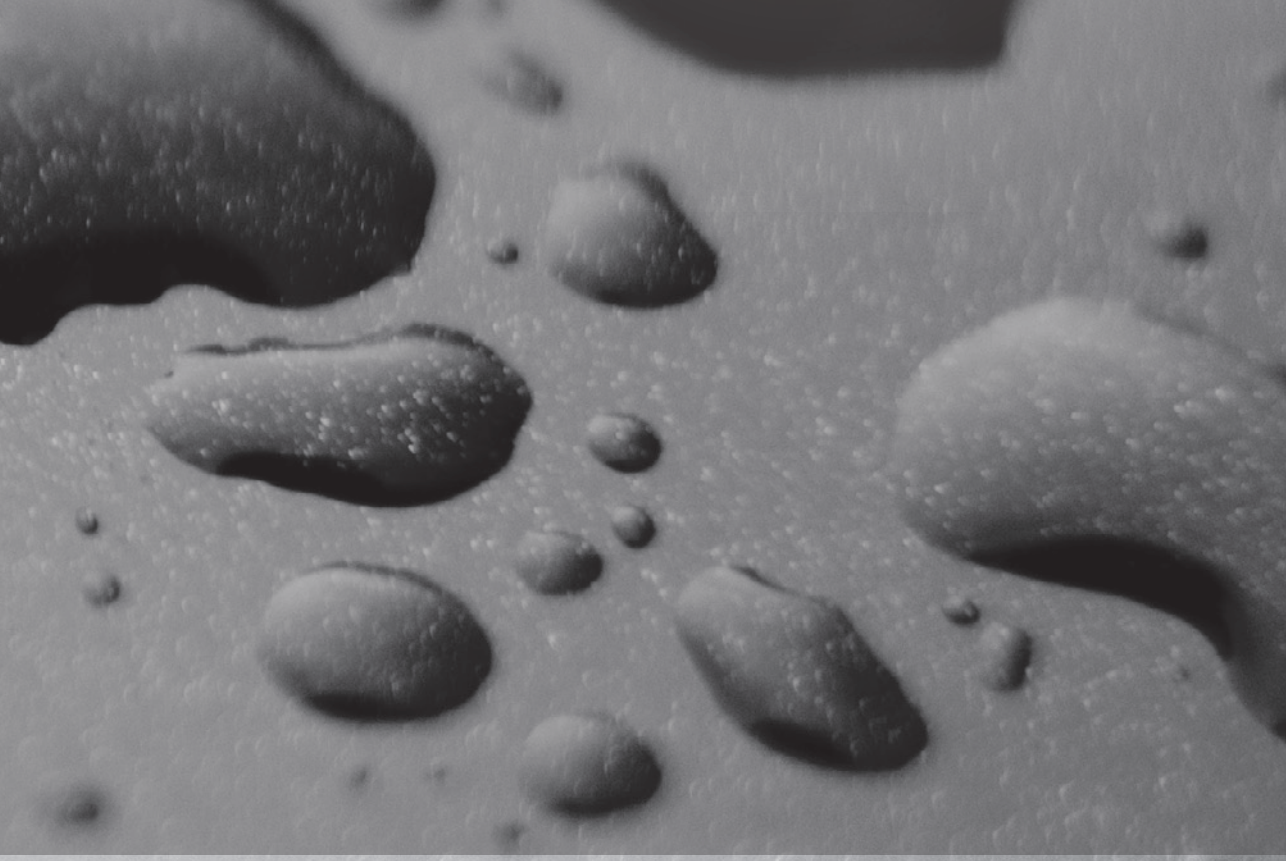
REFERENCES

1. Simpson H, Savine R, Sonksen P, Bengtsson BA, Carlsson L, Christiansen JS, Clemmons D, Cohen P, Hintz R, Ho K, Mullis P, Robinson I, Strasburger C, Tanaka T & Thorner M. Growth hormone replacement therapy for adults: into the new millennium. *Growth Horm IGF Res* 2002 **12** 1-33.
2. McGauley GA. Quality of life assessment before and after growth hormone treatment in adults with growth hormone deficiency. *Acta Paediatr Scand Suppl* 1989 **356** 70-72; discussion 73-74.
3. Badia X, Lucas A, Sanmarti A, Roset M & Ulied A. One-year follow-up of quality of life in adults with untreated growth hormone deficiency. *Clin Endocrinol (Oxf)* 1998 **49** 765-771.
4. Wiren L, Whalley D, McKenna S & Wilhelmsen L. Application of a disease-specific, quality-of-life measure (QoL-AGHDA) in growth hormone-deficient adults and a random population sample in Sweden: validation of the measure by rasch analysis. *Clin Endocrinol (Oxf)* 2000 **52** 143-152.
5. Rosilio M, Blum WF, Edwards DJ, Shavrikova EP, Valle D, Lamberts SW, Erfurth EM, Webb SM, Ross RJ, Chihara K, Henrich G, Herschbach P & Attanasio AF. Long-term improvement of quality of life during growth hormone (GH) replacement therapy in adults with GH deficiency, as measured by questions on life satisfaction-hypopituitarism (QLS-H). *J Clin Endocrinol Metab* 2004 **89** 1684-1693.
6. Ahmad AM, Hopkins MT, Thomas J, Ibrahim H, Fraser WD & Vora JP. Body composition and quality of life in adults with growth hormone deficiency; effects of low-dose growth hormone replacement. *Clin Endocrinol (Oxf)* 2001 **54** 709-717.
7. Blum WF, Shavrikova EP, Edwards DJ, Rosilio M, Hartman ML, Marin F, Valle D, van der Lely AJ, Attanasio AF, Strasburger CJ, Henrich G & Herschbach P. Decreased quality of life in adult patients with growth hormone deficiency compared with general populations using the new, validated, self-weighted questionnaire, questions on life satisfaction hypopituitarism module. *J Clin Endocrinol Metab* 2003 **88** 4158-4167.
8. Herschbach P, Henrich G, Strasburger CJ, Feldmeier H, Marin F, Attanasio AM & Blum WF. Development and psychometric properties of a disease-specific quality of life questionnaire for adult patients with growth hormone deficiency. *Eur J Endocrinol* 2001 **145** 255-265.
9. Murray RD, Skillicorn CJ, Howell SJ, Lissett CA, Rahim A & Shalet SM. Dose titration and patient selection increases the efficacy of GH replacement in severely GH deficient adults. *Clin Endocrinol (Oxf)* 1999 **50** 749-757.
10. Murray RD, Skillicorn CJ, Howell SJ, Lissett CA, Rahim A, Smethurst LE & Shalet SM. Influences on quality of life in GH deficient adults and their effect on response to treatment. *Clin Endocrinol (Oxf)* 1999 **51** 565-573.
11. Le Roith D, Bondy C, Yakar S, Liu JL & Butler A. The somatomedin hypothesis: 2001. *Endocr Rev* 2001 **22** 53-74.
12. Ho KK. Consensus guidelines for the diagnosis and treatment of adults with GH deficiency II: a statement of the GH Research Society in association with the European Society for Pediatric Endocrinology, Lawson Wilkins Society, European Society of Endocrinology, Japan Endocrine Society, and Endocrine Society of Australia. *Eur J Endocrinol* 2007 **157** 695-700.
13. Ghigo E, Aimaretti G, Gianotti L, Bellone J, Arvat E & Camanni F. New approach to the diagnosis of growth hormone deficiency in adults. *Eur J Endocrinol* 1996 **134** 352-356.
14. Varewijck AJ, Lamberts SW, Uitterlinden P, Hofland LJ & Janssen JA. IGF-I bioactivity better reflects growth hormone deficiency than total IGF-I. *J Clin Endocrinol Metab* 2011 **96** 2248-2254.
15. Clemmons DR. IGF-I assays: current assay methodologies and their limitations. *Pituitary* 2007 **10** 121-128.

16. Chen JW, Ledet T, Orskov H, Jessen N, Lund S, Whittaker J, De Meyts P, Larsen MB, Christiansen JS & Frystyk J. A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum. *Am J Physiol Endocrinol Metab* 2003 **284** E1149-1155.
17. Brugts MP, Ranke MB, Hofland LJ, van der Wansem K, Weber K, Frystyk J, Lamberts SW & Janssen JA. Normal values of circulating insulin-like growth factor-I bioactivity in the healthy population: comparison with five widely used IGF-I immunoassays. *J Clin Endocrinol Metab* 2008 **93** 2539-2545.
18. Janssen JAMJL. IGF-I: Pro 's and Con's of a Bioassay. *Horm Res Paediatr* 2011 **76(suppl 1)** 106-110.
19. Cook DM, Yuen KC, Biller BM, Kemp SF & Vance ML. American Association of Clinical Endocrinologists medical guidelines for clinical practice for growth hormone use in growth hormone-deficient adults and transition patients - 2009 update. *Endocr Pract* 2009 **15 Suppl 2** 1-29.
20. Aaronson NK, Muller M, Cohen PD, Essink-Bot ML, Fekkes M, Sanderman R, Sprangers MA, te Velde A & Verrips E. Translation, validation, and norming of the Dutch language version of the SF-36 Health Survey in community and chronic disease populations. *J Clin Epidemiol* 1998 **51** 1055-1068.
21. Ware JE, Jr., Gandek B, Kosinski M, Aaronson NK, Apolone G, Brazier J, Bullinger M, Kaasa S, Leplege A, Prieto L, Sullivan M & Thunedborg K. The equivalence of SF-36 summary health scores estimated using standard and country-specific algorithms in 10 countries: results from the IQOLA Project. International Quality of Life Assessment. *J Clin Epidemiol* 1998 **51** 1167-1170.
22. Ware JE, Jr. & Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. *Med Care* 1992 **30** 473-483.
23. Elmlinger MW, Kuhnel W, Weber MM & Ranke MB. Reference ranges for two automated chemiluminescent assays for serum insulin-like growth factor I (IGF-I) and IGF-binding protein 3 (IGFBP-3). *Clin Chem Lab Med* 2004 **42** 654-664.
24. Taft C, Karlsson J & Sullivan M. Do SF-36 summary component scores accurately summarize subscale scores? *Qual Life Res* 2001 **10** 395-404.
25. Webb SM. Measurements of quality of life in patients with growth hormone deficiency. *J Endocrinol Invest* 2008 **31** 52-55.
26. Gasco V, Corneli G, Beccuti G, Prodam F, Rovere S, Bellone J, Grottoli S, Aimaretti G & Ghigo E. Retesting the childhood-onset GH-deficient patient. *Eur J Endocrinol* 2008 **159 Suppl 1** S45-52.
27. Filipsson Nystrom H, Barbosa EJ, Nilsson AG, Norrman LL, Ragnarsson O & Johannsson G. Discontinuing Long-Term GH Replacement Therapy--A Randomized, Placebo-Controlled Crossover Trial in Adult GH Deficiency. *J Clin Endocrinol Metab* 2012 **97** 3185-3195.
28. Molitch ME, Clemmons DR, Malozowski S, Merriam GR, Shalet SM, Vance ML & Stephens PA. Evaluation and treatment of adult growth hormone deficiency: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* 2006 **91** 1621-1634.
29. Valassi E, Brick DJ, Johnson JC, Biller BM, Klibanski A & Miller KK. Effect of growth hormone replacement therapy on the quality of life in women with growth hormone deficiency who have a history of acromegaly versus other disorders. *Endocr Pract* 2012 **18** 209-218.
30. Deijen JB, Arwert LI, Witlox J & Drent ML. Differential effect sizes of growth hormone replacement on Quality of Life, well-being and health status in growth hormone deficient patients: a meta-analysis. *Health Qual Life Outcomes* 2005 **3** 63.
31. Webb SM, Resmini E, Santos A & Badia X. Quality of Life in Acromegaly and Growth Hormone Deficiency In *Growth Hormone Related Diseases and Therapy*, ch. 12, pp 237-250. Ed H Ken. New York: Humana Press, 2011.
32. Rietveld I, Janssen JA, Hofman A, Pols HA, van Duijn CM & Lamberts SW. A polymorphism in the IGF-I gene influences the age-related decline in circulating total IGF-I levels. *Eur J Endocrinol* 2003 **148** 171-175.

33. Deal C, Ma J, Wilkin F, Paquette J, Rozen F, Ge B, Hudson T, Stampfer M & Pollak M. Novel promoter polymorphism in insulin-like growth factor-binding protein-3: correlation with serum levels and interaction with known regulators. *J Clin Endocrinol Metab* 2001 **86** 1274-1280.
34. Attanasio AF, Lamberts SW, Matranga AM, Birkett MA, Bates PC, Valk NK, Hilsted J, Bengtsson BA & Strasburger CJ. Adult growth hormone (GH)-deficient patients demonstrate heterogeneity between childhood onset and adult onset before and during human GH treatment. Adult Growth Hormone Deficiency Study Group. *J Clin Endocrinol Metab* 1997 **82** 82-88.



Changes in IGF-IR Bioactivity do not parallel changes in Total IGF-during GH treatment of GH-deficient adults

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Submitted

ABSTRACT

Aim: Previously we demonstrated that IGF-IR bioactivity offers advantages in the diagnostic evaluation of adult GHD. It is unknown whether IGF-IR bioactivity can be used to monitor GH therapy. The aim of the present study was to investigate the value of IGF-IR bioactivity for monitoring GH therapy.

Methods: 106 patients (54 m; 52 f) diagnosed with GHD by GH-provocative tests were included; 22 were GH-naive, 84 were already on GH treatment and discontinued therapy 4 weeks before baseline values were established. IGF-IR bioactivity was determined by the IGF-IR KIRA assay, total IGF-I by immunoassay (Immulite). GH doses were titrated to achieve total IGF-I levels within the normal range.

Results: After 12 months, total IGF-I and IGF-IR bioactivity significantly increased (total IGF-I from 8.1 [95%CI 7.3-8.9] to 14.9 [95%CI 13.5-16.4] nmol/l; IGF-IR bioactivity from 115 [95%CI 104-127] to 181 [95%CI 162-202] pmol/l). Total IGF-I was within the normal range in 81% of patients, IGF-IR bioactivity in 51% and remained below normal in more than 40% of patients in whom total IGF-I had normalized. IGF-IR bioactivity was positive related to beta-cell function, while total IGF-I was inversely related to percentage body fat.

*Conclusions***:** During 12 months of GH treatment of GH deficient patients, changes in IGF-IR bioactivity did not parallel changes in total IGF-I. Despite normalization of total IGF-I, IGF-IR bioactivity remained subnormal in a considerable proportion of patients suggesting a relative undertreatment of GHD. At present it is unclear whether our results have any consequences for GH therapy in clinical practice.

INTRODUCTION

Growth hormone deficiency (GHD) in adults is characterised by perturbations in body composition, carbohydrate and lipid metabolism, bone mineral density, cardiovascular risk profile and quality of life[1-2]. At present, diagnosis is made on the basis of one (or two) abnormal provocative tests (GHRH-arginine test or insulin tolerance test[3]. Although GH is considered to be the main regulator of circulating insulin-like growth factor-I (IGF-I), there is a significant overlap of IGF-I levels between healthy and GH deficient subjects. Up to half of all individuals with confirmed GHD have IGF-I levels within the low-normal age-dependent range[4-6]. Recent studies have demonstrated that increased IGF-I levels in healthy subjects are predictive of increased risk of breast-, colon and prostate cancer[7-10].

During GH therapy GH dose is titrated against total IGF-I levels and the consensus guidelines advise that "values should be kept in the age-related normal range"[3, 11]. Thus, the values of total IGF-I can be normal to begin with and yet that is the goal of therapy. Previous studies have shown that the relationship between serum IGF-I response during GH treatment and other treatment effects such as metabolic endpoints and body composition is poor[12-13]. In addition it has been reported that long-term monitoring of GH therapy by using age- and sex-adjusted normal serum total IGF-I values, might lead to different dose adjustments when different total IGF-I immunoassays are used[14].

Many of the methods currently used for measurement of circulating total IGF-I are hampered by interferences of IGF-binding proteins (IGFBPs) that remain after extraction[15]. On the other hand, by extracting IGFBPs, the modifying effects of these proteins on IGF-I action are ignored. In 2003, a kinase receptor activation (KIRA) bioassay was developed by Chen et al. to measure IGF-IR bioactivity at physiological conditions[16]. The principle of this assay is based on quantification of IGF-I receptor (IGF-IR) activation after stimulation with serum in vitro[16]. In this way bioavailable IGF-I is quantified while taking into account the modifying effects of IGFBPs. Previously, we demonstrated that IGF-IR bioactivity offers advantages over total IGF-I in the diagnostic evaluation of adult GHD[6]. As such it can be hypothesized that the measurement of IGF-IR bioactivity can also be used to monitor GH therapy. The aim of the present study was to investigate the value of IGF-IR bioactivity for monitoring GH therapy.

PATIENTS AND METHODS

Study population

This study population has been previously described[17]. Briefly, 106 Patients diagnosed with GHD by GH-provocative tests were included in the present study. 84 patients were diagnosed as Adult Onset GH deficiency (AO-GHD) and 22 as Childhood onset GH de-

ficiency (CO-GHD). 84 patients had already been treated with recombinant human GH, 22 patients were GH-naive. 4 Patients had isolated GD, 11 patients had one additional pituitary hormone deficiency, 23 patients had two additional pituitary hormone deficiencies, 68 patients had had three or more additional pituitary hormone deficiencies.

For the diagnosis of GHD, patients with multiple pituitary hormone deficits had to have a serum GH peak below the cut-off value in one GH provocative test (arginine-GHRH test: GH peak< 16.5 µg/L, insulin tolerance test: GH peak< 5.0 µg/L) and patients with isolated GHD needed two GH provocative tests. Inclusion criteria were: 1. Male or female patients between 18-80 years, 2. Written voluntary informed consent, 3. Subjects using hormone replacement therapy for additional pituitary deficits had to be on an optimized treatment regimen for at least three months prior to inclusion. Exclusion criteria were: 1. Patients who had received certain types of therapies for other reasons such as radiotherapies, surgeries, chemo-or immunotherapies in the three months prior to study start, 2. Female patients who were pregnant or lactating, or who wanted to become pregnant within one year. 3. Subjects who, in the judgement of the investigators, were likely to be non-compliant or uncooperative during the study. From all subjects informed consent was obtained and the study was approved by the medical ethics committee of Erasmus MC.

Study design

After inclusion, all 84 patients who had already been treated with recombinant human GH were asked to discontinue GH treatment for 4 weeks. All patients were studied at baseline and 12 months. The 84 patients were using different brands of recombinant human GH (Genotropin (Pfizer, Capelle aan de Ijssel, The Netherlands) (n=4), Humatrope (Eli Lilly, Houten, The Netherlands) (n=68). Norditropin (Novo Nordisk, Alphen aan de Rijn, The Netherlands) (n=8), Nutropinaq (Ipsen, Hoofddorp, The Netherlands) (n=1) and Zomacton (Ferring, Hoofddorp, The Netherlands) (n=3). After 4 weeks baseline values were established and patients restarted their GH treatment. No patient was switched from one recombinant GH product to another. The 22 GH-naive patients were studied before starting GH therapy and 12 months thereafter. In 14 of the GH-naive patients Norditropin was started, while in 8 patients Nutropinaq was started. GH dose was adjusted targeting serum total IGF-I concentrations to the middle (Z-score=0) of the normal gender- and age-related reference values for the healthy population[18]. The following laboratory assessments were conducted in the fasting state: bioactive IGF-I, total IGF-I, insulin, glucose, IGFBP-1, and IGFBP-3. Moreover, body weight, height and blood pressure were measured. Fat mass percentage was assessed by Bioelectrical Impedance (BIA) (using an Omron Body Composition Monitor with a hand to hand method, Omron, Hoofddorp, The Netherlands). Body mass index (BMI), Waist-to Hip ratio (WHR) were calculated. The updated homeostasis model assessment (HOMA-2) was used to assess insulin resistance

(HOMA-IR), and beta-cell function (HOMA-B) from pairs of fasting glucose and insulin levels[19] . Percentage of IGF-IR bioactivity over total IGF-I was calculated by dividing IGF-IR bioactivity (nmol/L) by total IGF-I (nmol/L) and multiplying by 100. Duration of GHD was defined as the time that had elapsed since GHD diagnosis (confirmed by a GH provocative test) up to the date of inclusion in this study.

Blood measurements

The IGF-IR KIRA assay has been previously described[16, 20]. Briefly, IGF-I binding to the IGF-IR results in autophosphorylation of tyrosine residues located within the intracellular kinase domain, being the first step in the intracellular signalling cascade. The IGF-IR KIRA assay uses a human embryonic kidney (HEK) cell line that is stably transfected with the human IGF-IR gene (HEK IGF-IR) and quantifies phosphorylation of tyrosine residues of the transfected IGF-IR to assess IGF-IR bioactivity. After 48 hours of culture HEK IGF-IR cells were stimulated for 15 minutes at 37°C with increasing known amounts of human recombinant IGF-I (Invitrogen, Breda, The Netherlands) in a range of 0.06-1.0 nmol/L and study serum samples. In addition, 2 control serum samples were tested on every plate to ensure optimal performance. Standards and serum samples were diluted in Krebs-Ringer bicarbonate (KRB) buffer adjusted to pH 7.4 by CO_2 and supplemented with 0.1% human serum albumin (HSA) (Octalbine®) (Octopharma, Lachen, Switzerland). After stimulation cells were lysed. Crude lysates were transferred to a sandwich assay. Wells were coated with a monoclonal antibody directed against the IGF-IR (MAD1) (Novozymes-Gropep, Aidelade, Australia) that was used as capture antibody in a concentration of 5.0µg/mL. An europium labelled monoclonal anti-phosphotyrosine antibody (Eu-PY20) (Perkin-Elmer Life sciences, Groningen, the Netherlands) was used as a detection antibody in a concentration of 1.25µg/mL. Contents were read in a time-resolved fluorometer (Victor2 multilabel counter) (Perkin-Elmer life sciences, Groningen, The Netherlands). Assays were performed in 48 well plates (Corning, NY, USA). For measurements of IGF-IR bioactivity, an IGF-I standard, two internal control samples were included on each culture plate. Serum samples were diluted 1/10. All measurements were done in duplicate. The intra-assay and inter-assay CVs were below 15%.

Serum total IGF-I, IGFBP-3 and insulin were measured by a solid-phase, enzyme-labelled chemiluminescent immunometric assays (Immulite 2000 supplied by Siemens Medical Solutions Diagnostics, Los Angeles, USA)

Both total IGF-I concentrations and IGF-IR bioactivity were compared with the age-specific normative range values for IGF-I that have been published before[20-21]. For total IGF-I normal values have been established in serum samples collected from 1584 healthy individuals, neonates, infants, children, adolescents and adults up to the age of 88 years by an enzyme-labelled chemiluminescent immunometric assays (Immulite 2000)[21]. In the present study, total IGF-I concentrations were also measured by an automated

chemiluminescent assay system (Immulite 2000). The normal ranges for IGF-IR bioactivity have been previously published by our group and have been determined by using the same IGF-IR KIRA bioassay that was used in this study[20]. These normal values were established by measuring IGF-IR bioactivity in serum samples from healthy, non-fasting blood donors (total n = 427), whose ages ranged from 18 to 79 years (median: 44 years).

Both for total IGF-I and IGF-IR bioactivity individual Z-scores were calculated using the following formula: Z-score = (x–average x/S.D.) where x is the actual total IGF-I level or IGF-IR bioactivity, average x is the mean total IGF-I level or IGF-IR bioactivity at that age, and S.D. is standard deviation for the mean at that age.

Serum glucose, total cholesterol, LDL cholesterol and HDL cholesterol were determined with standard laboratory methods. Cholesterol parameters were only measured after 12 months GH treatment.

Statistics

The clinical characteristics of the study population are presented as mean with ranges or SE. The Kolmogorov- Smirnov-test was used to test normality of variables (data were considered to be normally distributed when P> 0.05). For data that did not meet the criteria for normality, logarithmic transformations were applied and are presented as geometric mean with ranges or 95% CI or if logarithmic transformation did not normalize data as median with interquartile range. An unpaired t-test/ one-way ANOVA or Mann-Whitney/ Kruskal-Wallis test were used to test differences in continuous variables within a visit, differences in categorical variables were tested by using the Chi-Squared test. A paired t-test or a Wilcoxon Signed Ranks test were used to test differences between baseline and 12 months of GH therapy. Age-adjusted Pearson 's correlations were calculated between variables.

A P-value of 0.05 or less was considered statistically significant. Data were analysed using SPSS 17 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Baseline characteristics

The left column of table 1 shows the clinical baseline characteristics of the study population. As reported previously[17], at baseline 38% of all patients had total IGF-I levels >-2SD despite a proven GHD by a decreased GH response in provocative test, while this was 22% for IGF-IR bioactivity. At baseline total IGF-I was positively correlated to IGF-IR bioactivity ($r=0.42$, $p<0.001$) and IGFBP-3 ($r=0.67$, $p<0.001$) and inversely related to IGFBP-1 (-0.38, $p=<0.001$). IGF-IR bioactivity was also positively related to IGFBP-3 ($r=0.35$, $p<0.001$) and inversely related to IGFBP-1 (-0.22, $p=0.03$).

Table 1. Changes in clinical parameters during GH treatment

Characteristics study population	**Baseline (mean and range)**	**1 year GH treatment (mean and range)**	**p-value**
Age (yrs)	54.0 # (18.0-79.4)	-	-
Duration of GHD (yrs)	9.5# (0-22)	-	-
GHD-categories (N of subjects)			
AO-GHD	84		
CO- GHD	22		
		-	-
GH-naive	22		
Previous GH treatment	84		
Physical Measurements			
BMI (kg/m^2)	28.4 (17.9-49.8)	28.3 (17.5-50.3)	0.32
Body fat (%)	32.5 (11.9-46.7)	32.0 (5.6-47.3)	0.02*
Waist (cm)	99 (63-154)	96 (70-150)	<0.001*
WHR (cm/cm)	0.98 (0.74-1.19)	0.96 (0.76-1.11)	0.002*
Systolic BP (mmHg)	128 (96-163)	134 (91-195)	0.001*
Diastolic BP (mmHg)	80 (58-102)	82 (57-130)	0.04*
Laboratory testing			
Fasting Glucose level (mmol/L)	4.7# (3.0-11.2)	5.1# (3.9-9.7)	0.001*
Insulin (pmol/L)	36# (13-909)	53# (12-324)	0.03*
HOMA-B score	82# (30-648)	91#(32-323)	0.29
HOMA-IR score	0.7# (0.3-9.3)	1.0#(0.3-6.8)	0.008*
Total IGF-I (nmol/L)	8.1 (2.7-23.0)	14.9 (2.7-39.6)	<0.001*
IGF-IR bioactivity (pmol/L)	115 (20-337)	183 (39-606)	<0.001*
IGF-IR bioactivity/ total IGF-I (%)	1.4 (0.28-5.63)	1.2 (0.35-7.69)	0.003
IGFBP-1 (µg/L)	23.0 (0.4-131.2)	17.2 (0.4-124.6)	<0.001*
IGFBP-3 (mg/L)	3.4 (1.2-7.1)	4.0 (1.5-7.5)	<0.001*

Changes in clinical parameters during GH treatment are shown as mean (or median (#)) and range.
BMI= Body Mass Index, Body Fat (as % of body mass), Systolic BP= Systolic Blood Pressure, Diastolic BP= Diastolic Blood Pressure, WHR= Waist-to-Hip Ratio, HOMA-B= Homeostasis Model Assessment of beta-cell function, HOMA-IR score= Homeostasis Model Assessment of insulin resistance.
* differences between baseline and 12 months of GH treatment statistically significant.

Changes in IGF-I parameters after 12 months of GH therapy

The right column of table 1 shows the changes in IGF-I parameters. Total IGF-I increased from 8.1 nmol/L [95%CI 7.3-8.9] at baseline to 14.9 nmol/L [95% 13.5-16.4] at 12 months (p<0.001). IGF-IR bioactivity increased from 115 pmol/L [95%CI 103-127] to 183 pmol/L [95%CI 164-204], (p<0.001) after 12 months. The percentage of IGF-IR bioactivity divided by total IGF-I decreased van 1.4% to 1.2% (p=0.003). IGFBP-3 increased from 3.3 mg/L [95%CI 3.1-3.5] at baseline to 4.0 mg/L (95%CI 3.9-4.3] after 12 months (p<0.001). IGFBP-1

decreased from 23.0 µg/L [95%CI 20.1-29.9] at baseline to 17.2 µg/L [95%CI 14.3-22.5] at 12 months (p<0.001).

Mean Z-score for total IGF-I increased from -2.3 ± 0.1 SD at baseline to -0.6 ± 0.1 SD at 12 months (p<0.001). Mean Z-score for IGF-IR bioactivity increased from -2.5 ± 0.1 SD to -1.8 ±0.1 SD after 12 months (p<0.001) while mean Z-score for IGFBP-3 increased -1.6 ± 0.1SD at baseline to: -0.6± 0.1SD (p<0.001). The percentage of patients with total IGF-I levels within the normal range increased from 38% to 81% (Figure 1). For IGF-IR bioactivity this percentage increased from 22% to 51% (Figure 1).

IGF-IR bioactivity remained below normal in more than 40% of patients in whom total IGF-I had normalized.

At 12 months the mean GH dose was 0.20 mg/day (range: 0.05-2.1). Patients in whom total IGF-I had normalized used lower doses of GH compared to those in whom it had not normalized (0.19 compared to 0.28 mg/day, p=0.02). Also they had higher IGFBP-3 levels (4.2 vs. 3.4 mg/L, p=0.001) and IGF-IR bioactivity (202 vs. 113 pmol/L, p=<0.001). In contrast there was no difference in IGFBP-1 levels (15.5 vs. 27.2 µg/L, p=0.07).

GH dose did not differ between patients in whom IGF-IR bioactivity had normalized compared to those in whom it remained subnormal (0.22 vs. 0.19 mg/day, p=0.21). Patients in whom IGF-IR bioactivity had normalized, had significantly higher total IGF-I levels (17.7 vs. 12.6 nmol/L, p<0.001) and higher IGFBP-3 levels (4.3 vs. 3.8 mg/L, p=0.04). In contrast, IGFBP-1 levels were similar (0.15 vs. 0.17 µg/L, p=0.65) compared to those in whom IGF-IR bioactivity had not normalized.

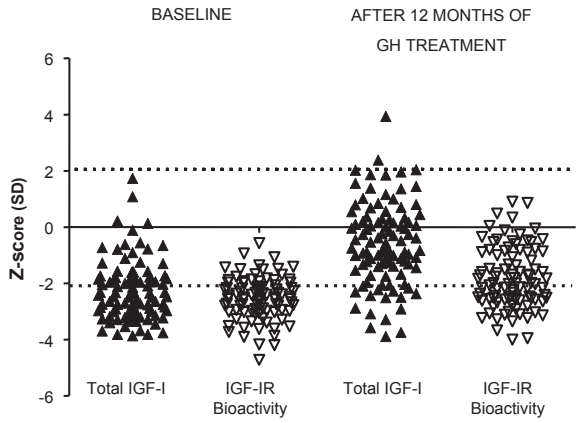

Figure 1.

Z-scores of total IGF-I (black triangles) and IGF-IR bioactivity (open triangles) at baseline (left) and after 12 months of GH therapy (right). Each triangle represents a patient. The horizontal dotted lines represent the normal range in the healthy population (from -2SD to +2 SD). At baseline 38% of all patients had total IGF-I levels >-2SD compared to 22% for IGF-IR bioactivity. After 12 months of GH treatment, this percentage increased to 81% for total IGF-I, while for IGF-IR bioactivity it increased to 51%.

At 12 months, total IGF-I was positively related to IGF-IR bioactivity (r=0.53, p=<0.001) and IGFBP-3 (r=0.50, p<0.001) and inversely related to IGFBP-1 (r=-0.20, p=0.05). There was no relationship between total IGF-I and GH dose (r=0.06, p=0.53). IGF-IR bioactivity was positively related to IGFBP-3 (r=0.29, p=0.005) but not to IGFBP-1 (r=-0.12, p=0.25). There was no relation between IGF-IR bioactivity and GH dose (r=0.15, p=0.16).

