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Publication status and date:
Published: 06/01/2010

Document Version
Publisher's PDF, also known as Version of record

Citation for the published version (APA):
Brugts, M. (2010). *IGF-I bioactivity in aging, health and disease*. [Doctoral Thesis, Erasmus University Rotterdam]. Erasmus Universiteit Rotterdam (EUR).

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Erasmus University Rotterdam
Making Minds Matter

IGF-I Bioactivity in Aging, Health and Disease

Michael Pascal Brugts

IGF-I Bioactivity in Aging, Health and Disease.
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ISBN: 978-90-8559-915-9

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IGF-I Bioactivity in Aging, Health and Disease

IGF-I bioactiviteit in relatie tot veroudering, gezondheid en ziekte

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.G. Schmidt
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 6 januari om 15.30 uur

door

Michael Pascal Brugts
geboren te Rotterdam

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The studies described in this thesis were performed at Erasmus Medical Center, Rotterdam, The Netherlands and were in part supported by a grant of ZonMW (NWO 948-00-003).

Printing of this thesis was financially supported by:
Ipsen Farmaceutica B.V., Goodlife Healthcare B.V., AstraZeneca Nederland B.V., Pfizer B.V., Boehringer Ingelheim B.V., Servier Nederland B.V., Roche B.V., Novartis Pharma B.V., PerkinElmer Nederland B.V., Novo Nordisk B.V., Erasmus Universiteit Rotterdam.

Voor de strijder van het licht bestaat,
de onmogelijke liefde niet.
Hij laat zich niet intimideren door zwijgen,
door onverschilligheid, of door afwijzing.
Hij weet dat achter een ijzig voorkomen,
een vurig hart schuilgaat.
Zonder liefde is hij niets

Paulo Coelho

Ter nagedachtenis aan Franciscus Adrianus Jansen,
mijn geliefde oom

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Chapter 1

Introduction and Aim of the Thesis

INTRODUCTION

1.1 The history on the discovery and nomenclature of Insulin-like Growth Factors

The existence of insulin-like growth factors (IGFs) in blood was first recognized by Wiliam D. Salmon Jr. and Wiliam H. Daughaday in 1956 [1]. Examining the role of pituitary-regulated growth-stimulating substances, these authors demonstrated that a growth hormone (GH) dependent factor in serum could stimulate labeled sulfate ($^{35}SO_4$) incorporation into chondroitin sulfate (cartilage) *in vitro*. In line with their findings, Salmon and Daughaday initially designated this factor as 'sulfation factor' [1]. Comparative studies were also performed *in vivo*. Hypophysectomy of rats markedly reduced labeled sulfate incorporation into chondroitin sulfate of epiphyseal cartilage, whereas injections of pituitary extracts and purified bovine GH effectively restored $^{35}SO_4$ incorporation [2,3]. The observations that the direct effect of bovine GH on costal cartilage was minimal, whereas serum from hypophysectomized rats treated with bovine GH stimulated ^{3}H-thymidine incorporation into cartilage led to the postulation that GH utilized the intermediary substance sulfation factor [4,5].

In 1963 a second line of research reported excess insulin-like activity (ILA) in normal serum. In this study Froesch et al. used an adipose tissue bioassay in which glucose uptake and net gas exchange were used as metabolic indexes of ILA in serum. They showed that in fasting subjects only 7% of serum ILA could be neutralized by insulin specific blocking antibodies [6]. Based on these findings Froesch et al. introduced the term 'non-suppressible insulin-like activity (NSILA)'.

In a third line of investigation Dulak and Temin described a polypeptide fraction produced by a cell line derived from fetal rat liver cells [7]. Dulak and Temin called this fraction 'multiplication-stimulating activity (MSA)' as it was able to stimulate growth of chicken embryo fibroblast *in vitro* [7,8].

As it became clear that sulfation factor and NSILA had similar (if not identical) actions, Daughaday and colleagues proposed the term 'somatomedin' as official designation for sulfation factor in 1972. An important argument for this; the accumulating evidence of sulfation factor/NSILA being an important mediator of GH (also known as somatotropin) induced actions [5].

In the following years it was found that somatomedin/NSILA actually consisted of a family of at least two peptides (respectively somatomedin A and C) [9-11]. The amino acid sequences of both polypeptides were published by Rinderknecht and Humbel in 1978 [12]. Moreover, in 1981 Marquard and colleagues showed that rat MSA differed only a few amino acids from human

somatomedin A [13]. As the structure of all proteins closely resembled that of pro-insulin, these proteins were officially renamed as Insulin-like growth factor I (IGF-I) and II (IGF-II) respectively in 1987 [14].

1.2 Evolutionary and structural aspects of insulin-like proteins

1.2.1 The insulin protein superfamily

Together with insulin, IGF-I and IGF-II are members of the so-called insulin protein superfamily [15]. This superfamily is comprised of small proteins that show similarity at the levels of their primary and tertiary structure. The primary peptide sequence of each family member is characterized by three domains; a carboxyl A chain joined to an amino terminal B chain by an intervening C chain (B-C-A) [15].

The insulin superfamily is an ancient family. Insulin and/or insulin-like proteins have been described in unicellular eukaryotes [16] as well as in primitive species (e.g. insects, tunicates, annelids, and others) [15-19]. Throughout evolution, the prototypical domain structure of these proteins appears to have been well conserved: especially the B and A chains share relatively high sequency homology between species and exhibit a pattern of distinct and highly conserved cysteine motifs which characterize the family. For instance, the motif (CC-3X-C-8X-C) present in the A peptide has been termed the so-called insulin signature [15].

In mammals the insulin protein superfamily originally comprised of four members: insulin, IGF-I, IGF-II, and relaxin. Relaxin was identified in the 1920s by Frederick Hisaw from serum of female guinea pigs as a factor that induced widening of the birth canal [20]. In addition relaxin has been found to have a variety of other roles, e.g. endometrial differentiation during embryo implantation, stimulation of angiogenesis, wound healing and lactation and being a modulating factor in cardiovascular responses [21]. Additional members of the insulin superfamily have been identified more recently, being predominantly expressed in tissues of the reproductive system [15]; Leydig insulin-like peptide precursor 3 or INSL3 [22], early placenta insulin-like peptide, placentin or INSL4 [23,24], relaxin/insulin-like factor 2 or INSL5 [25] and, relaxin/insulin-like factor 1 or INSL6 [26,27]. Of all the members of the insulin protein family, insulin and the IGFs are the most closely related members in terms of primary structure and biological activities (see below) [28].

Based on their type of target receptors, the members of the insulin protein superfamily can be roughly divided in two distinct groups; those that activate specific tyrosine kinase receptors (insulin, IGF-I and IGF-II), and those

that act through G-protein-coupled seven transmembrane receptors (GPCR) (relaxin H1, H2 and INSL3-6). Two orphan leucine-rich repeat-containing G-protein-coupled receptors, LGR7 and LGR8, have been identified as being relaxin receptors [15].

1.2.2 Structural and evolutionary aspects of IGF proteins

The term insulin-like growth factor (IGF) was proposed to denote a class of proteins whose structure was highly similar to that of pro-insulin [29,30]. The mature human IGF-I protein consists of 70 amino acids with a molecular weight of 7.65 kDa [12]. It is synthesized and cleaved from a larger prepro-molecule (prepro-IGF-I), containing classic A,B,C and D domains, together with a 48 amino acid signal peptide and a carboxy-terminal extension (E) peptide. The signal and E peptide are cleaved off during processing of the mature IGF-I peptide [31]. In contrast to insulin, the mature IGF-I protein retains the C peptide and the extended carboxy terminal D-domain, thereby remaining a contiguous amino acid chain (Figure 1). In general, human IGF-I, IGF-II and insulin have approximately 50% of their amino acids in common [31]. The human IGF-I protein shares 48% amino acid sequence homology with human pro-insulin [12,30]. The A and B domains of IGF-I and insulin share up to 60-70% of their amino acids [29]. The mature human IGF-II protein consists of 67 amino acids with a molecular weight of 7.5 kD [32]. Like IGF-I, it is also synthesized mainly in the liver and cleaved from a larger prepro-molecule

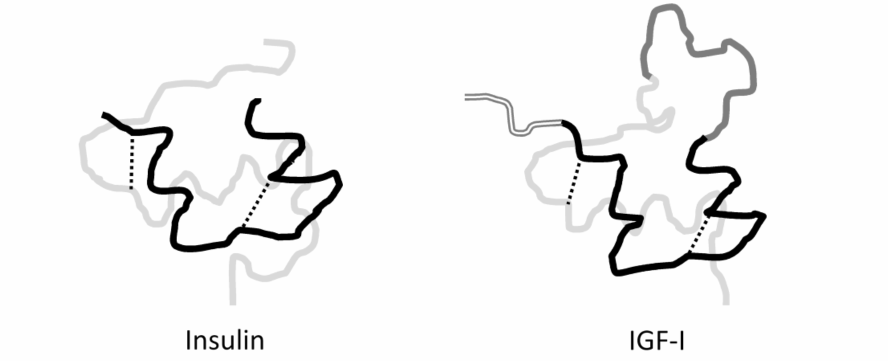

Figure 1. Figure of human insulin and IGF-I. The mature insulin peptide is composed of an A-region (black) linked to a B-region (light grey) by cysteine bridges (dashed lines). IGF-I and IGF-II (not shown) are also composed of an A and B region, but contain two extra regions compared to insulin; the C (dark grey) region between the B and A domains and the D region (open lines) at the C-terminus. Adapted from Brzozowski et al. Biochemistry 2002; 41:9389-9397 and LeRoith et al. 1992, 116(10):854-62.

(prepro-IGF-II), containing A,B,C and D domains, an amino acid signal peptide and a carboxy-terminal extension (E) peptide [15]. In humans IGF-II has about 70% structure homology with IGF-I [32].

The high amino acid sequence homology and the overlap of biological activities (see below) between IGFs and insulin suggests a high probability of an evolutionary link between these hormones. Complementary DNAs encoding prepro-IGF-I have been cloned from several vertebrate species including mammals, birds (e.g. chicken), amphibians and fish (e.g. salmon) [33]. Focusing on primates, IGF-I amino acid sequences are strongly conserved; with the exception of new word monkeys (e.g. Callithrix jacchus or Marmoset) the sequences of all the mature primate IGF-Is are identical to that of human. Also mature dog IGF-I is identical to that of man. In addition, the existence of IGF-I, IGF-II and insulin as distinct proteins occurs in all vertebrates except for the primitive jawless fish (Agnatha). Concerning jawless fish, although separate IGF and insulin genes have been described in these organisms, there are no separate genes for IGF-I and IGF-II [34]. Amphioxus, a protochordate expresses a hybrid insulin/IGF molecule. This strongly suggests that IGFs diverged from the line of insulin evolution at an early stage of vertebrate evolution, antedating the primitive jawless vertebrates [35]. From these studies the hypothesis emerged that duplications of an ancestral insulin(-like) gene, that functioned primarily as a "mitogenic" growth factor, allowed insulin to develop as a primarily metabolic regulator while the mitogenic activity was retained by the IGF gene [36].

1.3 The human IGF-I-gene

IGF-I is encoded by a single gene copy that in man resides on the long arm of chromosome 12 (12q 22–24.1). It comprises six exons spanning a region of about 85 kilo base-pairs (kb) [37]. A schematic overview of the IGF-I gene is shown in Figure 2. IGF-I gene expression is initiated from either one of two promoter regions (P1 and P2, respectively exons 1 and 2) in a tissue specific manner. In the liver and the kidney IGF-I transcription is initiated mainly from P2 and is highly GH dependent, whereas in other tissues P1 is the most important initiation site [33]. Exons 1, 2, 5 and 6 are alternatively spliced. Exons 1 and 3 and exons 2 and 3 code for the signal peptides, whereas exons 3 and 4 code for the mature protein. The signal peptide is later cleaved off. Exons 5 and 6 encode distinct so-called carboxy-terminal E regions, of which there is no equivalent in insulin. Within the inactive pre-pro-IGF-I precursors these E-regions play an important role in the maturation of the IGF-I peptide. For human IGF-I three different E-domains (Ea (35 residues), Eb (77 residues)

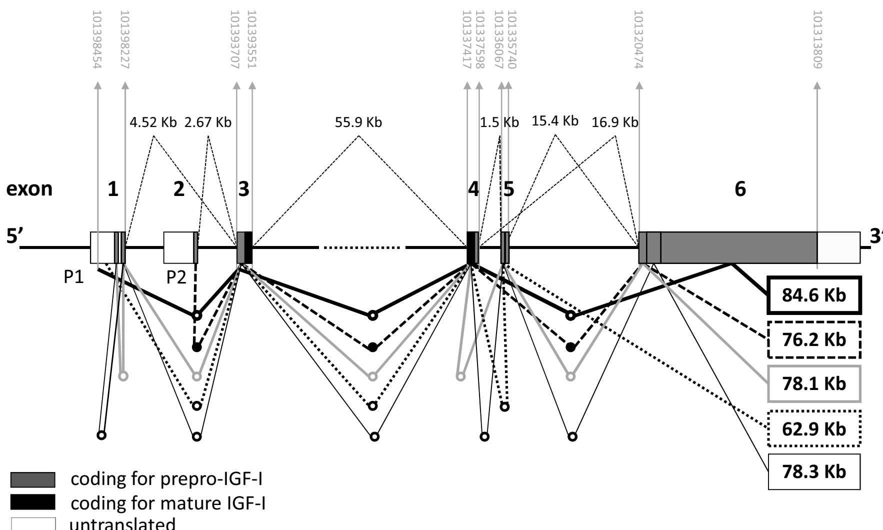

Figure 2. Schematic overview of the human IGF-I gene. The human IGF-I gene is located on chromosome 12 and comprises of six exons (1-6). Exon 1 and 2 respectively contain the two promoter regions from which RNA transcription is initiated (P1 and P2). Several IGF-I mRNA precursors have been isolated. Five of these are shown in this figure ranging from ~63 to ~85 Kb. Exons 5 and 6 give rise to the 3' untranslated region (3'UTR) from which three different E-domains are transcribed. All mRNA transcripts give rise to the same IGF-I molecule. This mature IGF-I protein is encoded by exons 3 and 4. In grey the chromosomal positions are shown. Also distance between exons is shown.

and Ec) result from differential splicing of the transcript [33]. Specific biological activities have been reported from each E-domain, which however goes beyond the scope of this thesis.