Changes in clinical parameters after 12 months of GH therapy

The right column of table 1 shows the changes in clinical parameters. After 12 months of GH therapy, fat mass percentage, waist and WHR significantly decreased. In addition, systolic and diastolic blood pressure significantly increased. Also glucose concentrations, insulin levels and insulin resistance significantly increased, while beta-cell function did not change.

Table 2 shows age-adjusted interrelationships between IGF-I parameters and several metabolic parameters. Total IGF-I was inversely related to fat mass percentage (r=-0.29, p=0.02) but not to other parameters (Table 2). IGF-IR bioactivity was positively related to HOMA-B (r=0.28, p=0.02) (Table 2). There was no relationship between IGF-IR bioactivity and insulin, glucose or HbA_{1C} (Table 2). IGF-IR bioactivity was also not related to lipid parameters.

There were no differences in metabolic parameters between patients in whom total IGF-I or IGF-IR bioactivity had normalized compared to those in whom it had not.

DISCUSSION

We recently reported, in the same study population, that IGF-IR bioactivity in untreated GH-deficient patients was more frequently below the normal range than total IGF-I, demonstrating that measurement of IGF-IR bioactivity may offer advantages over the measurement of total IGF-I in the diagnosis of GHD[6]. Previously, it as been suggested that the IGF-IR KIRA assay is more sensitive than common total IGF-I immunoassays to detect differences in clinical state during monitoring of therapeutic interventions[22]. The most striking finding in our present study was that during 12 months of GH treatment, changes in IGF-IR bioactivity did not parallel changes in total IGF-I. Interestingly, IGF-IR bioactivity remained subnormal despite normalization of total IGF-I in a considerable proportion of patients.

We observed a significant positive relationship between IGF-IR bioactivity and beta-cell function as assessed by HOMA. GH has been shown to stimulate pancreatic beta-cell proliferation directly or via IGF-I[23-24]. IGF-I is considered to play an essential role in enhancing beta-cell function[23-25] .We observed no relationship between IGF-IR bioactivity

Table 2. Relationships (age-adjusted) between IGF-I and clinical parameters after 12 months of GH treatment

	BMI	Waist	WHR	Body Fat	Systolic BP	Diastolic BP	Glucose	HbA_{1C}	Insulin	HOMA-B	HOMA-IR	Cholesterol	HDL-cholesterol	LDL-cholesterol
Total IGF-I (p-value)	-0.002 (0.99)	0.03 (0.78)	0.14 (0.24)	-0.29 (0.02*)	0.14 (0.23)	0.09 (0.48)	-0.12 (0.32)	0.04 (0.75)	0.13 (0.29)	0.16 (0.18)	0.10 (0.39)	-0.04 (0.77)	0.07 (0.54)	-0.03 (0.81)
IGF-IR bioactivity (p-value)	0.03 (0.82)	0.02 (0.87)	0.03 (0.82)	0.03 (0.84)	0.03 (0.81)	-0.07 (0.57)	0.12 (0.32)	-0.20 (0.10)	0.15 (0.21)	0.28 (0.02*)	0.14 (0.25)	0.14 (0.23)	0.11 (0.36)	0.12 (0.30)

BMI= Body Mass Index, WHR= Waist-to-Hip Ratio, Body Fat (as percentage of body mass), Systolic BP= Systolic Blood Pressure, Diastolic BP= Diastolic Blood Pressure , HOMA-B= Homeostasis Model Assessment of beta-cell function, HOMA-IR score= Homeostasis Model Assessment of insulin resistance, HDL= High Density Lipoprotein, LDL= Low Density Lipoprotein.

* Statistically significant ($p<0.05$)

and glucose or insulin resistance. In this respect our study has limitations; firstly, we used fasting insulin and glucose based indices only, rather than the hyperinsulinemic clamp to assess insulin sensitivity. Secondly, total IGF-I concentrations were used to titrate GH doses, while changes in IGF-IR bioactivity were not taken into account. As a consequence total IGF-I concentrations had normalized in 81% of patients while IGF-IR bioactivity had normalized in only 51% of patients after 12 months of GH treatment.

In our study, most patients were not GH-naive and had already been substituted for GH and other pituitary hormonal deficits for many years. Nevertheless, after 12 months of GH treatment total IGF-I was inversely related to fat mass percentage suggesting an improvement in body composition. But despite a significant decrease in fat mass percentage after 12 months of GH treatment, waist circumference and WHR, fasting glucose, fasting insulin levels and insulin sensitivity as measured by HOMA increased. Total IGF-I was not related to waist circumference, WHR or insulin sensitivity. Thus the improvement in body composition did not coincide with improvements in insulin sensitivity. This may be explained by the action of GH to induce insulin resistance, independent of IGF-I[26]. Also, probably as a consequence of this latter action of GH, long-term studies in which GH doses were titrated to normalise total IGF-I levels have reported a decrease, no change or an increase in insulin sensitivity[27-31].

The question arises why changes in IGF-IR bioactivity did not parallel changes in total IGF-I. First of all, as discussed above, the IGF-IR KIRA bioassay takes into account the modifying effects of IGFBPs on IGF-I action, while in the presently available IGF-I immunoassays these modifying effects are ignored due to extraction procedures. However, interestingly, both at baseline and after 12 month of GH treatment, total IGF-I was more strongly related to IGFBPs than IGF-IR bioactivity.

An alternative explanation could be, that kinetics of IGF-IR bioactivity in the circulation differed strikingly from those of total IGF-I during GH therapy. In favour of this latter possibility, it was previously found that GH administration to GH deficient patients caused only a parallel increase in circulating (immunoreactive) free IGF-I and total IGF-I levels the first 10 hrs after subcutaneous injection[32]. However, thereafter free IGF-I started to decrease while total IGF-I concentrations levelled off[32]. Furthermore, a close temporal relationship has been reported between serum GH and free IGF-I levels: GH serum peaked concomitantly with the rise in free IGF-I, and at the time when GH was not longer detectable in serum, free IGF-I started to decrease[32]. Such relationship was not found for total IGF-I. It is very likely that such relationship also exists between GH and IGF-IR bioactivity. After subcutaneous injection maximum serum GH is reached after about 260 minutes[33]. In our study, fasting blood samples were drawn in the morning, while the last GH injection had been given the evening before, thus long after maximum serum GH concentrations were reached. However, the fact that in our study population IGF-IR

bioactivity was already lower than total IGF-I concentrations before starting GH treatment, argues against the possibility that differences in kinetics caused the discrepancy between total IGF-I and IGF-IR bioactivity during GH therapy.

As mentioned above, GH doses were titrated against circulating total IGF-I concentrations and not against IGF-IR bioactivity. A third explanation for the observed discrepancy between total IGF-I and IGF-IR bioactivity during GH therapy may be that total IGF-I concentrations did not adequately reflect the actual IGF-I levels. The assay for total IGF-I in our study has been calibrated against the WHO International Reference Reagent for IGF-I Immunoassays (WHO IRR 87/518). The WHO IRR 87/518 is of low purity (44%) and the assigned IGF-I protein content is higher than the value that can be determined by quantitative analysis[34]. This may have resulted in falsely elevated total IGF-I concentrations. As a consequence, the effect of GH treatment on total IGF-I concentrations may have been (systematically) overestimated. This latter option is supported by our findings that in a considerable proportion of patients IGF-IR bioactivity was still subnormal after 12 months of GH treatment. In contrast to the total IGF-I immunoassay, the IGF-IR KIRA assay has been calibrated against a recombinant human IGF-I standard of high purity (>97%). Thus IGF-IR bioactivity measured by the IGF-IR KIRA assay may better correspond to the actual (bioactive) IGF-I present in the circulation than total IGF-I assays reflect actual total IGF-I levels. As a consequence, subnormal IGF-IR bioactivity might be real and reflect a relative undertreatment of GHD despite normalization of total IGF-I concentrations.

Quarmby et al.[34] previously showed that calibration against WHO IRR 87/518 was the cause for systemic discrepancies between the Genentech total IGF-I normal ranges (not calibrated against WHO IRR 87/518) and the normal ranges of several other commercially available total IGF-I assays (all calibrated against WHO IRR 87/518). Recently, a new WHO Reference Standard has become available (IS 02/254). In contrast to the WHO IRR 87/518, the new Reference Standard is a >97%-pure recombinant and has been well characterized by the NIBSC[35]. It is at present unknown as to whether or not introduction of this new IGF-I standard in total IGF-I immunoassays will be able to eliminate the observed discrepancy between total IGF-I and IGF-IR bioactivity in our study.

In order to finally answer the question whether IGF-IR bioactivity could be a valuable tool to monitor GH therapy in GH deficient patients and whether titrating GH dose against IGF-IR bioactivity results in a better (metabolic) clinical outcome than titrating against total IGF- concentrations, we believe it will be necessary to perform a prospective randomized placebo- controlled study in which in one group GH dose is titrated against IGF-IR bioactivity, while in the other group GH dose is titrated against total IGF-I concentrations.

In conclusion, during 12 months of GH treatment of GH deficient patients, changes in IGF-IR bioactivity did not parallel changes in total IGF-I. Despite normalization of total

IGF-I, IGF-IR bioactivity remained subnormal in a considerable proportion of patients suggesting a relative undertreatment of GHD. At present it is unclear whether our results have any consequences for GH therapy in clinical practice.

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REFERENCES

1. Simpson H, Savine R, Sonksen P, Bengtsson BA, Carlsson L, Christiansen JS, Clemmons D, Cohen P, Hintz R, Ho K, Mullis P, Robinson I, Strasburger C, Tanaka T & Thorner M. Growth hormone replacement therapy for adults: into the new millennium. *Growth Horm IGF Res* 2002 **12** 1-33.
2. Rosilio M, Blum WF, Edwards DJ, Shavrikova EP, Valle D, Lamberts SW, Erfurth EM, Webb SM, Ross RJ, Chihara K, Henrich G, Herschbach P & Attanasio AF. Long-term improvement of quality of life during growth hormone (GH) replacement therapy in adults with GH deficiency, as measured by questions on life satisfaction-hypopituitarism (QLS-H). *J Clin Endocrinol Metab* 2004 **89** 1684-1693.
3. Molitch ME, Clemmons DR, Malozowski S, Merriam GR & Vance ML. Evaluation and treatment of adult growth hormone deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab* 2011 **96** 1587-1609.
4. Svensson J, Johannsson G & Bengtsson BA. Insulin-like growth factor-I in growth hormone-deficient adults: relationship to population-based normal values, body composition and insulin tolerance test. *Clin Endocrinol (Oxf)* 1997 **46** 579-586.
5. Ghigo E, Aimaretti G, Gianotti L, Bellone J, Arvat E & Camanni F. New approach to the diagnosis of growth hormone deficiency in adults. *Eur J Endocrinol* 1996 **134** 352-356.
6. Varewijck AJ, Lamberts SW, Uitterlinden P, Hofland LJ & Janssen JA. IGF-I bioactivity better reflects growth hormone deficiency than total IGF-I. *J Clin Endocrinol Metab* 2011 **96** 2248-2254.
7. Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, Hennekens CH & Pollak M. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science* 1998 **279** 563-566.
8. Hankinson SE, Willett WC, Colditz GA, Hunter DJ, Michaud DS, Deroo B, Rosner B, Speizer FE & Pollak M. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. *Lancet* 1998 **351** 1393-1396.
9. Shaneyfelt T, Husein R, Bubley G & Mantzoros CS. Hormonal predictors of prostate cancer: a meta-analysis. *J Clin Oncol* 2000 **18** 847-853.
10. Juul A. Serum levels of insulin-like growth factor I and its binding proteins in health and disease. *Growth Horm IGF Res* 2003 **13** 113-170.
11. Consensus guidelines for the diagnosis and treatment of adults with growth hormone deficiency: summary statement of the Growth Hormone Research Society Workshop on Adult Growth Hormone Deficiency. *J Clin Endocrinol Metab* 1998 **83** 379-381.
12. Johannsson G, Grimby G, Sunnerhagen KS & Bengtsson BA. Two years of growth hormone (GH) treatment increase isometric and isokinetic muscle strength in GH-deficient adults. *J Clin Endocrinol Metab* 1997 **82** 2877-2884.
13. Johannsson G, Sverrisdottir YB, Ellegard L, Lundberg PA & Herlitz H. GH increases extracellular volume by stimulating sodium reabsorption in the distal nephron and preventing pressure natriuresis. *J Clin Endocrinol Metab* 2002 **87** 1743-1749.
14. Anckaert E, Schiettecatte J, Vanbesien J, Smitz J, Velkeniers B & De Schepper J. Variability among five different commercial IGF-1 immunoassays in conditions of childhood-onset GH deficiency and GH therapy. *Acta Clin Belg* 2006 **61** 335-339.
15. Clemmons DR. IGF-I assays: current assay methodologies and their limitations. *Pituitary* 2007 **10** 121-128.
16. Chen JW, Ledet T, Orskov H, Jessen N, Lund S, Whittaker J, De Meyts P, Larsen MB, Christiansen JS & Frystyk J. A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum. *Am J Physiol Endocrinol Metab* 2003 **284** E1149-1155.

17. Varewijck AJ, Lamberts SWJ, Neggers SJCMM, Hofland LJ & Janssen JAMJL. IGF-I bioactivity might reflect different aspects of Quality of Life than Total IGF-I in GH Deficient patients during GH treatment. *J Clin Endocrinol Metab 2013* ***98*** *761-768.*
18. Cook DM, Yuen KC, Biller BM, Kemp SF & Vance ML. American Association of Clinical Endocrinologists medical guidelines for clinical practice for growth hormone use in growth hormone-deficient adults and transition patients - 2009 update. *Endocr Pract* 2009 **15 Suppl 2** 1-29.
19. Levy JC, Matthews DR & Hermans MP. Correct homeostasis model assessment (HOMA) evaluation uses the computer program. *Diabetes Care* 1998 **21** 2191-2192.
20. Brugts MP, Ranke MB, Hofland LJ, van der Wansem K, Weber K, Frystyk J, Lamberts SW & Janssen JA. Normal values of circulating insulin-like growth factor-I bioactivity in the healthy population: comparison with five widely used IGF-I immunoassays. *J Clin Endocrinol Metab* 2008 **93** 2539-2545.
21. Elmlinger MW, Kuhnel W, Weber MM & Ranke MB. Reference ranges for two automated chemiluminescent assays for serum insulin-like growth factor I (IGF-I) and IGF-binding protein 3 (IGFBP-3). *Clin Chem Lab Med* 2004 **42** 654-664.
22. Janssen JAMJL. IGF-I: Pro 's and Con's of a Bioassay. *Horm Res Paediatr* 2011 **76(suppl 1)** 106-110.
23. Hugl SR, White MF & Rhodes CJ. Insulin-like growth factor I (IGF-I)-stimulated pancreatic beta-cell growth is glucose-dependent. Synergistic activation of insulin receptor substrate-mediated signal transduction pathways by glucose and IGF-I in INS-1 cells. *J Biol Chem* 1998 **273** 17771-17779.
24. Rhodes CJ. IGF-I and GH post-receptor signaling mechanisms for pancreatic beta-cell replication. *J Mol Endocrinol* 2000 **24** 303-311.
25. Kulkarni RN, Holzenberger M, Shih DQ, Ozcan U, Stoffel M, Magnuson MA & Kahn CR. beta-cell-specific deletion of the Igf1 receptor leads to hyperinsulinemia and glucose intolerance but does not alter beta-cell mass. *Nat Genet* 2002 **31** 111-115.
26. Bondy CA, Underwood LE, Clemmons DR, Guler HP, Bach MA & Skarulis M. Clinical uses of insulin-like growth factor I. *Ann Intern Med* 1994 **120** 593-601.
27. Arafat AM, Mohlig M, Weickert MO, Schofl C, Spranger J & Pfeiffer AF. Improved insulin sensitivity, preserved beta cell function and improved whole-body glucose metabolism after low-dose growth hormone replacement therapy in adults with severe growth hormone deficiency: a pilot study. *Diabetologia* 2010 **53** 1304-1313.
28. Yuen K, Cook D, Ong K, Chatelain P, Fryklund L, Gluckman P, Ranke MB, Rosenfeld R & Dunger D. The metabolic effects of short-term administration of physiological versus high doses of GH therapy in GH deficient adults. *Clin Endocrinol (Oxf)* 2002 **57** 333-341.
29. Claessen KM, Appelman-Dijkstra NM, Adoptie DM, Roelfsema F, Smit JW, Biermasz NR & Pereira AM. Metabolic Profile in Growth Hormone-Deficient (GHD) Adults after Long-Term Recombinant Human Growth Hormone (rhGH) Therapy. *J Clin Endocrinol Metab* 2012.
30. Yuen KC & Dunger DB. Therapeutic aspects of growth hormone and insulin-like growth factor-I treatment on visceral fat and insulin sensitivity in adults. *Diabetes Obes Metab* 2007 **9** 11-22.
31. Arwert LI, Roos JC, Lips P, Twisk JW, Manoliu RA & Drent ML. Effects of 10 years of growth hormone (GH) replacement therapy in adult GH-deficient men. *Clin Endocrinol (Oxf)* 2005 **63** 310-316.
32. Lee PD, Durham SK, Martinez V, Vasconez O, Powell DR & Guevara-Aguirre J. Kinetics of insulin-like growth factor (IGF) and IGF-binding protein responses to a single dose of growth hormone. *J Clin Endocrinol Metab* 1997 **82** 2266-2274.
33. Janssen YJ, Frolich M & Roelfsema F. The absorption profile and availability of a physiological subcutaneously administered dose of recombinant human growth hormone (GH) in adults with GH deficiency. *Br J Clin Pharmacol* 1999 **47** 273-278.

34. Quarmby V, Quan C, Ling V, Compton P & Canova-Davis E. How much insulin-like growth factor I (IGF-I) circulates? Impact of standardization on IGF-I assay accuracy. *J Clin Endocrinol Metab* 1998 **83** 1211-1216.
35. Clemmons DR. Consensus statement on the standardization and evaluation of growth hormone and insulin-like growth factor assays. *Clin Chem* 2011 **57** 555-559.

The Value of Circulating IGF-IR Bioactivity in Untreated Acromegaly

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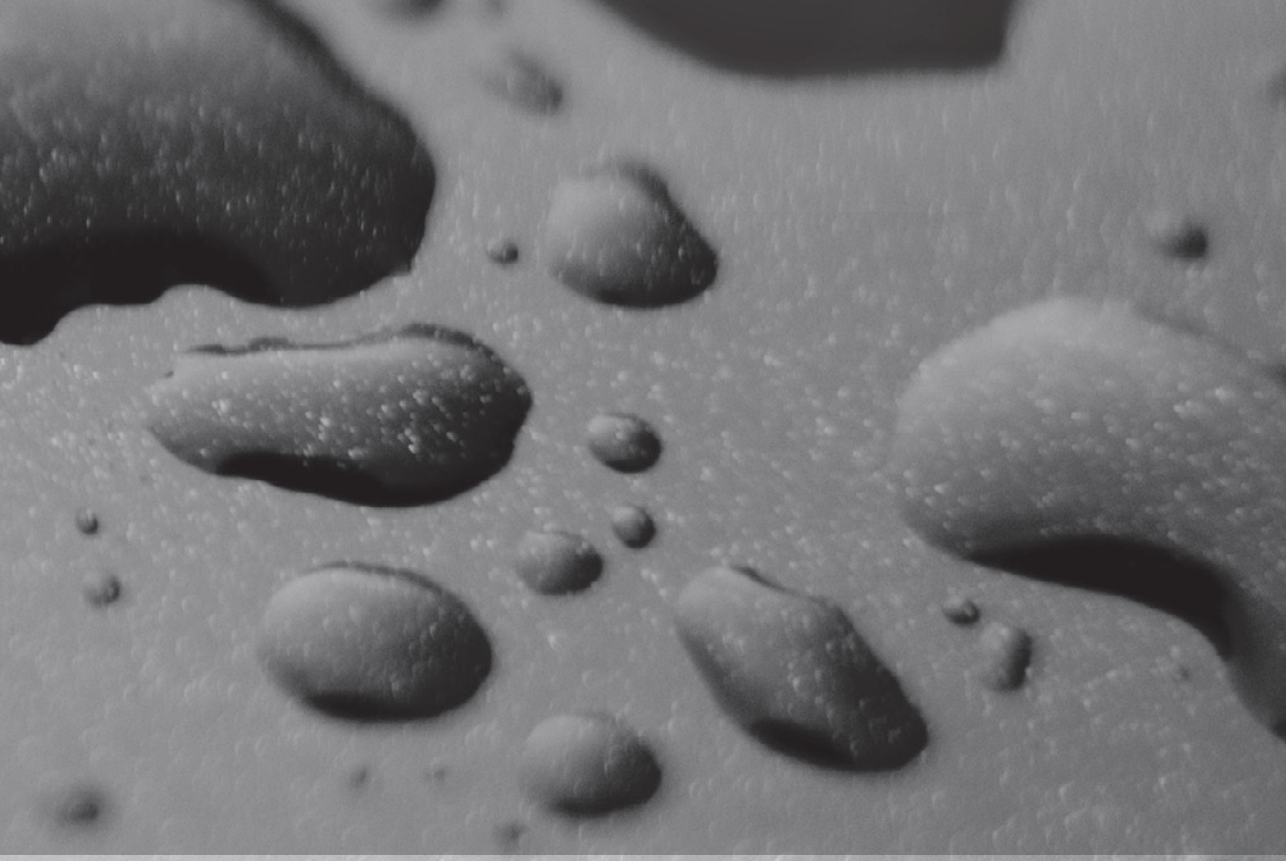
Submitted



Circulating IgGs may modulate IGF-I receptor stimulating activity in a subset of patients with Graves' Ophthalmopathy

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ABSTRACT

Aim: There is a close association between levels of TSH binding inhibitory immunoglobulins (TBII) and Graves' Ophthalmopathy (GO). In addition to the TSH receptor, the IGF-I receptor (IGF-IR) has been proposed to be a second autoantigen that plays a role in the pathogenesis of GO. The aim of this study was to investigate relationships between TBII and serum IGF-IR stimulating activity in relationship to age in patients with GO.

Methods: Prospective study of 70 patients with GO (26 euthyroid, 39 subclinical hyperthyroid, 5 hyperthyroid; m: 8, f: 62; age: 47.9 ± 1.0 yrs). Patients were graded according to Clinical Activity Score (CAS). IGF-IR stimulating activity was determined by IGF-IR Kinase Receptor Activation (KIRA) assay; TBII by immunoassay (Trak). Protein G magnetic beads were used to deplete serum of IgGs.

Results: TBII and CAS were positively related (r=0.30, p=0.01). In subjects with TBII above mean+1SD, IGF-IR stimulating activity was positively related to age (r=0.43, p=0.05), while such relationship was absent for subjects with TBII below mean+1SD (r=-0.04, p=0.81). Depletion of IgGs from sera of patients with both TBII above mean+1SD and IGF-IR stimulating activity above mean-1SD decreased IGF-IR stimulating activity, while depletion in patients with TBII above mean+1SD but IGF-IR stimulating activity below mean-1SD did not change IGF-IR stimulating activity.

Conclusions: In subjects with TBII above mean+1SD we observed an increase of IGF-IR stimulating activity with age. In a subgroup of these patients, depletion of IgGs significantly decreased IGF-IR stimulating activity suggesting that, in a subset of patients with GO, IgGs may have IGF-IR stimulating activities.

INTRODUCTION

Graves' disease (GD) is an autoimmune thyroid disorder in which immunoglobulins are produced that may directly stimulate the TSH-receptor (TSH-R) in the thyroid gland leading to hyperthyroidism. These TSH-R stimulating immunoglobulins may not only influence thyroid function but likely also mediate extrathyroidal manifestations of GD such as Graves' ophthalmopathy (GO).

More than 20 years ago it was reported that immunoglobulins of GD patients (GD-IgGs) had the ability to immunoprecipitate with the insulin-like growth factor-I (IGF-I) receptor (IGF-IR)[1]. In addition, these immunoglobulins could displace radiolabelled IGF-I from IGF-IR binding sites on orbital fibroblasts[2]. Smith et al. previously reported that GD-IgGs could stimulate the production of the T-cell chemoattractants interleukin (IL)-16, RANTES (regulated upon activation normal T-cell expressed and secreted) and hyaluronan in orbital fibroblasts of patients with GD[3-4]. These effects were blocked by monoclonal antibodies directed against the IGF-IR suggesting that these actions were mediated through pathways independent of the TSH-R.

These findings have raised the hypothesis that in (a subset of) Graves' patients immunoglobulins may stimulate the IGF-IR and contribute to GO. However, independent confirmation of this hypothesis has yet not been obtained.

Recently, a specific IGF-IR kinase receptor activation (KIRA) assay was developed to determine serum IGF-IR activating capacity (IGF-IR stimulating activity)[5-6]. The principle of this assay is based on quantification of autophosphorylation of tyrosine residues of the IGF-IR in response to stimulation with human serum in vitro[5].

In the present study, by using the IGF-IR KIRA assay, we studied whether sera of patients with GO could differentially stimulate the IGF-IR.

MATERIALS AND METHODS

Study Population

We performed a prospective clinical study in 70 consecutive GO patients, referred to the combined thyroid– eye clinic in the Academic Medical Center in Amsterdam between 2005 and 2010. 26 patients were euthyroid under antithyroid therapy (TSH 0.5-4.0 mU/L, fT4 <23 pmol/L and T3 <2.75nmol/L), whereas 39 patients had subclinical hyperthyroidism (TSH <0.5mU/L, fT4 <23pmol/L and T3 <2.75nmol/L); 5 patients were still hyperthyroid (TSH <0.5mU/L, fT4 <23pmol/L but T3 >2.75 nmol/L). In a single day all patients visited the laboratory, the endocrinologist, the orthoptist, and the ophthalmologist. They underwent CT scans and if necessary a visual field examination. At the end of the

day, patients were evaluated in a combined session by consultants in ophthalmology and endocrinology. All patients were classified according to the Clinical Activity Score (CAS). Informed consent was obtained from all patients, and the study was approved by the Medical Ethics Committee of the Academic Medical Center in Amsterdam, The Netherlands.

Blood Measurements

The IGF-IR KIRA assay has been previously described[5-6]. Briefly, IGF-I binding to the IGF-IR results in autophosphorylation of tyrosine residues located within the intracellular kinase domain, being the first step in the intracellular signalling cascade. The IGF-IR KIRA assay uses a HEK cell line that is stably transfected with the human IGF-IR gene (HEK IGF-IR) and quantifies phosphorylation of tyrosine residues of the transfected IGF-IR to assess IGF-IR stimulating activity. After 48 hours of culture, HEK IGF-IR cells were stimulated for 15 minutes at 37°C with increasing amounts of human recombinant IGF-I (Invitrogen, Breda, The Netherlands) in a range of 0.06-1.0 nmol/L or serum samples. In addition, 2 control serum samples were tested on every plate to ensure optimal performance. Standards and serum samples were diluted in Krebs-Ringer bicarbonate (KRB) buffer adjusted to pH 7.4 by CO_2 and supplemented with 0.1% human serum albumin (HSA) (Octalbine®) (Octopharma, Lachen, Switzerland). After stimulation cells were lysed. Crude lysates were transferred to a 96–wells plate. The wells were coated with a monoclonal antibody directed against the IGF-IR (MAD1) (Novozymes-Gropep, Aidelade, Australia) that was used as capture antibody in a concentration of 5.0 µg/mL diluted in antibody coating buffer. A biotinylated anti-phospho-tyrsosine monoclonal antibody (BAM1676) (R&D Systems Europe Ltd, Abingdon, UK) in a concentration of 0.2 µg/mL together with streptavidin labelled europium (DELFIA Eu-N1) (Perkin-Elmer Life sciences, Groningen, the Netherlands) in a concentration of 50 pmol/L were used as detection antibodies. Contents were read in a time-resolved fluorometer (Victor2 multilabel counter) (Perkin-Elmer life sciences, Groningen, The Netherlands). Assays were done in 48 well plates (Corning, NY, USA). Serum samples were diluted 1/10. All measurements were done in duplicate. The intra- and interassay coefficients of variation were <15%.

Total serum IGF-I was measured by a solid-phase, enzyme-labelled chemiluminescent immunometric assays (intra-assay CVs was 3.9% and inter-assay CVs was 7.7%) (Immulite 2000 supplied by Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA).

Both total IGF-I levels and IGF-IR stimulating activity were compared with the age-specific normative range values for IGF-I that have been published previously[7-8]. For total

IGF-I normal values have been established in serum samples collected from 1584 healthy individuals, neonates, infants, children, adolescents and adults up to the age of 88 years by an enzyme-labelled chemiluminescent immunometric assays (Immulite 2000)[8]. In the present study, total IGF-I levels were also measured by an automated chemiluminescent assay system (Immulite 2000). The normal ranges for IGF-IR stimulating activity have been previously published by our group[7]. These normal values were established by measuring IGF-IR stimulating activity in serum samples from healthy, non-fasting blood donors (total n = 427), whose ages ranged from 18 to 79 years (median: 44 years). Both for total IGF-I and IGF-IR stimulating activity individual Z-scores were calculated using the following formula: Z-score = (x–average x/S.D.) where x is the actual total IGF-I level or IGF-IR stimulating activity, average x is the mean total IGF-I level or IGF-IR stimulating activity at that age, and S.D. is standard deviation for the mean at that age.

TSH plasma and fT4 levels were determined with a solid phase time-resolved fluoroimmunoassay (Delfia, Wallac Oy, Turku, Finland). Total T3 plasma levels were determined by in-house RIA[9]. TBII were measured by an immunoassay (TSH-Rezeptor Antikörper assay (Trak), Brahms Diagnostica, Berlin, Germany).

Depletion IgGs

For IgG depletion from serum samples protein G magnetic beads were used according to manufacturer's protocol (Millipore, Billerica, USA). Efficiency of IgG depletion was tested in 10 serum samples by immunoturbidimetric analysis. Mean depletion achieved was 81.8% (range: 62.0-98.6%). To control for specificity of IgG depletion on IGF-IR stimulating activity by protein G magnetic beads, blood samples from two healthy controls without thyroid disease were also tested before and after IgG depletion.

Statistics

The clinical characteristics of the study population are presented as mean with SE. For data that did not meet the criteria for normality, logarithmic transformations were applied and are presented as geometric mean with 95% CI or if logarithmic transformation did not normalize data as median with interquartile range. The Kolmogorov- Smirnov-test was used to test normality of variables (data were considered to be normally distributed when P> 0.05). Spearmans' correlation coefficients were calculated to assess the associations between variables.

IGF-IR stimulating activity before and after IgG depletion were compared by using the Wilcoxon signed ranks test.

RESULTS

General characteristics of the study population are shown in Table 1. Mean age of the total group was 47.9 yrs [range 14-92 yrs]. As expected, there were more females than males (f: 62; m: 8). Median duration of thyroid disease was 3 yrs [1-7.3 yrs], median duration of GO was 1 yr [0-2 yrs]. Mean TBII level was 6.3 U/L [range 0.9-604.0 U/L].

Table 1. General Characteristics

	Mean	Range
Age (yrs)	47.9	14-92
Gender (% female)	89%	
Smoking (%)	42%	
Time after diagnosis GD (yrs)	7.0 (3.0*)	1-7.3**
Time after diagnosis GO (yrs)	2.4 (1.0*)	0-2**
CAS	1.6 (1*)	0-5
TBII (U/L)	6.3	0.9-604
fT4 (pmol/L)	16.2	2.7-23.0
T3 (nmol/L)	1.8	1.2-6.0
TSH (mU/L)	0.67 (0.20*)	0.01-3.30

* Median, ** Interquartile range

GD= Graves' Disease, GO= Graves' Ophthalmopathy, CAS= Clinical Activity Score, TBII= TSH Binding Inhibitory Immunoglobulins

There was a significant inverse relationship between TBII and TSH (r=-0.31, p=0.01), a positive correlation between TBII and T3 (r=0.35, p=0.003) and between TBII and CAS (r=0.30, p=0.01).

Mean IGF-IR stimulating activity was 238 pmol/L [95%CI 216-261] and mean total IGF-I concentration was 20.9 nmol/L [95%CI 19.1-23.3]. When these values were compared to normal values[7-8], IGF-IR stimulating activity was found to be low-normal (Z-score: -1.5±0.1 SD), while total serum IGF-I was normal (Z-score: 0.6±0.2 SD) (Figure 1).

Overall, there was a no relationship between age and total IGF-I or IGF-IR stimulating activity (total IGF-I: r=-0.22, p=0.08; IGF-IR stimulating activity: r=0.10, p=0.39). There was a significant inverse relationship between total IGF-I and CAS (r=-0.27, p=0.03) and between total IGF-I and TBII (r= -0.29, p=0.02), but not for IGF-IR stimulating activity (r=-0.17, p=0.17 and r=-0.02, p= 0.86, respectively). There was no relationship between TSH and total IGF-I (r=0.05, p=0.70) or IGF-IR stimulating activity (r=-0.15, p=0.23) nor between T3 and total IGF-I (r=-0.20, p=0.11) or IGF-IR stimulating activity (r=-0.04, p=0.74).

Previously it has been found that GO autoimmune disease activity correlates with TBII titers. Therefore we hypothesized that subjects with relatively high TBII titers and GO autoimmune activity would likely to be those with high titers of (other) autoantibodies,

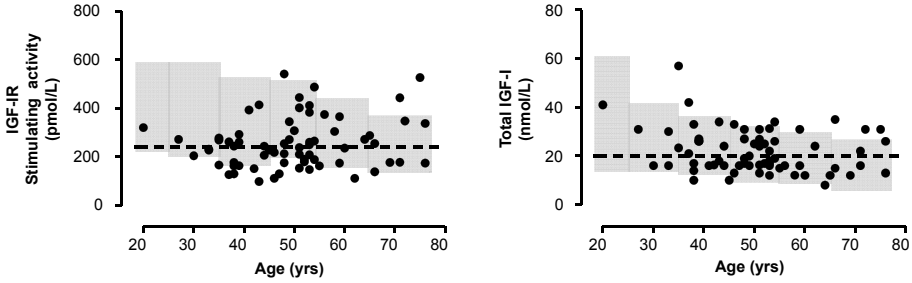

Figure 1.
Age distribution of IGF-IR stimulating activity (left panel) and total IGF-I concentrations (right panel) in 70 patients diagnosed with GO (black dots). The shaded area depicts the 95% confidence interval in healhty subjects per decade of age. The dashed horizontal line shows the median.

such as stimulating antibodies directed against the IGF-IR. Since the TBII titer was not normally distributed but severely skewed to the right, we stratified subjects into two groups: those with TBII titers above the mean + 1 SD (> 12 U/L) and those with TBII titers below the mean + 1SD (<12 U/L).

In subjects with TBII above mean+1SD, there was a positive relationship between IGF-IR stimulating activity and age (r=0.43, p=0.05), while such relationship was absent in subjects with TBII below mean+1SD (r=-0.04, p= 0.81) (Figure 2A).

In contrast to IGF-IR stimulating activity, total IGF-I was not related to age in patients with TBII above mean+1SD (r=-0.05, p=0.84), while in subjects with TBII below mean+1SD, this relationship just missed statistical significance (r=-0.27, p=0.07) (Figure 2B).

Figure 3A shows the relationship between TBII and CAS. In patients with TBII above mean+1SD, TBII was significantly correlated to CAS (r=0.48, p=0.03), whereas this was not found below mean+1SD (r=0.25, p=0.10). Moreover, the median CAS in subjects with TBII titers above the mean + 1 SD was higher (median=2, range 0-5) than in subjects with TBII titers below the mean + 1 SD (median=1, range 0-5), although this difference missed statistical significance (p=0.15). In addition, in the latter group the relationship between TBII and CAS showed a negative direction (r=-0.14, p=0.36) , while in the group with relatively high TBII this relationship showed a positive direction (r=0.26, p= 0.24).

Figure 3B shows the relationship between TBII and T3. In patients with TBII above mean+1SD, TBII was significantly correlated to T3 (r=0.44, p=0.04) whereas this was not found in patients with TBII below mean+1SD (r=0.09, p=0.55).

So, although IGF-IR stimulating activity in healthy subjects, like total IGF-I, is known to decrease with age, there was a positive correlation between IGF-IR stimulating and age in patients with TBII above mean+1SD (>12U/L). Therefore we hypothesized that in this subgroup circulating IgGs may contribute to the activation of the IGF-IR. To test this

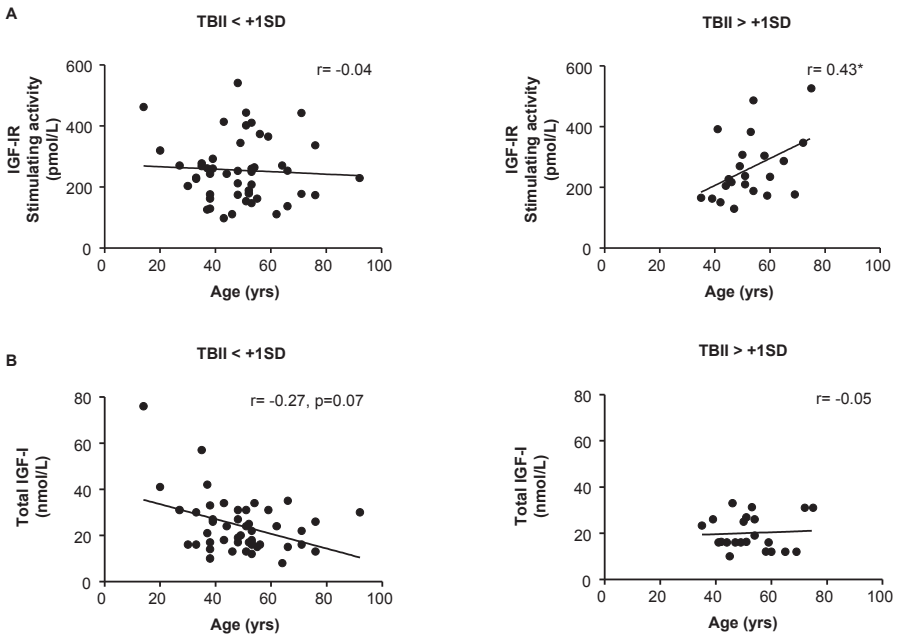

Figure 2.
Correlations between IGF-IR stimulating activity and age (A) and total IGF-I levels and age (B). Study population was divided into two groups; those with TBII titers below the mean + 1SD (<12U/L) (left) and those with TBII titers above the mean + 1 SD (>12UI/L) (right).
*p<0.05.

hypothesis we measured IGF-IR stimulating activity before and after depleting serum samples from IgGs. Overall, IgG depletion did not change IGF-IR stimulating activity (Figure 4A). However, in 10 out of 20 patients tested, IGF-IR stimulating activity decreased after IgG depletion (Figure 4B). Interestingly, this effect was particularly prominent in patients with relatively high IGF-IR stimulating activity (above -1SD). In this latter group median IGF-IR stimulating activity decreased after IgG depletion from 383 [304-487] to 322 [250-477] pmol/L (Z-scores from 0.1±0.5 to -0.5±0.5) (Figure 4B, right panel), while in the group with relatively low IGF-IR stimulating activity (below -1SD) this decline was small (from 205 [165-234] to 199[181-288] pmol/L, Z-scores from -2.0±0.2 to -1.8±0.2) (Figure 4B, left panel).

To control for specificity of IgG depletion on IGF-IR stimulating activity by protein G magnetic beads, IGF-IR stimulating activity was measured before and after IgG depletion in serum samples of two healthy controls. In both samples, no differences were found in IGF-IR stimulating activity before and after IgG depletion (Subject 1; before: 264 pmol/L, after: 254 pmol/L; Subject 2; before: 244 pmol/L, after: 263 pmol/L).

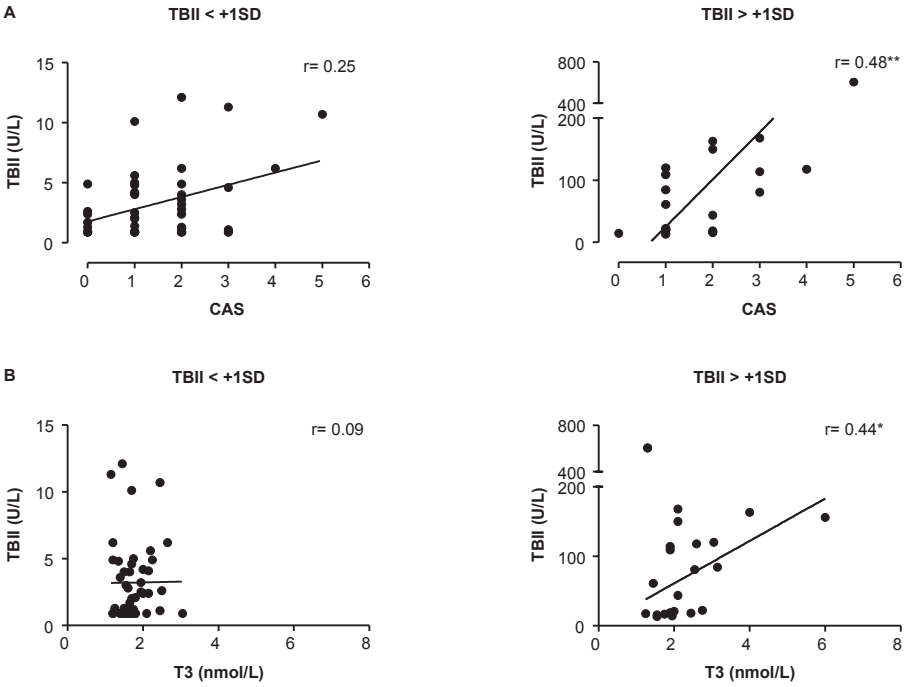

Figure 3.

Correlations between TBII and CAS (A) and between TBII and T3 levels (B). Study population was divided into two groups; those with TBII titers below the mean + 1SD (<12U/L) (left) and those with TBII titers above the mean + 1 SD (>12UI/L) (right).

*p<0.05, **p<0.01

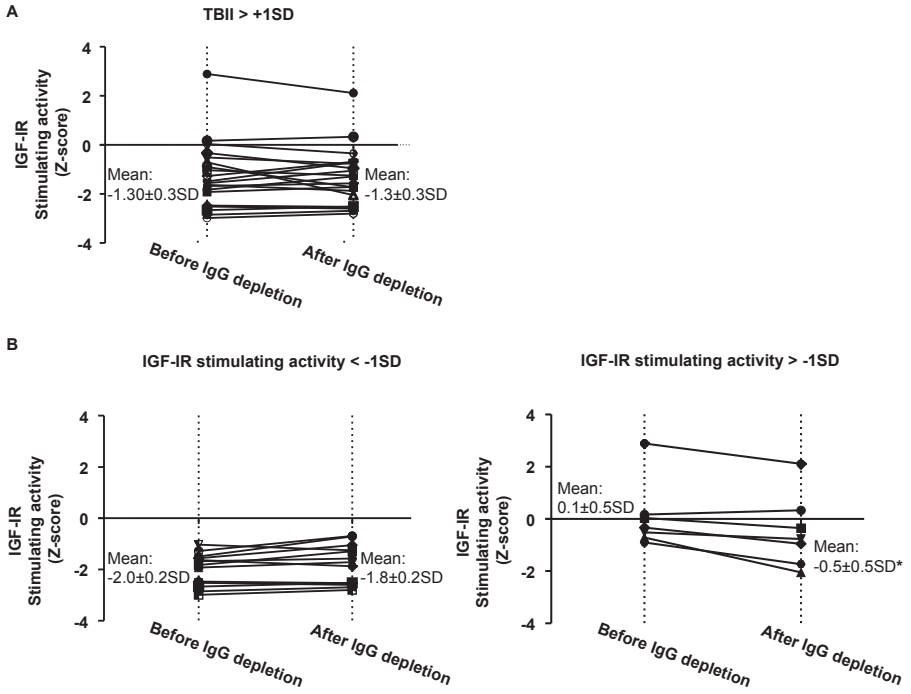

Figure 4.
Effects of IgG depletion on IGF-IR stimulating activity in all patients with TBII above mean+1SD. A. Overall effects. B. Effects of IgG depletion when all patients with TBII above mean+1SD were divided in those with IGF-IR stimulating activity below mean-1SD (left) and those with IGF-IR stimulating activity above mean-1SD (right). The effect of IgG depletion was particularly prominent in the latter group.
* $p<0.05$.

DISCUSSION

Since Graves' immunoglobulins may directly activate the IGF-IR, the IGF-IR has been suggested to be a second auto-antigen in GO. We therefore investigated, in patients with GO, whether there was a relationship between the circulating TBII and IGF-IR stimulation. At a first glance we found no relationship between TBII levels in blood and IGF-IR stimulation.

In our study, we measured TSH-R antibody levels by an immunoassay. These assays display high sensitivity and specificity for TSH-R autoantibodies. However, they do not measure the functional activity of these immunoglobulins, neither do they distinguish between stimulatory, blocking and neutral activity[10]. Previously a close relationship between TBII and CAS was reported[11-14]. It has been found that TBII are significantly higher in patients with a severe course of GO compared with patients with a mild course of GO[11, 14]. In the present study a close relationship between TBII and CAS was also found.

Recent evidence suggests that other self-antigens than the TSH-R, such as the IGF-IR, may contribute to GO[15]. To our best knowledge no method is available at present, which can specifically measure circulating IGF-IR antibodies and distinguish them from TBII. Theoretically the same antibodies that bind to TSH-R could also activate the IGF-IR. However, due to clear differences in the molecular structure of the TSH-R and the IGF-IR, it is more likely that the two receptors are activated by two discrete antibodies: those directed against the IGF-IR and those directed against the TSH-R.

In clinical medicine, extreme cases have often provided new insights into specific aetiologic factors and disease pathogenesis. Previously it has been found that GO autoimmune disease activity correlates with TBII titers. We therefore hypothesized that subjects with relatively high TBII titers and GO autoimmune activity would likely to be those with high titers of (other) autoantibodies, such as stimulating antibodies directed against the IGF-IR. Since the TBII titer was not normally distributed but severely skewed to the right, we stratified subjects into two groups: those with TBII titers above the mean + 1 SD (> 12 U/L) and those with TBII titers below the mean + 1SD (<12 U/L).

Only in patients with relatively high TBII titers a positive correlation was found between IGF-IR stimulating activity and age. This positive relationship was unexpected since IGF-IR stimulating activity in healthy subjects, like total IGF-I, is known to decrease with age[7]. We hypothesized that this positive correlation may be the result of the presence of circulating IGF-IR activating immunoglobulins. Our results suggest that the relative contribution of these antibodies progresses with age. In this situation, IGF-IR stimulating activity, as measured by the KIRA assay, seems to reflect the overall effect of circulating endogenous IGF-I and IGF-IR stimulating activity induced by IgGs (Figure 5). Interestingly, an age-associated increase in both organ-specific autoantibodies in humans has

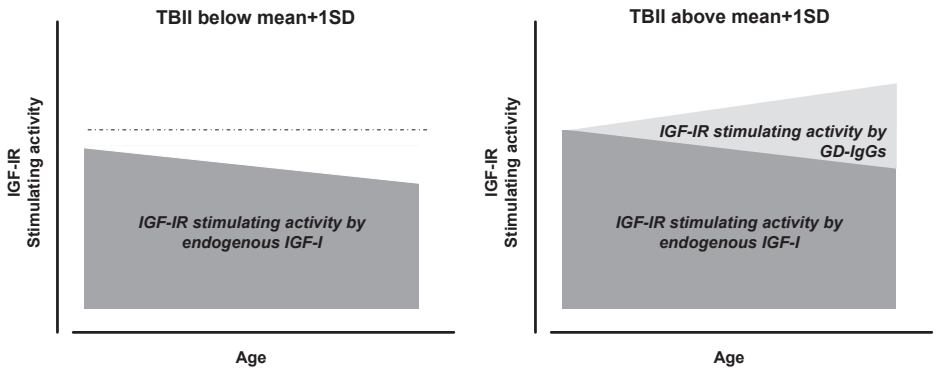

Figure 5.
The *suggested* relationship between GD-IgGs, circulating IGF-IR stimulating activity and age in patients with GO. The relative contribution of GD-IgGs to IGF-IR stimulating activity progresses with age. For further explanation see the Discussion section.

been consistently reported[16]. Moreover, Perros et al. previously reported an association between severity of thyroid-associated ophthalmopathy and advancing age[17].

Overall there was no relationship between IGF-IR stimulating activity and CAS. However, when the study population was stratified by TBII titers, median CAS in subjects with TBII titers above the mean + 1 SD was higher than in subjects with TBII titers below the mean + 1 SD, although this difference missed statistical significance. In addition, overall there was no relationship between TBII and IGF-IR stimulating activity. But when the study population was stratified by TBII titers, the relationship between IGF-IR stimulating activity and TBII showed a positive direction in subjects with TBII titers above the mean + 1 SD, while this relationship showed a negative direction in subjects with TBII titers below the mean + 1 SD. As previously discussed, the TBII assay measures immunoglobulins in patients with GD that inhibits TSH-R activation. However, this method has not been developed to measure stimulating IGF-IR stimulating activity. In addition, the small number of subjects in our study may explain why the relationship between TBII titers and IGF-IR stimulating activity missed statistical significance. Another factor may be that the measured IGF-IR stimulating activity probably reflects the sum of endogenous IGF-I and circulating GD-IgGs with IGF-IR stimulating activity (see Figure 5). Thus only a fraction of "total" serum IGF-IR stimulating activity is directly related to IGF-IR stimulating antibodies.

We measured IGF-IR stimulating activity before and after IgG depletion in the subgroup with high TBII levels. Interestingly, IGF-IR stimulating activity substantially decreased in patients with relatively high Z-scores for IGF-IR stimulating activity before IgG depletion. In contrast, there were only small changes in patients with relatively low Z-scores for IGF-IR stimulating activity before this treatment. This suggests that, significant levels of IGF-IR stimulating antibodies were present in the former group.

Our results are in variance with previous studies which reported anti-IGF-IR antibody activities in a greater percentage of patients[2-4]. This variance may be due to differences in assay sensitivities and/or differences in fundamental peculiarities of cells used to measure IGF-IR activity. Previously it was found that, in contrast to fibroblasts from healthy donors, fibroblasts from patients with GD showed a response to GD-specific IgG[3]. We did not use cells of GD patients but our experiments were performed using HEK cells that were stably transfected with the human IGF-IR. Moreover, in contrast to the present study, in which ten times diluted serum samples were used to measure IGF-IR stimulating activity, others have used highly purified IgGs isolated from serum samples to measure IGF-IR stimulating effects[3-4]. Finally, in our study, not post-receptor effects or inhibition of [^{125}I]IGF-I binding was measured, but rather phosphorylation of tyrosine residues of the IGF-IR as primary read out to assess IGF-IR stimulating activity[2-4].

The question arises how relevant our observations are since the relative contribution of IgGs to total serum IGF-IR stimulating activity was maximally 'only' 16.5%. However, our study shows that the combination of IgGs plus endogenous IGF-I produced greater stimulation than either agent alone. Moreover, antibody-induced receptor activation may show tissue-specific distribution and/or activity. In favour of this latter possibility orbital fibroblasts in patients with GO express higher levels of IGF-IR than normal fibroblasts[15]. In addition, human antibodies typically have a half-life greater than 1 week[18]. Due to their increased half-life, IGF-IR stimulating antibodies may induce prolonged activation of the IGF-IR as compared to endogenous IGF-I.

Although it has been suggested that the IGF-IR KIRA assay is more sensitive than common total IGF-I immunoassays to detect differences in clinical state[19], it should be stressed that, as for all in vitro systems, the IGF-IR KIRA assay does not mimic the exact in vivo conditions. IGF-IR expression may vary in a tissue-specific manner and inter-individual differences in the levels of proteins of the IGF-IR system may function as critical signalling determinants[20]. This may be especially the case when studying GO, since orbital fibroblasts in GD express higher levels of the IGF-IR than normal fibroblasts[15]. Thus, our findings in serum samples may underestimate the real local effects of IgGs on IGF-IR signalling in the orbits of Graves' patients.

In conclusion, only in subjects with TBII levels above mean+1SD we observed an increase of IGF-IR stimulating activity with age. In addition, in a subgroup of these patients, depletion of IgGs significantly decreased IGF-IR stimulating activity. These results suggest that, in a subset of patients with GO, circulating IgGs may have IGF-IR stimulating activities.

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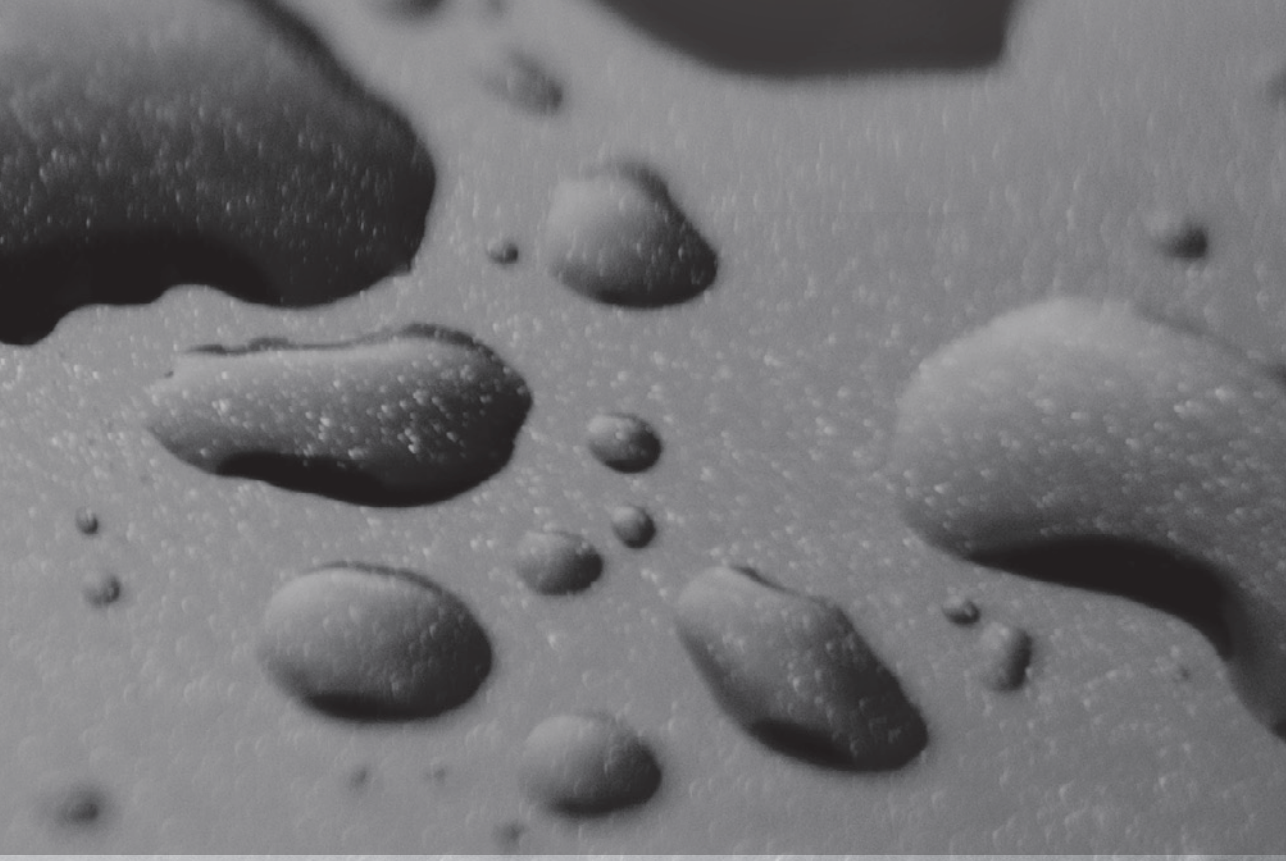
DISCLOSURES

All authors have nothing to disclose.

REFERENCES

1. Tramontano D, Cushing GW, Moses AC & Ingbar SH. Insulin-like growth factor-I stimulates the growth of rat thyroid cells in culture and synergizes the stimulation of DNA synthesis induced by TSH and Graves'-IgG. *Endocrinology* 1986 **119** 940-942.
2. Weightman DR, Perros P, Sherif IH & Kendall-Taylor P. Autoantibodies to IGF-1 binding sites in thyroid associated ophthalmopathy. *Autoimmunity* 1993 **16** 251-257.
3. Pritchard J, Han R, Horst N, Cruikshank WW & Smith TJ. Immunoglobulin activation of T cell chemoattractant expression in fibroblasts from patients with Graves' disease is mediated through the insulin-like growth factor I receptor pathway. *J Immunol* 2003 **170** 6348-6354.
4. Smith TJ & Hoa N. Immunoglobulins from patients with Graves' disease induce hyaluronan synthesis in their orbital fibroblasts through the self-antigen, insulin-like growth factor-I receptor. *J Clin Endocrinol Metab* 2004 **89** 5076-5080.
5. Chen JW, Ledet T, Orskov H, Jessen N, Lund S, Whittaker J, De Meyts P, Larsen MB, Christiansen JS & Frystyk J. A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum. *Am J Physiol Endocrinol Metab* 2003 **284** E1149-1155.
6. Brugts MP, van den Beld AW, Hofland LJ, van der Wansem K, van Koetsveld PM, Frystyk J, Lamberts SW & Janssen JA. Low circulating insulin-like growth factor I bioactivity in elderly men is associated with increased mortality. *J Clin Endocrinol Metab* 2008 **93** 2515-2522.
7. Brugts MP, Ranke MB, Hofland LJ, van der Wansem K, Weber K, Frystyk J, Lamberts SW & Janssen JA. Normal values of circulating insulin-like growth factor-I bioactivity in the healthy population: comparison with five widely used IGF-I immunoassays. *J Clin Endocrinol Metab* 2008 **93** 2539-2545.
8. Elmlinger MW, Kuhnel W, Weber MM & Ranke MB. Reference ranges for two automated chemiluminescent assays for serum insulin-like growth factor I (IGF-I) and IGF-binding protein 3 (IGFBP-3). *Clin Chem Lab Med* 2004 **42** 654-664.
9. Wiersinga WM & Chopra IJ. Radioimmunoassay of thyroxine (T4), 3,5,3'-triiodothyronine (T3), 3,3',5'-triiodothyronine (reverse T3, rT3), and 3,3'-diiodothyronine (T2). *Methods Enzymol* 1982 **84** 272-303.
10. Ponto KA, Kanitz M, Olivo PD, Pitz S, Pfeiffer N & Kahaly GJ. Clinical relevance of thyroid-stimulating immunoglobulins in graves' ophthalmopathy. *Ophthalmology* 2011 **118** 2279-2285.
11. Gerding MN, van der Meer JW, Broenink M, Bakker O, Wiersinga WM & Prummel MF. Association of thyrotrophin receptor antibodies with the clinical features of Graves' ophthalmopathy. *Clin Endocrinol (Oxf)* 2000 **52** 267-271.
12. Acuna OM, Athannassaki I & Paysse EA. Association between thyroid-stimulating immunoglobulin levels and ocular findings in pediatric patients with Graves disease. *Trans Am Ophthalmol Soc* 2007 **105** 146-150; discussion 150-141.
13. Dragan LR, Seiff SR & Lee DC. Longitudinal correlation of thyroid-stimulating immunoglobulin with clinical activity of disease in thyroid-associated orbitopathy. *Ophthal Plast Reconstr Surg* 2006 **22** 13-19.
14. Eckstein AK, Plicht M, Lax H, Neuhauser M, Mann K, Lederbogen S, Heckmann C, Esser J & Morgenthaler NG. Thyrotropin receptor autoantibodies are independent risk factors for Graves' ophthalmopathy and help to predict severity and outcome of the disease. *J Clin Endocrinol Metab* 2006 **91** 3464-3470.
15. Smith TJ. The putative role of fibroblasts in the pathogenesis of Graves' disease: evidence for the involvement of the insulin-like growth factor-1 receptor in fibroblast activation. *Autoimmunity* 2003 **36** 409-415.

16. Grolleau-Julius A, Ray D & Yung RL. The role of epigenetics in aging and autoimmunity. *Clin Rev Allergy Immunol* 2010 **39** 42-50.
17. Perros P, Crombie AL, Matthews JN & Kendall-Taylor P. Age and gender influence the severity of thyroid-associated ophthalmopathy: a study of 101 patients attending a combined thyroid-eye clinic. *Clin Endocrinol (Oxf)* 1993 **38** 367-372.
18. Bhaskar V, Goldfine ID, Bedinger DH, Lau A, Kuan HF, Gross LM, Handa M, Maddux BA, Watson SR, Zhu S, Narasimha AJ, Levy R, Webster L, Wijesuriya SD, Liu N, Wu X, Chemla-Vogel D, Tran C, Lee SR, Wong S, Wilcock D, White ML & Corbin JA. A fully human, allosteric monoclonal antibody that activates the insulin receptor and improves glycemic control. *Diabetes* 2012 **61** 1263-1271.
19. Janssen JAMJL. IGF-I: Pro 's and Con's of a Bioassay. *Horm Res Paediatr* 2011 **76(suppl 1)** 106-110.
20. Eckardt K, May C, Koenen M & Eckel J. IGF-1 receptor signalling determines the mitogenic potency of insulin analogues in human smooth muscle cells and fibroblasts. *Diabetologia* 2007 **50** 2534-2543.



Discussion

The aim of this thesis was to investigate the (patho)physiologic role of insulin receptor (IR) and IGF-I receptor (IGF-IR) bioactivity in health and disease under several conditions like diabetes, growth hormone deficiency (GHD), acromegaly and Graves' ophthalmopathy.

11.1 FROM BIOASSAYS TO IMMUNOASSAYS AND BACK?

Insulin elicits its various biological responses by binding to the IR, which is then followed by activation of its intrinsic tyrosine residues[1]. In the human body, due to alternative splicing of exon 11 of the IR gene, two IR transcripts are generated, resulting in IR-A (lacking exon 11) and in IR-B (full length)[2]. The relative abundance of mRNAs encoding the IR-A and IR-B isoforms is regulated in a tissue-specific manner[3] and also differs by stage of cell development and differentiation. The IR-A is the predominant isoform in foetal tissues and cancer cells, while the IR-B is the classical receptor for insulin with metabolic effects in muscle, liver and adipose tissues[3-4]. Although studies have suggested that differences between IR-A and IR-B in terms of receptor activation and signalling may result in different functions of each IR isoform[2], it appears that most cells have both IR isoforms and that the ratio of the two seems to be very important[2].

In *Chapter 2* we describe the development and validation of two Kinase Receptor Activation (KIRA) assays specific for the IR-A and IR-B, respectively, which quantify ligand/serum induced phosphorylation of the tyrosine residues within the IRs.

We found that the signal of both IR KIRA assays was relatively independent of serum dilution as long as the serum dilutions were kept below 1:16. A comparable phenomenon has been described for the IGF-IR specific KIRA assay[5] and has been ascribed to the buffering capacity of the IGFBPs which liberate IGF-I during dilution. We showed, in both IR KIRA assays, that specific antibodies directed against IGF-I and IGF-II could partially reduce the signal. This suggests that IGF-I and IGF-II in human serum may contribute to IR-A and IR-B signalling and contribute substantially to total insulin-like bioactivity. Here, it is good to recall that already more than 50 years ago, Yalow and Berson showed that serum contained higher insulin-like activity than immunoreactive insulin concentrations[6]. In addition, Froesch showed that specific anti-insulin antibodies could only block a small portion of total insulin-like activity[7]. Moreover, the presence of insulin antibodies or insulin analogues or excessive amounts of proinsulin may cause considerable discrepancies between immunological insulin concentrations and bioactive insulin[4]. However, probably enforced by the introduction of the RIA (first) for insulin and (later) for IGFs, these observations have long been ignored. This may not be surprising since insulin bioassays showed highly variable results. Over time several attempts have been made to introduce new bioassays to measure insulin-like activity but so far they have not been able to determine human serum bioactivity on human cells with high sensitivity and

low intra- and interassay variability. However, the two IR KIRA assays show a reasonably sensitivity (detection limits of 32 pmol/L of insulin) and for bioassays exceptionally low intra- and inter-assay variations (<12% and <15%, respectively).