1.3.1 IGF-I gene polymorphisms

A polymorphism is defined as a variation in the nucleotide sequence of genomic DNA that is too common to be due merely to a new mutation, thereby having a prevalence of at least 1 percent. Within the IGF-I gene region two common classes of polymorphisms are present: variable number of tandem repeats (VNTR) and single nucleotide polymorphisms (SNPs). VNTRs consist of a repetitive number of small nucleotide sequences (ranging from one to several) of which the array length can vary. SNPs are defined as a single nucleotide substitution by another nucleotide and have a genomic frequency in average of 1 per 100-1000 nucleotides. Another familiar concept in the research field focused on genetic variation is linkage disequilibrium (LD), being the co-occurrence of closely linked areas (or alleles) more

commonly than expected by chance only. The explanation for this is that genomic recombination during meiosis does not occur at random throughout the length of the genome, but at certain privileged sites. Thus, areas without these recombination sites are relatively well conserved and thereby tend to be inherited together as one block. A combination of such LD blocks give rise to haplotypes. An advantage of this approach is that to 'cover' the variation within a haplotype it may not be necessary to determine all polymorphisms within the region spanned by the LD blocks within that haplotype, but only those that 'tag' this variation. Such SNPs are therefore called tagging SNPs. This possibility exists because of the HapMap project, an international project investigating LD blocks throughout the genome and in different ethnicities (http://www.hapmap.org/).

In the last decade there has been done extensive research in the field of genetic polymorphisms, including numerous studies focusing on IGF-I gene polymorphisms/haplotypes, with outcomes ranging from associations with levels to complex diseases like diabetes and cancer. It is therefore impossible to cover all of this information. A few studies will be highlighted that are to some extent of interest in relation to studies included in this thesis. Vaessen et al. found in 900 Caucasian elderly participants of the Rotterdam study that variance in length of a VNTR microsatellite (ca-repeat) polymorphism at -1 kb of the IGF-I gene P1 promoter was associated with several phenotypes [38]. Absence of the commonest 192-base pair allele was associated with reduced serum IGF-I levels, reduced height, an increased risk of type 2 diabetes mellitus (OR 1.7, 95% CI 1.1–2.7) and MI (OR 1.7, 95% CI 1.1-2.5) later on in life. Others however could not replicate these findings in another study cohort [39]. In a case-control study by Nielsen et al. comprising 694 type 2 diabetics (DM type 2) and 218 glucose-tolerant control subjects no associations were found between the lack of the common promoter allele and DM type 2. The common wild-type allele of the P1 promoter polymorphism was associated with increased levels of fasting serum triglycerides in glucose-tolerant whites [40]. Rietveld et al. studied this VNTR polymorphism in 346 individuals of the Rotterdam study in relation to the age-related decline of total IGF-I levels [41]. They found that homozygous carriers of the common allele demonstrated significant decline in serum IGF-I with age as observed in the general population, whereas heterozygous and non-carriers did not. An identical pattern was observed for levels of IGFBP-3. The authors concluded that the P1 promoter ca-repeat polymorphism might directly or indirectly influence GH-mediated regulation of IGF-I secretion. Hovind et al. published a prospective follow-up study of a cohort of 277 patients with newly diagnosed type 1 diabetes. They

found that subjects with the variant type of the P1 IGF-I ca-repeat polymorphism had, during follow-up, a higher risk of development of microalbuminuria (MA) compared subjects with the wild type genotype (192/192). A similar finding was reported by Rietveld et al. in 1069 participants of the Rotterdam study, in which it was shown that in the presence of abnormal glucose tolerance a higher risk for MA was observed in variant carriers compared to carriers of the wild type genotype of this IGF-I gene polymorphism [42].

In a large case-control study in premenopausal women Patel et al. studied IGF-I haplotypes bases on tagging SNPs in relation to IGF-I, IGFBP-1 and IGFBP-3 levels and risk on breast cancer [43]. Thirty tagging SNPs were analyzed to cover most common haplotypes, including four haplotype blocks for IGF-I and three for IGFBP-1 and IGFBP-3. Certain IGF-I SNPs were associated with change in circulating total IGF-I levels. SNPs in IGF-I block 1 (covering the promoter region) were most closely associated with circulating total IGF-I levels; the variant alleles were significantly associated with higher circulating IGF-I levels (trend $p = 0.0075$ for rs7965399 and $p = 0.0262$ for rs35767). Certain IGFBP-3 SNPs were associated up to 12% change in circulating IGFBP-3 levels. Logistic regression analyses however found no significant associations between breast cancer and individual SNPs or haplotypes in located within the regions spanning the IGF-I, IGFBP-1, or IGFBP-3 genes.

1.4 The IGF system and its functional properties in health and disease

The IGF system is a complex molecular network acting in an endocrine, paracrine and autocrine fashion. Traditionally it includes two growth factors (IGF-I and IGF-II), six IGF-binding proteins (IGFBP-1 to -6), nine IGFBP-related proteins (IGFBP-rPs) and two cell surface receptors (IGF-IR and the IGF-II mannose-6-phosphate receptor (M-6-PR). The IGF-IR functions as biological target for both IGF-I and IGF-II. A schematic overview of the IGF system is shown in Figure 3 [32]. Figure 3 also shows insulin and the insulin receptor (IR), this because of the close relationship between IGFs and insulin as well as between their receptors, both qua structure and function. Indeed, it has been shown that IGFs and insulin can bind each other's receptors, and also hybrids of the IGF-IR and the IR exist (see below).

The axis in which IGFs have major participation is characterized by its pleiotrophic functions; it is involved in cell growth, proliferation, differentiation and cell survival as well as in the regulation of glucose, fat and protein metabolism. In this way the IGF system affects nearly every organ system in the body [32]. Figure 4 summarizes most of the established functions of IGF-I.

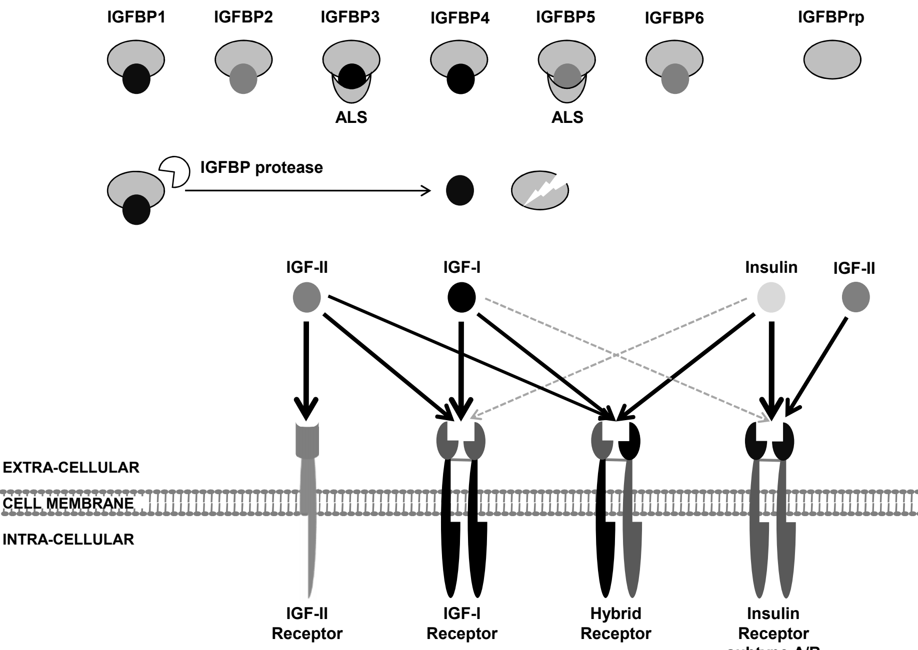

Figure 3. Schematic overview of the IGF system. Classically the IGF system is composed of two Insulin Like Growth Factors (IGF-I (black) and IGF-II (grey)), six high affinity Insulin-Like Growth Factor Binding Proteins (IGFBP-1 to -6), several related IGFBPs (IGFBPrp), IGFBP proteases and two receptors; the IGF-I receptor (IGF-IR) and the IGF-II receptor. All IGFBPs can bind both IGF-I and IGF-II (however with different binding affinity for some). Only the unbound forms of IGFs are thought to interact with the IGF-IR and the IGF-II receptor. Only the IGF-IR is bioactive, and next to IGF-I also binds IGF-II. Next to IGFs also insulin and the insulin receptor (IR) are shown. As IGFs and insulin as well as the IGF-IR and the IR share high sequence homology they are able to bind and activate each other's cognate receptors but with different affinity (denoted by the thickness of the arrows). Interestingly, several IGF-IR/IR hybrid receptors are known which can be bound by both IGFs and insulin.

1.4.1 The original Somatomedin Hypothesis: 'the endocrine paradigm'

Originally IGF-I was recognized as part of a classical endocrine hormone system being dominated by hypothalamic/pituitary factors, thereby regulating growth and tissue differentiation [44]. *In vitro* studies showed that IGF-I stimulates cell growth and differentiation in nearly all systems [45]. *In vivo* it was found that human fetal serum had readily detectable concentrations of somatomedins/IGFs [46-51], that fetal tissues possessed cell membrane somatomedin/IGF receptors of relatively high affinity [47], and that somatomedin levels in cord serum correlated with size at birth [52].

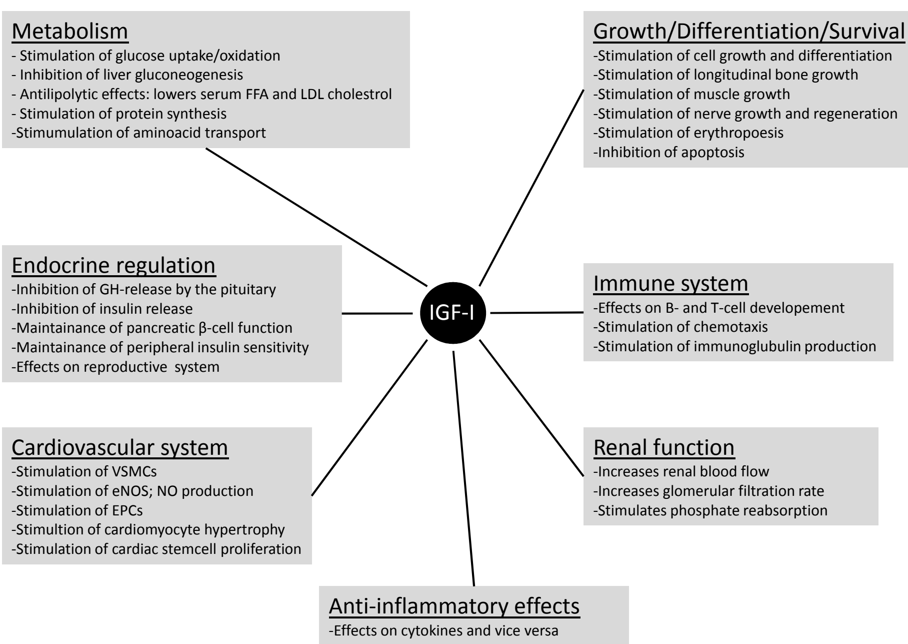

Figure 4. Overview of the main known functions of IGF-I.

IGF-I was thought being almost solely produced by the liver upon GH stimulation, itself produced by the pituitary in a pulsatile fashion. To some extent, GH also stimulates the production of IGF-II, as IGF-II levels are decreased about 20% on average in GH-deficiency [32]. The synthesis and pulsatory secretion of GH by the pituitary is influenced by several hypothalamic and pituitary factors [53]. Two transcription factors PROP1 and PIT1, that regulate the differentiation of pituitary cells into somatotrophs during embryogenesis, stimulate GH synthesis and its release by the pituitary. In addition, GH release is stimulated by transduction signal, activated by the binding of GH releasing hormone (GHRH) to the GHRH receptor. Ghrelin, produced in the hypothalamic arcuate nucleus and acting through the growth hormone secretagogue receptor, also stimulates the release of GH [54]. Growth hormone binds specifically to the GHR and thereby mediates the production of IGF-I by the liver [55]. The regulation of IGF-I production by GH appears to be mediated entirely by signalling through the Janus kinase (JAK) 2 pathway, via the phosphorylation of the transcription factor, signal transducer and activator of transcription (STAT) 5b [56]. Via the circulation IGF-I reaches its target tissues (e.g. bone, cartilage and muscle). In return, IGF-I inhibits GH secretion, thereby providing a negative feedback loop within the IGF system [57]. Thus, while it was recognized that IGFs had high structural similarity with

insulin - suggesting a metabolic function – at first instance the general idea predominated that the functional role of IGFs was primarily to act as growth factors in an endocrine fashion. This idea has been postulated as the original somatomedin hypothesis [44].

1.4.2 The Somatomedin Hypothesis 50 years later: 'the endocrine paradigm revised'

1.4.2A Growth/development

Today - more than half a century after the discovery of IGF-I - it has become clear that the original endocrine paradigm about the GH/IGF system as described is insufficient [44,55]. First evidence for this came from D'Ercole and collegues in 1980 who published data about extra hepatic IGF-I production. In these studies, the authors showed production of IGF-I in addition to liver tissue by different explants of fetal mouse tissues solely cultured in serum free medium (e.g. lung, brain, kidney and intestine). From this it was concluded that this evidence of local/peripheral IGF-I production strongly suggested autocrine/paracrine effects of IGFs [58]. Studies on IGF-I mRNA expression patterns in different species supported the idea of wide spread IGF-I production: it was found that the IGF-I gene was expressed in almost all (if not all) mammalian tissues, being not only limited to embryonic tissues, but also in the post-natal phase [59,60]. Furthermore it was found that not solely the liver, but also other tissues were able to produce IGF-I upon GH stimulation: in hypophysectomized rats being injected with GH IGF-I specific mRNA expression could be induced in multiple non-hepatic tissues (e,g. skeletal muscle, lung, heart, kidney and white adipose tissue) [61,62].