As discussed previously, it has been suggested that the IR and IGF-IR act at identical portals to the regulation of gene expression, with differences between insulin and IGF effects due to a modulation of the signal created by the specific ligand-receptor interactions[8]. As a consequence it is almost impossible in most in vitro cell lines to disentangle the individual contribution of each type receptor to the final downstream event. However, both KIRA assays distinguish serum insulin-like stimulating activity for the IR-A and the IR-B. This may help to gain more insight in the role of the two IR isoforms in health and disease.

Insulin and insulin analogues have variable cross-reactivity in insulin immunoassays. In addition, it has been reported that antibody cross-reactivity for insulin within an assay system can be concentration dependent[9-10]. In our hospital we currently make use of a solid-phase, enzyme-labelled chemiluminescent immunometric assay (Immulite 2000) to measure immunoreactive insulin concentrations. When samples with known amounts of insulin analogues (range 100, 1.000, 10.000 and 100.000 pmol/L) were tested, we found that this insulin immunoassay, only measured the real concentrations in samples when these samples contained very high (100.000 pmol/L) i.e. supraphysiological concentrations of an insulin analogue (Figure 1, unpublished data). In contrast, both IR KIRA assays were able to detect a more or less equal IR activation by human (native) insulin and insulin analogues over the whole range of concentrations tested[11] (Figure 2). Only insulin detemir induced a lower IR activation than human insulin[11]. This, however, is in agreement with clinical practice where the formulation of insulin detemir is 4-fold more concentrated than insulin in order to match biological potency[12].

So, the immunoassay measures the ability of analogues to cross-react in the assay, whereas the IR KIRA assays seem to be able to detect the real and actual bioactivity of the insulin analogues, which is usually the main goal of clinicians when measuring insulin[13]. Accordingly, the IR KIRA assays may permit the measurement of the "total" circulating insulin bioactivity in serum from patients receiving treatment with both insulin and insulin analogues.

Although the operational range of the IR KIRA assays allows to quantify the overall effect of total insulin-like factors in serum, it should be acknowledged that the KIRA assays (as for all in-vitro systems) do not mimic the exact in vivo situation. The KIRA assays only provide a crude, albeit convenient, measure of IR activation. IR KIRA assay results do not necessarily reflect bioactivity at the local tissue level, with the notable exception of vascular endothelial cells. Moveover, the contribution of various circulating peptides in activating the IRs in vivo depends not only on their concentrations and bioavailability, but also on the relative and absolute concentrations of the two IR present on various

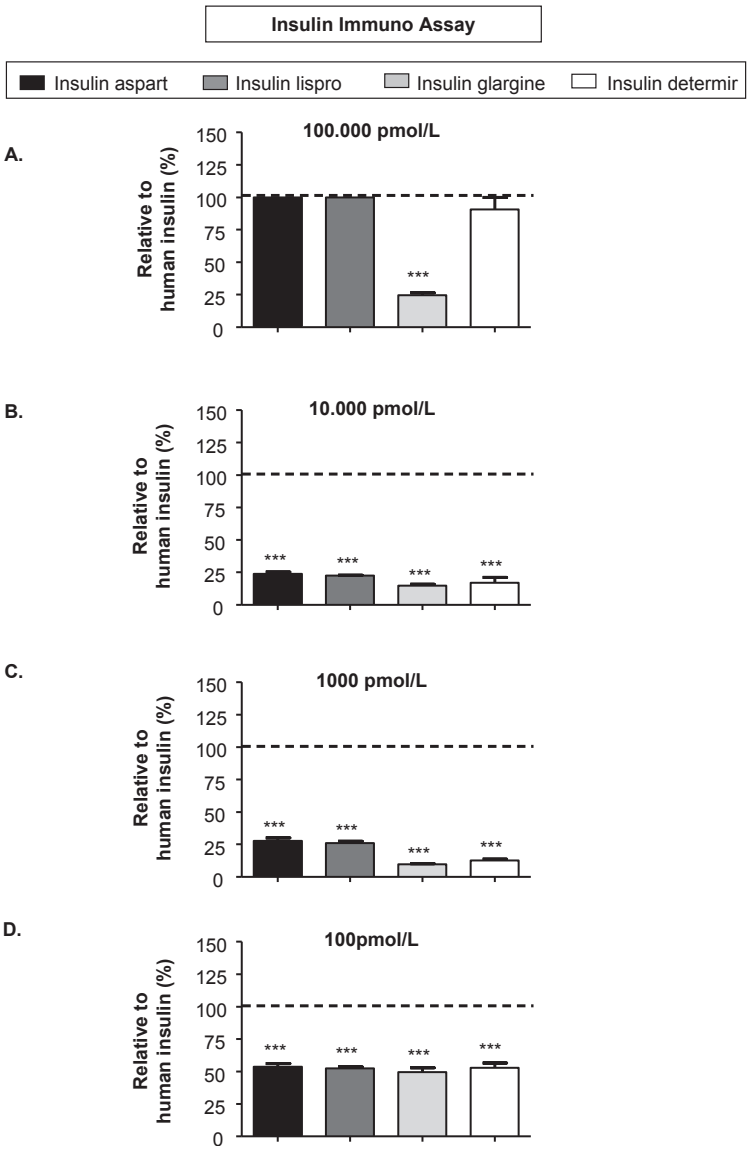


Figure 1.

Detection of insulin and insulin analogues by an insulin immunoassay. Samples which contained known amounts (100 (A), 1000 (B), 10.000 (C) and 100.000 (D) pmol/L) of insulin or insulin aspart (black bars), insulin lispro (dark grey bars), insulin glargine (light grey bars) or insulin detemir (white bars) in KRB 0.1% HSA were measured by a commercially available insulin assay. Samples containing insulin were arbitrarily set at 100% to test cross-reactivity with insulin analogues. Data are shown as mean (+SEM) percentage of three independent experiments. ***P<0.001 for significant different cross-reactivity with insulin.

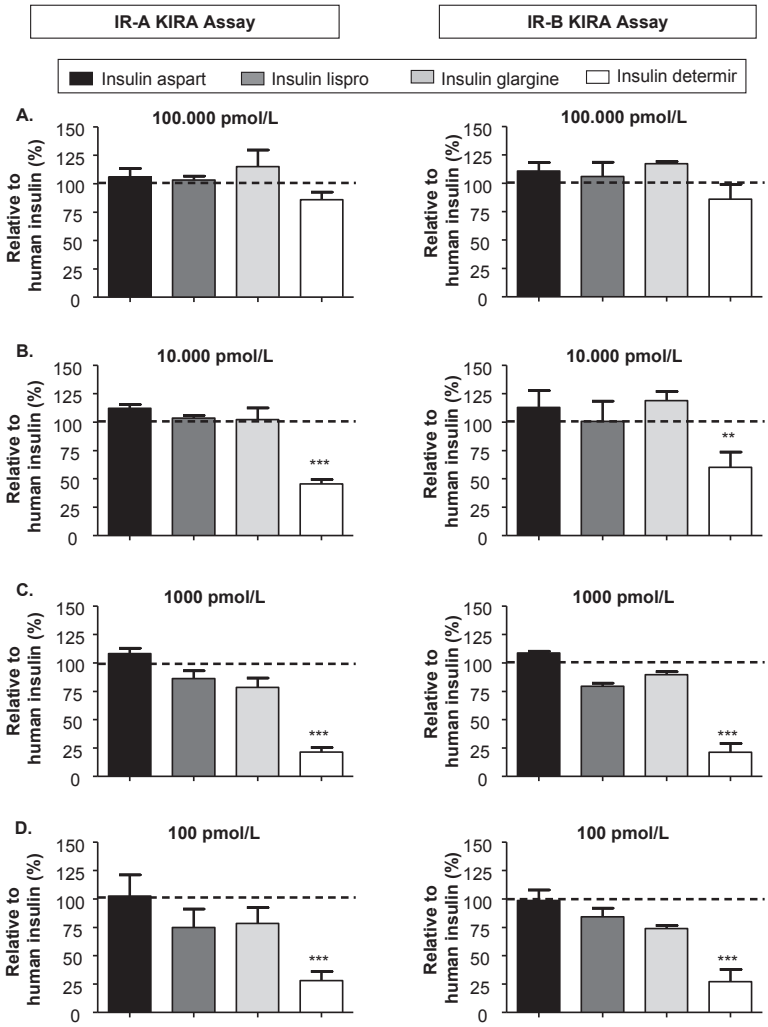


Figure 2.
Detection of insulin and insulin analogues by the IR-A (left) and IR-B (right) KIRA assay. Cells were stimulated with samples which contained known amounts (100, 1000, 10.000 and 100.000 pmol/L) of insulin or insulin aspart (black bars), insulin lispro (dark grey bars), insulin glargine (light grey bars) or insulin detemir (white bars) in KRB 0.1% HSA. Protocol was followed as described in the Methods section in Chapter 2. IR activation induced by insulin was arbitrarily set at 100%. Data are shown as mean (+SEM) percentage of three independent experiments. ** P<0.01, ***P<0.001 for significant different IR activation compared to insulin.

target tissues. In most tissues and cells where significant levels of both IRs and IGF-IRs are present, hybrids may be formed. These hybrids are heterodimeric receptors consisting of an IR alpha/beta monomer and an IGF-IR alpha/beta monomer linked by disulfide bonds. Such hybrids are probably formed during normal post- translational processing of both receptors[14] and are widely expressed in normal tissues and often aberrantly expressed in cancer cells[15]. Since both IR isoforms and IGF-IRs are endogenously expressed in our cell lines, theoretically at least three hybrids may be formed: IR-A/IR-B, IR-A/IGF-IR and IR-B/IGF-IR. This could have influenced our findings. However, we also demonstrated that our cell lines have very low endogenous levels of both IR-isoforms and the IGF-IRs compared to the transfected IR-isoform. Thus, the endogenous IRs and IGF-IRs are most probably out-competed in our IR KIRA assays following the high expression levels of the exogenous IRs achieved by transfection. In addition, although the precise biological role of the hybrids is still unclear, functional studies have demonstrated that IR/IGF-IR hybrids behave more like IGF-IRs than IRs[2]. So, although hybrid receptors may well play an important role in vivo, their relative contribution to the endpoint signal of both KIRA assays is assumed to be low but cannot be completely excluded.

Finally, the IRs have up to 6 key tyrosine residues and the antibody used in our study to detect the tyrosine residues may not necessarily recognize all residues with the same affinity. As the distinct roles of the different tyrosine residues remain to be clarified, this aspect may be of importance.

In conclusion, although some recognizable limitations, IR KIRA assays quantify human serum insulin-like activity in a reliable manner and are able to provide significant other and novel information (next to immunoassays) on the insulin system by taking into account both IR isoforms and the modifying effects of the IGFs on this system.

11.2 DO MODIFICATIONS OF HUMAN INSULIN STRUCTURE CHANGE POTENCY OF INSULIN ANALOGUES TO ACTIVATE THE IGF-IR, IR-A AND IR-B AND DOES THIS CHANGE SERUM BIOACTIVITY?

The structures of the IR and IGF-IR resemble each other to such an extent that insulin and IGFs can interact with each others receptor, although with quite different affinities. Structural modification of the insulin molecule may result in altered binding affinities and activities to the IR and/or the IGF-IR. As a consequence, insulin analogues may have an increased/decreased metabolic action and an increased/decreased mitogenic action compared to human insulin. The amino acid residues in the insulin molecule that are essential for binding to the IR have been identified[16]. Especially modifications at positions of the C-terminus of the B-chain, do not seem to significantly influence insulin binding

to the IR[17-18]. On the other hand, substitutions of amino acids in the B- chain do result in insulin molecules, which show increased structural homology with IGF-I, and as a consequence have an increased affinity for the IGF-IR[16-17].

In 2009 four observational studies were published of which three suggested that use of insulin glargine was associated with an increased risk of cancer[19-22]. The FDA reviewed these four studies and concluded that 'the evidence presented was inconclusive, due to limitations in how the studies were designed, carried out and in the data available for analysis'[23]. Nevertheless these findings did raise concerns as to whether or not insulin glargine promotes cell proliferation and growth of neoplasms. Especially since, in vitro, at high concentrations, insulin glargine was also found to be more potent than human insulin in stimulating the IGF-IR[17, 24-25]. In contrast, short-acting insulin analogues did not seem to bring additive risks in this respect[26].

In *Chapter 3* we studied whether short- and long-acting insulin analogues differed from human insulin in their potency to activate the IGF-IR, IR-A and IR-B in vitro by using the KIRA assays specific for the respective receptors (the IGF-IR specific KIRA assay has been developed in 2003 by Chen et al.[5]). In line with a previous study by Kurtzhals et al.[17], when we assessed stimulating activity for the IGF-IR, IR-A and IR-B in vitro, we found that short-acting insulin analogues (insulin lispro and insulin aspart) did not differ substantially from human insulin, nor from each other. In contrast, insulin glargine, at high concentrations, was more potent than human insulin in activating the IGF-IR in vitro.

A considerable fraction of insulin glargine is metabolized in vivo before it enters the circulation (see below). This makes it questionable whether in vitro results of insulin glargine may be extrapolated and thus are representative for its actions in vivo.

In *Chapter 4* we measured serum IGF-IR bioactivity (by the IGF-IR specific KIRA assay) in 104 type 2 diabetic patients randomized for insulin glargine or NPH insulin therapy for a prolonged time. We found that at baseline and after 36 weeks of insulin therapy, there was no difference in IGF-IR bioactivity between the two treatment groups[27]. We hypothesized that a potential explanation for the discrepancies between the previous in vitro results and these in vivo results of insulin glargine might lay in the dosing of insulin glargine in vivo. Although at this time insulin concentrations were not measured in our study, by extrapolating results from previous studies[12, 28] to ours, it seemed unlikely that free plasma insulin glargine concentrations exceeded 500-850 pmol/l during treatment. The latter suggests that in vivo concentrations of insulin glargine do probably not reach concentrations at which we and others had observed differences in IGF-IR activation in vitro[11, 17, 24-25, 29].

Another explanation for the discrepancies between the in vitro and in vivo results of insulin glargine could be that insulin glargine is metabolized in vivo. The metabolites of insulin glargine may be less potent than native insulin glargine to activate the IGF-IR. The findings presented in *Chapter 5* support the latter hypothesis.

At a first glance, quite surprisingly, we found that prolonged insulin treatment, instead of increasing IGF-IR bioactivity, further decreased IGF-IR bioactivity, while it did not change total IGF-I concentrations. There is considerable evidence to suggest that exogenous insulin (via subcutaneous insulin injection) has feedback inhibition on endogenous insulin secretion (secretion by the pancreas)[30]. Insulin enhances hepatic IGF-I production by regulating hepatic GH expression[31] and/or by a permissive effect on GH mediated postreceptor effects[32]. Insulin also has an important role in controlling IGF-IR bioactivity by regulating circulating concentrations of IGFBPs, especially IGFBP-1[33]. So, suppression of portal insulin by exogenous insulin therapy may have reduced portal insulin levels leading to (decreased hepatic IGF-I production and) reduced bioavailable IGF-I. In favour of this latter possibility, we observed in a previous study in critically ill children a significantly lower IGF-IR bioactivity and lower C-peptide levels and higher IGFBP-1 levels after intensive insulin therapy than after conventional insulin therapy[34]. In an earlier study by Arafat et al.[35], acute hyperinsulinemia also suppressed IGF-IR bioactivity. The decrease in circulating IGF-IR bioactivity in this study was attributed to insulin-mediated effects on circulating IGFBPs: insulin suppressed IGFBP-4 and IGFBP-1 and increased IGFBP-2 concentrations[35]. Unfortunately, in our study, we did not measure IGFBPs.

In contrast to IGF-IR bioactivity, total IGF-I levels did not change during insulin treatment in our study and in both previous studies[34-35]. However, immunoasssays not always accurately measure total IGF-I levels due to problems with assay standardization and/or with assay methodology[36]. In this respect, it has been suggested that the IGF-IR KIRA assay is more sensitive than the common total IGF-I immunoassays to detect differences in clinical state[37]. Moreover, the IGF-IR KIRA assay often seems to be superior to common IGF-I immunoassays to monitor therapeutic interventions[37]. Our results seem to confirm this.

It has been reported that IGFBP-3 protease activity is increased in untreated diabetic patients and decreases after several days of insulin treatment[38-39]. The latter effect may also have attributed to the observed decrease in IGF-IR bioactivity in our study during insulin treatment.

Another point that should be stressed is, that in our study the decline in serum IGF-IR bioactivity during insulin treatment was found under co-medication with relatively high doses of metformin. Metformin is a widely prescribed anti-diabetic drug which is recommended as the initial pharmacological therapy together with lifestyle interventions for type 2 diabetes[40]. All patients in our study were already treated with metformin for at

least three months before being randomly assigned to either insulin glargine or NPH insulin therapy. Several studies have shown that metformin treatment is associated with a lower cancer risk[22, 41-42]. Therefore, concomitant metformin use has been suggested to be a potential confounder when it comes to estimating the risks of insulin therapy for cancer[26]. However, it has been implied that a large number of studies reporting reductions in the incidence of or mortality from cancer after use of metformin, are afflicted with time-related biases[43]. Therefore, recently the potential beneficial effects of metformin on cancer were even seriously questioned[43]. Nevertheless, the in vivo-evidence supporting a beneficial effect of metformin are strong[43-44].

Metformin reverses endogenous hyperinsulinemia through its effects on glucose homeostasis and therefore may directly have antiproliferative effects[45]. Indirectly, a reduction of endogenous insulin levels may also lower IGF-IR bioactivity and improve insulin sensitivity[46]. Most importantly, metformin activates the AMP-activated protein kinase (AMPK) signalling pathway[44]. One of the major growth regulatory pathways controlled by AMPK is the mammalian target of rapamycin (mTOR) pathway and its downstream substrates[47-48]. In addition, in NIH-3T3 cells, stimulation of AMPK inhibits the ability of IGF-I to activate ras and its downstream targets but phosphorylation of the activated IGF-IR appeared to be unaffected by this increase in AMPK activity[49]. So, metformin is suggested to interfere with IGF-I signalling at the postreceptor level and not at the receptor level. Yet, we cannot exclude that concomitant metformin therapy has influenced our findings. On the other hand, in our study we merely addressed IGF-IR bioactivity and not cancer incidence.

Our study suggests that treatment with insulin glargine in vivo does not result in an increased IGF-IR signalling compared to human insulin. It is important to emphasize that with the IGF-IR specific KIRA assay merely phosphorylation of the tyrosine residues within the beta-subunits of the IGF-IR is quantified. It cannot be assessed whether activation of the IGF-IR by an insulin analogue results in a normal (i.e. balanced metabolic and mitogenic) activity at cellular level in vivo. The IGF-IR may differentially elicit biological effects and intracellular signalling upon binding of different insulin analogues. In this respect it has been suggested that intracellular signalling induced by insulin analogues differs from human insulin: insulin analogues preferentially activate the ERK (mitogenic) pathway rather than the AKT (metabolic) pathway[50]. Thus there is still a chance that (subtle) differences in the molecular structure of insulin analogues may affect in vivo signalling at the postreceptor level and thereby induce an abnormal metabolic:mitogenic ratio[2]. Our findings are in line with a recently published paper in which, in an animal model of type 2 diabetes, no differences were demonstrated in the degree of colonic epithelial proliferation between animals treated with insulin glargine or NPH insulin[51]. Nevertheless, in that study insulin treatment did result in a higher degree of colonic

epithelial proliferation, thereby pointing towards the potential mitogenic properties of all insulins, irrespective of the type of insulin[51]. Our results are furthermore supported by recent findings from a French study among 70,027 patients aged 40-79 yrs with a median follow-up of 2.67 yrs[52] and by a recent meta-analysis[53] who reported no increased cancer risk in patients treated with insulin glargine compared to other insulins.

The discussion about enhanced proliferative activity of certain insulin analogues has mainly focused on increased signalling via the IGF-IR. However, the IR and IGF-IR expression vary in a tissue-specific manner and inter-individual differences in the levels of proteins of the IGF-IR system may also function as a critical determinant of the mitogenic potency of insulin analogues[25].

Hansen et al.[54] have shown that an increased mitogenic potency of insulin analogues may not only be due to an increased affinity for the IGF-IR but may also result from slow ligand dissociation from the IR. IRs, like IGF-IRs, are overexpressed in many human malignancies[55]. Interestingly, especially the IR-A isoform is overexpressed in cancer and has the peculiar characteristic to bind not only insulin but also IGF-II and, although to a lesser extent, IGF-I[2, 56]. Thus, overexpression of IR-A may play a key role in the formation and progression of human cancers after starting treatment with insulin or insulin analogues.

In *Chapter 5* we investigated the in vitro potency of insulin glargine, its metabolites (M1, M2), IGF-I and NPH insulin to activate the IGF-IR, IR-A and IR-B by using the KIRA assays specific for the respective receptors. We found that, in contrast to insulin glargine, (also at supraphysiological concentrations) M1 and M2 induced equal IGF-IR activation as compared to human insulin. M1 was also equally potent as compared to human insulin in activating the IR-A and IR-B. In contrast, M2, at supraphysiological concentrations, was more potent than human insulin in activating both IR isoforms. Our findings are supported by previous studies, which have demonstrated that M1 has a lower mitogenic potential than insulin glargine, which is even lower than that of human insulin. Sommerfeld et al., found a higher affinity of insulin glargine for the IGF-IR than insulin and a more potent stimulation of thymidine incorporation in Saos-2 cells[57]. In contrast, M1 and M2 were significantly less active in binding to and activation of the IGF-IR and their mitogenicity in Saos-2 cells was equal to that of human insulin[57]. In another study, IGF-I and insulin glargine more efficiently stimulated phosphatidyl-inositol 3 phosphate PIP(3) production in breast cancer-derived MCF-7 cells as compared to human insulin[58]. In contrast, as compared to insulin, M1 and M2 showed lower potency in stimulating hybrid receptors (IR/IGF-IR), they induced less PIP(3) production, less Akt and Erk1/2 phosphorylation and less DNA synthesis in MCF-7 cells. These in vitro results are in keeping with our findings of slightly greater IR-A, IR-B and IGF-IR activation at high

(supraphysiological) concentrations by insulin glargine compared to human insulin, and with data showing that M1 and M2 do not share these properties of insulin glargine.

We also measured insulin glargine, M1 and M2 concentrations in plasma of 57 type 2 diabetic patients during insulin therapy. Moreover, we compared serum induced IR-A and IR-B activation (by IR-A and IR-B specific KIRA assays, respectively) of 104 patients treated with insulin glargine or NPH insulin. Interestingly we found that only M1, and not insulin glargine nor M2, could be detected in the plasma of these patients. This may (at least partly) explain why we previously[27] (as discussed in Chapter 4) were not able to detect differences in serum-induced IGF-IR activation between patients treated with insulin glargine or human insulin. Similarly, this may (partly) explain why we could not find differences in potency of serum to activate the IR-A or IR-B. These results do not support the idea that treatment with insulin glargine in type 2 diabetes leads to a stronger stimulation of the IR than NPH insulin. However, we did find a positive relationship between insulin dose and serum-induced IR-A activation for both treatment groups.

In contrast to what would be expected, we did not find insulin therapy to increase serum-induced IR-B activation. The absence of a change in IR-B and IR-A activation during insulin therapy is at first glance unexpected since the decrease in fasting glucose and HbA_{1C} levels after 36 weeks insulin therapy point towards an enhanced insulin action. Several mechanisms could explain this finding. First of all, it should be stressed that, as discussed above, this result was found under co-medication of metformin. Thus overall, the glucose lowering effect of insulin may have increased due to a metformin-induced improvement in insulin sensitivity. In addition, starting insulin therapy may have further improved insulin sensitivity by eliminating hyperglycaemia and by reducing free fatty acids[59]. Moreover, also in this study population, serum ALAT significantly decreased during insulin therapy[60]. Hepatic fat content usually correlates with ALAT[61] and increases hepatic insulin resistance[62] and as a consequence, with insulin requirements[62-63]. So, starting insulin therapy most likely increased insulin sensitivity in our study (by a combination of metformin use, decreased glucose toxicity and lipotoxicity), which lowers the need for circulating insulin(-like) activity. In addition, the prevailing circulating insulin activity mirrors not only the insulin subcutaneously administered and endogenous insulin secretion but also (hepatic) insulin clearance. An improvement in hepatic insulin extraction after starting insulin therapy may have contributed to an absence of a change in IR-B and IR-A activation[64]. Also, as reported in Chapter 4, by increasing the peripheral exogenous insulin concentration (and by lowering glucose levels), basal endogenous insulin secretion by the pancreas may decrease leading to a decrease of the portal insulin concentration[65]. One of the problems in the past concerned the difficulty of measuring the sum of the total effects of endogenous insulin and exogenous insulin (analogue)

levels. Our study suggests that with the IR specific KIRA assays, such measurement is probably possible. Absolute insulin bioactivity was higher for the IR-B than for the IR-A at baseline and after 36 weeks of insulin therapy. This latter difference is consistent with the intrinsically greater in vitro kinase activity of the IR-B than IR-A reported previously[66]. However, the difference may be also (partly) due to the lower number of IRs in the IR-A HEK cell line than in the IR-B HEK cell line which are used for the IR KIRA assays[67].

It needs to be addressed that serum samples from our studies performed in type 2 diabetic patients, were obtained from the LANMET study. The LANMET study was a multicenter, open, randomized, parallel-group study published in 2006[60] in which efficacy and safety of bedtime insulin glargine and metformin were compared to NPH insulin and metformin treatment in insulin-naive poorly controlled type 2 diabetic patients. So, the LANMET study was not primarily designed and performed to study the effects of insulin therapy on IR-A or IR-B or IGF-IR stimulating activities. Bioactivity could thus only be studied during a relatively short follow-up period and in subjects who were already treated with metformin. Therefore the post-hoc analysis in our study was inevitable.

In conclusion, the results in Chapter 3-5 show that in vitro results of insulin glargine may not be extrapolated to its in vivo actions. In vivo, insulin glargine is metabolized into M1 and M2 of which only the former can be detected in serum from patients treated with insulin glargine. M1 does not differ from human insulin in potency to activate the IGF-IR, IR-A and IR-B in vitro.

Serum IGF-IR, IR-A and IR-B bioactivity did not differ between patients treated with either insulin glargine or NPH insulin. These results do not support the idea that treatment with insulin glargine in type 2 diabetes leads to a stronger stimulation of the IGF-IR or IR than NPH insulin. However, the positive relationship between insulin dose and serum-induced IR-A activation for both treatment groups suggests that, irrespective of insulin type, there may be an enhanced IR-A signalling in subjects who are treated with relatively high insulin doses. Nevertheless, at present it is unclear whether this may have any consequences for insulin dosing in clinical practice.

It would be interesting to study the modifying effects of insulin therapy on IGF-IR bioactivity and IR bioactivity in type 1 diabetic subjects since they have decreased portal insulin concentrations on beforehand.

Moreover, future studies will have to investigate whether insulin therapy has similar effects on IR and IGF-IR bioactivity in the postprandial state (which is characterized by relatively high glucose levels) and whether bioactivity is related to the development of diabetic complications.

11.3 TOTAL IGF-I DEFICIENCY OR EXCESS; WHAT ABOUT IGF-IR BIOACTIVITY?

Growth hormone (GH) is considered the main regulator of circulating total IGF-I. Circulating total IGF-I is therefore routinely used for diagnosis and monitoring treatment of GH deficiency (GHD) and acromegaly. Nevertheless, discrepancies between clinical findings, GH and circulating total IGF-I levels are frequently encountered in clinical practice.

Many of the methods currently used for measurement of circulating total IGFs are hampered by interferences of IGFBPs remaining after extraction[68].

The most important reason that total IGF-I levels are still used as a measure of IGF-IR bioactivity has been the lack of reliable assays to measure IGF-IR bioactivity. In 2003, an IGF-IR specific KIRA assay was developed as an alternative method to evaluate levels of bioavailable IGF-I[5]. The principle of the IGF-IR KIRA assay is based on quantification of serum (or ligand)-induced IGF-IR phosphorylation in cells transfected with the human IGF-IR[5, 69]. In 2008, normal values for IGF-IR bioactivity have been established in our laboratory by using the IGF-IR KIRA assay[70]. Compared to current IGF-I immunoassays, this assay theoretically has the advantage of measuring the net effects of serum on IGF-IR activation since it does not ignore the modifying effects of IGFBPs and IGFBP proteases on the interaction between IGFs and the IGF-IR[37, 70]. Nevertheless, one should be cautious in interpreting IGF-IR bioactivity at the tissue level from circulating concentrations[71]. In addition to the circulating IGFBP profile and BP protease activity in the circulation, IGFBPs and BP proteases at the tissue level may also influence IGF-IR activation[71].

11.3.1. Growth hormone deficiency

With increasing age, the contribution of GH to circulating total IGF-I levels appears to diminish. In a substantial fraction of patients diagnosed as GH deficient, total IGF-I levels remain within the normal range, especially in patients above 40 yr of age[72-73]. It has been proven that the diagnosis of GH disorders cannot solely rely on determination of total IGF-I. Therefore, current diagnostic testing uses provocative tests of GH secretion[74].

In *Chapter 6* we investigated the diagnostic value of IGF-IR bioactivity (by the IGF-IR specific KIRA assay) in 94 patients with proven GHD, determined by GH provocative tests. 81 Patients had already been treated with recombinant human GH and were asked to discontinue GH treatment for one month before baseline values were established. 13 patients were GH-naive.

We found that only 62% of confirmed untreated GH deficient patients had total IGF-I levels below the normal range, whereas for IGF-IR bioactivity this percentage was 82%. Moreover, for total IGF-I this percentage decreased to 57% in patients aged above 40 years (compared to 88% below 40 years of age), while for IGF-IR bioactivity this percentage remained high in patients aged above 40 years of age (74% above 40 years,

84% below 40 years of age). This suggests that IGF-IR bioactivity can better distinguish untreated GH deficient patients from healthy subjects and thus offers advantages over the measurement of total IGF-I in the diagnosis of GHD. Moreover it suggests that circulating IGF-IR bioactivity is a better marker of GH action throughout lifespan. In favour of this argument, in our study we observed that IGF-IR bioactivity significantly decreased with longer duration of GHD, while such relationship was not found for total IGF-I.

It has been reported that the combination of ≥3 pituitary hormone deficits and a serum total IGF-I concentration <11nmol/L predicts adult GHD with 95% accuracy[75]. However, many patients with GHD have <3 pituitary hormone deficits. Our study shows that before starting GH therapy, the percentage of GH deficient patients with IGF-IR bioactivity below the normal range was high and independent of the number pituitary deficits, whereas for total IGF-I this percentage decreased with a decreasing number of pituitary deficits. Again, this suggests that circulating IGF-IR bioactivity is a better marker for diagnosing GHD than total IGF-I.

Although the cross-reactivity for IGF-II in this IGF-IR KIRA assay is only 12%[5], the contribution of IGF-II in untreated GH deficient subjects may be relatively more significant considering the fact that IGF-II production is relatively GH independent[76]. Indeed we found that addition of an IGF-II neutralizing antibody (80nmol/L) to ten fold diluted pooled sera from healthy subjects and from GH deficient subjects decreased the IGF-IR KIRA assay signal by 16% vs. 64% (Figure 3, unpublished data). Although IGF-II concentrations were not measured in our study, these results support the idea that the relative contribution of IGF-II is more important in GH deficient patients than in healthy subjects. In addition, this underlines again that the IGF-IR KIRA assay is more sensitive than the common total IGF-I immunoassays to detect differences in clinical state[37].