In 1982 the original paradigm of circulating IGF-I as an important component for longitudinal growth was challenged significantly by findings by Isakson et al. [63]. In their studies the authors showed that unilateral infusion of human GH into the cartilage growth plate of hypophysectomized rats resulted in significant longitudinal growth of bone at the respective site, but no effect was observed in the contralateral limb. Similar results were obtained by others [64,65]. Although the suggestion has been made that GH has direct, non-IGF-I dependent, proliferative effects on growth plate germinal zone cells - which indeed has been observed by others [66,67] - the dwarfism phenotype of *igf-I/IGF-IR* null mice clearly shows the significance of IGF-I effects in longitudinal growth [68,69]. In addition, it was found that IGF-I knockout mice are even smaller than GHR knockout mice [70]. Also in humans it has been found that IGF-I gene deletion results in a phenotype that is undisputedly dominated by

an extreme short stature [71]. The IGF system is thus intimately involved in prenatal and postnatal growth, although production of both IGF-I and IGF-II in utero is essentially independent of GH. Through an, as yet, unidentified molecular 'switch', IGF-I production at around the time of birth transitions from its GH-independent mode to a profoundly GH-dependent mode [56].

The contribution of paracrine/autocrine IGF production in relation to growth/development was emphasized by Yakar et al. who studied mouse models in which liver-specific IGF-I gene deletion could be introduced. In these models circulating GH levels were increased up to 4-fold and circulating IGF-I levels were decreased to about 25% in the knockout mice compared to the wild-type. Surprisingly, body length, femoral length and weight of organs did not differ between liver-specific knockouts and wild-type littermates [72,73]. It has to be noticed, however, that circulating free IGF-I levels were found to be normal in liver-specific IGF-I knockout (LID) mice. Interestingly, LID mice show a 4-fold increase in serum insulin levels and abnormal glucose clearance after insulin injection. Furthermore the insulin insensitivity in the LID mice was shown to be muscle specific. Recombinant human IGF-I treatment of the LID mice caused a reduction in insulin levels and an increase in insulin sensitivity [74].

Based on the above, not surprisingly, the question was raised whether the endocrine/circulating component of the IGF system is of significance in relation to organism growth/development. However, it was shown that if circulating levels of IGF-I dropped to 10-15% of normal by knocking out the acid-labile subunit (ALS; see below) next to the IGF-I gene in mice, the linear growth of this mouse model was reported to be significantly reduced. The proximal growth plates of the tibiae of these double knockout mice were smaller in total height as well as in the height of the proliferative and hypertrophic zones of chondrocytes. There was also a 10% decrease in bone mineral density and about 35% decrease in cortical thickness in these mice. The authors concluded that a certain threshold concentration of circulating IGF-I is necessary for normal bone growth and development [75]. Ueki et al. reported in ALS deficient mice that GH effects (body weight gain, linear growth) mediated by endocrine IGF-I were largely dependent on the absence of ALS, whereas the GH-dependent stimulation of IGF-I expression in liver, adipose tissue and skeletal muscle was unaffected. Recently, Wu et al. introduced another interesting mouse model. In order to further determine the function of endocrine versus tissue produced IGF-I they developed the KO-HIT mice which have no tissue IGF-I but exhibit increased serum IGF-I levels due to hepatic over-expression. The authors showed that in the total absence of tissue IGF-I, elevated levels of

serum IGF-I could support normal body size at puberty and postpubertal ages, but were insufficient to fully develop the female reproductive system [76].

In conclusion: It has become clear that the IGF system is widely spread throughout the body where it has both endocrine and paracrine/autocrine properties. Up to 70% of circulating IGF-I is produced by the liver. Paracrine/autocrine IGF has a large contribution in the regulation of growth/development and reproduction, although endocrine IGF also has input of significance. GH and IGF-I have both dependent and solitary effects on growth. However, normal postnatal growth requires the combined actions of GH and IGF-I [77].

1.4.2B Metabolic aspects of IGFs and their counteractions on GH effects

The original somatomedin hypothesis mainly addressed the mitogenic aspects of IGFs (e.g. growth/differentiation) [44]. However, IGFs were originally (also) identified by their insulin-like effects (referred to as NSILAs) suggesting IGFs having important metabolic functions/effects [6]. Indeed, IGFs share high sequence homology with pro-insulin (see paragraph 1.2.2 in this thesis). In paired euglycemic clamp studies, Boulware et al. showed that IGF-I, when compared to insulin, had almost identical effects on glucose uptake, glucose production, plasma free fatty acid concentrations, and fat oxidation rates [78]. Similar to insulin, IGF-I stimulates peripheral glucose uptake [78-80], inhibits gluconeogenesis, enhances adipogenesis and inhibits proteolysis, although it appears to be less effective than insulin [81,82]. The effects of IGF-I on muscle and total body glucose metabolism have been studied extensively in animals studies [79,80,83,84] and have been confirmed in humans [78,85-90]. Concerning support of evidence for the lipogenic properties of IGF-I: most leading observations come from studies of treatment of GH-insensitive subjects with IGF-I. In such subjects Laron et al. have shown that long-term recombinant IGF-I therapy (up to 12 years) increased body adipose tissue to 2-3 fold of the normal values [91].

In line with the metabolic actions of IGFs, nutrition has been recognized as a major determinant of circulating IGF levels, but also of its actions. Nutritional deprivation decreases hepatic IGF-I production by diminishing IGF-I gene expression and increases the clearance and degradation of serum IGF-I through changes in the levels of circulating IGFBPs [92]. Decreased levels of total IGF-I have been observed in states of undernutrition, including marasmus, anorexia nervosa, celiac disease and HIV infection [92-94], whereas IGF-I levels rise when malnourished patients are refeeded [95,96]. One of the nutritional factors that is clearly associated with IGF-I activity is protein intake. Elevated protein intake is able to prevent the mentioned IGF-I decrease in hypocaloric

states [97], whereas protein restriction leads to target organ insensitivity for IGF-I [98,99]. An interesting observation in this field comes from Schurch et al. in a double-blind placebo-controlled study in elderly individuals with a recent hip fracture and low total IGF-I levels [100]. The authors observed that a daily protein repletion (20 g) for 6 months resulted in increased total IGF-I and IGFBP-3 levels within 1 week (and later on also in prealbumin and IgM), increased muscle strength and lowered incidence of medical complications (e.g. infections). The authors hypothesized that protein intake might improve immune status through restoration of IGF-I actions on the immune system. Indeed IGF-I has been shown to stimulate proliferation of immune cells and modulates immunoglobulin secretion [101]. Next to protein intake, the carbohydrate content of the diet is also a major determinant of responsiveness of IGF-I to GH and might be even of superior importance; while the protein intake is able to increase IGF-I in the presence of adequate energy, there is a threshold energy requirement below which optimal protein intake fails to raise IGF-I after fasting [92].

The physiological effects of GH are complex and pleiotrophic. Amongst the metabolic effects of GH are gluconeogenesis, ketogenesis and lipolysis, all being opposite to those of IGFs [55]. Thus, the original suggestion that GH exerts its effects on non-osseous tissues through IGF-I (or IGF-II) as mediator does not hold completely: e.g. effects of IGF-I on muscle and other tissues are the opposite of those of GH itself. Kaplan and Cohen described an augmentative/counteractive system for GH-IGF. 'Because IGFs have insulin-like effects that stimulate protein synthesis and inhibit proteolysis while countering the hyperglycemic and lipolytic effects of GH, this mechanism serves to enhance the anabolic effects of GH' [55].

1.4.3 IGF binding proteins in relation to IGF-dependent and independent actions

Unlike insulin, which circulates in an unbound form, IGFs circulate in the plasma complexed to a group of IGF specific binding proteins (IGFBPs). The IGFBPs circulate in molar excess of the IGFs. In this way over 99% of present IGFs in the circulation are bound, meaning less than 1% of IGFs circulates in a free form [32,102].

Six high affinity IGFBPs are known, referred to as IGFBP-1 to -6, of which IGFBP-1 to -3 are more abundant in serum, whereas IGFBP-4 to -6 are more tissue specific and show low circulatory levels (Table 1). In humans, all six IGFBPs share about 50% amino acid sequence homology, with the highest degree of homology in the N- and C-terminal regions. The IGFBP genes share a common structural organization; four conserved exons are located within

Table 1: Overview of the Insulin-like Growth Factor Binding Proteins

Binding Protein	Chromosome	Molecular Weight (kDa)	Amino acid residues	Modulation of IGF action	Relative IGF affinity	Comments
IGFBP-1	7	28	234	Inhibition and/or Potentiation	I > II	Regulated by insulin RGD sequence Phosphorylated Integrin binding
IGFBP-2	2	34	289	Inhibition	I < II	Major IGFBP in semen RGD sequence Heparin binding
IGFBP-3	7	29	264	Inhibition and/or Potentiation	I = II	Major IGFBP in serum Regulated by GH ALS binding N-glycosylation Phosphorylated Insulin binding Inhibition of IR-autophosphorylation Inhibition of mitogenesis Heparin binding Cell binding Nuclear localization
IGFBP-4	17	26	237	Inhibition	I = II	N-glycosylation 2 extra cysteines
IGFBP-5	2	29	252	Potentiation	I < II	Major IGFBP in bone ALS binding Phosphorylated Heparin binding Cell binding Nuclear localization
IGFBP-6	12	28	216	Inhibition	I < II	Mainly present in CSF O-glycosylation 2 less cysteines

This table was modified after: Sue M. Firth and Robert C. Baxter: Cellular Actions of the Insulin-Like Growth Factor Binding Proteins, Endocrine Reviews, 2001, 23(6):824–854, Sujatha Rajaram, David J. Baylink, and Subburaman Mohan: Endocrine Reviews 1997 18(6): 801–831 and Calum Livingstone: Insulin-like growth factor-related proteins and diabetic complications, The British Journal of Diabetes and Vascular Disease, 2003, 3(5): 326-331.

these genes ranging from 5 kb (IGFBP-1) to more than 30 kb (IGFBP-2 and IGFBP-5) [103,104]. A conserved amino-terminal domain contains six disulfide bonds in all IGFBPs except for IGFBP-6, which has five (Table 1). Important IGF-binding residues are found in the amino-terminal domain, as well as in the carboxy terminal domain. The central domain of the IGFBPs shows essentially no structural conservation among any members of the family [104,105].

IGFBPs are found in the circulation in an unbound form or in complex with IGFs; the binary complex. IGFBP-3 and IGFBP-5 also form ternary complexes, consisting of a binary complex of the respective IGFBP with IGFs in complex

with a leucine-rich glycoprotein termed the acid labile subunit (ALS) [106,107]. Free or binary-complexed IGFBPs are believed to exit the circulation rapidly, whereas ternary complexes appear to be essentially confined to the vascular compartment [108-110].

Being originally recognized as (passive) circulating transport proteins for IGF-I and IGF-II, IGFBPs are now recognized as playing a variety of roles in the circulation, the extracellular environment and even inside the cell: **1.** IGFBPs extend half-life of circulating IGFs by preventing their degradation by proteolysis. The half-life of unbound/free IGF-I has been reported to be in the range of less than 10 minutes [111]. However, when forming a ternary complex with IGFBP-3 and ALS, the half-life of IGF-I is extended to approximate 12-16 hours [112]. **2.** IGFBPs predominate distribution and bio-availability of IGFs, thereby modulating IGF interaction with the IGF-IR [113]. In general IGFBPs are thought to inhibit IGF actions most probably by binding competition with the IGF target receptors. IGFBPs have also been shown to be able to potentiate IGF action presumably by facilitating IGF delivery to target tissues [32,114,115]. Although multiple studies describe the potentiating and inhibiting actions of IGFBPs on IGF activity, currently there is no unifying mechanism that explains these opposite actions. **3.** In addition to IGF related/dependent actions, IGFBPs also have been shown to have their own intrinsic bioactivity by binding nuclear receptors (e.g. inhibition of growth and induction of apoptosis by IGFBP-3) [105,115,116]. Interestingly in this respect, in the genome all IGFBPs are in close proximity to the Homeobox gene clusters. Hox genes are coding for DNA binding proteins that are involved in the regulation of organism morphogenesis. The possibility has been postulated that IGFBPs and Hox genes originate from ancestral single genes that underwent controlled duplication and translocation, suggesting evolutionary linkage [32,117-119].

1.4.3A IGF actions in relation to IGFBP-1

Insulin-like growth factor binding protein 1 became the first characterized member of the IGF binding proteins [120-122]. Next to that of IGFBP-3, the human IGFBP-1 gene is located on chromosome 7 (7p14-p12), in close proximity to the Hox-A gene [123-125]. IGFBP-1 is a 25-28 kDa protein. Twelve N-terminal and 6 C-terminal cystein residues, which are conserved in mammalian IGFBP-1 sequences and other IGFBPs, are important for IGF binding [104]. IGFBP-1 is produced in the liver, kidneys and decidua and the ovary [126]. Serum levels of IGFBP-1 are highly dependent on metabolic factors/status, and thereby highly fluctuate in accordance with food intake. The highest levels of circulating IGFBP-1 levels are found in a fasting state (>100 ng/

dL) inducing a peak at night, and the lowest after food intake (<10 ng/dL) [32]. Next to high intra-individual variation, IGFBP-1 levels also vary considerably between healthy subjects [127]. In relation to other IGFBPs, IGFBP-1 is thus unique in having dramatically variable kinetics in the circulation [126].

The main determinant of IGFBP-1 levels is insulin, through inhibition of hepatic IGFBP-1 gene transcription probably via hepatic nuclear factor 3 (HNF3) [126,128]. In this way IGFBP-1 is involved in the regulation of glucose metabolism. Low circulating levels of IGFBP-1 indeed have been associated with glucose tolerance, features of the metabolic syndrome and insulin resistance, obesity and the development of cardiovascular disease [129-133]. It has been found that in situations of severe stress (e.g. infection, trauma, surgery and burn injury) the relation between IGFBP-1 and insulin diminishes. Cytokine control of IGFBP-1 production may be an explanation for these observations [134,135]. In addition to insulin, IGF-I and IGF-II probably also inhibit IGFBP-1 production, whereas glucagon and especially glucocorticoids are important stimulatory factors for IGFBP-1 production [126].

In relation to IGF function, IGFBP-1 appears to be a primary and acute determinant of IGF-I bioavailability [105]. It has been suggested that IGFBP-1 facilitates IGF-I transport to the extra vascular compartment, thus thereby potentially increasing IGF-I availability/activity in the target tissues [102].

Both potentiating and inhibitory effects of IGFBP-1 on IGF-I actions have been described. The positive or negative modulation of IGF-I bioavailability by IGFBP-1 is thought to be related to the phosphorylation status of serine residues [32,105,136]. The affinity of phosphorylated human IGFBP-1 for IGF-I is six-fold higher than for the nonphosphorylated protein [136]. In humans, phosphorylated forms in general inhibit IGF-I actions, whereas dephosphorylated IGFBP-1 has stimulatory activity [120,137,138]. The impacts of IGFBP-1 phosphorylation on its function *in vivo* are however uncertain.

1.4.3B IGF actions in relation to IGFBP-3

IGFBP-3 is the major IGFBP in human serum. Its molecular weight varies from 29 kDa (nonglycosylated) to 44 kDa due to the possibility of glycosylation [139]. IGFBP-3 forms in which various N-glycosylation sites have been altered by mutagenesis reveal that decreasing glycosylation tends to increase cell surface association [140]. The functional implications of this are not certain however. Like IGFBP-1, IGFBP-3 can be secreted as a phosphoprotein, which in contrast to IGFBP-1 does not affect IGF-I binding capacity, however [141]. GH is the main regulator of IGFBP-3 production by the liver. Other stimulators are

interleukin-1, tumor necrosis factor-alpha, transforming growth factor-beta, retinoic acid, parathormone , estradiol, glucocorticoids and p53 [32].

Being the most abundant IGFBP, the major IGF transport function can be attributed to IGFBP-3, binding about 75-90% of circulating IGFs. In healthy adults, about 90% of IGFBP-3 circulates in the ternary 150 kD complex which cannot cross the vascular endothelium [104]. The circulating levels of IGFBP-3 are dependent on age, being low at birth and peaking at puberty after which they decrease in further life [139].

The relationship between IGFs and IGFBP-3 is highly complex. Inhibiting actions predominate, however IGF potentiating actions by IGFBP-3 have been demonstrated in several studies and have been reviewed extensively [32,104,105,139]. In general, *in vitro* studies show that IGFBP-3 in solution decreases (growth) stimulatory effects of IGFs via high affinity sequestration of the ligands. The affinity of IGF to IGFBP-3 is higher than to the IGF-IR [142], thereby preventing IGF-IR interaction. In contrast, preincubation of cells with IGFBP-3 followed by IGF-I treatment has been found to enhance the presentation of IGF to the IGF-IR. Several concepts have been reported: IGFBP-3 cell association and processing to a form of reduced IGF affinity [143,144], IGFBP-3 induced protection of cells from IGF-I mediated IGF-IR down-regulation [145], mediation of IGF actions by IGFBP-3 through the phosphatidyl inositol 3 (PI3)-kinase pathway [146] and/or release of IGFs from IGFBP-3 via specific proteases for IGFBP-3 [147,148]. Interestingly, 5 amino acids present in a 18-residue basic domain (containing a heparin-binding motif) that is implicated in IGFBP-3 cell binding play an important role in ALS binding. From this it may be speculated that the endothelial cell surface and ALS compete for the same residues on IGFBP-3, a process that is involved in the dissociation of the ternary complex and the release of IGFs [104].

1.4.4 IGF Receptors

The IGF receptor family comprises two trans-membrane proteins. Most actions of both IGF-I and IGF-II are mediated through the type I IGF receptor (IGF-IR), being the cognate receptor for these ligands [32]. IGF-II also binds the mannose-6-phosphate receptor with high affinity. Most studies demonstrate that this receptor, having no intrinsic activity, functions mainly as a scavenger for IGF-II [32,149].

IGFs also have binding capacity for the insulin receptor (IR). A segment localized to the C-terminus of the IR α-subunit is subject to tissue-specific alternative splicing, thereby resulting in two IR isoforms, the IR-A (minus exon 11) and IR-B, respectively. IGF-II has been reported to bind the IR isoform A

Table 2. Receptor subtypes of the IGF system

	Homotetramers			Hybrids		
Receptor	IR-B/IR-B	IR-A/IR-A	IGF-IR/IGF-IR	IR-B/IR-A	IR-B/IGF-IR	IR-A/IGF-IR
Name	IR-B	IR-A	IGF-IR	HIR-AB	HR-B	HR-A
Ligand(s)	Insulin	Insulin IGF-II	IGF-I IGF-II	Insulin IGF-II	IGF-I IGF-II	IGF-I IGF-II Insulin

Adapted from 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.

with high affinity. Therefore, IGF-II might use both the IGF-IR and the IR-A to exert its effects. Hybrid receptors, formed by one IR hemicomplex and one IGF-IR hemicomplex, have been observed [150,151]. Functional studies suggest that these hybrids behave like IGF-IR homotetramers rather than as IR homotetramers. IR/IGF-IR hybrid receptors bind IGF-I with an affinity similar to that of the IGF-IR, whereas they bind insulin with a much lower affinity [152,153]. The IR isoform in hybrid receptors does not affect affinity of IGF-I, IGF-II and insulin for hybrid receptors [154]. Hence, hybrid receptors may play a role in switching signaling from insulin to IGF-I (Table 2). Very recently a review has been published about this interesting subject by Belfiore et al [155].

1.4.4A Structure of the IGF-IR

The IGF-IR is a member of the insulin tyrosine kinase class of cell-surface receptors, together with the IR and the orphan insulin receptor-related receptor [156]. The human IGF-IR gene is located on chromosome 15q26 and encodes a single polypeptide of 1367 amino acids (440 kDa) that is constitutively expressed in most tissues. However, IGF-IRs are very low expressed in liver or adipose tissue, in contrast to IRs [157].

The IGF-IR shares approximately 70% amino acid sequence homology with the IR, and their genes exhibit obvious homology in terms of size and exon organization. The IGF-IR is synthesized as single-chain pre-proreceptor, with a 30-residue signal peptide that is cleaved co-translationally. Proreceptors are glycosylated, folded and dimerized by chaperone proteins. In the Golgi apparatus the receptors are processed at a tetrabasic Arg-Lys-Arg-Arg furin-protease cleavage site to generate the α (130–135 kDa) and β (90–97 kDa) subunits of the mature receptor [158].

The mature IGF-IR consists of two disulfide $\alpha\beta$-half receptors, linked by disulfide bridges, giving rise to the mature $\alpha_2\beta_2$-heteroterameric holoreceptor

even in the absence of ligand. The extracellular α-subunits, containing cysteine-rich regions, form the binding site for IGFs [159]. The β-subunits cross the cell membrane, being extracellularly linked to the α-subunits, while the intracellular region of the β-subunits possesses tyrosine kinase activity. The kinase domain of the IGF-IR shares 85% homology with that of the IR, and the ATP binding cleft is 100% conserved. IGF-IR activation leads to autophosphorylation on tyrosines 1131, 1135 and 1136 in the kinase domain, followed by phosphorylation of juxtamembrane tyrosines and carboxy-terminal serines. This is followed by recruitment of specific docking intermediates, including insulin-receptor substrate-1 (IRS-1) and SH2 containing protein (Shc). These molecules link the IGF-IR to diverse signalling pathways.

1.4.4B IGF-IR signaling pathways

Two main well known signaling pathways are activated through the IGF-IR; 1) the lipid kinase phosphatidylinositol-3-kinase (PI3K) / V-akt murine thymoma viral oncogene homolog 1 (AKT1) pathway (AKT1 is also known as protein kinase B (PKB)) - and 2) the Ras / Mitogen-Activated Protein Kinase (MAPK)

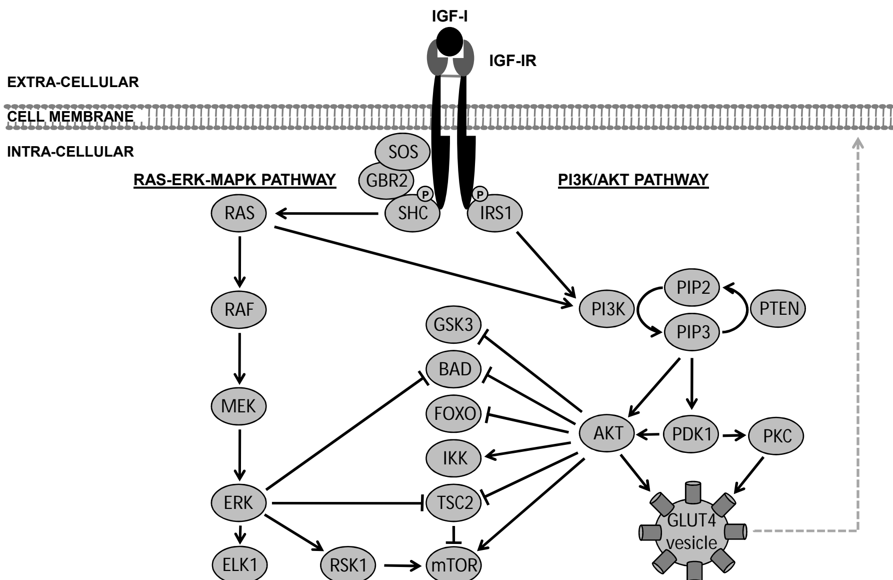

Figure 5. Schematic overview of the IGF-IR signaling pathways. After activation of the IGF-IR by binding of IGF-I or –II, the autophosphorylation process of the kinase subunits leads to phosphorylation of adaptor proteins. Two main intracellular pathways are activated, the RAS-ERK-MAPK pathway and the PI3/AKT pathway. Both pathways are described in detail in the text of *paragraph 1.4.4B.*

pathway [160]. A schematic diagram of these pathways is illustrated in Figure 5. In addition the IGF-IR can modify calcium-dependent signaling pathways [160,161].

PI3K-AKT pathway: IRS-1 plays a key role in transmitting signals from the IGF-IR to intracellular pathways PI3K / AKT and ERK / MAPK pathways [162]. IGF-IR tyrosine phosphorylation induces binding of IRS-1 to the tyrosine kinase catalytic unit through its src homology 2 domain (SH2). Tyrosine-phosphorylated IRS-1 interacts with PI3Ks - also having a SH2 domain - that catalyze the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3) [163]. PIP3 binds AKT1 and phosphoinositide dependent kinase-1 (PDK-1). PDK-1 is a crucial kinase for activation of both AKT1 and subtypes of protein kinase C (PKC). The importance of PDK-1 has been demonstrated by experiments in homozygous knock-out mice, which die during early embryonic development [164]. Activated PKCs and AKT1 facilitate glucose transporter-4 (GLUT4) translocation from GLUT4 vesicles to the membrane, thereby enhancing glucose uptake [165]. The serine-threonine protein kinase AKT1 froms a key protein in IGF-IR signaling. AKT1 phosphorylates and inactivates several cytoplasmatic proteins (Figure 5). Next to GLUT4 translocation AKT1 mediates glucose (and protein) homeostasis via inhibition of glycogen synthase kinase-3 (GSK3), thereby indirectly promoting glycogen (and protein) synthesis [165,166]. A primary function of AKT1 is phosphorylation of B-cell lymphoma 2 (Bcl-2)-associated death promoter (BAD), thereby inhibiting its pro-apoptotic properties. Phosphorylation of BAD results in dissociation of the anti-apoptotic proteins Bcl-2 and B-cell lymphoma-extra large (Bcl-xl) from the BAD-Bcl2-Bcl-xl complex [167]. Active AKT1 also inhibits pro-apoptotic members of the forkhead transcription factor family (FOXO) by phosphorylation [168]. Brunet et al. showed that human FKHR1 - also known as FOXO1 – is a direct target of AKT1 preventing FHKR1 induced apoptosis via genes such as Fas ligand [169]. Trough activation of Inhibitor of KB (I-KB) kinases (IKKs), AKT1 allows the dissociation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) from I-KB. In the nucleus NF-KB acts as an transcription factor which activates several including anti-apoptotic genes [170,171]. The serine/threonine protein kinase mammalian target of rapamycin (mTOR) is also a AKT1 target directly or indirectly (by blocking mTOR inhibitor Tuberous sclerosis protein 2 (TSC2)) [172,173]. Via several downstream targets mTOR stimulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription.

Ras/ERK pathway: IGF-IR activation can also lead to activation of a complex composed of SH2 containing protein (Shc), growth factor receptor bound protein-2 (GRB2) and the guanine nucleotide exchange factor 'son of sevenless' (SOS). The activated SHC-GRB2-SOS complex activates the small GTPase Ras which initiates sequential phosphorylation cascades involving serine/threonine kinase Raf, MAPK Kinases (MEK1/2), and extracellular signal-regulated kinases (ERK) or MAPK. An endpoint of the Ras / MAPK pathway is modification of transcription factor activity, such as activation of Elk transcription factors [174]. This target pathway of IGF-IR signaling has been mostly associated with cell proliferation, differentiation and migration, but in some cases can also inhibit apoptosis [160]. In accordance with AKT1, ERKs can inhibit apoptosis via phosporylation of BAD and can indirectly stimulate mTOR activity (Figure 5).

1.5 The IGF system, aging and longevity

Aging is characterized by a diminished capacity to control homeostasis, which associates with an increased susceptibility to develop life-threatening diseases and disabilities, such as cancer, cardiovascular disease, diabetes and neurodegenerative disorders. A significant and increasing proportion of the population is elderly, especially in the developed countries, making it of increasing importance to gain insights on how aging predisposes individuals to disease. Interestingly, peculiar metabolic characteristics are endowed in long-lived people. Some of these include lower than expected body mass index, fasting plasma glucose, plasma insulin/IGF-I, insulin resistance as well as preserved pancreatic B-cell function [175].