Untreated GHD is recognized to result in alterations in body composition, glucose and lipid metabolism, bone metabolism and physical performance[77]. The beneficial effects of long-term GH replacement on body composition and metabolism in patients with GHD are well documented[77]. In addition to metabolic disturbances in GHD, QoL is impaired[78-81]. Evaluations of QoL, one of the key clinical endpoints, have shown a high degree of variability although it has been found to improve with GH replacement therapy when determined by disease specific questionnaires[81-86].

During GH therapy, GH dose is nowadays titrated against total IGF-I concentrations. According to consensus guidelines, total IGF-I values should be kept in the age-related normal range[73, 87-88]. However, as discussed above, in a substantial fraction of patients diagnosed as GH deficient, total IGF-I concentrations are within the normal range before starting GH therapy[72, 89]. Yet, no consistent relation has been found between improvement of QoL and total IGF-I during GH therapy.

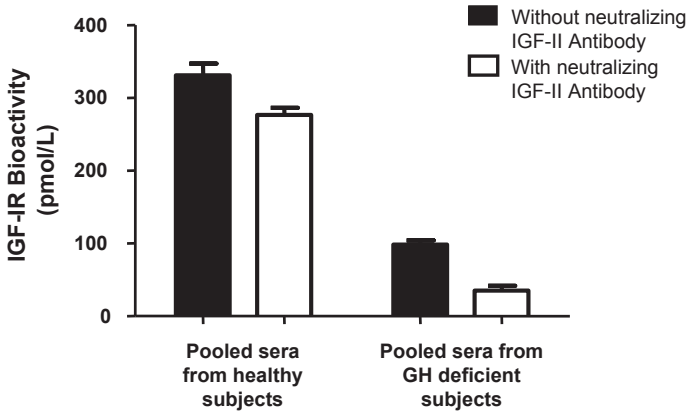


Figure 3.
The contribution of IGF-II present in serum in healthy subjects compared to GH deficient subjects. An IGF-II neutralizing antibody (80nmol/L) was incubated with ten fold diluted pooled sera from healthy subjects and from GH deficient subjects for 1 hour at 37°C. After incubation, protocol was followed as described in the Methods section in Chapter 6. Addition of an IGF-II neutralizing antibody to pooled sera from healthy subjects resulted in a 16% decrease of the IGF-IR KIRA signal. Addition of an IGF-II neutralizing antibody to pooled sera from GH deficient subjects resulted in a 64% decrease of the IGF-IR KIRA signal.

In *Chapter 7* we investigated the relationship between IGF-IR bioactivity (by the IGF-IR specific KIRA assay) and QoL during 12 months of GH treatment of 106 GH deficient patients (22 were GH-naive, 84 were already on GH treatment and discontinued therapy 4 weeks before baseline values were established). QoL was measured by using the Questions on Life Satisfaction-Hypopituitarism (QLS-H) Module and the Short form (SF) Health Survey-36 questionnaire (SF-36Q). The QLS-H module has been suggested to more likely identify impairments specifically related to GHD and (eventually) benefits of GH treatment than the SF-36Q. In contrast to the QLS-H, the SF-36Q was designed to evaluate QoL in the general population[81, 90].

After 12 months of GH replacement therapy, we did not find a significant improvement in disease specific QoL as measured by the QLS-H module. Our results are in contrast to previously published data from Rosilio et al., who reported a significant increase in QLS-H Z-scores after 1 yr of GH treatment in a much larger cohort of GHD patients[81]. The study by Rosilio et al. was performed in 7 countries including the Netherlands. In contrast to that study, the majority of patients in our study was not GH treatment naive at the moment of inclusion and had already been substituted for GH and other pituitary hormonal deficits for many years.

In our study baseline QLS-H Z-scores were established after 1 month off GH treatment. One month off GH treatment is generally considered to be long enough to restore baselines values for biochemical parameters (such as IGF-IR bioactivity and total IGF-I),

that existed before the start of the GH therapy[91]. However, our study suggests that one month off GH treatment may not be long enough to obtain QoL values back to the level before GH therapy was first started. As to yet there is no clear consensus how long a minimal withdrawal period from GH-treatment should be to obtain QoL values back to the level before GH therapy was first started. A recent study reported significant changes in QoL after 4 months of GH withdrawal[92].

The potential degree of improvement in QoL is generally proportional to the deviation from normality before start of GH therapy[93]. Poorer pretreatment QoL is usually associated with a greater improvement in QoL after administration of GH[94]. Thus the relatively high baseline QLS-H Z-scores in our study at baseline may have been an important factor for the lack of improvement in QoL after restarting GH therapy. However, QLS-H Z-scores also did not significantly increase after 12 months of GH replacement therapy in GH-naive patients. This may be due to the small sample size of GH-naive patients since GH replacement has been found to improve QoL only with a small effect size[95] .

Despite the fact that disease specific QLS-H Z-score had not improved after 12 months of GH treatment, QLS-H Z-score was significantly correlated to IGF-IR bioactivity but not to total IGF-I.

After 12 months, IGF-IR bioactivity was still below normal in more than 40% of patients in whom total IGF-I had normalized. Most strikingly we found, that in patients in whom IGF-IR bioactivity had not normalized after 12 months, QLS-H Z-scores were significantly lower compared to patients in whom IGF-IR bioactivity had normalized. This discrepancy was not found for total IGF-I.

In our study, GH dose was titrated against total IGF-I concentrations and in accordance to recent guidelines, serum IGF-I concentrations were targeted to the middle (Z-score=0)[96]. In this respect, it would be interesting to study QLS-H Z-scores using IGF-IR bioactivity as parameter to titrate GH dose.

12 Months of GH therapy also did not improve QoL when measured by the generic SF-36Q. The physical component summary of this questionnaire, but not the mental component summary, was positively related to both total IGF-I and IGF-IR bioactivity.

Patients with total IGF-I concentrations within the normal range scored significantly higher on the mental and physical component summary of the SF-36Q than patients with IGF-I concentrations below the normal range, while for IGF-IR bioactivity such differences were not found. So, total IGF-I was more strongly related to changes in the generic SF-36Q than IGF-IR bioactivity. In contrast, IGF-IR bioactivity was more strongly related to changes in the disease specific QLS-H module.

Several limitations of our study should be addressed. Our study did not have a randomized placebo-controlled design. Further GH was titrated on the results of total IGF-I con-

centrations, while IGF-IR bioactivity was not taken into account. Evaluation of effects of GH replacement is complex: IGF-I concentrations and IGF-IR bioactivity are regulated by many factors other than GH such as nutrition, adequate insulin secretion, the immune system, several tissue-specific factors such as gonadotrophins, sex steroids, cortisol and thyroid function and last but not least genetic factors[97-99]. Many factors other than IGF-I determine QoL. They include (abnormal) body composition, decreased exercise capacity, metabolic disturbances, other hormonal deficits and their treatments, possible neuroendocrine effects in the central nervous system and comorbidities[100].

There is evidence that the clinical manifestations and 'experience' of GHD in adulthood differs according to whether the patients acquired their disease in childhood or adulthood[101]. Unfortunately, the study was not powered to assess differences in QoL between subjects with adult-onset vs. childhood-onset GHD or to access differences between patients with different underlying disease or to study the effect of co-replaced hormones. In addition, the duration of follow-up was relatively short and some GH-mediated effects may only become manifest after long-term GH treatment.

As discussed above, during GH therapy GH dose is titrated against total IGF-I levels and the consensus guidelines advise that "values should be kept in the age-related normal range"[73, 87-88]. Thus, the values of total IGF-I can be normal to begin with and yet that is the goal of therapy. Previous studies have shown that the relationship between serum IGF-I response during GH treatment and other treatment effects such as metabolic endpoints and body composition is poor[102-103]. In addition it has been reported that long-term monitoring of GH therapy by using age- and sex-adjusted normal serum total IGF-I values, might lead to different dose adjustments when different total IGF-I immunoassays are used[104].

In *Chapter 8* we investigated the value of IGF-IR bioactivity for monitoring GH therapy (during 12 months) in 106 GH deficient patients.

The most striking finding in our study was, that during 12 months of GH treatment, changes in IGF-IR bioactivity did not parallel changes in total IGF-I. Interestingly, IGF-IR bioactivity remained subnormal despite normalization of total IGF-I in a considerable proportion of patients.

In addition, we found a significant positive relationship between IGF-IR bioactivity and beta-cell function as assessed by homeostasis model assessment (HOMA). GH has been shown to stimulate pancreatic beta-cell proliferation directly or via IGF-I[105-106]. IGF-I is considered to play an essential role in enhancing beta-cell function[105-107]. We observed no relationship between IGF-IR bioactivity and glucose or insulin resistance. In this respect our study has limitations; firstly, we used fasting insulin and glucose based indices

only, rather than the hyperinsulinemic clamp to assess insulin sensitivity. Secondly, total IGF-I concentrations were used to titrate GH doses, while changes in IGF-IR bioactivity were not taken into account. As a consequence total IGF-I concentrations had normalized in 81% of patients while IGF-IR bioactivity had normalized in only 51% of patients after 12 months of GH treatment.

In our study, most patients were not GH treatment naive and had already been substituted for GH and other pituitary hormonal deficits for many years. Nevertheless, after 12 months of GH treatment total IGF-I was inversely related to fat mass percentage suggesting an improvement in body composition. But despite a significant decrease in fat mass percentage after 12 months of GH treatment, waist circumference and WHR, fasting glucose, fasting insulin levels and insulin sensitivity as measured by HOMA increased. Total IGF-I was not related to waist circumference, WHR or insulin sensitivity. Thus, the improvement in body composition did not coincide with improvements in insulin sensitivity. This may be explained by the action of GH to induce insulin resistance, independent of IGF-I[108]. Also, probably as a consequence of this latter action of GH, long-term studies in which GH doses were titrated to normalise total IGF-I levels have reported a decrease, no change or an increase in insulin sensitivity[109-113].

The question arises why changes in IGF-IR bioactivity did not parallel changes in total IGF-I. First of all, as discussed above, the IGF-IR KIRA bioassay takes into account the modifying effects of IGFBPs on IGF-I action, while the IGF-I immunoassays do not. However, interestingly, both at baseline and after 12 months of GH treatment, total IGF-I was more strongly related to IGFBPs (IGFBP-1 and IGFBP-3) than IGF-IR bioactivity.

An alternative explanation could be, that kinetics of IGF-IR bioactivity in the circulation differed strikingly from those of total IGF-I during GH therapy. In favour of this latter possibility, it was previously found that GH administration to GH deficient patients caused only a parallel increase in circulating (immunoreactive) free IGF-I and total IGF-I levels the first 10 hrs after subcutaneous injection[114]. However, thereafter free IGF-I started to decrease while total IGF-I concentrations levelled off[114]. Furthermore, a close temporal relationship has been reported between serum GH and free IGF-I levels: GH serum peaked concomitantly with the rise in free IGF-I, and at the time when GH was not longer detectable in serum, free IGF-I started to decrease[114]. Such relationship was not found for total IGF-I. It is very likely that such relationship also exists between GH and IGF-IR bioactivity. After subcutaneous injection maximum serum GH is reached after about 260 minutes[115]. In our study, fasting blood samples were drawn in the morning, while the last GH injection had been given the evening before, thus long after maximum serum GH concentrations were reached. However, the fact that in our study population IGF-IR bioactivity was already lower than total IGF-I concentrations before starting GH

treatment, argues against the possibility that differences in kinetics caused the discrepancy between total IGF-I and IGF-IR bioactivity during GH therapy.

A third explanation for the observed discrepancy between total IGF-I and IGF-IR bioactivity during GH therapy may be that total IGF-I concentrations did not adequately reflect the actual IGF-I levels. As mentioned above, GH doses were titrated against circulating total IGF-I concentrations and not against IGF-IR bioactivity. The assay for total IGF-I in our study has been calibrated against the WHO International Reference Reagent for IGF-I Immunoassays (WHO IRR 87/518). The WHO IRR 87/518 is of low purity and the assigned IGF-I protein content is higher than the value that can be determined by quantitative analysis[116]. This may have resulted in falsely elevated total IGF-I concentrations. As a consequence, the effect of GH treatment on total IGF-I concentrations may have been (systematically) overestimated. This latter option is supported by our findings that in a considerable proportion of patients IGF-IR bioactivity was still subnormal after 12 months of GH treatment. In contrast to the total IGF-I immunoassay, the IGF-IR KIRA assay has been calibrated against a recombinant human IGF-I standard of high purity. Thus IGF-IR bioactivity measured by the IGF-IR KIRA assay may better correspond to the actual (bioactive) IGF-I present in the circulation than total IGF-I assays. As a consequence, subnormal IGF-IR bioactivity might be real and reflect a relative undertreatment of GHD despite normalization of total IGF-I concentrations.

Quarmby et al.[116] previously showed that calibration against WHO IRR 87/518 was the cause for systemic discrepancies between the Genentech total IGF-I normal ranges (not calibrated against WHO IRR 87/518) and the normal ranges of several other commercially available total IGF-I assays (all calibrated against WHO IRR 87/518).

Recently, a new WHO Reference Standard (IS 02/254) has become available. In contrast to the WHO IRR 87/518, this new standard is a >97%-pure recombinant and has been well characterized by the NIBSC[117]. It is at present unknown as to whether or not introduction of this new IGF-I standard in total IGF-I immunoassays will be able to eliminate the observed discrepancy between total IGF-I and IGF-IR bioactivity in our study. Future studies will hopefully provide an answer to this question.

In conclusion, the results in Chapter 6-8 suggest a role for IGF-IR bioactivity in the evaluation of adult GHD. Especially in the diagnosis of GHD, determination of IGF-IR bioactivity may offer advantages over total IGF-I.

During GH treatment (aiming at 'normal' total IGF-I concentrations), changes in total IGF-I did not parallel changes in IGF-IR bioactivity. In contrast to total IGF-I, IGF-IR bioactivity remained below the normal range in a considerable proportion of patients. Nevertheless, IGF-IR bioactivity was more strongly related to changes in the disease specific QLS-H module than total IGF-I, suggesting that IGF-IR bioactivity may better reflect changes in disease-specific QoL in GHD. On the other hand, both total IGF-I and IGF-IR

bioactivity were only related to body fat content or beta-cell function, respectively. In order to finally answer the question whether IGF-IR bioactivity could also be a valuable tool to monitor GH therapy in GHD and whether titrating GH dose against IGF-IR bioactivity results in a better (metabolic) clinical outcome than titrating against total IGF-I concentrations, we believe it will be necessary to perform a prospective randomized placebo- controlled study in which in one group GH dose is titrated against IGF-IR bioactivity, while in the other group GH dose is titrated against total IGF-I concentrations.

11.3.2. Acromegaly

Growth hormone and IGF-I are the 'classical' biochemical parameters used to diagnose acromegaly and to assess disease activity during treatment. Nowadays, the diagnosis of active acromegaly is based on clinical presentation, unsuppressed GH levels during an oral glucose tolerance test (OGTT), and elevated age- and gender-matched total IGF-I levels (and radiological detection of a pituitary tumour)[118]. During treatment IGF-I is considered to be the most feasible assessment[118-120]. If patients still have symptoms of active acromegaly, mean or single GH measurements can still be helpful (unless treated with Pegvisomant)[118-120]. Discordance between GH and total IGF-I levels has been noted in active acromegaly and in those following treatment[119-120].

In acromegaly, QoL has been reported to be significantly reduced in active untreated disease and in controlled treated disease, even after long-term follow-up. From the patient's perspective, QoL is one of the most important parameters of disease control. So the goals of treatment are to normalize IGF-I and GH levels and activity, and improve QoL[121]. However, normalization of levels of total serum IGF-I and GH do not necessarily reflect optimal QoL, nor relief of symptoms in patients with acromegaly[122-127].

In *Chapter 9* we investigated the value of circulating IGF-IR bioactivity in 15 patients with untreated acromegaly. QoL was assessed by the Acromegaly quality of life questionnaire (AcroQoL), by the Patient-Assessed Acromegaly Symptom Questionnaire (PASQ) and by the Short form (SF) Health Survey-36 questionnaire (SF-36Q).

In all acromegalic patients total IGF-I levels were above the upper limit of normal. However, in contrast, in 67% and 47% of patients respectively, IGF-IR bioactivity and IGFBP-3 were within the normal range.

The question arises why we found this discrepancy between total IGF-I levels and values for IGF-IR bioactivity? As already discussed above, total IGF-I measures are subjected to several pitfalls[117]. The discrepancy between total IGF-I and IGF-IR bioactivity could be due to falsely elevated total IGF-I levels due to the fact that the total IGF-I immunoassay used has been calibrated against the WHO IRR 87/518 reference reagent while the IGF-IR KIRA assay has been calibrated against a recombinant human IGF-I standard of high purity. On the other hand, most patients also had abnormal GH secretion and clinical

symptoms of active acromegaly. So, this could mean that total IGF-I is a better parameter to diagnose active acromegaly than IGF-IR bioactivity. However, all patients in our study were selected on high basal total IGF-I levels and not on high basal IGF-IR bioactivity. This may have introduced a bias.

An alternative possibility that may explain the discrepancy between total IGF-I and IGF-IR bioactivity, is the possible existence of only a mild degree of GH oversecretion in our study population. Ribeiro-Oliveira et al.[120] recently found that 63% of patients with untreated acromegaly had relatively low mean 24 hour GH levels despite high total IGF-I levels. It could be that our study population (in this respect) is comparable to that of Ribeiro-Oliveira et al. Although we did not measure 24 hour GH levels, in favour of this latter possibility, IGF-IR bioactivity was positively related to random GH measurements, while such relationship was absent for total IGF-I. GH as well as IGF-I may, independently from each other, cause signs and symptoms of acromegaly. Previously, it has been suggested by Van der Lely et al. that many acromegalics have a relatively low GH secretion and a considerable amount of total IGF-I, and vice versa[128]. Until now it has been impossible to show specific clinical characteristics between such groups. The measurement of IGF-IR bioactivity may be of help to distinguish between these two groups.

Our finding that IGF-IR bioactivity was within the normal range in a considerable number of patients is in agreement with previous results obtained in untreated acromegaly with IGF-I bioassays based on the measurement of sulphate incorporation in rat cartilage cells[129].

Normal reference ranges for total IGF-I and IGF-IR bioactivity are based on estimates of the central 95 percentile limits of measurements in healthy subjects[130]. Hence, the variation in the population determines the width of the 'normal' population-based reference range[131]. The 'normal' population-based reference range is an artificial concept since in biology no clear boundaries exist. In addition, during life, total IGF-I concentrations and IGF-IR bioactivity change with age. At each age, the population-based reference ranges for total IGF-I and IGF-IR bioactivity in serum are broad. The ability of laboratory reference ranges to detect an abnormal test result in an individual depends on the balance between contributors to the biologic variation[131]. If the major part of the overall variation is the result of variation within individuals, while differences between individual set-points are small, then a population-based reference range matches variations in each of any individual[131]. Conversely, if the overall variation is mainly caused by narrow individual variation around dispersed individual set-points, then a population-based reference range is unlikely to detect minor deviations from the individual set-point. This latter seems the case for IGF-I. Borofsky et al. found that most of the IGF-I variability was between individuals rather than within individuals[132]. This suggests that the ratio of the within- to between- individual variation for IGF-I is low. In other words, when the individual variation in serum IGF-IR bioactivity in healthy subjects is relatively narrow

compared to the 'normal' laboratory reference ranges, an IGF-IR bioactivity within the 'normal' reference range can be considerable abnormal (i.e. outside the 'individual' reference range). We previously demonstrated that common gene polymorphisms within the IGF-I gene may be an important factor responsible for the fact that IGF-I values fluctuate around a fixed setpoint in a single subject[98, 133]. Thus, it may be difficult to discriminate between active and adequately controlled acromegaly when IGF-I (bioactivity) is within the 'normal' reference range. Simultaneous measurement of GH secretion and IGF-I may help to solve this problem.

It has been suggested that patients' perception of his/her QoL cannot be inferred directly from hormonal levels[134]. Nevertheless, although the PASQ still requires validation before it can be used in everyday clinical practice, in our study, both total IGF-I and IGF-IR bioactivity were significantly related to the disease-specific PASQ. This latter relationship was stronger for total IGF-I than for IGF-IR bioactivity. Moreover, there was an inverse relationship between the physical component summary of the SF-36Q and IGF-IR bioactivity and total IGF-I. This latter relationship was stronger for IGF-IR bioactivity than for total IGF-I. Finally, IGF-IR bioactivity, but not total IGF-I, was significantly related to the physical dimension of the disease-specific AcroQoL. So, although IGF-IR bioactivity (in contrast to total IGF-I) was not above the normal range in all patients, IGF-IR bioactivity was more strongly related to health related QoL measures than total IGF-I, suggesting that IGF-IR bioactivity may better reflect physical limitations perceived by untreated acromegalic patients.

It should be stressed that our study has a considerable number of limitations. First of all only a small number of acromegalic patients was studied and it had a cross-sectional study design. Moreover, only fasting hormone levels were measured and there was no information on (24 hour) GH secretion.

In conclusion, despite the fact acromegalic patients had total IGF-I levels above the reference range, a considerable number of patients had IGF-IR bioactivity within the reference range. Yet, IGF-IR bioactivity within the reference range may still be abnormal for an individual. IGF-IR bioactivity was more strongly related to measures of QoL than total IGF-I. This suggests that IGF-IR bioactivity may better reflect physical limitations perceived by untreated acromegalic patients. At present it is unclear which target value for IGF-IR bioactivity should be considered for an individual indicative for recovery. Measures of QoL may be helpful in this respect. To obtain reference values characterizing a single subject is to use the subjects' previous values as a reference for any future value. An alternative strategy may be to combine GH and IGF-I values in combination with QoL measures.

11.4 DOES THE IGF-IR PLAY A ROLE IN THE PATHOGENESIS OF GRAVES' OPHTHALMOPATHY?

As discussed in Chapter 10, previous findings have raised the hypothesis that in (a subset of) Graves' patients immunoglobulins may stimulate the IGF-IR which may contribute to Graves' ophthalmopathy (GO) and suggest that the IGF-IR may be another important aetiologic autoantigen in GO.

In *Chapter 10* we studied the relationships between TSH binding inhibitory immunoglobulins (TBII) and serum IGF-IR stimulating activity in relationship to age in 70 patients with GO.

At a first glance we found overall no relationship between TBII levels in blood and IGF-IR stimulation. However, in this study, TSH-R antibody levels were measured by an immunoassay. These assays display high sensitivity and specificity for TSH-R autoantibodies. However, they do not measure the functional activity of these immunoglobulins to TSH-R, neither do they distinguish between stimulatory, blocking and neutral activity[135].

Since it was previously found that GO autoimmune disease activity correlates with TBII titers[136-139] we hypothesized that subjects with relatively high TBII titers and GO autoimmune activity would likely to be those with high titers of (other) autoantibodies (such as e.g. stimulating antibodies directed against the IGF-IR).

The TBII titer was not normally distributed but severely skewed to the right and therefore we stratified subjects into two groups: those with TBII titers above the mean + 1 SD (> 12 U/L) and those with TBII titers below the mean + 1SD (<12 U/L). Interestingly, we found that only in patients with relatively high TBII titers there was a positive correlation between IGF-IR stimulating activity and age. This positive relationship was unexpected since IGF-IR stimulating activity in healthy subjects, like total IGF-I, is known to decrease with age[70]. We hypothesized that this positive correlation was the result of the presence of circulating IGF-IR activating immunoglobulins. In this situation, IGF-IR stimulating activity, as measured by the KIRA assay, seems to reflect the sum of circulating endogenous IGF-I and IGF-IR stimulating activity induced by IgGs.

Moreover, the positive relationship between IGF-IR stimulating activity and age suggests that the relative contribution of these antibodies progresses with age. Perros et al. previously reported an association between severity of thyroid-associated ophthalmopathy and advancing age[140]. In addition, an age-associated increase in organ-specific autoantibodies in humans has been consistently reported[141].

Overall there was no relationship between IGF-IR activity and CAS. When the study population was stratified by TBII titers, median CAS in subjects with TBII titers above

the mean + 1 SD was higher than in subjects with TBII titers below the mean + 1 SD, although this latter difference missed statistical significance. In addition, overall there was no relationship between TBII and IGF-IR stimulating activity. However, when the study population was stratified by TBII titers, the relationship between IGF-IR stimulating activity and TBII was positive in subjects with TBII titers above the mean + 1 SD, while this relationship was negative in subjects with TBII titers below the mean + 1 SD.

The observed lack of statistical significance for the relationship between IGF-IR activity and CAS may be due to the small number of subjects included in our study. In addition, GO is considered active in patients with a CAS ≥ 3[142]. In our study the number of patients with CAS ≥ 3 was low. Thus, in a considerable number of patients the eye disease was not longer in the most active phase of GO. Another factor could be that IGF-IR stimulating activity probably reflects the sum of endogenous IGF-I and circulating Graves' disease (GD)-IgGs with IGF-IR stimulating activity. Thus, only a fraction of "total" serum IGF-IR activity is directly related to IGF-IR stimulating antibodies.

Nevertheless, our results point towards the presence of circulating IGF-IR activating immunoglobulins in the subgroup of patients with relatively high TBII. To test this, we measured IGF-IR stimulating activity before and after IgG depletion in the subgroup with high TBII levels. Interestingly, serum IGF-IR stimulating activity substantially decreased in patients with relatively high Z-scores for IGF-IR stimulating activity before IgG depletion. In contrast, there were only small changes in patients with relatively low Z-scores for serum IGF-IR stimulating activity before this treatment. This suggests that significant levels of IGF-IR stimulating antibodies were present in the former group.

Our results are in variance with previous studies which reported anti-IGF-IR antibody activities in a greater percentage of patients[143-145]. This variance may be due to differences in assay sensitivities and/or differences in fundamental peculiarities of cells used to measure IGF-IR activity. Previously it was found that, in contrast to fibroblasts from healthy donors, fibroblasts from patients with GD showed a response to GD-specific IgG[143]. We did not use cells of GD patients but our experiments were performed using HEK cells that were stably transfected with the human IGF-IR. Moreover, in contrast to the present study, in which ten times diluted serum samples were used to measure IGF-IR activity, others have used highly purified IgGs isolated from serum samples to measure IGF-IR stimulating effects[143-144]. Finally, in our study, not post-receptor effects or inhibition of[^{125}I]IGF-I binding was measured, but rather phosphorylation of tyrosine residues of the IGF-IR as primary read out to assess IGF-IR stimulating activity[143-145].

The question arises how relevant our observations are since the relative contribution of IgGs to total serum IGF-IR stimulating activity was maximally 'only' 16.5%. However, our study shows that the combination of IgGs plus endogenous IGF-I produced greater

stimulation than either agent alone. Moreover, antibody-induced receptor activation may show tissue-specific distribution and/or activity. In the eye the local circumstances may significantly differ from those in the circulation. In favour of this latter possibility orbital fibroblasts in patients with GO express higher levels of IGF-IR than normal fibroblasts[146]. In addition, human antibodies typically have a half-life greater than 1 week[147]. Due to their increased half-life, IGF-IR stimulating antibodies may induce prolonged activation of the IGF-IR as compared to endogenous IGF-I.

There is no simple relationship between the binding sites of antibodies and their biological effects[148]. In theory, stimulating IGF-IR antibodies may bind at the IGF-IR binding site (the orthosteric site). Orthosteric antibodies that mimic ligand signalling have also been reported for other receptors[147, 149]. However, in most cases, these antibodies probably block binding of the endogenous ligand and thereby block receptor activation[147]. It has also been reported that allosteric antibodies, antibodies that do not bind at the specific ligand binding site of receptors, can also activate cell signalling[150]. In favour of this latter mechanism, Kumar et al. found that the monoclonal TSH-R stimulating antibody M22 (like IGF-I) enhances phosphorylation of Akt, whereas a specific blocking antibody directed against the IGF-IR inhibited phosphorylation of Akt induced by M22 or IGF-I[151]. They suggested that the stimulation of hyaluronic acid production by GD-IgG reported by Smith et al. reflected TSHR stimulating autoantibodies rather than putative IGF-IR autoantibodies but that TSHR and IGF-IR signalling may be closely linked in the GO orbit. Tsui et al. demonstrated a physical relationship between TSHR and IGF-IR and showed that blocking of IGF-IR could block signalling provoked by both TSH and GD-IgGs[152]. So, the same antibodies that bind to TSH-R could also activate the IGF-IR. On the other hand, due to clear differences in the molecular structure of the TSH-R and the IGF-IR, it may also be likely that the two receptors are activated by two discrete antibodies: those directed against the IGF-IR and those directed against the TSH-R. It is important to emphasize that in our study, by using the IGF-IR KIRA assay, phosphorylation of the tyrosine residues within the beta-subunits of the IGF-IR is quantified and not subsequent intracellular signalling. So, although it may be well possible that the TSH-R and IGF-IR signalling pathways are closely linked, our findings show that in a subset of Graves' patients, circulating IgGs can directly modulate IGF-IR phosphorylation and signalling at the receptor level.

Very recently, Minich et al. have developed a luminescent immunoprecipitation assay (performed on human embryonic kidney cells stably transfected with the human IGF-IR) to detect IGF-IR antibodies in human serum[153]. By using this assay, they found that circulating IGF-IR autoantibodies display a wide concentration range in both control individuals and patients with GO[153]. However, the average (median) IGF-IR autoantibody concentrations were not different in controls and GO patients with untreated active

disease[153]. In addition, there was no association between IGF-IR autoantibodies levels and severity or activity of GO[153].

What accounts for the widely divergent results obtained in our study and that of Minich et al.?

The assay developed by Minich et al., in a certain way, resembles the TBII assay: this assay -like the TBII assay- quantifies antibody titer but does do not give any information about the biological effect of the IGF-IR antibodies (stimulating, neutral or inhibiting effects). The lower limits for this assay were based on arithmetical arguments and not on proven biological mechanisms. Another important limitation of the assay, is the possibility that relatively low affinity antibodies may have gone undetected in their assay. Important examples of low affinity antibodies have been described in the literature. In the study by Minich et al. when IGF-IR autoantibodies were added to hepatocarcinoma HepG2 cells no autophosphorylation of the IGF-IR was observed, but instead inhibition of the IGF-IR was found. This suggests, but does not prove, that in the assay of Minich et al. especially orthosteric antibodies were measured. Moreover, the likely diverse nature of antibodies present in sera from patients with GO may block or modify interactions with the IGF-IR in some cells while promoting it in others and may change in time.

In conclusion, by using the IGF-IR KIRA assay we found that in a subset of patients with GO, significant levels of IGF-IR stimulating antibodies were present in the circulation.

It would be interesting to study the relationship in (more active) GO between (high titers of) IGF-IR antibodies, determined by the assay by Minich et al., and IGF-IR stimulating activity, determined by our IGF-IR KIRA assay. Moreover, it would be interesting to study the relationship between stimulating TSH-R antibodies and IGF-IR stimulating activity in active GO patients.

REFERENCES

1. Okada Y, Yokono K, Katsuta A, Yoshida M, Morita S, Irino H, Goto T, Baba S, Roth RA & Shii K. Development of an assay for bioactive insulin. *Anal Biochem* 1998 **257** 134-138.
2. Belfiore A, Frasca F, Pandini G, Sciacca L & Vigneri R. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. *Endocr Rev* 2009 **30** 586-623.
3. Moller DE, Yokota A, Caro JF & Flier JS. Tissue-specific expression of two alternatively spliced insulin receptor mRNAs in man. *Mol Endocrinol* 1989 **3** 1263-1269.
4. Mosthaf L, Grako K, Dull TJ, Coussens L, Ullrich A & McClain DA. Functionally distinct insulin receptors generated by tissue-specific alternative splicing. *Embo J* 1990 **9** 2409-2413.
5. Chen JW, Ledet T, Orskov H, Jessen N, Lund S, Whittaker J, De Meyts P, Larsen MB, Christiansen JS & Frystyk J. A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum. *Am J Physiol Endocrinol Metab* 2003 **284** E1149-1155.
6. Yalow RS & Berson SA. Immunoassay of endogenous plasma insulin in man. *J Clin Invest* 1960 **39** 1157-1175.
7. Froesch ER, Buergi H, Ramseier EB, Bally P & Labhart A. Antibody-Suppressible and Nonsuppressible Insulin-Like Activities in Human Serum and Their Physiologic Significance. An Insulin Assay with Adipose Tissue of Increased Precision and Specificity. *J Clin Invest* 1963 **42** 1816-1834.
8. Boucher J, Tseng YH & Kahn CR. Insulin and insulin-like growth factor-1 receptors act as ligand-specific amplitude modulators of a common pathway regulating gene transcription. *J Biol Chem* 2010 **285** 17235-17245.
9. Glenn C & Armston A. Cross-reactivity of 12 recombinant insulin preparations in the Beckman Unicel DxI 800 insulin assay. *Ann Clin Biochem* 2010 **47** 264-266.
10. Heald AH, Bhattacharya B, Cooper H, Ullah A, McCulloch A, Smellie S & Wark G. Most commercial insulin assays fail to detect recombinant insulin analogues. *Ann Clin Biochem* 2006 **43** 306-308.
11. Varewijck AJ, Goudzwaard JA, Brugts MP, Lamberts SW, Hofland LJ & Janssen JA. Insulin glargine is more potent in activating the human IGF-I receptor than human insulin and insulin detemir. *Growth Horm IGF Res* 2010 **20** 427-431.
12. Owens DR & Bolli GB. Beyond the era of NPH insulin--long-acting insulin analogs: chemistry, comparative pharmacology, and clinical application. *Diabetes Technol Ther* 2008 **10** 333-349.
13. Lazarus NR. Insulin and insulin-like substances in the circulation. *Clin Endocrinol Metab* 1972 **1** 623-644.
14. LeRoith D, Adamo D, Werner H & Roberts Jr CT. Molecular and cellular biology of the insulin-like growth factors. In *Molecular Endocrinology, Basic Concepts and Clinical Correlations*, ch. 13, pp 181-183. Ed W DE, 1995.
15. Pandini G, Frasca F, Mineo R, Sciacca L, Vigneri R & Belfiore A. Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. *J Biol Chem* 2002 **277** 39684-39695.
16. Slieker LJ, Brooke GS, DiMarchi RD, Flora DB, Green LK, Hoffmann JA, Long HB, Fan L, Shields JE, Sundell KL, Surface PL & Chance RE. Modifications in the B10 and B26-30 regions of the B chain of human insulin alter affinity for the human IGF-I receptor more than for the insulin receptor. *Diabetologia* 1997 **40 Suppl 2** S54-61.
17. Kurtzhals P, Schaffer L, Sorensen A, Kristensen C, Jonassen I, Schmid C & Trub T. Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. *Diabetes* 2000 **49** 999-1005.

18. Nakagawa SH & Tager HS. Role of the COOH-terminal B-chain domain in insulin-receptor interactions. Identification of perturbations involving the insulin mainchain. *J Biol Chem* 1987 **262** 12054-12058.
19. Hemkens LG, Grouven U, Bender R, Gunster C, Gutschmidt S, Selke GW & Sawicki PT. Risk of malignancies in patients with diabetes treated with human insulin or insulin analogues: a cohort study. *Diabetologia* 2009 **52** 1732-1744.
20. Jonasson JM, Ljung R, Talback M, Haglund B, Gudbjornsdottir S & Steineck G. Insulin glargine use and short-term incidence of malignancies-a population-based follow-up study in Sweden. *Diabetologia* 2009 **52** 1745-1754.
21. Colhoun HM. Use of insulin glargine and cancer incidence in Scotland: a study from the Scottish Diabetes Research Network Epidemiology Group. *Diabetologia* 2009 **52** 1755-1765.
22. Currie CJ, Poole CD & Gale EA. The influence of glucose-lowering therapies on cancer risk in type 2 diabetes. *Diabetologia* 2009 **52** 1766-1777.
23. FDA. FDA Drug Safety Communication: Update to ongoing safety review of Lantus (insulin glargine) and possible risk of cancer. http://www.fda.gov/Drugs/DrugSafety/ucm239376.htm, 2011.
24. Sciacca L, Cassarino MF, Genua M, Pandini G, Le Moli R, Squatrito S & Vigneri R. Insulin analogues differently activate insulin receptor isoforms and post-receptor signalling. *Diabetologia* 2010 **53** 1743-1753.
25. Eckardt K, May C, Koenen M & Eckel J. IGF-1 receptor signalling determines the mitogenic potency of insulin analogues in human smooth muscle cells and fibroblasts. *Diabetologia* 2007 **50** 2534-2543.
26. Smith U & Gale EA. Does diabetes therapy influence the risk of cancer? *Diabetologia* 2009 **52** 1699-1708.
27. Varewijck AJ, Janssen JA, Vahatalo M, Hofland LJ, Lamberts SW & Yki-Jarvinen H. Addition of insulin glargine or NPH insulin to metformin monotherapy in poorly controlled type 2 diabetic patients decreases IGF-I bioactivity similarly. *Diabetologia* 2012 **55** 1186-1194.
28. Vehkavaara S & Yki-Jarvinen H. 3.5 years of insulin therapy with insulin glargine improves in vivo endothelial function in type 2 diabetes. *Arterioscler Thromb Vasc Biol* 2004 **24** 325-330.
29. Weinstein D, Simon M, Yehezkel E, Laron Z & Werner H. Insulin analogues display IGF-I-like mitogenic and anti-apoptotic activities in cultured cancer cells. *Diabetes Metab Res Rev* 2009 **25** 41-49.
30. DeFronzo RA, Binder C, Wahren J, Felig P, Ferrannini E & Faber OK. Sensitivity of insulin secretion to feedback inhibition by hyperinsulinaemia. *Acta Endocrinol (Copenh)* 1981 **98** 81-86.
31. Baxter RC & Turtle JR. Regulation of hepatic growth hormone receptors by insulin. *Biochem Biophys Res Commun* 1978 **84** 350-357.
32. Maes M, Underwood LE & Ketelslegers JM. Low serum somatomedin-C in insulin-dependent diabetes: evidence for a postreceptor mechanism. *Endocrinology* 1986 **118** 377-382.
33. Suikkari AM, Koivisto VA, Rutanen EM, Yki-Jarvinen H, Karonen SL & Seppala M. Insulin regulates the serum levels of low molecular weight insulin-like growth factor-binding protein. *J Clin Endocrinol Metab* 1988 **66** 266-272.
34. Gielen M, Mesotten D, Brugts M, Coopmans W, Van Herck E, Vanhorebeek I, Baxter R, Lamberts S, Janssen JA & Van den Berghe G. Effect of intensive insulin therapy on the somatotropic axis of critically ill children. *J Clin Endocrinol Metab* 2011 **96** 2558-2566.
35. Arafat AM, Weickert MO, Frystyk J, Spranger J, Schofl C, Mohlig M & Pfeiffer AF. The role of insulin-like growth factor (IGF) binding protein-2 in the insulin-mediated decrease in IGF-I bioactivity. *J Clin Endocrinol Metab* 2009 **94** 5093-5101.

36. Chestnut RE & Quarmby V. Evaluation of total IGF-I assay methods using samples from Type I and Type II diabetic patients. *J Immunol Methods* 2002 **259** 11-24.
37. Janssen JAMJL. IGF-I: Pro 's and Con's of a Bioassay. *Horm Res Paediatr* 2011 **76(suppl 1)** 106-110.
38. Bang P, Brismar K & Rosenfeld RG. Increased proteolysis of insulin-like growth factor-binding protein-3 (IGFBP-3) in noninsulin-dependent diabetes mellitus serum, with elevation of a 29-kilodalton (kDa) glycosylated IGFBP-3 fragment contained in the approximately 130- to 150-kDa ternary complex. *J Clin Endocrinol Metab* 1994 **78** 1119-1127.
39. Bereket A, Lang CH, Blethen SL, Gelato MC, Fan J, Frost RA & Wilson TA. Effect of insulin on the insulin-like growth factor system in children with new-onset insulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* 1995 **80** 1312-1317.
40. Nathan DM, Buse JB, Davidson MB, Ferrannini E, Holman RR, Sherwin R & Zinman B. Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes Care* 2009 **32** 193-203.
41. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR & Morris AD. Metformin and reduced risk of cancer in diabetic patients. *Bmj* 2005 **330** 1304-1305.
42. Bowker SL, Majumdar SR, Veugelers P & Johnson JA. Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. *Diabetes Care* 2006 **29** 254-258.
43. Suissa S & Azoulay L. Metformin and the Risk of Cancer: Time-related biases in observational studies. *Diabetes Care* 2012 **35** 2665-2673.
44. Jalving M, Gietema JA, Lefrandt JD, de Jong S, Reyners AK, Gans RO & de Vries EG. Metformin: taking away the candy for cancer? *Eur J Cancer* 2010 **46** 2369-2380.
45. Goodwin PJ, Pritchard KI, Ennis M, Clemons M, Graham M & Fantus IG. Insulin-lowering effects of metformin in women with early breast cancer. *Clin Breast Cancer* 2008 **8** 501-505.
46. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer* 2008 **8** 915-928.
47. Shaw RJ, Bardeesy N, Manning BD, Lopez L, Kosmatka M, DePinho RA & Cantley LC. The LKB1 tumor suppressor negatively regulates mTOR signaling. *Cancer Cell* 2004 **6** 91-99.
48. Ning J & Clemmons DR. AMP-activated protein kinase inhibits IGF-I signaling and protein synthesis in vascular smooth muscle cells via stimulation of insulin receptor substrate 1 S794 and tuberous sclerosis 2 S1345 phosphorylation. *Mol Endocrinol* 2010 **24** 1218-1229.
49. Kim J, Yoon MY, Choi SL, Kang I, Kim SS, Kim YS, Choi YK & Ha J. Effects of stimulation of AMP-activated protein kinase on insulin-like growth factor 1- and epidermal growth factor-dependent extracellular signal-regulated kinase pathway. *J Biol Chem* 2001 **276** 19102-19110.
50. Vigneri R, Squatrito S & Sciacca L. Insulin and its analogs: actions via insulin and IGF receptors. *Acta Diabetol* 2010 **47** 271-278.
51. Nagel JM, Staffa J, Renner-Müller I, Horst D, Vogeser M, Langkamp M, Hoeflich A, Göke B, Kolligs FT & Mantzoros CS. Insulin Glargine and NPH Insulin Increase to a Similar Degree Epithelial Cell Proliferation and Aberrant Crypt Foci Formation in Colons of Diabetic Mice *Hormones and Cancer* 2010 **1** 320-330.
52. Fagot JP, Blotiere PO, Ricordeau P, Weill A, Alla F & Allemand H. Does Insulin Glargine Increase the Risk of Cancer Compared With Other Basal Insulins?: A French nationwide cohort study based on national administrative databases. *Diabetes Care* 2012.
53. Du X, Zhang R, Xue Y, Li D, Cai J, Zhou S, Huang Z, Yu R & Liu Y. Insulin glargine and risk of cancer: a meta-analysis. *Int J Biol Markers* 2012 **27** e241-246.

54. Hansen BF, Danielsen GM, Drejer K, Sorensen AR, Wiberg FC, Klein HH & Lundemose AG. Sustained signalling from the insulin receptor after stimulation with insulin analogues exhibiting increased mitogenic potency. *Biochem J* 1996 **315 (Pt 1)** 271-279.
55. Frasca F, Pandini G, Sciacca L, Pezzino V, Squatrito S, Belfiore A & Vigneri R. The role of insulin receptors and IGF-I receptors in cancer and other diseases. *Arch Physiol Biochem* 2008 **114** 23-37.
56. Denley A, Bonython ER, Booker GW, Cosgrove LJ, Forbes BE, Ward CW & Wallace JC. Structural determinants for high-affinity binding of insulin-like growth factor II to insulin receptor (IR)-A, the exon 11 minus isoform of the IR. *Mol Endocrinol* 2004 **18** 2502-2512.
57. Sommerfeld MR, Muller G, Tschank G, Seipke G, Habermann P, Kurrle R & Tennagels N. In vitro metabolic and mitogenic signaling of insulin glargine and its metabolites. *PLoS One* 2010 **5** e9540.
58. Pierre-Eugene C, Pagesy P, Nguyen TT, Neuille M, Tschank G, Tennagels N, Hampe C & Issad T. Effect of insulin analogues on insulin/IGF1 hybrid receptors: increased activation by glargine but not by its metabolites M1 and M2. *PLoS One* 2012 **7** e41992.
59. Glaser B & Cerasi E. Early intensive insulin treatment for induction of long-term glycaemic control in type 2 diabetes. *Diabetes Obes Metab* 1999 **1** 67-74.
60. Yki-Jarvinen H, Kauppinen-Makelin R, Tiikkainen M, Vahatalo M, Virtamo H, Nikkila K, Tulokas T, Hulme S, Hardy K, McNulty S, Hanninen J, Levanen H, Lahdenpera S, Lehtonen R & Ryysy L. Insulin glargine or NPH combined with metformin in type 2 diabetes: the LANMET study. *Diabetologia* 2006 **49** 442-451.
61. Westerbacka J, Corner A, Tiikkainen M, Tamminen M, Vehkavaara S, Hakkinen AM, Fredriksson J & Yki-Jarvinen H. Women and men have similar amounts of liver and intra-abdominal fat, despite more subcutaneous fat in women: implications for sex differences in markers of cardiovascular risk. *Diabetologia* 2004 **47** 1360-1369.
62. Seppala-Lindroos A, Vehkavaara S, Hakkinen AM, Goto T, Westerbacka J, Sovijarvi A, Halavaara J & Yki-Jarvinen H. Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. *J Clin Endocrinol Metab* 2002 **87** 3023-3028.
63. Ryysy L, Hakkinen AM, Goto T, Vehkavaara S, Westerbacka J, Halavaara J & Yki-Jarvinen H. Hepatic fat content and insulin action on free fatty acids and glucose metabolism rather than insulin absorption are associated with insulin requirements during insulin therapy in type 2 diabetic patients. *Diabetes* 2000 **49** 749-758.
64. Polonsky KS, Given BD, Hirsch L, Shapiro ET, Tillil H, Beebe C, Galloway JA, Frank BH, Karrison T & Van Cauter E. Quantitative study of insulin secretion and clearance in normal and obese subjects. *J Clin Invest* 1988 **81** 435-441.
65. Bratusch-Marrain PR & Waldhausl WK. Suppression of basal, but not of glucose-stimulated insulin secretion by human insulin in healthy and obese hyperinsulinemic subjects. *Metabolism* 1985 **34** 188-193.
66. Kosaki A, Pillay TS, Xu L & Webster NJ. The B isoform of the insulin receptor signals more efficiently than the A isoform in HepG2 cells. *J Biol Chem* 1995 **270** 20816-20823.
67. Varewijck AJ, Brugts MP, Frystyk J, Goudzwaard JA, Uitterlinden P, Waaijers AM, Feng Y, Dimitrov DS, Lamberts SW, Hofland LJ & Janssen JA. Circulating insulin-like growth factors may contribute substantially to insulin receptor isoform A and insulin receptor isoform B signalling. *Mol Cell Endocrinol* 2013 **365** 17-24.
68. Clemmons DR. IGF-I assays: current assay methodologies and their limitations. *Pituitary* 2007 **10** 121-128.

69. Sadick MD, Intintoli A, Quarmby V, McCoy A, Canova-Davis E & Ling V. Kinase receptor activation (KIRA): a rapid and accurate alternative to end-point bioassays. *J Pharm Biomed Anal* 1999 **19** 883-891.
70. Brugts MP, Ranke MB, Hofland LJ, van der Wansem K, Weber K, Frystyk J, Lamberts SW & Janssen JA. Normal values of circulating insulin-like growth factor-I bioactivity in the healthy population: comparison with five widely used IGF-I immunoassays. *J Clin Endocrinol Metab* 2008 **93** 2539-2545.
71. Brismar K & Lewitt MS. The IGF and IGFBP system in insulin resistance and diabetes. In *IGF-I and Nutrition in Health and Disease* pp 251-270. Eds MS Houston, JMP Holly & EL Feldman: Humana Press Inc, Totowa, NJ, 2005.
72. Ghigo E, Aimaretti G, Gianotti L, Bellone J, Arvat E & Camanni F. New approach to the diagnosis of growth hormone deficiency in adults. *Eur J Endocrinol* 1996 **134** 352-356.
73. Ho KK. Consensus guidelines for the diagnosis and treatment of adults with GH deficiency II: a statement of the GH Research Society in association with the European Society for Pediatric Endocrinology, Lawson Wilkins Society, European Society of Endocrinology, Japan Endocrine Society, and Endocrine Society of Australia. *Eur J Endocrinol* 2007 **157** 695-700.
74. Molitch ME. Diagnosis of GH deficiency in adults--how good do the criteria need to be? *J Clin Endocrinol Metab* 2002 **87** 473-476.
75. Hartman ML, Crowe BJ, Biller BM, Ho KK, Clemmons DR & Chipman JJ. Which patients do not require a GH stimulation test for the diagnosis of adult GH deficiency? *J Clin Endocrinol Metab* 2002 **87** 477-485.
76. Rajaram S, Baylink DJ & Mohan S. Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. *Endocr Rev* 1997 **18** 801-831.
77. Simpson H, Savine R, Sonksen P, Bengtsson BA, Carlsson L, Christiansen JS, Clemmons D, Cohen P, Hintz R, Ho K, Mullis P, Robinson I, Strasburger C, Tanaka T & Thorner M. Growth hormone replacement therapy for adults: into the new millennium. *Growth Horm IGF Res* 2002 **12** 1-33.
78. McGauley GA. Quality of life assessment before and after growth hormone treatment in adults with growth hormone deficiency. *Acta Paediatr Scand Suppl* 1989 **356** 70-72; discussion 73-74.
79. Badia X, Lucas A, Sanmarti A, Roset M & Ulied A. One-year follow-up of quality of life in adults with untreated growth hormone deficiency. *Clin Endocrinol (Oxf)* 1998 **49** 765-771.
80. Wiren L, Whalley D, McKenna S & Wilhelmsen L. Application of a disease-specific, quality-of-life measure (QoL-AGHDA) in growth hormone-deficient adults and a random population sample in Sweden: validation of the measure by rasch analysis. *Clin Endocrinol (Oxf)* 2000 **52** 143-152.
81. Rosilio M, Blum WF, Edwards DJ, Shavrikova EP, Valle D, Lamberts SW, Erfurth EM, Webb SM, Ross RJ, Chihara K, Henrich G, Herschbach P & Attanasio AF. Long-term improvement of quality of life during growth hormone (GH) replacement therapy in adults with GH deficiency, as measured by questions on life satisfaction-hypopituitarism (QLS-H). *J Clin Endocrinol Metab* 2004 **89** 1684-1693.
82. Ahmad AM, Hopkins MT, Thomas J, Ibrahim H, Fraser WD & Vora JP. Body composition and quality of life in adults with growth hormone deficiency; effects of low-dose growth hormone replacement. *Clin Endocrinol (Oxf)* 2001 **54** 709-717.
83. Blum WF, Shavrikova EP, Edwards DJ, Rosilio M, Hartman ML, Marin F, Valle D, van der Lely AJ, Attanasio AF, Strasburger CJ, Henrich G & Herschbach P. Decreased quality of life in adult patients with growth hormone deficiency compared with general populations using the new, validated, self-weighted questionnaire, questions on life satisfaction hypopituitarism module. *J Clin Endocrinol Metab* 2003 **88** 4158-4167.

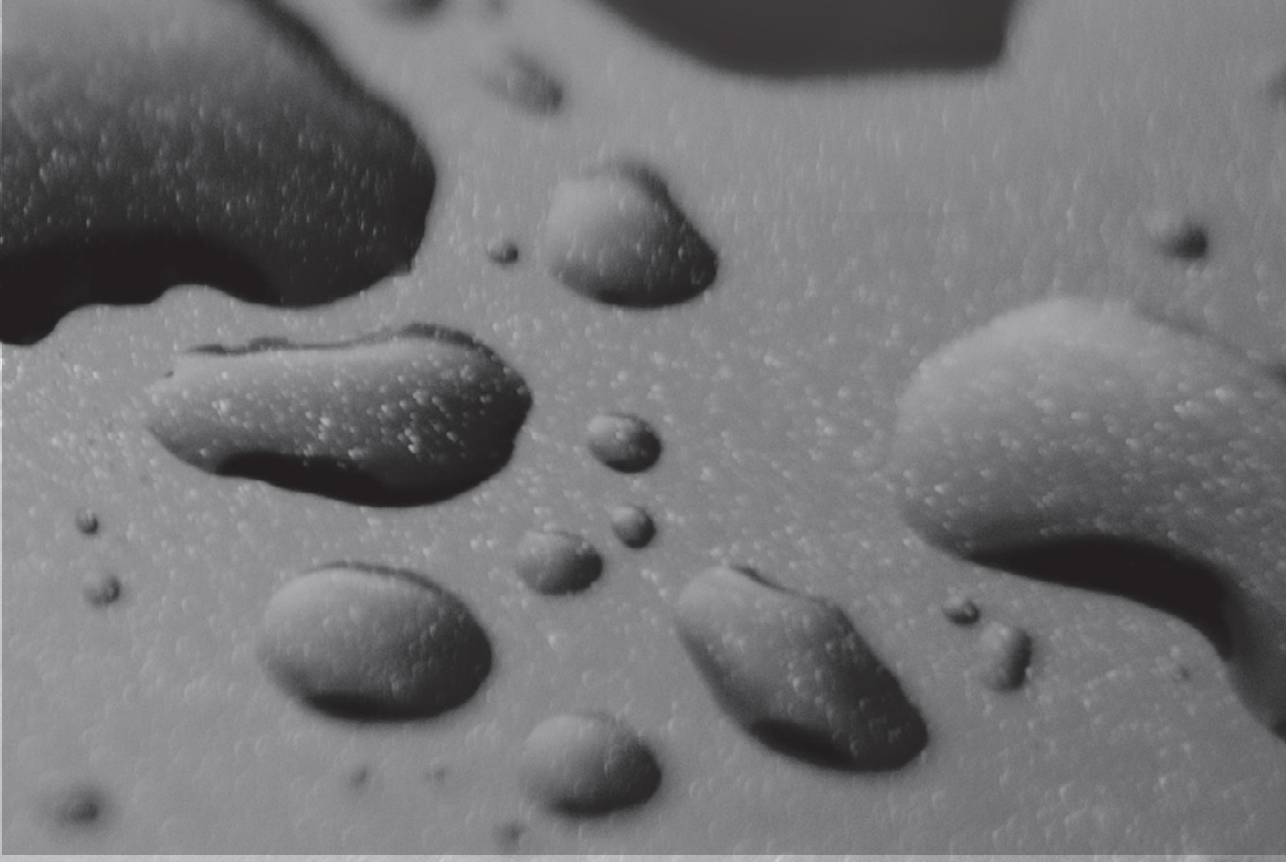
84. Herschbach P, Henrich G, Strasburger CJ, Feldmeier H, Marin F, Attanasio AM & Blum WF. Development and psychometric properties of a disease-specific quality of life questionnaire for adult patients with growth hormone deficiency. *Eur J Endocrinol* 2001 **145** 255-265.
85. Murray RD, Skillicorn CJ, Howell SJ, Lissett CA, Rahim A & Shalet SM. Dose titration and patient selection increases the efficacy of GH replacement in severely GH deficient adults. *Clin Endocrinol (Oxf)* 1999 **50** 749-757.
86. Murray RD, Skillicorn CJ, Howell SJ, Lissett CA, Rahim A, Smethurst LE & Shalet SM. Influences on quality of life in GH deficient adults and their effect on response to treatment. *Clin Endocrinol (Oxf)* 1999 **51** 565-573.
87. Consensus guidelines for the diagnosis and treatment of adults with growth hormone deficiency: summary statement of the Growth Hormone Research Society Workshop on Adult Growth Hormone Deficiency. *J Clin Endocrinol Metab* 1998 **83** 379-381.
88. Molitch ME, Clemmons DR, Malozowski S, Merriam GR & Vance ML. Evaluation and treatment of adult growth hormone deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab* 2011 **96** 1587-1609.
89. Varewijck AJ, Lamberts SW, Uitterlinden P, Hofland LJ & Janssen JA. IGF-I bioactivity better reflects growth hormone deficiency than total IGF-I. *J Clin Endocrinol Metab* 2011 **96** 2248-2254.
90. Webb SM. Measurements of quality of life in patients with growth hormone deficiency. *J Endocrinol Invest* 2008 **31** 52-55.
91. Gasco V, Corneli G, Beccuti G, Prodam F, Rovere S, Bellone J, Grottoli S, Aimaretti G & Ghigo E. Retesting the childhood-onset GH-deficient patient. *Eur J Endocrinol* 2008 **159 Suppl 1** S45-52.
92. Filipsson Nystrom H, Barbosa EJ, Nilsson AG, Norrman LL, Ragnarsson O & Johannsson G. Discontinuing long-term GH replacement therapy--a randomized, placebo-controlled crossover trial in adult GH deficiency. *J Clin Endocrinol Metab* 2012 **97** 3185-3195.
93. Molitch ME, Clemmons DR, Malozowski S, Merriam GR, Shalet SM, Vance ML & Stephens PA. Evaluation and treatment of adult growth hormone deficiency: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* 2006 **91** 1621-1634.
94. Valassi E, Brick DJ, Johnson JC, Biller BM, Klibanski A & Miller KK. Effect of growth hormone replacement therapy on the quality of life in women with growth hormone deficiency who have a history of acromegaly versus other disorders. *Endocr Pract* 2012 **18** 209-218.
95. Deijen JB, Arwert LI, Witlox J & Drent ML. Differential effect sizes of growth hormone replacement on Quality of Life, well-being and health status in growth hormone deficient patients: a meta-analysis. *Health Qual Life Outcomes* 2005 **3** 63.
96. Cook DM, Yuen KC, Biller BM, Kemp SF & Vance ML. American Association of Clinical Endocrinologists medical guidelines for clinical practice for growth hormone use in growth hormone-deficient adults and transition patients - 2009 update. *Endocr Pract* 2009 **15 Suppl 2** 1-29.
97. Juul A. Serum levels of insulin-like growth factor I and its binding proteins in health and disease. *Growth Horm IGF Res* 2003 **13** 113-170.
98. Rietveld I, Janssen JA, Hofman A, Pols HA, van Duijn CM & Lamberts SW. A polymorphism in the IGF-I gene influences the age-related decline in circulating total IGF-I levels. *Eur J Endocrinol* 2003 **148** 171-175.
99. Deal C, Ma J, Wilkin F, Paquette J, Rozen F, Ge B, Hudson T, Stampfer M & Pollak M. Novel promoter polymorphism in insulin-like growth factor-binding protein-3: correlation with serum levels and interaction with known regulators. *J Clin Endocrinol Metab* 2001 **86** 1274-1280.
100. Webb SM, Resmini E, Santos A & Badia X. *Quality of Life in Acromegaly and Growth Hormone Deficiency* 2011.

101. Attanasio AF, Lamberts SW, Matranga AM, Birkett MA, Bates PC, Valk NK, Hilsted J, Bengtsson BA & Strasburger CJ. Adult growth hormone (GH)-deficient patients demonstrate heterogeneity between childhood onset and adult onset before and during human GH treatment. Adult Growth Hormone Deficiency Study Group. *J Clin Endocrinol Metab* 1997 **82** 82-88.
102. Johannsson G, Grimby G, Sunnerhagen KS & Bengtsson BA. Two years of growth hormone (GH) treatment increase isometric and isokinetic muscle strength in GH-deficient adults. *J Clin Endocrinol Metab* 1997 **82** 2877-2884.
103. Johannsson G, Sverrisdottir YB, Ellegard L, Lundberg PA & Herlitz H. GH increases extracellular volume by stimulating sodium reabsorption in the distal nephron and preventing pressure natriuresis. *J Clin Endocrinol Metab* 2002 **87** 1743-1749.
104. Anckaert E, Schiettecatte J, Vanbesien J, Smitz J, Velkeniers B & De Schepper J. Variability among five different commercial IGF-1 immunoassays in conditions of childhood-onset GH deficiency and GH therapy. *Acta Clin Belg* 2006 **61** 335-339.
105. Hugl SR, White MF & Rhodes CJ. Insulin-like growth factor I (IGF-I)-stimulated pancreatic beta-cell growth is glucose-dependent. Synergistic activation of insulin receptor substrate-mediated signal transduction pathways by glucose and IGF-I in INS-1 cells. *J Biol Chem* 1998 **273** 17771-17779.
106. Rhodes CJ. IGF-I and GH post-receptor signaling mechanisms for pancreatic beta-cell replication. *J Mol Endocrinol* 2000 **24** 303-311.
107. Kulkarni RN, Holzenberger M, Shih DQ, Ozcan U, Stoffel M, Magnuson MA & Kahn CR. beta-cell-specific deletion of the Igf1 receptor leads to hyperinsulinemia and glucose intolerance but does not alter beta-cell mass. *Nat Genet* 2002 **31** 111-115.
108. Bondy CA, Underwood LE, Clemmons DR, Guler HP, Bach MA & Skarulis M. Clinical uses of insulin-like growth factor I. *Ann Intern Med* 1994 **120** 593-601.
109. Arafat AM, Mohlig M, Weickert MO, Schofl C, Spranger J & Pfeiffer AF. Improved insulin sensitivity, preserved beta cell function and improved whole-body glucose metabolism after low-dose growth hormone replacement therapy in adults with severe growth hormone deficiency: a pilot study. *Diabetologia* 2010 **53** 1304-1313.
110. Arwert LI, Roos JC, Lips P, Twisk JW, Manoliu RA & Drent ML. Effects of 10 years of growth hormone (GH) replacement therapy in adult GH-deficient men. *Clin Endocrinol (Oxf)* 2005 **63** 310-316.
111. Claessen KM, Appelman-Dijkstra NM, Adoptie DM, Roelfsema F, Smit JW, Biermasz NR & Pereira AM. Metabolic Profile in Growth Hormone-Deficient (GHD) Adults after Long-Term Recombinant Human Growth Hormone (rhGH) Therapy. *J Clin Endocrinol Metab* 2012.
112. Yuen K, Cook D, Ong K, Chatelain P, Fryklund L, Gluckman P, Ranke MB, Rosenfeld R & Dunger D. The metabolic effects of short-term administration of physiological versus high doses of GH therapy in GH deficient adults. *Clin Endocrinol (Oxf)* 2002 **57** 333-341.
113. Yuen KC & Dunger DB. Therapeutic aspects of growth hormone and insulin-like growth factor-I treatment on visceral fat and insulin sensitivity in adults. *Diabetes Obes Metab* 2007 **9** 11-22.
114. Lee PD, Durham SK, Martinez V, Vasconez O, Powell DR & Guevara-Aguirre J. Kinetics of insulin-like growth factor (IGF) and IGF-binding protein responses to a single dose of growth hormone. *J Clin Endocrinol Metab* 1997 **82** 2266-2274.
115. Janssen YJ, Frolich M & Roelfsema F. The absorption profile and availability of a physiological subcutaneously administered dose of recombinant human growth hormone (GH) in adults with GH deficiency. *Br J Clin Pharmacol* 1999 **47** 273-278.
116. Quarmby V, Quan C, Ling V, Compton P & Canova-Davis E. How much insulin-like growth factor I (IGF-I) circulates? Impact of standardization on IGF-I assay accuracy. *J Clin Endocrinol Metab* 1998 **83** 1211-1216.