In the last two decades the insulin/IGF signaling pathway has been of great interest in relation to organism aging and longevity, including that of humans. Transduction of insulin/IGF signals may be critical determinants of lifespan in lower rank organisms. Genetic studies performed in the nematode *Caenorhabditis elegans* identified insulin/insulin-like (receptor) pathway genes (age-I, daf-2, daf-16 and ins-7) as regulators of its lifespan [176-179]. Daf-2 encodes the nematode homologue of the insulin/insulin-like receptors in mammals. Disruption of the insulin/IGF-I like daf-2 pathway significantly extended lifespan of these organisms up to 30-100% dependent on the mutation [180]. Soon afterwards, it was shown that the evolutionary highly conserved insulin/insulin-like pathways also influence lifespan in yeast and insects in a comparative way [181-183]. Referring to the later organisms, the fruit fly *Drosophila melanogaster* insulin-like receptor gene (InR) is a homologue of *C.elegans* daf-2 and mammalian insulin receptors. Although

genotypes homozygous for mutant InR have been reported to be lethal, several heteroallelic combinations of InR alleles produce viable adults with a dwarfism phenotype [184]. Measurement of basal and insulin stimulated kinase activity indicated that InRp5545 (P-element insertion in exon-1) and InRE19 alleles both resulted in reduced receptor signaling function. Tatar et al. found the heteroallelic, hypomorphic InRp5545/InRE19 genotype to live 85% longer than wild-type controls. Interestingly sexual dimorphism existed as this was only true in female flies [183]. InRp5545/InRE19 males had higher mortality as early adults but reduced mortality at late ages.

To study the insulin/IGF signaling in relation to mammalian longevity several rodent models have been used [180]. Of interest are heterozygous models of GH/IGF-I deficiency and IGF-IR knockouts. Both Snell (PIT-1 mutation [185]), and Ames (PROP-1 mutation [186]) mice demonstrate GH/IGF-I deficiency, a dwarfism phenotype and increased longevity in both males and females relative to wild-type controls [187,188]. Results have to be interpreted with caution however; next to GH/IGF-I multiple other primary endocrine deficiencies (prolactin, TSH) and hormonal/metabolic changes (insulin sensitivity, glucocorticoids) are present in Snell and Ames mice which could be of importance in relation to organism longevity [180]. Analogous to *Drosophila melanogaster,* IGF-IR and IR homozygous knock-out mice are not vital, but die at or shortly after birth due to respiratory insufficiency and metabolic complications [68]. Holzberger et al. developed a IGF-IR mutant mouse model (IGF-IR$^{+/-}$) harboring heterozygous deletion of exon 3. This exon is coding for the ligand binding domain of the IGF-IR and deletion resulted in 50% reduction in receptor levels [189-191]. Post-natal growth curves did not differ between IGF-IR$^{+/-}$ and wild-type littermates, and only slight differences developed afterwards being in favor of wild-type controls and more pronounced in males than in females. Fertility, consumption and energy expenditure were unaffected. Survival analysis revealed a significantly longer lifespan in IGF-IR$^{+/-}$ mice compared to wild-types, but this was only significant in females (+33%). Interestingly IGF-IR$^{+/-}$ mice displayed increased resistance towards methyl viologen-induced intracellular oxidative stress. Again these results showed the same sex-dimorphic pattern [189].

Since genetic alterations in IGF-I/insulin (or homologous) signaling pathways seem to influence longevity in organisms, ranging from yeast to rodents, the question was raised whether the low insulin/IGF-I signaling paradigm is also applicable to human longevity. Findings are characterized by controversy and studies are limited (also due to ethical limits). Both isolated GH deficiency and GH overproduction in acromegaly have been associated with reduced

lifespan [192,193]. However, individuals with GH insensitivity or Laron syndrome (GHR resistance) have a dwarfism phenotype with high GH and low IGF-I levels which is not obviously associated with reduced or increased lifespan [194]. In a study performed in healthy centenarians, Paolisso et al. observed circulating IGF-I levels being equal to subjects aged over 75 years, whereas centenarians had lower IGFBP-3 levels. They suggested that in centenarians the bioavailability of IGF-I is enhanced despite its lower levels [195]. Ruiz-Torres et al. studied the pattern of sex-specific decline of IGF-I levels with age in 205 individuals aged 19-93 years. In contrast to women, decline in IGF-I levels with advancing age in men showed a higher slope in individuals aged <55 years and a lower slope onwards. They concluded that the regression line observed in aged men was artificially modified by mortality since the lowest IGF-I levels measured were higher than those predicted, suggesting increased mortality among men with relatively low circulating IGF-I levels [196].

Other information comes from studies on genetic variability at the loci of insulin and IGF-I/II genes. Insulin/IGF gene polymorphisms in relation to human longevity were first studied in the region spanning the insulin-IGF-II loci. De Luca et al. reported that restriction enzyme determined FokI-AvaII haplotypes were found to associate with longevity in Italian centenarians, however this was only found in men [197]. Whether and how this polymorphism is involved in altered insulin/IGF activity, however, is not known. In the Ashkenazi ethnic group an association was found between an ApaI IGF-II polymorphism and longevity, but here only in females [198]. In contrast no association between variability at the insulin gene locus and lifespan was observed in an aging Dutch population in the Leiden 85-plus study [199]. At the human IR locus a haplotype was associated with longevity in a group of Japanese subjects older than 105 years [200]. Bonafè et al. found genotypic combinations at codon 1013 of IGF-IR and at −359 bp from the starting codon of phosphoinositide-3-OH kinase (PI3KCB) were associated with longevity and with low IGF-I plasma levels in Italian centenarians [201]. The synonymous polymorphism (rs2229765) in the IGF-IR gene consists of a G to A transition leading to the amino acid change Glu>Glu. Recently, Albani et al. studied this IGF-IR gene polymorphism into more detail within another Italian population [202]. They found that males showed an age-related increase in the A-allele of rs2229765 and a change in the plasma level of IGF-I, which dropped significantly after 85 years of age (85+ group; A/A homozygous subjects had the lowest plasma IGF-1 level). Whether this polymorphism effects IGF-IR activity was however not reported.

Together these observations suggest that the insulin/IGF-I system might be involved in longevity, although results are sparse and sometimes conflicting especially for studies in man. Furthermore, information on how certain factors influence IGF-I bioactivity in relation to human survival is absent and prospective data (which is of course very difficult to obtain in the elderly) are not available.

1.6 IGF-I and insulin sensitivity, diabetes mellitus and cardiovascular disease

1.6.1 Insulin sensitivity, obesity and diabetes mellitus type 2

Several observations suggest that IGF-I plays a primary role in insulin physiology. Liver-specific IGF-I deficient (LID) mice - characterized by their low circulating IGF-I levels - have high GH levels, glucose intolerance and insulin resistance [203]. Also, mice with disturbed IGF-IR function display features of severe insulin resistance and early onset of type 2 diabetes mellitus (DM type 2) [204], whereas over-expression of these receptors in skeletal muscle produces a less severe phenotype [205]. Mechanisms that could explain these observations are IGF-I-induced enhancement of peripheral insulin actions via IGF-IR signaling (e.g. skeletal muscle) and/or suppression of pituitary GH secretion. Another interesting observation in this field comes from pancreatic β-cell specific IGF-IR knock-out mice, which show severely impaired insulin secretion upon glucose stimulation compared to controls, which is accompanied by glucose intolerance [206,207].

The role of IGF-I in relation to insulin sensitivity has also been recognized in humans. A rare state of IGF-I deficiency related to a homozygous partial deletion in the IGF-I gene is associated with severe insulin resistance which was normalized after administration of IGF-I [208,209]. Using clamp studies Cusi et al. showed that in healthy subjects IGF-I enhanced insulin sensitivity in the fasting state [210]. In addition, several studies showed that administration of IGF-I (either alone or in combination with IGFBP-3) improved insulin sensitivity in healthy subjects [78,85,89,203,211], in those with existing insulin resistance [212,213] as well as in subjects with DM type 2 [214,215].

Although these studies point towards a significant role of IGF-I in glucose homeostasis, there is lack of unanimity when assessing studies focusing on the relationship between the IGF-axis and obesitas and/or DM type 2. It is well known that especially obese subjects are prone to develop DM type 2. Some studies have reported increased circulating total IGF-I levels in obese subjects and type 2 diabetics [216,217]. However, several other studies reported IGF-I levels within the (lower) normal range in obesity-related DM type 2 [218-221].

Conversely, lower circulating total IGF-I levels have been associated with poorer glycaemic control in DM type 2 [222]. In addition, low circulating IGF-I levels also have been found to be independently associated with the presence of features of the metabolic syndrome, including insulin resistance [223]. For levels of free IGF-I a different profile has been found, being relatively high in obese subjects with and without DM type 2 when compared to non-diabetic lean controls [224]. Obesity also affects IGF-II, IGFBP-2 and IGFBP-3; circulating total and free IGF-II are relatively high in obese subjects, as is IGFBP-3, which tend to further rise if type 2 DM is present. In contrast, IGFBP-2 levels are decreased in subjects with obesitas and are even lower in those having type 2 DM [224]. In contrast to measures of IGF-I, those of GH and IGFBP-1 show more clear patterns in relation to obesity and/or insulin resistance. Obesity is associated with GH hyposecretion and reduced synthesis and circulating levels of IGFBP-1 are observed in states of insulin resistance, such as obesity [131,225,226]. Elevated serum levels of free IGF-I and -II are likely to increase the feedback inhibition of the pituitary GH secretion and this provides an explanation for the hyposecretion of GH in obese subjects. Still, despite GH hyposecretion, obese subjects show no sign of GHD [224]. Actually it is thought that obese subjects are GH hypersensitive since short-term treatment with low dose GH has been shown to increase serum total IGF-I significantly more in obese than in lean subjects [227]. The increased responsiveness to GH in obese subjects could be explained by an up-regulated hepatic GHR density, as circulating GH-binding protein (GHBP), which originates from cleavage of the extracellular part of the GHR, is elevated in obese subjects [224,228].

Prospective data about the association between the risk of DM type 2 and the IGF-axis are limited. Sandhu et al. found an inverse relationship between circulating total IGF-I levels and the risk of DM type 2 after an average follow-up of 4.5 years among about 600 subjects aged 45-65 years [229]. Those having IGF-I levels above the median had half the risk of DM type 2 compared to those below the median.

1.6.2 The IGF-axis in relation to cardiovascular disease

An enormous number of studies has been published that focused on the question whether and (if so) how the IGF system is associated with the risk of cardiovascular disease (CVD). The link between IGF-I and CVD comes from observations that subjects with primary GH deficiency and those with acromegaly are characterized by increased mortality attributable to CVD [230-232]. In general, previous studies point to IGF-I as a pro-atherogenic mediator. For instance, IGF-I has been shown to stimulate growth and migration of vascular

smooth muscle cells (VSMC) [233-235], and these cells express high numbers of both IGF-IR and IGF-IR/IR hybrids [90]. More recently, it has been shown that activation of the PI3K/AKT pathway potentiates platelet aggregation [102,103]. On the other hand there are several studies that claim opposite effects for IGF-I in the development of atherosclerosis. Tumor necrosis factor α (TNFα), a pro-atherogenic factor, produced by activated macrophages in atherosclerotic plaques has been shown to actively inhibit IGF-I gene expression [236]. In addition, IGF-I may have directly beneficial effects on the vascular system through stimulation of nitric oxide production (activated AKT1 stimulates endothelial nitric oxide synthase) [237]. Third, insulin resistance has been convincingly linked to hypertension, obesity, dyslipidemia and cardiovascular disease [238]. Therefore, via its beneficial effects on glucose homeostasis and insulin sensitivity (as described in paragraph 1.6.2) IGF-I could have important anti-atherosclerotic effects.

Several observational studies report significant associations between low circulating total and/or free IGF-I levels and increased risk of CVD [132,239-242]. Low circulating total IGF-I has been correlated with increased risk of MI and AP [243-245]. In a nested case-control study within a prospective study Juul et al. measured total circulating IGF-I and IGFBP-3 in individuals who on average had a diagnosis of ischemic heart disease (IHD, N=231) 7.6 years after blood sampling and in age and sex matched controls [242]. The authors reported that subjects in the lowest quartile of total IGF-I at baseline on average had a 1.94 (95% CI 1.03-3.66) times higher risk of IHD during the 15 years of follow-up when compared to those in the highest quartile. For individuals in the highest quartile of IGFBP-3 this was 2.16 (1.18-3.95) times higher than those in the lowest quartile. Individuals with low IGF-I and high IGFBP-3 showed a relative risk of 4.07 (1.48-11.22) of having IHD compared to controls. The authors concluded that IGF-I may influence the development of IHD, in which individuals with low levels of total IGF-I and high IGFBP-3 levels are at risk.

1.7 Measurement of IGF-I: IGF-I immunoassays and IGF-I bioassays

1.7.1 Immunoassays to determine total circulating IGF-I levels

All assays for measurement of circulating IGF-I were initially conventional radioimmunoassays (RIA). These assays are based on competition between a fixed amount of radiolabeled IGF-I, a fixed of IGF-I specific antibodies and a variable amount of IGF-I in the (unknown) blood sample. As almost all IGF-I in blood is bound with high affinity to IGFBPs techniques have been developed

to remove or inactivate IGFBPs to overcome assay interference [246]. The gold standard technique for removal of IGFBPs is that of acid gel filtration column chromatography [247]. Limitations of applicability of this technique are sample volume expansion, reproducibility and labor intensiveness [248]. A widely used method of IGFBP removal has been acid/ethanol precipitation [249]. By use of this technique IGF-I is especially extracted from IGFBPs, which form a ternary complex with ALS (IGFBP-3 and IGFBP-5). Smaller IGFBPs, however, (e.g. IGFBP-1) remain in the sample and this still can produce significantly residual signal interference, especially in pathophysiological conditions (e.g. diabetes) [250]. To minimize this problem, Blum et al. developed a method that utilized addition of IGF-II to saturate remaining IGFBP binding capacity for IGF-I after acid/ethanol precipitation [251]. Most of the currently used IGF-I immunoassays have a 2-step antibody design, rather than using competitive binding assays. An IGF-I capture antibody is linked to a solid phase (e.g. beads, microparticles, coated tube), whereas a second labeled IGF-I antibody - directed against another epitope of the IGF-I molecule than the capture antibody – is used for detection. The read-out technique used is dependent on the type of label linked to the detection antibody; radioactive nuclide (immunoradiometric assay (IRMA)), enzyme (enzyme-linked immunoabsorbant assay (ELISA)) [252], chemiluminescent molecules (Chemiluminescence immunoassay (CLIA)). Of notification, although in theory these assays have the potential to eliminate IGFBP interference without necessity of IGFBP extraction and saturation techniques, they can still be potentially altered by the presence of IGFBPs [248,253]. Furthermore other determinants such as the utilized IGF-I standard or antibodies, as well as sample storage conditions may have impact on assay outcomes [248,254-256].