117. Clemmons DR. Consensus statement on the standardization and evaluation of growth hormone and insulin-like growth factor assays. *Clin Chem* 2011 **57** 555-559.
118. Katznelson L, Atkinson JL, Cook DM, Ezzat SZ, Hamrahian AH & Miller KK. American Association of Clinical Endocrinologists medical guidelines for clinical practice for the diagnosis and treatment of acromegaly--2011 update. *Endocr Pract* 2011 **17 Suppl 4** 1-44.
119. Giustina A, Chanson P, Bronstein MD, Klibanski A, Lamberts S, Casanueva FF, Trainer P, Ghigo E, Ho K & Melmed S. A consensus on criteria for cure of acromegaly. *J Clin Endocrinol Metab* 2010 **95** 3141-3148.
120. Ribeiro-Oliveira A, Jr., Faje AT & Barkan AL. Limited utility of oral glucose tolerance test in biochemically active acromegaly. *Eur J Endocrinol* 2011 **164** 17-22.
121. Neggers SJ, Biermasz NR & van der Lely AJ. What is active acromegaly and which parameters do we have? *Clin Endocrinol (Oxf)* 2012 **76** 609-614.
122. Biermasz NR, Dekker FW, Pereira AM, van Thiel SW, Schutte PJ, van Dulken H, Romijn JA & Roelfsema F. Determinants of survival in treated acromegaly in a single center: predictive value of serial insulin-like growth factor I measurements. *J Clin Endocrinol Metab* 2004 **89** 2789-2796.
123. Neggers SJ, van Aken MO, de Herder WW, Feelders RA, Janssen JA, Badia X, Webb SM & van der Lely AJ. Quality of life in acromegalic patients during long-term somatostatin analog treatment with and without pegvisomant. *J Clin Endocrinol Metab* 2008 **93** 3853-3859.
124. Biermasz NR, Pereira AM, Smit JW, Romijn JA & Roelfsema F. Morbidity after long-term remission for acromegaly: persisting joint-related complaints cause reduced quality of life. *J Clin Endocrinol Metab* 2005 **90** 2731-2739.
125. Bonapart IE, van Domburg R, ten Have SM, de Herder WW, Erdman RA, Janssen JA & van der Lely AJ. The 'bio-assay' quality of life might be a better marker of disease activity in acromegalic patients than serum total IGF-I concentrations. *Eur J Endocrinol* 2005 **152** 217-224.
126. Hua SC, Yan YH & Chang TC. Associations of remission status and lanreotide treatment with quality of life in patients with treated acromegaly. *Eur J Endocrinol* 2006 **155** 831-837.
127. Rowles SV, Prieto L, Badia X, Shalet SM, Webb SM & Trainer PJ. Quality of life (QOL) in patients with acromegaly is severely impaired: use of a novel measure of QOL: acromegaly quality of life questionnaire. *J Clin Endocrinol Metab* 2005 **90** 3337-3341.
128. van der Lely AJ, de Herder WW, Janssen JA & Lamberts SW. Acromegaly: the significance of serum total and free IGF-I and IGF-binding protein-3 in diagnosis. *J Endocrinol* 1997 **155 Suppl 1** S9-13; discussion S15-16.
129. Wiedemann E, Uthne E, Tang RG, Spencer M, Saito T & Linfoot JA. Serum somatomedin activity by rat cartilage bioassay and human placental membrane radioreceptor assay in acromegaly and Cushings disease. In *Somatomedins and Growth*, pp 289-294. Eds G Giordano, JJ Van Wyk & F Minuto. New York: Academic Press 1979.
130. O'Brien PC & Dyck PJ. Procedures for setting normal values. *Neurology* 1995 **45** 17-23.
131. Andersen S, Bruun NH, Pedersen KM & Laurberg P. Biologic variation is important for interpretation of thyroid function tests. *Thyroid* 2003 **13** 1069-1078.
132. Borofsky ND, Vogelman JH, Krajcik RA & Orentreich N. Utility of insulin-like growth factor-1 as a biomarker in epidemiologic studies. *Clin Chem* 2002 **48** 2248-2251.
133. Vaessen N, Janssen JA, Heutink P, Hofman A, Lamberts SW, Oostra BA, Pols HA & van Duijn CM. Association between genetic variation in the gene for insulin-like growth factor-I and low birthweight. *Lancet* 2002 **359** 1036-1037.

134. Webb SM, Resmini E, Santos A & Badia X. Quality of Life in Acromegaly and Growth Hormone Deficiency In *Growth Hormone Related Diseases and Therapy*, ch. 12, pp 237-250. Ed H Ken. New York: Humana Press, 2011.
135. Ponto KA, Kanitz M, Olivo PD, Pitz S, Pfeiffer N & Kahaly GJ. Clinical relevance of thyroid-stimulating immunoglobulins in graves' ophthalmopathy. *Ophthalmology* 2011 **118** 2279-2285.
136. Acuna OM, Athannassaki I & Paysse EA. Association between thyroid-stimulating immunoglobulin levels and ocular findings in pediatric patients with Graves disease. *Trans Am Ophthalmol Soc* 2007 **105** 146-150; discussion 150-141.
137. Dragan LR, Seiff SR & Lee DC. Longitudinal correlation of thyroid-stimulating immunoglobulin with clinical activity of disease in thyroid-associated orbitopathy. *Ophthal Plast Reconstr Surg* 2006 **22** 13-19.
138. Eckstein AK, Plicht M, Lax H, Neuhauser M, Mann K, Lederbogen S, Heckmann C, Esser J & Morgenthaler NG. Thyrotropin receptor autoantibodies are independent risk factors for Graves' ophthalmopathy and help to predict severity and outcome of the disease. *J Clin Endocrinol Metab* 2006 **91** 3464-3470.
139. Gerding MN, van der Meer JW, Broenink M, Bakker O, Wiersinga WM & Prummel MF. Association of thyrotrophin receptor antibodies with the clinical features of Graves' ophthalmopathy. *Clin Endocrinol (Oxf)* 2000 **52** 267-271.
140. Perros P, Crombie AL, Matthews JN & Kendall-Taylor P. Age and gender influence the severity of thyroid-associated ophthalmopathy: a study of 101 patients attending a combined thyroid-eye clinic. *Clin Endocrinol (Oxf)* 1993 **38** 367-372.
141. Grolleau-Julius A, Ray D & Yung RL. The role of epigenetics in aging and autoimmunity. *Clin Rev Allergy Immunol* 2010 **39** 42-50.
142. Mourits MP, Prummel MF, Wiersinga WM & Koornneef L. Clinical activity score as a guide in the management of patients with Graves' ophthalmopathy. *Clin Endocrinol (Oxf)* 1997 **47** 9-14.
143. Pritchard J, Han R, Horst N, Cruikshank WW & Smith TJ. Immunoglobulin activation of T cell chemoattractant expression in fibroblasts from patients with Graves' disease is mediated through the insulin-like growth factor I receptor pathway. *J Immunol* 2003 **170** 6348-6354.
144. Smith TJ & Hoa N. Immunoglobulins from patients with Graves' disease induce hyaluronan synthesis in their orbital fibroblasts through the self-antigen, insulin-like growth factor-I receptor. *J Clin Endocrinol Metab* 2004 **89** 5076-5080.
145. Weightman DR, Perros P, Sherif IH & Kendall-Taylor P. Autoantibodies to IGF-1 binding sites in thyroid associated ophthalmopathy. *Autoimmunity* 1993 **16** 251-257.
146. Smith TJ. The putative role of fibroblasts in the pathogenesis of Graves' disease: evidence for the involvement of the insulin-like growth factor-1 receptor in fibroblast activation. *Autoimmunity* 2003 **36** 409-415.
147. Bhaskar V, Goldfine ID, Bedinger DH, Lau A, Kuan HF, Gross LM, Handa M, Maddux BA, Watson SR, Zhu S, Narasimha AJ, Levy R, Webster L, Wijesuriya SD, Liu N, Wu X, Chemla-Vogel D, Tran C, Lee SR, Wong S, Wilcock D, White ML & Corbin JA. A fully human, allosteric monoclonal antibody that activates the insulin receptor and improves glycemic control. *Diabetes* 2012 **61** 1263-1271.
148. O'Brien RM, Soos MA & Siddle K. Monoclonal antibodies to the insulin receptor stimulate the intrinsic tyrosine kinase activity by cross-linking receptor molecules. *Embo J* 1987 **6** 4003-4010.
149. Zick Y, Rees-Jones RW, Taylor SI, Gorden P & Roth J. The role of antireceptor antibodies in stimulating phosphorylation of the insulin receptor. *J Biol Chem* 1984 **259** 4396-4400.

150. Cazorla M, Arrang JM & Premont J. Pharmacological characterization of six trkB antibodies reveals a novel class of functional agents for the study of the BDNF receptor. *Br J Pharmacol* 2011 **162** 947-960.
151. Kumar S, Iyer S, Bauer H, Coenen M & Bahn RS. A stimulatory thyrotropin receptor antibody enhances hyaluronic acid synthesis in graves' orbital fibroblasts: inhibition by an IGF-I receptor blocking antibody. *J Clin Endocrinol Metab* 2012 **97** 1681-1687.
152. Tsui S, Naik V, Hoa N, Hwang CJ, Afifiyan NF, Sinha Hikim A, Gianoukakis AG, Douglas RS & Smith TJ. Evidence for an association between thyroid-stimulating hormone and insulin-like growth factor 1 receptors: a tale of two antigens implicated in Graves' disease. *J Immunol* 2008 **181** 4397-4405.
153. Minich WB, Dehina N, Welsink T, Schwiebert C, Morgenthaler NG, Kohrle J, Eckstein A & Schomburg L. Autoantibodies to the IGF1 Receptor in Graves' Orbitopathy. *J Clin Endocrinol Metab* 2012.



Summary
Samenvatting

SUMMARY

There are great functional and structural similarities between insulin and the IGFs on the one hand, and the insulin receptor (IR) and IGF-I receptor (IGF-IR) on the other hand. It may therefore not be surprising that more than fifty years ago it was already found that serum contained a higher amount of total insulin-like bioactivity than immunoreactive insulin and that anti-insulin antibodies could only block a small portion of total insulin bioactivity of serum. However, due to the highly variable results of bioassays, they were replaced by insulin radioimmunoassays (RIAs). Although the RIA for insulin has greatly increased knowledge of the physiology of glucose homeostatasis and of the various causes of diabetes mellitus it neglects the potential contributions of IGFs to insulin-like signalling. Moreover, insulin immunoassays do not assess potential biological effects of circulating insulin-like factors on the two IR isoforms (IR-A and the IR-B).

In 2003, Chen et al. developed a Kinase Receptor Activation (KIRA) bioassay specific for the IGF-IR. The principle of this assay is based on quantification of IGF-IR activation after stimulation with serum (or ligands) in vitro. The IGF-IR KIRA assay has proven to be sensitive to the modifying influences of circulating IGF binding proteins (IGFBPs) and IGFBP-proteases and to have an overall precision that is comparable with the traditional IGF-I immunoassays. Such KIRA assays had not yet been described for the human IR-A and IR-B.

The aim of this thesis was to investigate the (patho)physiologic role of IR and IGF-IR bioactivity in health and disease, specifically diabetes, growth hormone deficiency (GHD), acromegaly and Graves' ophthalmopathy (GO).

Chapter 1 First the history of the different pathways to the same summit, namely identification of insulin and the IGFs, is described. Then an overview on the insulin- IGF system is provided with specific attention to the impact of disease (diabetes, GHD, acromegaly and GO and of treatment on this system.

In **Chapter 2** we describe the development and validation of two KIRA assays specific for the IR-A and IR-B, respectively, which quantify ligand/serum induced phosphorylation of the tyrosine residues within the IRs.

Both KIRA assays showed a reasonable sensitivity and for bioassays exceptionally low intra- and inter-assay variations. The operational range of both assays allowed the for determination of total insulin-like activity in human serum. Analysis of serum samples showed IGF-I and IGF-II present in human serum may contribute substantially to IR-A and IR-B signalling in vitro. So, the two IR KIRA assays may be useful tools to disentangle the relative contribution of the IGFs present in serum on IR specific signalling.

In **Chapter 3** we studied whether short- and long-acting insulin analogues differ from human insulin in their potency to activate the IGF-IR, IR-A and IR-B in vitro by using KIRA assay specific for the respective receptors.

We found that insulin glargine, at supraphysiological levels, was more potent than human insulin in activating the IGF-IR in vitro. However, in vivo, a considerable fraction of insulin glargine is metabolized before it enters the circulation (see Chapter 5). This makes it questionable whether in vitro results of insulin glargine may be extrapolated and representative for its actions in vivo.

In **Chapter 4** we measured serum IGF-IR bioactivity (by using the IGF-IR KIRA assay) in 104 type 2 diabetic patients randomized for insulin glargine or NPH insulin for 36 weeks.

We found that at baseline and after 36 weeks of insulin therapy, there was no difference in IGF-IR bioactivity between the two treatment groups. We hypothesized that a potential explanation for the discrepancies between the previous insulin glargine in vitro results and these in vivo results could be, that in vivo concentrations of insulin glargine do probably not reach concentrations at which we and others had observed differences in IGF-IR activation in vitro. Another explanation for the discrepancies between the in vitro and in vivo results of insulin glargine could be that insulin glargine is metabolized in vivo. The metabolites of insulin glargine are less potent than native insulin glargine to activate the IGF-IR. The findings presented in Chapter 5 support the latter hypothesis.

Moreover, we found that prolonged insulin treatment, instead of increasing IGF-IR bioactivity, decreased IGF-IR bioactivity. Suppression of portal insulin by exogenous insulin may have been a mechanism that led to the (decreased hepatic IGF-I production and) reduced bioavailable IGF-I. Reduced bioavailable IGF-I has also been found during insulin therapy or hyperinsulinemic euglycaemic clamp studies performed previously and has been attributed to lower C-peptide levels and/or changes in IGFBPs. Unfortunately, in our study, IGFBPs were not measured. In accordance to these studies, we found that in contrast to IGF-IR bioactivity, total IGF-I levels did not change during insulin treatment. However, immunoasssays not always accurately measure total IGF-I levels due to problems with assay standardization and/or with assay methodology. In this respect, it has been suggested that the IGF-IR KIRA assay is more sensitive than the common total IGF-I immunoassays to detect differences in clinical state.

In **Chapter 5** we investigated the in vitro potency of insulin glargine, its metabolites (M1, M2), IGF-I and NPH insulin to activate the IGF-IR, IR-A and IR-B by using KIRA assays specific for the respective receptors. In addition, we measured concentrations of insulin glargine, M1 and M2 concentrations (by liquid chromatography–mass spectrometry) in plasma of 57 type 2 diabetic patients during insulin therapy. Finally, we compared serum

induced IR-A and IR-B activation (by using IR KIRA assays) of 104 patients treated with insulin glargine or NPH insulin.

We found that, in contrast to insulin glargine, M1 and M2 induced equal IGF-IR activation as human insulin. M1 was also equally potent as human insulin in activating the IR-A and IR-B. In contrast, M2, at supraphysiological concentrations, was more potent than human insulin in activating both IR isoforms.

Only M1, and not insulin glargine nor M2, could be detected in the plasma of the treated patients. This may (at least partly) explain why we were not able to detect differences in serum-induced IR-A or IR-B activation between both treatment groups and why we previously (discussed in Chapter 4) found no differences in serum induced IGF-IR activation.

We found a positive relationship between insulin dose and serum-induced IR-A activation for both treatment groups. This suggests that, irrespective of type of insulin therapy chosen, there may be an enhanced IR-A signalling in subjects who are treated with relatively high insulin doses.

After insulin therapy we found serum-induced IR-B or IR-A activation to be unchanged. The latter is unexpected since fasting glucose and HbA_{1C} levels did decrease pointing towards an enhanced insulin action. Several mechanisms could explain this finding. Starting insulin therapy most likely increased insulin sensitivity which lowers the need for circulating insulin(-like) activity. In addition, an improvement in hepatic insulin extraction after start of the insulin therapy may have contributed to the unchanged IR-B and IR-A activation. Also, as reported in Chapter 4, exogenous insulin (therapy) may suppress portal insulin levels. One of the problems inherent in the past concerns the difficulty of measuring the sum of the total effects of endogenous insulin and exogenous insulin (analogue) levels. Our study suggests that with the IR specific KIRA assays, such measurement is probably possible. In this respect, it would be interesting to study the modifying effects of insulin therapy on circulating IGF-IR bioactivity and IR bioactivity in type 1 diabetic subjects since they have decreased portal insulin concentrations on beforehand.

In **Chapter 6**, we investigated the diagnostic value of IGF-IR bioactivity (by using the IGF-IR KIRA assay) in 94 patients with proven GHD, diagnosed by GH provocative tests.

We found that IGF-IR bioactivity was more frequently below the normal range in untreated GH deficient patients than total IGF-I levels, especially in patients >40 years of age. This suggests that IGF-IR bioactivity can better distinguish untreated GH deficient patients from healthy subjects and thus offers advantages over the measurement of total IGF-I in the diagnosis of GHD. Moreover it suggests that circulating IGF-IR bioactivity is a better marker of GH action in untreated GH deficient subjects throughout lifespan. In favour of this argument, in our study we observed that IGF-IR bioactivity significantly

decreased with longer duration of GHD, while such relationship was not found for total IGF-I. Finally, we found that with a decreasing number of additional pituitary deficits, total IGF-I levels more frequently remained within the normal range, whereas the percentage below the normal range was high for IGF-IR bioactivity and independent of additional deficits. These results suggest that determination of IGF-IR bioactivity may offer advantages in the evaluation of adult GHD compared to total IGF-I as bioactivity better reflects GHD as defined by GH stimulation tests, especially in subjects >40 years of age.

In **Chapter 7** we investigated the relationship between IGF-IR bioactivity (by using the IGF-IR KIRA assay) and quality of life (QoL) during 12 months of GH treatment of 106 GH deficient patients (22 were GH-naive, 84 were already on GH treatment and discontinued therapy 4 weeks before baseline values were established). QoL was measured by using the disease specific QLS-H module and the generic SF-36 questionnaire.

Despite a significant increase in IGF-IR bioactivity and total IGF-I after 12 months of GH treatment, we did not find a significant improvement in QoL as measured by the QLS-H module. The relatively high QLS-H Z-scores in our study suggest that one month off GH treatment may not be long enough to obtain QoL values back to the level before GH therapy was first started and may have been an important factor for the lack of improvement in QoL after restarting GH therapy. However, in our study, QLS-H Z-scores also did not significantly increase in GH-naive patients. This may be due to the small sample size of GH-naive patients since GH replacement has been found to improve QoL only with a small effect size.

Despite the fact that disease specific QLS-H Z-score had not improved after 12 months of GH treatment, IGF-IR bioactivity was significantly correlated to QLS-H Z-score. Moreover, after 12 months, IGF-IR bioactivity was still below normal in more than 40% of patients in whom total IGF-I had normalized. In patients in whom IGF-IR bioactivity had not normalized after 12 months, QLS-H Z-scores were significantly lower compared to patients in whom IGF-IR bioactivity had normalized. This discrepancy was not found for total IGF-I. In this respect, it would be interesting to study QLS-H scores using IGF-IR bioactivity as parameter to titrate GH dose.

12 Months of GH therapy also did not improve QoL when measured by the SF-36 questionnaire. The physical component summary (PCS) of this questionnaire was positively related to both total IGF-I and IGF-IR bioactivity. Patients with total IGF-I concentrations within the normal range scored significantly higher on the SF-36 questionnaire than patients with IGF-I concentrations below the normal range, while for IGF-IR bioactivity such differences were not found.

Our study suggests that IGF-IR bioactivity may better reflect changes in disease-specific QoL in GH-deficient patients than total IGF-I and that both measurements may reflect different aspects of QoL.

In **Chapter 8** we investigated the value of IGF-IR bioactivity (by using the IGF-IR KIRA assay) for monitoring GH therapy (during 12 months) in 106 GH deficient patients.

We found that changes in IGF-IR bioactivity did not parallel changes in total IGF-I; after 12 months of GH therapy, IGF-IR bioactivity remained subnormal despite normalization of total IGF-I in a considerable proportion of patients.

After 12 months of GH treatment, we observed a significant positive relationship between IGF-IR bioactivity and beta-cell function as assessed by HOMA. We observed no relationship between IGF-IR bioactivity and glucose or insulin resistance. However, we used fasting insulin and glucose based indices only, rather than hyperinsulinemic clamp to assess insulin sensitivity. Moreover, only total IGF-I concentrations were used to titrate GH doses and not changes in IGF-IR bioactivity.

After 12 months of GH treatment total IGF-I was inversely related to body fat mass percentage, suggesting an improvement in body composition. However, despite a significant decrease in fat mass percentage, waist circumference and WHR, there was an increase in fasting glucose, fasting insulin levels and insulin sensitivity. Total IGF-I was not related to waist circumference, WHR or insulin sensitivity. Thus the improvement in body composition did not coincide with improvements in insulin sensitivity. This may be explained by the action of GH to induce insulin resistance, independent of IGF-I.

Why did changes in IGF-IR bioactivity not parallel changes in total IGF-I? First, as discussed above, the IGF-IR KIRA bioassay takes into account the modifying effects of IGFBPs on IGF-I action, while IGF-I immunoassays do not. Secondly, kinetics of IGF-IR bioactivity in the circulation may differ from those of total IGF-I during GH therapy. Finally, total IGF-I concentrations may have not adequately reflected the actual IGF-I levels. GH doses were titrated only against total IGF-I concentrations and not against IGF-IR bioactivity. The IGF-I immunoassay used in our study has been calibrated against a WHO International Reference Reagent for IGF-I Immunoassays which is of low purity. In contrast, the IGF-IR KIRA assay has been calibrated against a recombinant human IGF-I standard of high purity. As a consequence, the effect of GH treatment on total IGF-I concentrations may have been (systematically) overestimated and the subnormal IGF-IR bioactivity might be real and reflect a relative undertreatment of GHD.

In order to finally answer the question whether IGF-IR bioactivity could be a valuable tool to monitor GH therapy in GHD and whether titrating GH dose against IGF-IR bioactivity results in a better (metabolic) clinical outcome than titrating against total IGF- concentrations, we believe it is necessary to perform a prospective randomized

placebo- controlled study in which in one group GH dose is titrated against IGF-IR bioactivity, while in the other group GH dose is titrated against total IGF-I concentrations.

In **Chapter 9** we investigated the value of circulating IGF-IR bioactivity in 15 patients with untreated acromegaly. QoL was assessed by the AcroQoL, PASQ and by the SF-36 questionnaire.

In all acromegalic patients total IGF-I levels were above the upper limit of normal. However, in contrast, a considerable number of the patients had values for IGF-IR bioactivity and IGFBP-3 within the normal range. The question arises why we found this discrepancy between total IGF-I levels and values for IGF-IR bioactivity? As already discussed above, total IGF-I measures are subjected to several pitfalls. The discrepancy between total IGF-I and IGF-IR bioactivity could be due to falsely elevated total IGF-I levels due to the fact that the total IGF-I immunoassay used has been calibrated against the WHO IRR 87/518 reference reagent (see above). On the other hand, most patients also had abnormal GH secretion and clinical symptoms of active acromegaly. So, this could mean that total IGF-I is a better parameter to diagnose active acromegaly than IGF-IR bioactivity. However, all patients in our study were selected on high basal total IGF-I levels and not high basal IGF-IR bioactivity which may have introduced a bias.

An alternative possibility that may explain the discrepancy is the possible existence of only a mild degree of GH oversecretion in our study population. Previously, it has been suggested that many acromegalics have a relatively low GH secretion in combination with a considerable amount of total IGF-I, and vice versa. Until now it has been impossible to show specific clinical characteristics between such groups. The measurement of IGF-IR bioactivity may be of help to distinguish between these two groups.

We previously found that the normal range of IGF-IR bioactivity within the healthy population, over the whole age range, is very wide. This opens the possibility that IGF-IR bioactivity within the normal reference range, may still be too high for an (acromegalic) individual. Thus, it may be difficult to discriminate between active and adequately controlled acromegaly when IGF-IR bioactivity is within the 'normal' reference range. Simultaneous measurement of GH secretion and IGF-I may help to solve this problem.

Both total IGF-I and IGF-IR bioactivity were significantly related to the disease-specific PASQ. Moreover, there was an inverse relationship between the physical component summary of the SF-36 questionnaire. and IGF-IR bioactivity and total IGF-I. Finally, IGF-IR bioactivity, but not total IGF-I, was significantly related to the physical dimension of the disease-specific AcroQoL. So, although IGF-IR bioactivity (in contrast to total IGF-I) was not above the normal range in all patients, IGF-IR bioactivity was more strongly related to health related QoL measures than total IGF-I, suggesting that IGF-IR bioactivity may better reflect physical limitations perceived by untreated acromegalic patients. Thus,

also in untreated acromegaly, IGF-IR bioactivity may be of additional value in the evaluation of QoL.

In **Chapter 10** we studied the relationships between Thyrotrophin Binding Inhibiting Immunoglobulins (TBIIs) and serum IGF-IR stimulating activity in relationship to age in 70 patients with GO.

Overall we found no relationship between TBII levels in blood and IGF-IR stimulating activity and no relationship beween IGF-IR stimulating activity and clinical activity score (CAS). Previously it has been found that GO autoimmune disease activity correlates with TBII titers. We hypothesized that subjects with relatively high TBII titers and GO autoimmune activity would likely to be those with high titers of (other) autoantibodies, such as stimulating antibodies directed against the IGF-IR. The TBII titer was not normally distributed but severely skewed to the right. We therefore stratified subjects into two groups: those with high and low TBII titers.

In patients with high TBII titers we found a positive correlation between IGF-IR stimulating activity and age. This relationship was unexpected since IGF-IR stimulating activity in healthy subjects, like total IGF-I, is known to decrease with age.

In addition, after stratifying for TBII titers, the relationship between IGF-IR stimulating activity and TBII was positive in subjects with high TBII titers, while this relationship was negative in subjects with low TBII titers. Also, median CAS was higher in subjects with high TBII titers than in subjects with low TBII titers, although this difference was not statistically significant.

These results support the hypothesis of the presence of circulating IGF-IR activating immunoglobulins in a subgroup of patients with relatively high TBII titers. We measured IGF-IR stimulating activity before and after IgG depletion in the subgroup with high TBII levels. IGF-IR stimulating activity substantially decreased in patients with relatively high IGF-IR stimulating activity before IgG depletion and not in patients with relatively low IGF-IR stimulating activity before depletion. Our findings suggest that in a subset of Graves' patients, circulating IgGs can directly stimulate signalling at the IGF-IR level.

In **Chapter 11**, the general discussion, the results of all studies performed in this thesis are discussed. Our results show that determination of IR and IGF-IR bioactivity by KIRA assays helps to study normal physiology and pathologic changes of the insulin-IGF system, which may help to provide new insights in this system.

SAMENVATTING

Er zijn grote functionele en structurele overeenkomsten tussen insuline en insuline-achtige groeifactoren aan de ene kant en tussen de insuline receptor (IR) en insuline-achtige groeifactor-I receptor (IGF-IR) aan de andere kant. Het is daarom misschien niet verwonderlijk dat ruim 50 jaar geleden al werd ontdekt dat de hoeveelheid van insuline-(achtige) bioactiviteit in serum hoger was dan immunoreactief insuline en dat anti-insuline antistoffen slechts een kleine hoeveelheid van de totale insuline bioactiviteit konden blokkeren. Echter, door de weinig consistente resultaten van bioassays, werden deze assays in de loop van de tijd geheel vervangen door radioimmunoassays (RIAs). Alhoewel de introductie van de insuline RIA tot een grote toename van kennis heeft geleid over de glucose regulatie in het lichaam en diverse oorzaken van diabetes mellitus, is de potentiele contributie van IGFs op totale insuline (-achtige) werking op de achtergrond geraakt. Bovendien geven insuline immunoassays geen informatie over de mogelijke biologische effecten van circulerende insuline-achtige factoren op de twee verschillende insuline receptoren, (IR-A en IR-B), die in het lichaam voorkomen.

In 2003, hebben Chen et al. een Kinase Receptor Activation (KIRA) bioassay specifiek voor de IGF-IR ontwikkeld. Het principe van deze assay is gebaseerd op het kwantitatief meten van de phosphorylatie van tyrosine residuen van de IGF-IR na stimulatie met serum (of liganden) in vitro. Het is aangetoond dat de IGF-IR KIRA assay sensitief is voor de modificerende invloeden van circulerende IGF bindende proteinen (IGFBPs) en IGFBP- proteasen en dat de assay een precisie heeft die overeenkomt met de traditionele immunoassays. Zulke KIRA assays waren nog niet ontwikkeld voor de humane IR-A en IR-B.

Het doel van dit proefschrift was om de (patho)fysiologische rol van IR en IGF-IR bioactiviteit te onderzoeken in gezondheid en ziekten, en dit te doen bij diabetes, groeihormoon deficientie (GHD), acromegalie en Graves' ophthalmopathie (GO).

In **Hoofdstuk 1** wordt de geschiedenis beschreven van 'de verschillende wegen die naar uiteindelijk hetzelfde resultaat' hebben geleid, namelijk de identificatie van insuline en IGFs als afzonderlijke factoren. Daarna wordt een overzicht gegeven over het insuline-IGF systeem waarbij specifiek de impact van bepaalde ziekten en aandoeningen (diabetes, GHD, acromegalie en GO en de effecten van behandeling op dit systeem worden besproken.

In **Hoofdstuk 2** wordt de ontwikkeling en validatie van twee KIRA assays, specifiek voor de IR-A en de IR-B, besproken. Beide KIRA assays kunnen de door een ligand of serum geïnduceerde phosphorylatie van de tyrosine residuen van de IRen kwantitatief meten.

Beide KIRA assays toonden een redelijke sensitiviteit en (voor bioassays) lage intra- en inter-assay variaties. Het operationele bereik van beide assays was genoeg om insuline-achtige activiteit in humaan serum kwantitatief te meten. Analyse van humaan serum toonde aan dat IGF-IR en IGF-II in serum een substantiële bijdrage leveren aan het IR-A en IR-B signaal in vitro. Beide IR KIRA assays zouden daarom behulpzame nieuwe instrumenten kunnen zijn om de relatieve bijdrage van IGFs, aanwezig in serum, aan het IR signaal te onderzoeken.

In **Hoofdstuk 3** werd onderzocht of kort- en langwerkende insuline analogen verschillen van humaan insuline in hun potentie om de IGF-IR, IR-A en IR-B in vitro te activeren. Bij dit onderzoek werd gebruik gemaakt van de specifieke KIRA assays voor de IR-A en IR-B. Insuline glargine bleek, in suprafysiologische concentraties, krachtiger dan humaan insuline, de IGF-IR te activeren. Echter in vivo wordt een substantiele fractie van insuline glargine gemetaboliseerd voordat het in de circulatie terecht komt (zie Hoofdstuk 5). Derhalve is het de vraag of in vitro resultaten van insuline glargine zonder meer geextrapoleerd mogen worden en representatief zijn voor de werking van insuline glargine in vivo.

In **Hoofdstuk 4** werd serum IGF-IR bioactiviteit gemeten bij 104 patienten met type 2 diabetes die gerandomiseerd waren voor insuline glargine dan wel NPH insuline behandeling gedurende 36 weken.

Voor en na 36 weken insuline behandeling vonden wij bij deze patiënten geen verschil tussen beide groepen in de serum-geïnduceerde activatie van de IGF-IR. Het gevonden resultaat in vivo verschilde dus duidelijk van onze hierboven beschreven in vitro resultaten (Hoofdstuk 3). Dit zou mogelijk verklaard kunnen worden door het feit dat in vivo niet die concentraties worden bereikt waarbij wij (en anderen) in vitro verschillen tussen insuline glargine en humaan insuline in IGF-IR activatie vonden. Anderzijds, zouden deze verschillen ook verklaard kunnen worden omdat insuline glargine in vivo gemetaboliseerd wordt. De resultaten in Hoofdstuk 5 ondersteunen de laatste hypothese in hoge mate.