1.7.2 Free IGF-I assays

In line with the generally accepted idea that only the unbound IGF-I molecule is biologically active, assays have been developed to measure free IGF-I. Early methods for determination of free IGF-I were based on neutral size-exclusion chromatography, neutral high pressure liquid chromatograph (HPLC) and reverse phase chromatography [224]. It was however widely acknowledged that chromatography distorted the equilibrium between free and bound IGF-I and thereby grossly overestimated levels of unbound peptide [257].

Later Frystyk et al. developed an assay in order to determine free IGF-I levels in human serum. In this assay they used an ultrafiltration method, in which serum is filtered at a low-centrifugal force across a membrane allowing passage of unbound IGFs but not of IGFBPs. This is followed by a sandwich assay to

analyze IGF-I concentration in the ultrafiltrate compartment [258,259]. At the same time, another method for determination of free IGF-I was published by Takada et al. using an IRMA method [260]. In both assays temperature turned out to be an important determinant. Using the ultrafiltration method free IGF-I levels measured at 37°C were considerably higher compared to those measured at 5°C [259]. Frystyk et al. compared both methods. They found that the direct IRMA technique, next to temperature, was highly sensitive to incubation time. Furthermore it was realized that the free IGF-I levels measured by the direct IRMA were higher than those measured by the ultrafiltration method. This because the capture antibody used in the direct IRMA method next to binding unbound IGF-I probably also extracted IGF-I from IGFBPs [261].

While the relevance of free IGF-I to IGF activity is debated, it was found that circulating free rather than total IGF-I levels regulate the negative feedback loop with GH [262]. Thus, while these assays offer the possibility to determine the unbound fraction of circulating IGF-I, they are technically difficult to perform and outcomes are dependent on the type of assay used.

1.7.3 IGF-I Bioassays and the IGF-I Kinase Receptor Activation Assay (KIRA)

As counts for numerous biological systems - including for the IGF system - extracellular biological information is integrated at a cellular level through cell surface receptors. Receptor activation then leads to activation and / or inhibition of target signalling cascades, which eventually lead to cellular endpoint responses (e.g. proliferation, differentiation, survival, product secretion / uptake or apoptosis). A bioassay is essentially any technique that assesses the bioactivity of a given ligand by measuring its induction of a certain response by an intact cell. Outcomes ('responses') that are generally used in bioassays include proliferation, differentiation, survival, product secretion, fluorescence, cytotoxicity or apoptosis. All of these responses used in traditional bioassays are downstream events, initiated by the stimulation of a receptor by its ligand(s). In case of IGFs classical bioassays have been based on sulfate and ^{3}H-thymidin incorporation into cartilage [2,5,263] and proliferation of BALBc 3T3 fibroblast cells [264-266] or MCF- 7 human breast adenocarcinoma cells [267,268]. However, the weakness of these IGF-I bioassays lies within the assessment of cellular responses that are far downstream of the IGF-IR signaling cascade. This opens the possibility of interference or cross-links by alternative pathways that may lead to the same cellular response. In addition, in these assays IGF-I bioactivity might be biased by endogenous production of IGFs, IGFBPs, proteases or other factors produced by the read-out system due to long duration of ligand exposure. Thus, from the above the cautious

conclusion could be drawn that immunoassays also have been introduced to estimate IGF-I bioactivity due to the lack of alternative methods (bioassays) that met acceptable performance criteria to be available for measurement of IGF bioactivity in larger settings. It can thus be stated that the (additive) value and performance of bioassays in general greatly depends on their underlying design. As with immunoassays, performance of bioassays should be critically subjected to general assay terms as sensitivity, specificity and assay variability, but even more important, they should also provide meaningful and additive information of biological significance by coming as close as possible to the *in vivo* situation.

In 1999 Sadick et al. published a novel strategy for a rapid bioassay that in theory can be applied to every member of the tyrosine kinase receptor super family. This method was therefore termed as kinase receptor activation assay (KIRA). It employs a two-plate system, one cell culture plate for stimulation of intact cells, followed by receptor solubilization and a second plate for receptor capture and analysis of tyrosine phosphorylation by ELISA [269]. In this way the KIRA assay can be defined essentially as monospecific in that only the response of the receptor is determined for which it is designed [269].

In case of IGFs, the IGF-IR forms the key structure by connecting the extracellular IGF system to its intracellular targets. In this way, biological information harbored by the IGF system is integrated and translated into intracellular events at the level of the IGF-IR. Activation of the IGF-IR leads to conformational changes, allowing autophosphorylation of the intracellular kinase domains. Thereby IGF-IR autophosphorylation – the first intracellular event, only initiated by ligands able to activate the IGF-IR – forms the most specific intracellular target, that could be used to assess bioactivity generated by the IGF system. Based on the KIRA principle, Chen et al. refined an IGF-I KIRA developed by Sadick et al. and validated it for measurement of IGF-I bioactivity in human serum. As a biological read-out system, they used human embryonic renal cells (293 EBNA) stabile transfected with cDNA encoding the full-length human IGF-IR gene. Figure 6 shows a scheme of the IGF-I KIRA procedure designed by Chen et al.

The IGF-I KIRA uses a short (15-min) sample incubation time, and this minimizes the possible time for the cells to produce IGFBPs and proteases that may interfere with the ligand activity during sample incubation. In contrast to IGF immunoassays IGFBPs are not removed from serum samples, but are allowed to modulate IGF-IR activation via inhibition and/or delivery-of IGFs at physiological conditions as happens *in vivo*. Qua performance Chen et al showed that the IGF-I KIRA is highly sensitive, specific (cross-reactivity of insulin,

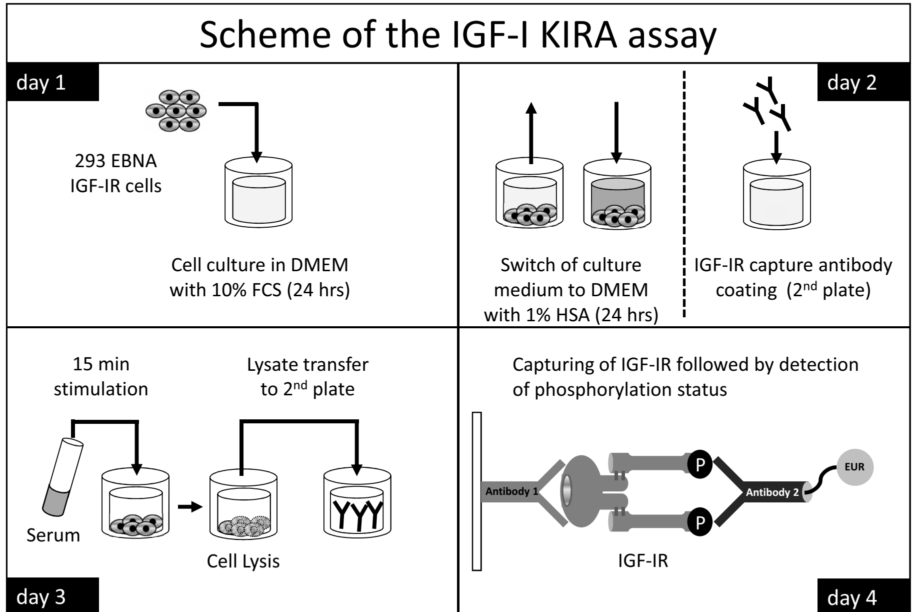

Figure 6. Schematic overview of the IGF-I Kinase Receptor Activation Assay (IGF-I KIRA). The assay is a 4-day procedure. On day 1 human embryonic kidney cells stabile transfected with the human IGF-IR are cultured in 48 well culture plates (200,000 cells per well) in medium containing 10% fetal calf serum (FCS). On day 2 the medium is replaced by medium containing 1% human serum albumin (HSA). A second 96 well plate is coated with an human IGF-IR capture antibody (MAD1). On day 3 cells are stimulated with serum for 15 minutes and then cells are lysed. Lysate is transferred to the washed 96 well plate that was coated with MAD1 on day 2. On day 4 the 96 well plate is washed and a second antibody is added (PY20), specifically recognizing phosphorylated residues located at the kinase domains of the IGF-IR. The PY20 antibody contains an europium label that can be detected by a time resolved fluorometer.

insulin analogs, and proinsulin was less than 1%; IGF-II cross-reactivity was 12%), and accurate (intra- and inter-assay coefficients of variation were about 7 and 15% respectively). In addition, co-incubation of recombinant IGF-I with increasing concentrations of IGFBP-1, -2, or -3 dose dependently reduced the IGF-I KIRA signal, showing that the IGF-I KIRA is sensitive for IGFBP modulation. Analysis of serially diluted serum samples showed that the signal was relatively independent of the dilution up to 1:20. Receptor-binding experiments with iodinated IGF-I further showed that IGF-IR-binding affinity for IGF-II and insulin was more than 10 and 200 times lower, respectively, than that for IGF-I.

By quantification of IGF-IR activation, the IGF-I KIRA is based on a different methodological principle when compared to IGF-I immunoreactive

assays [270]. Theoretically IGF-I KIRA measurements represent the outcome of the sum of all factors that modify IGF-IR activation, especially those factors that directly modulate interactions between IGF-I and the IGF-IR (IGFBPs, IGFBP proteases). This makes the IGF-I KIRA method a new and scientifically interesting tool to study IGF-I bioactivity.

1.7.4 IGF-I bioactivity; what's in a name?

As the IGF-IR by far has the highest affinity for IGF-I and outcomes of unknown serum samples are plotted against known concentrations of recombinant IGF-I as a reference, the method was named 'IGF-I KIRA', and its outcomes 'circulating IGF-I bioactivity'. However, IGF-II also binds and acts through the IGF-IR. Therefore it is to be expected that results of the IGF-I KIRA are also influenced by circulating IGF-II. As described, the contribution of IGF-II to the IGF-I KIRA signal was about 12% when compared to IGF-I at equal concentrations [270]. The contribution of IGF-II to IGF-I bioactivity could be biologically significant *in vivo* since circulating IGF-II levels are higher than those of IGF-I and do not decline with age as does IGF-I [252].

One could argue that sensitivity to IGF-II is a disadvantage of the IGF-I KIRA. This argument indeed holds when this phenomenon is interpreted as interference of IGF-II in the assay. On the other hand, the general aim of a bioassay is to give insight in the function of a biological system and not of single system parameters as such. Thus, when applied to the IGF system, input of IGF-II in the IGF-I KIRA results can also be seen as an advantage and should therefore not be ignored. In this way no biological information is lost about the regulation of IGF system biology when studied in relation to health and disease.

In conclusion, it is thus recognized by the author that the term IGF-I bioactivity is very well defendable (and from an historical point of view respected in this thesis), but when looked at in more detail it does not completely cover all of what is actually assessed by use of this bioassay; namely bioactivity that is generated through IGF-IR activation, being independent of the ligand (IGF-I or IGF-II and to some extend insulin) that facilitates this.

1.7.5 Studies on IGF-I bioactivity

At the moment of writing this thesis several studies have been published in which IGF-I bioactivity was studied. An overview of these studies will be given in this paragraph without discussing every detail.

In 8 GH-deficient (GHD) and 8 healthy age-matched controls Kanaley et al. studied whether exercise-induced GH release and administration affected the

circulating IGF-system [271]. Blood samples were drawn at baseline, during and post-excercise (45 min of cycle ergometer exercise. IGFBP-1 rose at 75 min post-exercise in all groups, and IGFBP-2 and -3 increased significantly over time in GHD subjects, but no change in IGF-I bioactivity was found during exercise or recovery in either group although there was a trend for a slight decrease in IGF-I bioactivity post-exercise.

Gravholt et al. studied the effects on the GH/IGF-system in 23 women with Turner's syndrome (TS) before and during hormone replacement therapy (HRT) in comparison to 24 healthy age-matched women [272]. They showed that while immunoreactive IGF-I was normal, IGF-I bioactivity was decreased in TS. Although during HRT both the basal increased IGFBP-3 proteolytic activity and decreased levels of IGFBP-3 carried within ternary complex were normalized, this did not affect IGF-I bioactivity in TS patients on HRT.

In an *in vitro* setting Møller et al. investigated the effect of six different glycosaminoglycans (GAGs) on serum levels of free IGF-I and IGF-II, bioactive IGF-I, total IGF-I, and IGFBPs [273]. Second, the effect of heparin was studied in serum from 10 pregnant and 10 post partum women, 8 normal subjects and 8 patients with type 1 DM. Basal free and bioactive IGF-I where significantly higher in pregnant women, but lower in type 1 diabetics when compared to controls. In diabetics total IGF-I was also significantly decreased. All GAGs significantly increased levels of free IGF-I in a dose-dependent manner, whereas total immunoreactive IGF-I and IGFBP levels remained unchanged. Heparin strongly increased free and bioactive IGF-I in all tested sera. This was most pronounced in sera of pregnant women and less in subjects with type 1 diabetics.

In a case-control study Chen et al. studied changes in circulating total, free, and bioactive IGF-I as well as in IGFBP-1 and -2, and IGFBP-1-bound IGF-I (binary complex) during an oral glucose tolerance test (OGTT) in 7 patients with liver cirrhosis and 7 matched healthy controls for age and BMI [274]. Blood samples were drawn at 0, 30, 60, 90, 120, 150, and 180 min. Baseline levels of total (-47%), free (-36%), and bioactive IGF-I (-51%) were significantly lower, while IGFBP-1 (+268%) was higher in liver cirrhotic patients. Despite unchanged concentrations of total and free IGF-I, bioactive IGF-I declined significantly, together with levels of IGFBP-1, IGF-I:IGFBP-1 binary complex and IGFBP-1 saturation index, after OGTT in patients with liver cirrhosis and the same tendency was observed in healthy subjects. The authors hypothesized this may be caused by the observed higher levels of unsaturated IGFBP-1, as well as the faster disappearance of 'readily dissociable IGF-I' present in IGF-I:IGFBP-1 binary complexes.