In plaats van een toename van de IGF-IR bioactiviteit vonden wij dat, tijdens exogene insuline toediening, de IGF-IR bioactiviteit afnam. Onderdrukking van de endogene insuline afgifte aan de vena porta door exogeen insuline (therapie) zou een mogelijke verklaring kunnen zijn voor (een verminderde hepatische IGF-IR productie) en de waargenomen afname van de IGF-IR bioactiviteit. Een afname van de IGF-IR bioactiviteit is ook eerder gevonden tijdens insuline therapie/hyperinsulinemische euglycaemische klem in voorgaande studies en werd toegeschreven aan verlaagde C-peptide levels en/of veranderingen in IGFBPs. Helaas hebben wij geen informatie over veranderingen in IGFBP concentraties in onze studie populatie. In overeenstemming met bevindingen

door anderen in eerdere studies vonden wij dat, in tegenstelling tot IGF-IR bioactiviteit, concentraties van totaal IGF-IR niet veranderden tijdens insuline therapie. Dit laatste zou kunnen samenhangen met het feit dat, immunoassays niet altijd in staat zijn de werkelijke IGF-IR concentraties te meten. Bij dit laatste spelen waarschijnlijk problemen met assay standaardisatie en/of methodologie van de IGF-IR immunoassays een belangrijke rol. Wat dit laatste betreft is het al eerder gesuggereerd dat de IGF-IR KIRA assay sensitiever is dan de IGF-I immunoassay om verschillen tussen personen tijdens ziekte en gezondheid op te sporen.

In **Hoofdstuk 5** hebben we in vitro de potentie onderzocht van insuline glargine en zijn metabolieten (M1 en M2), IGF-I en NPH insuline om de IGF-IR, de IR-A en de IR-B te activeren. Ook werden serum concentraties van insulin glargine, M1 en M2 gemeten in 57 type 2 diabeten die werden behandeld met insuline glargine. Tot slot werd de serum geïnduceerde IR-A en IR-B activatie onderzocht en vergeleken bij 104 type 2 diabeten die behandeld werden met insuline glargine of NPH insuline.

In tegenstelling tot insuline glargine hadden M1 en M2 een vergelijkbaar effect op de IGF-IR activatie als humaan insuline. M1 induceerde ook vergelijkbare effecten op de IR-A en IR-B activatie als humaan insuline terwijl M2, bij suprafysiologische concentraties, krachtiger dan humaan insuline de beide receptoren kon activeren.

Alleen M1, maar niet insuline glargine of M2, kon worden aangetoond in het plasma van patiënten die behandeld waren met insuline glargine. Dit zou (in iedergeval gedeeltelijk) kunnen verklaren waarom we in deze studie geen verschillen vonden in serum geïnduceerde IR-A en IR-B activatie. Bovendien kan dit verklaren waarom we eerder (zoals besproken in Hoofdstuk 4) geen verschillen vonden in serum geïnduceerde IGF-IR activatie.

Wij vonden een duidelijke positieve relatie tussen insuline dosis en de serum geïnduceerde IR-A activatie in zowel de groep die met insuline glargine als met NPH insuline behandeld werd. Dit suggereert dat, ongeacht de soort insuline, er mogelijk een toegenomen stimulatie van de IR-A optreedt in patiënten die worden behandeld met relatief hoge insuline doses.

We vonden dat na insuline therapie de activatie van de IR-A en IR-B niet toenam. Op het eerste gezicht lijkt dit tegenstrijdig omdat insuline therapie wel leidde tot een daling van nuchtere glucose spiegels en HBA_{1C} waarden hetgeen duidt op een toegenomen (betere) insuline activiteit. Er zijn verschillende mogelijke verklaringen voor deze bevinding. Het starten van insuline therapie heeft zeer waarschijnlijk geleid tot een toegenomen insuline gevoeligheid waardoor minder insuline (-achtige) activiteit nodig is. Daarbij heeft een toename van de hepatische insuline extractie mogelijk ook bijgedragen aan de gelijk gebleven activatie van de IR-A en IR-B. Ook kan, zoals al beschreven in Hoofdstuk 4, exogene insuline (therapie) de portale insuline concentraties

doen afnemen. Een van de problemen in het verleden was, dat het onmogelijk was om met immunoassays de som van de totale effecten van endogeen en exogeen insuline te meten. De resultaten in onze studie suggereren dat dit misschien wel mogelijk is met de IR specifieke KIRA assays. Wat dit laatste betreft zou het interessant zijn om de modificerende effecten van insuline therapie op IGF-IR bioactiviteit te meten in patiënten met type 1 diabetes, omdat deze patienten met en zonder insuline therapie alijd verlaagde l insuline spiegels in de vena porta hebben.

In **Hoofdstuk 6** werd onderzocht wat de diagnostische waarde van IGF-IR bioactiviteit is in GHD. We onderzochten daarom 94 patienten met een bewezen GHD. Onbehandeld lagen de waarden voor IGF-IR bioactiviteit (in vergelijking met de waarden voor totaal IGF-I) vaker onder de normaal waarden. Dit werd met name bij patienten ouder dan 40 jaar gezien. Onze resultaten suggereert dat IGF-IR bioactiviteit beter in staat is onderscheid te maken dan totaal IGF-I tussen patiënten met een onbehandelde GHD en gezonde personen. Dit laatste biedt mogelijk voordelen (t.o.v. totaal IGF-I) in de diagnose van GHD. Ook suggeseert het dat IGF-IR bioactiviteit een betere weerspiegeling is van GH actie in GHD gedurende het leven. Dit wordt ondersteund door het feit dat IGF-IR bioactiviteit, en niet totaal IGF-I, significant daalde met een langere duur van GHD.

Tot slot vonden we dat naarmate er minder uitval van andere hypofyse assen was, de waarden voor totaal IGF-I meer frequent normaal waren bij patiënten met bewezen GHD, terwijl dit percentage voor IGF-IR bioactiviteit geheel onafhankelijk was van het aantal aan uitgevallen (andere) hypofyse assen.

Deze studie suggereert dat het bepalen van IGF-IR bioactiviteit bij volwassenen voordelen kan bieden boven de bepaling van totaal IGF-I bij de diagnose GHD. Dit voordeel lijkt bij personen ouder dan 40 jaar het meest uitgesproken.

In **Hoofdstuk 7** bestudeerden wij de relatie tussen IGF-IR bioactiviteit en kwaliteit van leven (quality of life (QoL)) in 106 GH deficiente patienten die gedurende 12 maanden werden behandeld met GH (22 patienten waren GH naief, 84 waren al eerder behandeld met GH en stopten de behandeling gedurende 4 weken waarna de basale metingen werden verricht). QoL werd gemeten met behulp van de ziekte specificieke QLS-H module en met behulp van de meer op de algemene gezondheid afgestemde SF-36 vragenlijst.

Na 12 maanden GH behandeling vonden wij, ondanks een significante toename in IGF-IR bioactiviteit en in de totale IGF-I spiegels, geen significante verbetering van QoL gemeten met behulp van de QLS-H module. De relatieve hoge QLS-H Z-scores bij aanvang van de studie suggereert dat het stoppen van GH behandeling gedurende 1 maand misschien niet lang genoeg is om echte basaal waarden voor QoL te realiseren. Dit laatste zou een verklaring kunnen zijn voor het ontbreken van een verbetering in

QoL na het hervatten van de GH behandeling. Echter, in onze studie werd ook geen verbetering gevonden in QLS-H Z-scores in GH-naive patienten. Dit laatste zou ook verklaard kunnen worden door het kleine aantal geïncludeerde GH-naive patiënten in onze studie. Eerder is namelijk gevonden dat GH behandeling QoL verbetert maar dat het effect slechts klein is. Onze studie miste dus waarschijnlijk de statistische power om dit aspect te meten.

Ondanks het feit dat 12 maanden GH behandeling geen verbetering gaf van de ziekte specifieke QLS-H Z-score, was IGF-IR bioactiviteit wel significant gecorreleerd aan QLS-H Z-score. Na 12 maanden GH behandeling had 40% van de mensen waarbij totaal IGF-I genormaliseerd was, nog steeds IGF-IR bioactiviteit waarden beneden de normale referentie waarden. Patienten waarbij de IGF-IR bioactiviteit genormaliseerd was, hadden een significant betere QLS-H Z-score dan patiënten waarbij IGF-IR bioactiviteit niet genormaliseerd was. Dit verschil werd niet gevonden voor totaal IGF-I. Wat dit laatste betreft zou het interessant zijn om QLS-H scores te bestuderen als GH dosis getitreerd zou worden op geleide van de IGF-IR bioactiviteit.

12 Maanden GH behandeling leidde ook niet tot een verbetering in QoL gemeten middels de SF-36 vragenlijst. Echter, de fysieke component van deze vragenlijst was wel gecorreleerd aan zowel totaal IGF-I als IGF-IR bioactiviteit. Patienten waarbij totaal IGF-I genormaliseerd was scoorden significant hoger op de SF-36 vragenlijst dan patienten waarbij totaal IGF-I nog niet genormaliseerd was. Dit verschil werd niet gevonden voor IGF-IR bioactiviteit.

Onze studie suggereert dat, in GH deficiente patienten, IGF-IR bioactiviteit tijdens behandeling met GH beter veranderingen in ziekte specifieke QoL weerspiegelt dan totaal IGF-I en dat beide metingen waarschijnlijk verschillende aspecten van QoL weerspiegelen.

In **Hoofdstuk 8** onderzochten wij de waarde van IGF-IR bioactiviteit bij het monitoren van de effecten van GH behandeling (gedurende 12 maanden) van 106 volwassen patiënten met GHD.

Wij vonden dat veranderingen in IGF-IR bioactiviteit niet parallel verliepen aan de veranderingen in totaal IGF-I. Na 12 maanden GH behabdeling was IGF-IR bioactiviteit nog niet genormaliseerd in een substantieel aantal patiënten, terwijl totaal IGF-I in deze patiënten wel normaal was.

Na 12 maanden GH behandeling was er een positieve relatie tussen IGF-IR bioactiviteit en beta-cel functie, gemeten met de homeostasis model assessment (HOMA) methode. Wij vonden geen relatie tussen IGF-IR bioactiviteit en glucose of insuline resistentie. Echter, in deze studie hebben we alleen nuchtere waarden van insuline en glucose gemeten en was insuline resistentie niet bepaald door middel van een hyperinsulinemische klem

methode. Bovendien werd GH dosis getitreerd op geleide van totaal IGF-I en niet op geleide van IGF-IR bioactiviteit.

Na 12 maanden GH behandeling was er sprake van een negatieve relatie tussen de totale IGF-I spiegel en het percentage lichaamsvet hetgeen wijst op een verbetering van de lichaamssamenstelling. Echter, ondanks een afname van de vet maasa, taille omvang en taille/heup ratio, verslechterden de nuchtere insuline en glucose waarden en de insuline sensitiviteit. Totaal IGF-I was niet gerelateerd aan taille omvang, taille/heup ratio of insuline sensitiviteit. Dus, verbetering in lichaamssamenstelling ging niet gepaard met verbetering in insuline sensitiviteit. Dit zou verklaard kunnen worden door een direct van door GH geïncludeerde insuline resistentie, dus onafhankelijk van IGF-I.

Waarom verliepen de veranderingen in IGF-IR bioactiviteit niet parallel aan veranderingen in totaal IGF-I tijdens de behandeling met GH? Ten eerste, zoals hierboven beschreven, is de IGF-IR KIRA assay wel degelijk gevoelig voor de modificerende invloeden van IGFBPs op de werking van IGF-IR, terwijl dit niet het geval is voor IGF-I immunoassays. Ten tweede zou de kinetiek van IGF-IR bioactiviteit anders kunnen zijn dan van totaal IGF-I tijdens GH behandeling. Tot slot zou het zo kunnen zijn dat totaal IGF-I concentraties geen adequate weerspiegeling zijn van de actuele IGF-I concentraties.

Tijdens de studie werd de GH dosis bepaald op geleide van totaal IGF-I concentraties en niet op geleide van IGF-IR bioactiviteit. De IGF-I immunoassay die in onze studie gebruikt is, is gecalibreerd tegen een internationale WHO referentie reagens welke niet geheel zuiver is. De IGF-IR KIRA assay daarentegen is juist wel gecalibreerd tegen een IGF-I standaard die erg zuiver is. Om deze reden zou het effect van GH behandeling op totaal IGF-I systematisch overschat kunnen zijn. De gemeten subnormale IGF-IR bioactiviteit zou dus op een relatieve onderbehandeling van de patiënten met GHD kunnen wijzen.

Om de vraag te kunnen beantwoorden of IGF-IR bioactiviteit een waardevolle meting is om GH behandeling te monitoren in GH deficiente patienten en of het titreren van GH dosis tegen IGF-IR bioactiviteit in plaats van totaal IGF-I leidt tot een betere (metabole) uitkomst, zou het nodig zijn om in de toekomst een prospectieve gerandomiseerde placebo-gecontroleerde studie te verrichten waarbij in de ene groep GH getitreerd wordt tegen bioactiviteit terwijl in de andere groep GH getitreerd worden tegen totaal IGF-I.

In **Hoofdstuk 9** onderzochten wij de waarde van IGF-IR bioactiviteit in 15 onbehandelde patienten met acromegalie. QoL werd gemeten met behulp van verschillende vragenlijsten AcroQoL, de PASQ en de SF-36 questionnaire.

Alle patienten hadden verhoogde totaal IGF-I waarden, terwijl de waarden van IGF-IR bioactiviteit en IGFBP-3 bij meerdere patienten binnen de normale referentiewaarden vielen. Hoe kunnen we dit verschil tussen totaal IGF-I en IGF-IR bioactiviteit verklaren?

Zoals hierboven al besproken kent de meting van totaal IGF-I enkele valkuilen. Het zou bijvoorbeeld zo kunnen zijn dat de waarden voor totaal IGF-I vals verhoogd waren omdat de assay die gebruikt was in onze studie voor deze meting gecalibreerd is tegen de WHO IRR 87/518 (zie boven). Aan de andere kant hadden de meeste patienten een verhoogde GH secretie en de klinische symptomen van acromegalie. Dit zou erop kunnen wijzen dat totaal IGF-I een betere parameter is voor de diagnose van acromegalie dan IGF-IR bioactiviteit. Echter, alle patienten in onze studie werden geselecteerd op verhoogde totaal IGF-IR concentraties en niet op een verhoogde IGF-IR bioactiviteit, hetgeen mogelijk een bias heeft geintroduceerd.

Een alternatieve verklaring voor het verschil is de mogelijke aanwezigheid van een gering verhoogde GH overproductie in onze studie populatie. Eerder is gesuggereerd dat veel acromegalen een relatieve lage GH secretie kunnen hebben en toch een behoorlijk hoog totaal IGF-I, en omgekeerd. Tot op heden was het onmogelijk om deze groepen goed te onderscheiden op basis van specifieke klinische eigenschappen. IGF-IR bioactiviteit kan mogelijk een bijdrage leveren om deze twee groepen van elkaar te onderscheiden.

Eerder hebben we gevonden dat de referentie range van normaal waarden voor IGF-IR bioactiviteit in de gezonde populatie erg groot is. Dit betekent dat een bepaalde waarde voor IGF-IR bioactiviteit, gelegen binnen de normale referentie range, toch te hoog kan zijn voor een individuele patient (met acromegalie). Het kan dus moeilijk zijn om een onderscheid te maken tussen actieve en adequaat behandelde acromegalie als IGF-IR bioactiviteit binnen de normaal normale referentie range valt. Het gelijktijdig meten van GH en IGF-I zou in dit geval uitkomst kunnen bieden.

Zowel totaal IGF-I als IGF-IR bioactiviteit waren significant gerelateerd aan de ziekte specifieke PASQ. Ook was er voor zowel totaal IGF-I als voor IGF-IR bioactiviteit een omgekeerde relatie met de fysieke component van de SF-36 questionnaire. Bovendien was er een relatie tussen IGF-IR bioactiviteit en de fysieke dimensie van de ziekte specifieke AcroQoL terwijl dit niet het geval was voor totaal IGF-I. Samenvattend, ook al was IGF-IR bioactiviteit niet in alle patienten verhoogd, was IGF-IR bioactiviteit wel sterker gerelateerd aan gezondheids gerelateerde QoL dan totaal IGF-I. Dit suggereert dat IGF-IR bioactiviteit mogelijk beter de fysieke beperkingen, ervaren door onbehandelde acromegalen, weerspiegelt. Derhalve zou IGF-IR bioactiviteit, ook in onbehandelde acromegalie, van toegevoegde waarde kunnen zijn bij de evaluatie van QoL.

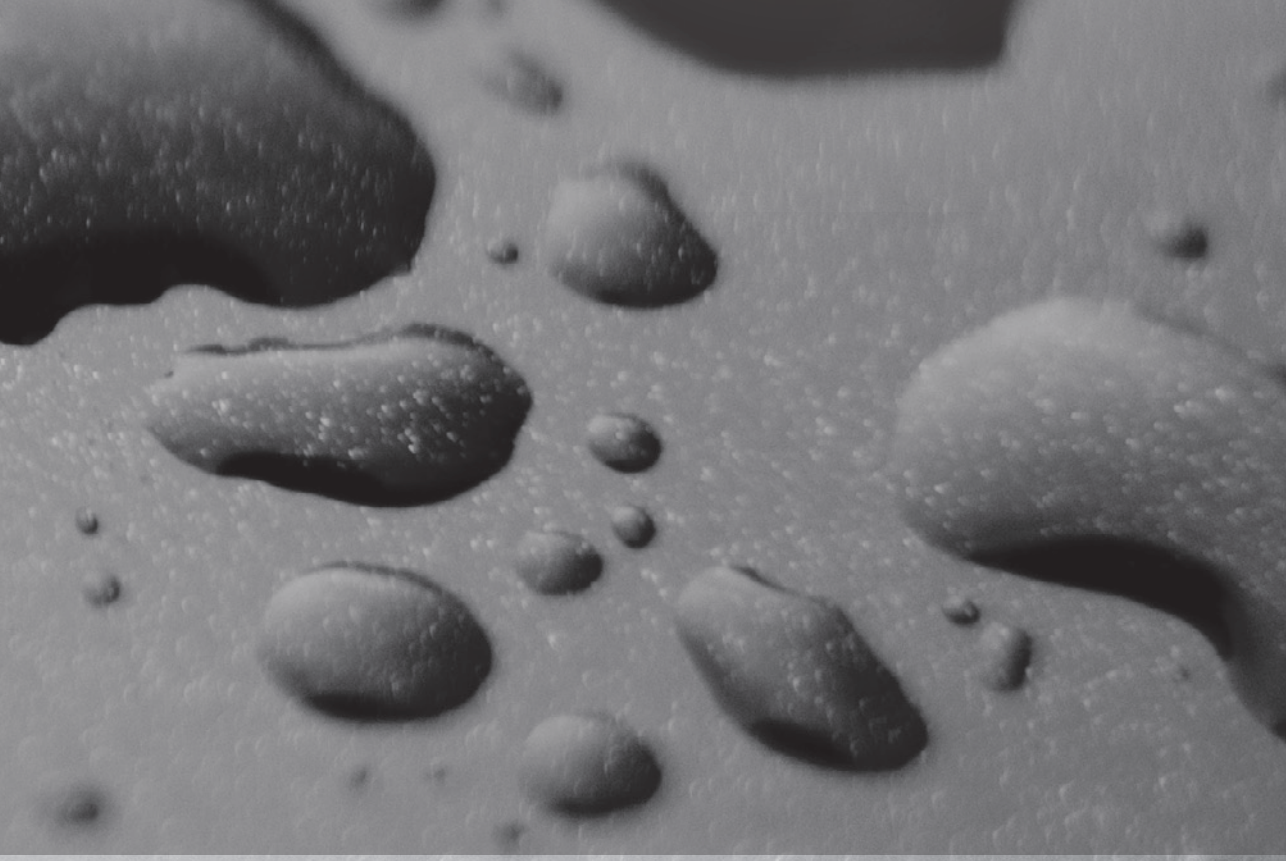
In **Hoofdstuk 10** bestudeerden wij de relatie tussen Thyrotropine Bindende Inhiberende Immunoglobulinen (TBIIs) en serum geïnduceerde IGF-IR stimulerende activiteit in relatie tot leeftijd in 70 patienten met GO.

In eeste instantie vonden wij geen duidelijke relatie tussen TBII concentraties in serum en serum geïnduceerde IGF-IR stimulerende activiteit of tussen serum geïnduceerde IGF-IR stimulerende activiteit en klinische activiteits score (CAS). Echter omdat de TBII concentraties niet normaal verdeeld waren, werd er gestratificeerd in twee groepen: een groep met relatief lage en een groep met relatief hoge TBII concentraties. Bovendien veronderstelden we dat patienten met de hoogste TBII titers ook de hoogste titer hadden voor andere autoantistoffen.

In patiënten met de hoogste TBII concentraties vonden we een positieve relatie tussen serum geïnduceerde IGF-IR stimulerende activiteit en leeftijd. Deze relatie was verrassend omdat we eerder hebben gevonden dat serum geïnduceerde IGF-IR stimulerende activiteit in gezonde personen (net als totaal IGF-I), met de leeftijd afneemt. Bovendien was er in patiënten met de hoogste TBII concentraties, een positieve relatie tussen de hoogte van de serum geïnduceerde IGF-IR stimulerende activiteit en TBII, terwijl deze relatie afwezig was in de groep met lage TBII concentraties. Ook was de gemiddelde CAS hoger in de groep patiënten met de hoogste TBII concentraties dan in de groep patiënten met de laagste TBII concentraties; echter, dit verschil was niet statistisch significant.

Deze resultaten ondersteunen de hypothese dat er in de subgroep met de hoogste TBII concentraties, immunoglobulinen aanwezig zijn die de IGF-IR kunnen activeren. Om deze hypothese verder te testen hebben we in deze subgroep de serum geïnduceerde IGF-IR stimulerende activiteit gemeten voor en na depletie van de IgG fractie. De serum geïnduceerde IGF-IR stimulerende activiteit daalde in patienten met vooraf relatief hoge serum geïnduceerde IGF-IR stimulerende activiteit maar niet in patiënten met vooraf een relatief lage serum geïnduceerde IGF-IR stimulerende activiteit. Onze bevindingen suggereren dat in een subgroep van Graves' patienten, circulerende IgGs de IGF-IR direct kunnen activeren.

In **Hoofdstuk 11**, de algehele discussie, worden de resultaten van alle beschreven studies in dit proefschrift besproken. Onze resultaten laten zien dat het bepalen van IR en IGF-IR bioactiviteit van waarde kunnen zijn om de fysiologie en pathofysiologische veranderingen van het insuline-IGF systeem te bestuderen, hetgeen zou kunnen bijdragen aan nieuwe inzichten in dit systeem.



LIST OF PUBLICATIONS

1. Insulin glargine is more potent in activating the human IGF-I receptor than human insulin and insulin detemir.
   Varewijck AJ, Goudzwaard JA, Brugts MP, Lamberts SW, Hofland LJ, Janssen JA.
   Growth Horm IGF Res. 2010 Dec;20(6):427-31.

2. IGF-I bioactivity better reflects growth hormone deficiency than total IGF-I.
   Varewijck AJ, Lamberts SW, Uitterlinden P, Hofland LJ, Janssen JA.
   J Clin Endocrinol Metab. 2011 Jul;96(7):2248-54.

3. Addition of insulin glargine or NPH insulin to metformin monotherapy in poorly controlled type 2 diabetic patients decreases IGF-I bioactivity similarly.
   Varewijck AJ, Janssen JA, Vähätalo M, Hofland LJ, Lamberts SW, Yki-Järvinen H.
   Diabetologia. 2012 Apr;55(4):1186-94. Epub 2012 Jan 10.

4. Low circulating IGF-I bioactivity is associated with human longevity: Findings in centenarians' offspring. Low circulating IGF-I bioactivity is associated with human longevity: Findings in centenarians' offspring.
   Vitale G, Brugts MP, Ogliari G, Castaldi D, Fatti LM, **Varewijck AJ**, Lamberts SW, Monti D, Bucci L, Cevenini E, Cavagnini F, Franceschi C, Hofland LJ, Mari D, Janssen J.
   Aging (Albany NY). 2012 Sep;4(9):580-9.

5. Insulin and its analogues and their affinities for the IGF1 receptor.
   Varewijck AJ, Janssen JA.
   Endocr Relat Cancer. 2012 Sep 5;19(5):F63-75

6. Circulating insulin-like growth factors may contribute substantially to insulin receptor isoform A and insulin receptor isoform B signalling.
   Varewijck AJ, Brugts MP, Frystyk J, Goudzwaard JA, Uitterlinden P, Waaijers AM, Feng Y, Dimitrov DS, Lamberts SW, Hofland LJ, Janssen JA.
   Mol Cell Endocrinol. 2013 Jan 5;365(1):17-24.

7. IGF-I bioactivity might better reflect Quality of Life than Total IGF-I in GH Deficient patients during GH treatment.
   Varewijck AJ, Lamberts SW, Neggers SJ, Hofland LJ, Janssen JA
   J Clin Endocrinol Metab. 2013 Feb;98(2):761-8.

8. Circulating IgGs may modulate IGF-I receptor stimulating activity in a subset of patients with Graves' Ophthalmopathy.
   Varewijck AJ, Boelen A., Lamberts SWJ, Fliers E, Hofland LJ, Wiersinga WM, Janssen JA
   J Clin Endocrinol Metab. 2013 Feb;98(2):769-76.

9. Concentrations of insulin glargine and its metabolites during long-term insulin therapy in type 2 diabetic patients and comparison of effects of insulin glargine, its metabolites, IGF-I and human insulin on insulin- and IGF-I receptor signaling.
   Varewijck AJ, Yki-Järvinen H, Schmidt R, Tennagels N, Janssen JAMJL
   Diabetes, 2013.

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PhD PORTFOLIO

Name PhD student:	Aimee J. Varewijck
Erasmus MC department	Internal Medicine, Endocrinology
Research School:	MolMed
PhD period:	November 2008 – May 2012
Promotors:	L.J. Hofland and S.W.J. Lamberts
Co-promotor:	J.A.M.J.L. Janssen

1. PhD Training	Year	Workload
1.1. General academic skills		
CPO minicursus; Methodologie van patient gebonden onderzoek en voorbereiding van subsidieaanvragen, Rotterdam, The Netherlands	2010	0.3
Course on Basic and Translational Oncology, Molmed, Rotterdam, The Netherlands	2010	1.5
1.2. Research skills		
NIHES course 'Classical Methods for Data-analysis', Rotterdam, The Netherlands	2009	5.7
Workshop 'moleculaire diagnostiek voor dokters', MolMed, Rotterdam, The Netherlands	2010	0.25
NIHES course 'Quality of Life Measurement', Rotterdam, The Netherlands	2011	0.9
1.3. (Inter) National Conferences- participation and presentations		
Endocrine Society Meeting, Washington, USA. Poster Presentation: Insulin Analogues Differ in Their Ability To Activate the Insulin Like-Growth Factor-I Receptor and Insulin-A Receptor In Vitro	2009	1.0
Bataafsch Genootschap der Proefondervinderlijke Wijsbegeerte, Afstudeer pijs 2009, Rotterdam, The Netherlands Oral presentation: Hebben Insuline Analoga een andere werking op de Insuline-achtige Groei Factor-I Receptor dan het natuurlijk insuline?	2009	1.0
Wetenschapsdagen Interne Geneeskunde, Antwerpen, Belgium Poster presentation: Insulin Analogues differ in their ability to activate the Insulin-B Receptor, Insulin-A Receptor and IGF-I Receptor in vitro	2010	0.8
Nederlandse Endocrinologie Dagen, Noordwijkerhout, The Netherlands Oral presentation: Treatment with insulin (analogues), for the better or for the worse?	2010	0.8
MolMed Day 2010, Rotterdam, The Netherlands Oral presentation: Treatment with insulin (analogues), For the better or for the worse?	2010	0.8
Endocrine Society Meeting, San Diego, USA. Poster Presentation: Novel Specific Kinase Receptor Activation Bioassays for the Human Insulin Receptor A (IR-A) and B (IR-B) To Study the Insulin-IGF System	2010	1.0
Wetenschapsdagen Interne Geneeskunde, Antwerpen, Belgium Poster presentation: IGF-I Bioactivity More Accurately Reflects Growth Hormone Deficiency than Total-IGF-I	2011	0.8

Nederlandse Endocrinologie Dagen, Noordwijkerhout, The Netherlands Oral presentation: IGF-I Bioactivity more Accurately reflects Growth Hormone Deficiency than Total IGF-I	2011	0.8
Endocrine Society Meeting, Boston, USA. 2 Oral presentations: IGF-I Bioactivity Better Reflects Growth Hormone Deficiency than Total IGF-I. IGF-I Bioactivity decreases during Insulin Therapy in Type 2 Diabetic Patients: Comparison of High Doses of Insulin Glargine and NPH Insulin in the LANMET study. 1 Poster presentation: Pasireotide Treatment in Cushing Disease: Effects on the IGF-I System	2011	2.0
Nederlandse Endocrinologie Dagen, Noordwijkerhout, The Netherlands Oral presentation: Insulin glargine and malignancy: an unwarranted alarm?	2012	0.8
Endocrine Society Meeting, Houston, USA. 1 Oral presentation: Changes in IGF-I Bioactivity Do Not Parallel Changes in Total IGF-I during GH Treatment of GH-Deficient Adults 2 Poster presentations: IGF-I Bioactivity Might Better Reflect Quality of Life Than Total IGF-I in GH-Deficient Patients during GH Treatment. Circulating IgGs May Modulate IGF-I Receptor Activity in a Subset of Euthyroid Patients with Graves' Ophthalmopathy	2012	2.0
1.4. Seminars and workshops Attending seminars of the Department of Internal Medicine/ Erasmus MC lectures, Rotterdam, The Netherlands	2008-2012	1.0
Attended INKEP (Ipsen Nordic Knowledge Exchange Program), Rotterdam, The Netherlands	2009	0.6
Attended ECE 2011 European Congress of Endocrinology, Rotterdam, The Netherlands	2011	1.0
2. Teaching Activities	**Year**	**Workload**
2.1. Supervising Bachelors Thesis Wouter de Wilde, Life Science student, Hogeschool Rotterdam, Rotterdam, The Netherlands Project title: Do differences in Receptor activation lead to differences in metabolic and proliferative effects?	2010	7.5
2.2. Supervising medical students Practicals endocrinology, Erasmus MC, Rotterdam, The Netherlands	2009-2011	2.4

ABOUT THE AUTHOR

Aimee Joan Varewijck was born on October 15th 1983, in Nieuw-Vennep, The Netherlands and raised by her beloved parents Catherine Mary Donger and André Varewijck. In 2002 she completed secondary school at the Herbert Vissers College in Nieuw-Vennep, after which she started her medical study at the Erasmus University Rotterdam. From 1996 to 2005 she was selected for Dutch Swimming Team (Nederlandse Culturele Sportbond, NCS) and participated in national training sessions and international swimming events.

In 2007-2008 her graduation research was performed at the Department of Internal Medicine, Erasmus Medical Center Rotterdam entitled 'Do insulin analogues differ in their ability to activate the Insulin-like Growth Factor-I Receptor and Insulin Receptor'. For this work she received an award for 'Best Student Research' by Bataafsch Genootschap der Proefondervindelijke Wijsbegeerte in 2009. In 2008 she finished her internships with an optional internship in the Department of Critical Care Medicine, Sick Kids Hospital, Toronto, Canada and obtained her medical degree. After this she started the work presented in this thesis at the Department of Internal Medicine at the Erasmus Medical Center Rotterdam, under the enthusiastic supervision of Dr. J.A.M.J.L. Janssen, Prof. dr. L.J. Hofland and Prof. dr. S.W.J. Lamberts. In 2011 she received the Women in Endocrinology Young Investigators Award and an Outstanding Abstract Award from the Endocrine Society and in both 2011 and 2012 she received a travel grant from the Dutch Endocrine Society. In May 2012 she started her training residencies in Internal Medicine at the Maasstad Hospital in Rotterdam under the supervision of Dr. M.A. van den Dorpel, which will be continued at the Erasmus Medical Center under supervision of Prof. J.L.C.M van Saase. In 2011 she married her love David van Rees Vellinga.