Laursen et al. established a model to analyze the regulatory network including the IGF-IR, IGF-I and -II, IGFBP-4 and -5 and their specific proteolytic enzyme pregnancy-associated plasma protein-A (PAPP-A) [275]. They showed that in cells treated with the respective IGFBPs in excess of IGF-I, a substantially higher level of IGF-IR activation was observed with IGFBP-4 compared with IGFBP-5, whereas when using equimolar amounts of these IGFBPs and IGF there was almost no difference. However, the progress of proteolysis was by far more pronounced in the second experiment. Binding analyses showed tighter binding of IGFs to intact IGFBP-5 than IGFBP-4. To test the hypothesis that proteolysis of IGFBP-4 occurred only after cleavage of IGFBP-5, IGF-IR activity was analyzed in relation to cleavage of wild-type IGFBP-4 or -5 and variants resistant to proteolytic activity. Indeed, proteinase-resistant IGFBP-5 completely blocked the release of bioactive IGFs from wild-type IGFBP-4.

Støving et al. published a longitudinal case-control study that focused on the relation between circulating IGF-I bioactivity and IGF-binding capacity (IGFBP-1/IGF-I binary complex formation) in patients with anorexia nervosa (AN) at different stages and in different subtypes of the disease [276]. The study was performed in 45 women with AN and 24 age-matched healthy controls. Despite increased IGFBP-1 concentrations, levels of IGFBP-1/IGF-I were normal in AN. Circulating IGF-I bioactivity was significantly decreased in patients with restricting and binge-purging subtypes of AN compared to controls. This was also the case for total and free IGF-I. Furthermore all three IGF-I parameters increased significantly after weight gain during 2–6 months. Furthermore, in AN patients, the three IGF-I measurements were tightly correlated. The authors concluded that in AN circulating total and free IGF-I levels mirrored IGF-I bioactivity.

Recently, Karl et al. published a cross-sectional study in a cohort of 44 normally active healthy young women to examine associations between factors reportedly associated with free and total IGF-I levels and bioactive IGF-I [277]. Regression models predicting bioactive IGF-I demonstrated that 61% of the variation in IGF-I bioactivity could be attributed to concentrations of total and free IGF-I, and IGFBP-1, -2 and -3. Age and alcohol intake were negatively correlated to all three IGF-I parameters. Estradiol and progesterone levels were positively correlated with both free and bioactive IGF-I levels. IGF-I bioactivity solitary showed a negative relationship with body fat and a positive relationship with habitual selenium intake. Only free IGF-I was positively associated with habitual isoflavone intake. The authors concluded that alcohol intake may blunt physiologic actions of the IGF-axis, that female sex-hormones may influence the IGF-I axis and that although IGF-I bioactivity

was not associated with total energy intake, associations with selenium and isoflavone intake may reflect a positive association between protein intake and the IGF-I axis.

In another study Frystyk et al. analyzed the relationship between IGF-I bioactivity and obesity in 34 healthy women [278]. Of them 11 were lean, 12 overweight, and 11 obese. Bioactive IGF-I, IGFBP-1 and GH were measured in 6h pools of serum. Mean 24h GH, peak stimulated GH and total IGF-I levels were inversely associated with BMI, whereas IGF-I bioactivity and IGFBP-3 levels were not. Total IGF-I was significantly reduced in obese subjects compared to those having overweight. IGF-I bioactivity showed a comparable trend but it was not significant. Percent bioactive IGF-I [(bioactive/total IGF-I) x 100] was significantly higher in obese than both lean and overweight subjects. Based on results not fitting their hypothesis the authors concluded that in obesity normal IGF-I activity is maintained and could be mediated by enhanced sensitivity to GH at the level of the liver.

AIM OF THE THESIS

1.8 Aim and Outline of the Thesis

The general aim of the research presented in this thesis is to evaluate whether circulating IGF-I bioactivity measured by the IGF-I-KIRA has the potential to produce new insights in the functional properties of the circulating IGF-I system in relation to human aging, health and disease.

Chapter 2: In order to determine the relation between circulating IGF-I bioactivity and circulating total IGF-I levels we measured IGF-I bioactivity in serum of a healthy study population of blood donors in which total IGF-I levels had been determined by use of five different IGF-I immunoassays. Dependency of this relation on the type of methodology used to measure circulating total IGF levels was evaluated. In addition, normal values of circulating IGF-I bioactivity were determined in the healthy population.

Chapter 3: The IGF system has been linked to organism survival. Therefore we studied the relationship between circulating total, free and bioactive IGF-I and human survival in an prospective study of elderly men. The main question was whether IGF-I bioactivity, being based on another methodological and theoretical principle than measurements of circulating immunoreactive IGF-I levels could provide new information in this field of research.

Chapter 4: The IGF system is greatly affected in individuals with chronic renal failure (CRF), especially in those that are in need of dialysis. Total IGF-I

has been suggested as a suitable marker for nutritional status in CRF patients, which are in general characterized by undernutrition. In this study we analyzed whether measures of total and bioactive IGF-I were affected by nutrition enriched dialysates in CRF patients on chronic abdominal peritoneal dialysis (CAPD).

Chapter 5: As IGF-I immunoassays are dependent on the interaction between IGF-I and highly specific antibodies in theory these measurements can be negatively or positively disturbed by the presence of serum heterophilic antibodies that interact with either the capture or the detection antibody or with both. Although this type of assay disturbance has been recognized for many immunoassays this has never been described for IGF-I immunoassays. In *chapter 5* we describe to our best knowledge the first case in the literature of heterophilic antibody interference of an IGF-I immunoassays. We also studied whether the IGF-I KIRA was sensitive for heterophilic antibody interference.

Chapter 6: Insulin and IGF activity are thought to influence each other's actions. In this respect IGF-I and or IGFBP levels have been studied in relation to glucose homeostasis, insulin resistance, diabetes mellitus and the metabolic syndrome (MS). In a cross-sectional study we analyzed whether and (if so) how circulating IGF-I bioactivity was related to glucose homeostasis, insulin resistance and to the MS in a group of individuals randomly selected from a population study.

Chapter 7: Several studies have found associations between IGF-I gene polymorphisms and circulating levels of total IGF-I. In a subcohort of the Rotterdam study whether single nucleotide polymorphisms (SNPs) and haplotypes of the IGF-I gene were related to levels of circulating total and bioactive IGF-I. Furthermore we also studied whether these polymorphisms/haplotypes were related to the development of MI and/or AP in the study population.

Chapter 8 covers the general discussion and the future perspectives.

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Chapter 2

Normal Values of Circulating IGF-I Bioactivity in the Healthy Population: Comparison with Five widely used IGF-I Immunoassays

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Based on: J Clin Endocrinol Metab. 2008 Jul;93(7):2539-45

ABSTRACT

Background: IGF-I immunoassays are primarily used to estimate IGF-I bioactivity. Recently, an IGF-I specific Kinase Receptor Activation Assay (KIRA) has been developed as an alternative method. However, no normative values have been established for the IGF-I KIRA.
Objective: To establish normative values for the IGF-I KIRA in healthy adults.
Design: Cross-sectional study in healthy non-fasting blood donors.
Study participants: 426 healthy individuals (310 M, 116 F; age range: 18 – 79 yrs)
Main outcome Measures: IGF-I bioactivity determined by the KIRA. Results were compared with total IGF-I, measured by five different IGF-I immunoassays.
Results: Mean (± SD) IGF-I bioactivity was 423 (± 131) pmol/L and decreased with age (β = -3.4 pmol/L/yr, $p < 0.001$). In subjects younger than 55 yrs mean IGF-I bioactivity was significantly higher in women than in men. Above this age this relationship was inverse, suggesting a drop in IGF-I bioactivity after menopause. This drop was not reflected in total IGF-I levels. IGF-I bioactivity was significantly related to total IGF-I (r_s varied between 0.46 – 0.52; P-values < 0.001).
Conclusions: We established age-specific normative values for the IGF-I KIRA. We observed a significant drop in IGF-I bioactivity in women between 50 and 60 years, which was not perceived by IGF-I immunoassays. The IGF-I KIRA, when compared to IGF-I immunoassays, theoretically has the advantage that it measures net effects of IGF-binding proteins on IGF-I receptor activation. However, it has to be proven whether information obtained by the IGF-I KIRA is clinically more relevant than measurements obtained by IGF-I immunoassays.

INTRODUCTION

Fifty years ago Salmon and Daughaday discovered a factor in serum which stimulated sulphate incorporation by cartilage *in vitro* [1]. This unknown factor, then called sulphation factor or somatomedin-C, was later renamed insulin-like growth factor-I (IGF-I) [2,3]. After generation of highly specific antibodies for IGF-I, it became possible to develop immunoassays for the assessment of circulating IGF-I levels [4-6]. Today, these IGF-I immunoassays are clinically widely used to assess IGF-I bioactivity in humans and are applicable to measure large numbers of blood samples.

In the circulation about 99% of circulating IGF-I is bound to six high affinity IGF-binding proteins (IGFBPs) [7]. IGFBPs interfere with antibody binding to IGF-I and therefore, in virtually all common IGF-I immunoassays an extraction method has to be used in order to remove these IGFBPs [8,9]. Remaining IGFBPs or their fragments may interfere and produce falsely increased or decreased circulating total IGF-I levels [10]. This latter phenomenon may especially occur in pathological conditions [11].

Another problem is that large differences in absolute circulating levels of total IGF-I are observed between different commercially available IGF-I immunoassays [8,12]. Recently it was suggested that this variability in assay performance and the use of inappropriate reference ranges undermine the applicability of international consensus criteria in local practice [11]. Nevertheless, introduction of IGF-I immunoassays has been proven to be useful in the diagnosis and treatment of acromegaly [13].

The IGFBPs are considered to regulate IGF-I bioavailability [7,14]. However, the commonly used IGF-I immunoassays in fact ignore the effects of IGFBPs on the interactions between IGF-I and the IGF-I receptor [10]. Frystyk et al. recently developed a kinase receptor activation assay (KIRA) specific for IGF-I [15,16]. This IGF-I KIRA quantifies phosphorylation of tyrosine residues of the activated IGF-I receptor (IGF-IR) as a measure for IGF-I bioactivity in serum [17]. In contrast to commonly used IGF-I immunoassays, the IGF-I KIRA is sensitive for modifications of IGF-IR activation by circulating IGFBPs and IGFBP-proteases [15,18]. Therefore, the IGF-I KIRA method might be an important advancement in measuring circulating IGF-I bioactivity, which could enhance insights in the IGF-I system both in normal and pathological conditions.

The aim of the present study was to establish normative values for the IGF-I KIRA in the healthy population. Results of the IGF-I KIRA were compared

with circulating total IGF-I levels obtained by five commonly used IGF-I immunoassays [19].

RESEARCH DESIGN AND METHODS

Subjects and measurements

The study population has been described previously [19]. Briefly, morning serum samples were taken from healthy non-fasting blood donors (N = 426; females N = 116). Age ranged from 18 to 79 years (median: 44 yrs). Height and weight were measured and the body mass index (BMI) was calculated. Mean ± SD for BMI was 25.3 ± 3.9 kg/m^2 (range: 15.8 – 42.2). All participants gave informed consent. The Ethics Committee of the Charité Humboldt University (Berlin, Germany) had approved this study.

Total IGF-I Immunoassays

Five different immunoassays were used to measure total circulating IGF-I in the healthy population. Three of these assays were immunometric assays, whereas two were conventional radioimmunoassays (RIAs). The following immunoassays were used: Assay A: IGF-I RIA, an in-house assay at University Children's Hospital, Tuebingen, Germany; Assay B: IGF-I RIA-CT™, Mediagnost, Tuebingen, Germany; Assay C: Diagnostic Systems Laboratories (DSL) 2800 Active® IGF-I-IRMA, Sinsheim, Germany; Assay D: Nichols Advantage® Chemiluminiscence IGF-I Immunometric assay, Nichols Institute Diagnostics, San Juan Capistrano, CA, USA.; Assay E: IGF-I CIA, Immulite®, Diagnostic Products Corp. (DPC). In all immunoassays recombinant human IGF-I was used as standard. After acidification an excess of IGF-II was used to eliminate residual interference with IGFBPs. For four of these immunoassays (Assays 1, 2, 3 and 4), the age-related reference ranges for circulating total IGF-I in this study population have been previously published [19]. Intra- (a) and inter- (b) assay coefficients of variation varied between (a) 3.1 – 7.0% and (b) 3.8 – 8.8% respectively [19]. Total IGF-I levels are expressed as nmol/L (to convert total IGF-I levels into μg/L, values have to be divided by 0.131).

IGF-I Kinase receptor activation assay (IGF-I KIRA)

Circulating IGF-I bioactivity was measured using an in-house IGF-I kinase receptor activation assay as was previously described [15]. This assay uses human embryonic renal cells stably transfected with cDNA of the human IGF-IR gene (293 EBNA IGF-IR). Cells were a kind gift from Prof. Pierre de Meyts,

(Receptor Biology Laboratory, Hagedorn Research Institute, Novo Nordisk, Gentofte, Denmark). After 48 ours of culture, cells were stimulated at 37°C with either recombinant IGF-I standards (Austral Biologicals, San Ramon, CA) or 10-fold diluted serum samples for 15 minutes and lysed afterwards. Crude lysates were transferred to a sandwich assay. For capture a monoclonal antibody directed against the human IGF-IR (MAD1, 1μg/well, Novozymes Gropep, Adelaide, Australia) was used. As tracer an europium-labelled monoclonal anti-phosphotyrosine antibody (PY20, Perkin-Elmer Life Sciences) was used. Contents were read in a time-resolved fluorometer (Victor^2 multilabel counter, Perkin-Elmer, Groningen, The Netherlands). Assays were performed in 48 well plates. IGF-I standards, 2 control samples, and unknown serum samples were included in duplicate on every plate. Intra-assay CV was 5.6%. The inter-assay CVs were respectively 6.8% and 12.6% for the two control samples, which averaged (mean ± SD) 414 ± 28 pmol/L and 1146 ± 144 pmol/L (N = 60 plates), respectively. Circulating IGF-I bioactivity is expressed as pmol/L (to convert IGF-I bioactivity into μg/L, values have to be divided by 131). Serum samples used in the IGF-I KIRA were kept at -80°C and had been thawed ones. From previous unpublished data we know that repetitive freezing and thawing of serum samples (up to several times) does not change results of the IGF-I KIRA. IGF-I KIRA measurements were performed 5 years after initial collection of serum.

To test whether estradiol (E2) affects results of the IGF-I KIRA we performed the following experiment; Serum was collected from a healthy male individual. Influence of estradiol (E2) was tested by adding 500 or 1000 pM final concentration to recombinant IGF-I standards and/or to a 1/10 diluted serum sample. Recombinant IGF-I standards and a 1/10 dilution of the serum to which no E2 was added were used as controls.

Statistical Analysis

Data were analyzed using SPSS for Windows, release 12.0 (SPSS, Chicago, Illinois) unless otherwise reported. For IGF-I bioactivity measurements means ± SD, medians, and the 95% confidence intervals (95% CI) are presented. The Kolmogorov-Smirnov test with the Dallal-Wilkinson-Lilliefors correction (K-S test) and the D'Agustino and Pearson omnibus test (A-P test: GraphPad version 5.0, GraphPad Software, Inc., San Diego) were used to test data for normality of distribution. When no normality of distribution was found, data were log-transformed. Correlations between IGF-I bioactivity and total IGF-I are presented as Spearman correlation coefficients (r_s). Nonparametric Mann-Whitney or Wilcoxon rank sum tests were used to compare IGF-I levels

between men and women categorized by age. Linear regression was used to calculate the relationship between IGF-I bioactivity and age. The coefficient of variation (CV) was calculated by using the formula: (SD/mean) × 100%. This CV standardizes the relative spread in data between IGF-I assays, so that a sensible comparison can be made. Curve estimation and regression analysis were performed to determine whether age-related changes were best fitted by a linear, exponential or polynomial function. Where more than one function was significant, the one with the highest R^2 value was considered the best fitting model. A P value < 0.05 was considered statistically significant.

RESULTS

Circulating IGF-I bioactivity was almost normally distributed (untransformed data: K-S test; $p = 0.07$, A-P test; $p = 0.04$, (Figure 1A)). Log-transformation of IGF-I bioactivity levels did not improve normality of the data distribution (log-transformed data: K-S test; $p = 0.0001$, A-P test; $p < 0.0001$). In contrast, circulating total IGF-I levels showed an asymmetric distribution in all five studied IGF-I immunoassays (untransformed data: K-S and A-P tests; $p < 0.0001$ for all immunoassays (Figure 1B – 1F)). After log-transformation of total IGF-I levels a normal distribution (K-S and A-P test: $p > 0.05$) was obtained in three out of five IGF-I immunoassays (data not shown). (Total IGF-I assays that did not show a normal distribution after log transformation were: DSL IGF-I IRMA (K-S test; $p = 0.003$, A-P test; $p = 0.005$) and Nichols IGF-I CIA (K-S test; $p = 0.03$ and A-P test; $p = 0.02$). Linear correlation and regression coefficients between the measurements of IGF-I immunoassays are shown in Table 1.

To estimate the spread in the data of IGF-I in the study population, we calculated the coefficient of variation (CV). The CV for the IGF-I KIRA was 31% and thereby lower than for the five studied IGF-I immunoassays (Mediagnost IGF-I RIA: 34%, In-house IGF-I RIA: 36%, DPC IGF-I CIA: 42%, Nichols IGF-I CIA: 45%, DSL IGF-I IRMA: 48%, respectively).

Mean (± SD) circulating IGF-I bioactivity was 423 (± 131) pmol/L and ranged from 57 to 875 pmol/L. In Table 2 mean values of circulating IGF-I bioactivity are presented after stratification for age. IGF-I bioactivity decreased significantly with age, which was best fitted by a linear model (slope $\beta = -3.4$ pmol/L/yr, (95% CI: -4.5 – -2.5); $p < 0.001$). There was no significant difference in β ($p = 0.16$) between men ($\beta = -2.9$ pmol/L/yr (95% CI: -3.8 – -1.9); $p < 0.0001$) and women ($\beta = -4.6$ pmol/L/yr (95% CI: -6.0 – -3.3); $p < 0.001$) (Figure 2)). With one exception (Nichols), the age-related decreases in total

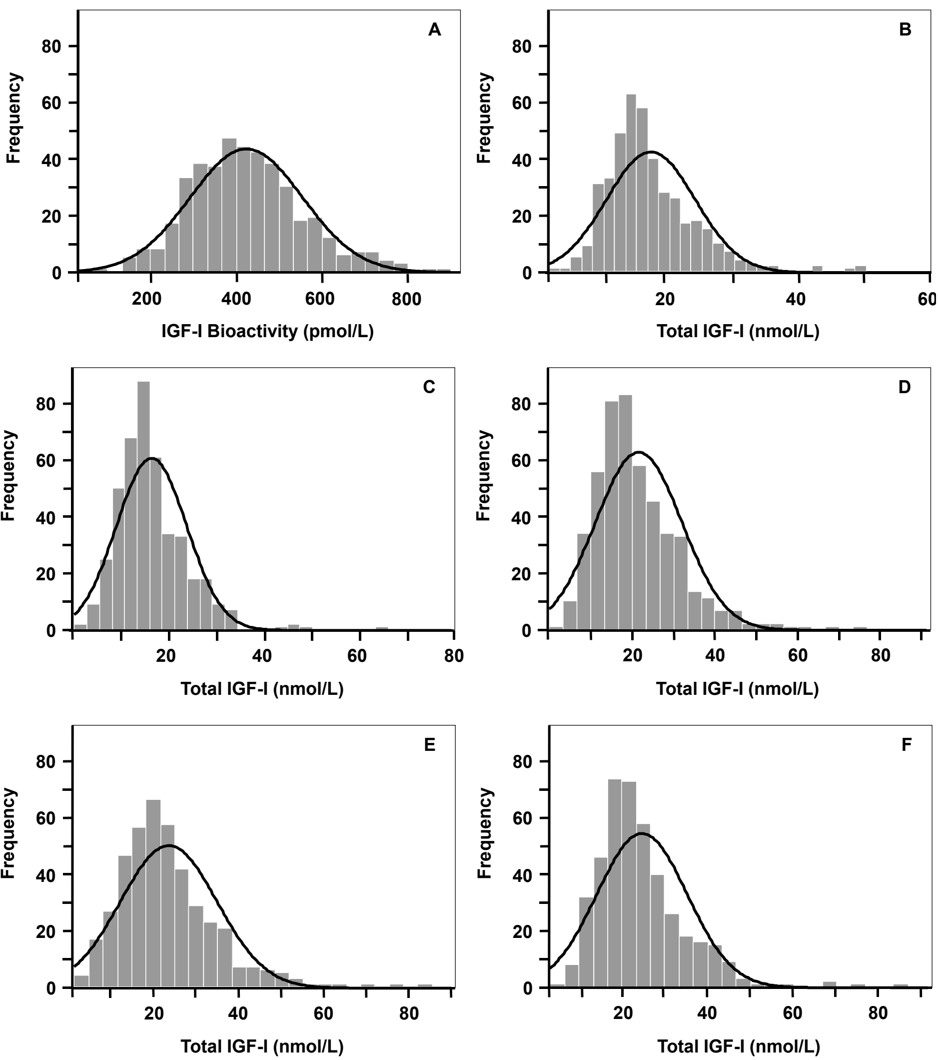

Figure 1. Distribution of IGF-I measurements in the study population: (**A**) IGF-I KIRA; (**B**) IGF-I RIA, an in-house assay at University Children's Hospital, Tuebingen, Germany; (**C**) IGF-I RIA-CTtm, Mediagnost; (**D**) IGF-I-IRMA 2800 Active®, DSL; (**E**) IGF-I CIA, Nichols Advantage®; (**F**) IGF-I CIA, Immulite® DPC. Data obtained by the IGF-I KIRA showed an almost normal distribution, in contrast to IGF-I immunoassays for which data were skewed leftwards. An ideal bell-shaped normal distribution curve is shown in each plot.

IGF-I were best fitted by polynomial (quadratic) functions (data not shown). The decrease of total IGF-I with age measured by the total IGF-I CIA (Nichols) was best fitted by a linear model (β = -0.48 nmol/L/yr, (95% CI: -0.54 – -0.42); $p < 0.001$).

Table 1: Linear regression equations: comparison of the relation between five IGF-I immunoassays

Dependent variable(Y)	Independent variable (X) A	B	C	D
A	***			
B	R^2 = 0.85 Y = 2.20x + 0.97	***		
C	R^2 = 0.85 Y = 1.43x – 5.38	R^2 = 0.80 Y = 1.32x – 5.58	***	
D	R^2 = 0.82 Y = 1.52x – 3.20	R^2 = 0.75 Y = 1.37x – 2.96	R^2 = 0.87 Y = 1.01x + 3.54	***
E	R^2 = 0.87 Y = 1.45x – 2.60	R^2 = 0.91 Y = 1.41x – 4.19	R^2 = 0.86 Y = 0.93x + 4.57	R^2 = 0.81 Y = 0.84 + 3.36

Regression models are based on total IGF-I levels expressed as nmol/L. In all models correlation was significant (P < 0.001 for all). IGF-I immunoassays: (**A**) IGF-I RIA, an in-house assay at University Children's Hospital, Tuebingen, Germany; (**B**) IGF-I RIA-CTTM, Mediagnost; (**C**) IGF-I-IRMA 2800 Active®, DSL; (**D**) IGF-I CIA, Nichols Advantage®; (**E**) IGF-I CIA, Immulite® DPC.

Table 2: Age-related reference ranges for IGF-I bioactivity in healthy adults (N = 426)

Age (years)	Number (N)	Mean (pmol/L)	SD	Median (pmol/L)	5th percentile	95th percentile
< 24	38	527	139	528	287	747
25 - 34	66	476	140	461	254	763
35 - 44	100	432	132	428	212	666
45 - 54	79	411	122	402	253	643
55 - 64	103	385	108	383	191	566
> 65	40	344	081	343	173	468

Overall, there were no gender specific differences in mean IGF-I bioactivity (men: mean = 420 pmol/L, (95% CI: 405 – 435) *vs.* women: mean = 433 (95% CI: 409 – 458); p = 0.36 (adjusted for age and BMI, Table 3)).

In subjects younger than 55 yrs, mean IGF-I bioactivity was significantly higher in women than in men (men (N = 207) mean = 436 pmol/L, (95% CI: 418 – 454) *vs.* (women (N = 76): mean = 484 (95% CI: 455 – 513); p = 0.007, (Figure 3)). Above the age of 55 years this relationship was opposite and mean IGF-I bioactivity in women was significantly lower than in men (men (N = 103) mean = 387 pmol/L, (95% CI: 366 – 408) *vs.* women (N = 40): mean 337 (95% CI: 313 – 361); p = 0.008). Table 3 shows means for IGF-I bioactivity in men and women stratified for age. In all five IGF-I immunoassays there were no gender-specific differences in mean circulating total IGF-I levels before age 55 yrs. Above age 55 years mean circulating total IGF-I levels were significantly lower in women than in men in all IGF-I immunoassays (data not

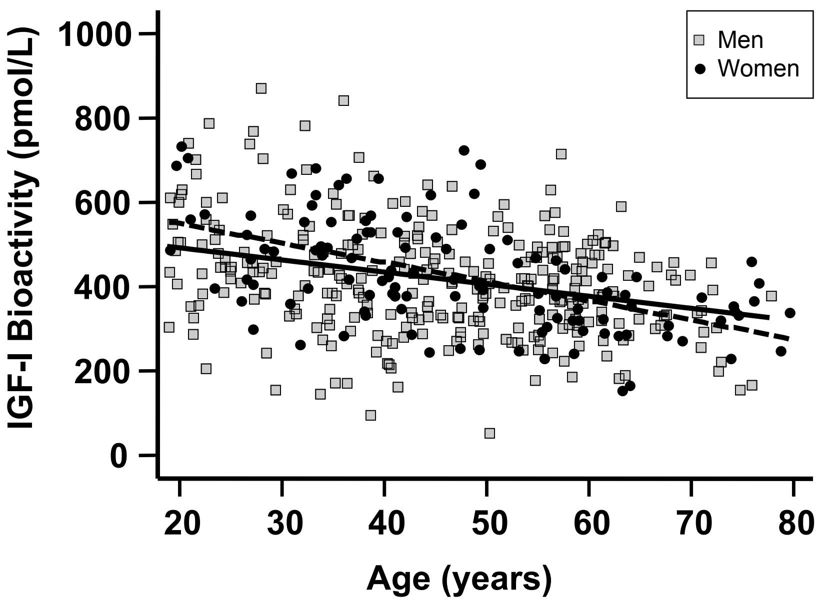

Figure 2. IGF-I bioactivity levels according to age. IGF-I bioactivity decreased significantly with age in both men (□) and women (●). Linear regression lines for men (——) and women (- - -) are shown.

shown). The statistically significant drop in IGF-I bioactivity in women around age 55 years was not observed for all five IGF-I immunoassays.

Circulating IGF-I bioactivity was significantly related to circulating total IGF-I levels (r_s varied between 0.46 – 0.52, $p < 0.001$ for all five IGF-I immunoassays, Figure 4A – 4E). Mean circulating IGF-I bioactivity calculated as percentage of total IGF-I averaged 1.8 to 2.4% (in-house RIA: 2.4% (0.42 – 5.82) (median

Table 3: Age-related reference ranges for IGF-I bioactivity in healthy individuals: men vs. women

Age (years)	Men			Women			P-value
	Number (N)	Mean (pmol/L)	SD	Number (N)	Mean (pmol/L)	SD	
18 - 79	310	420	131	117	433	131	0.36*
< 24	31	510	138	7	602	126	0.13**
25 - 34	46	470	150	20	489	119	0.47**
35 - 44	70	417	140	30	467	106	0.04**
45 - 54	60	396	106	19	461	150	0.008**
55 - 64	79	400	110	24	332	083	0.009**
> 65	24	344	093	16	344	063	0.50**

*Adjusted for age and BMI. **Adjusted for BMI.

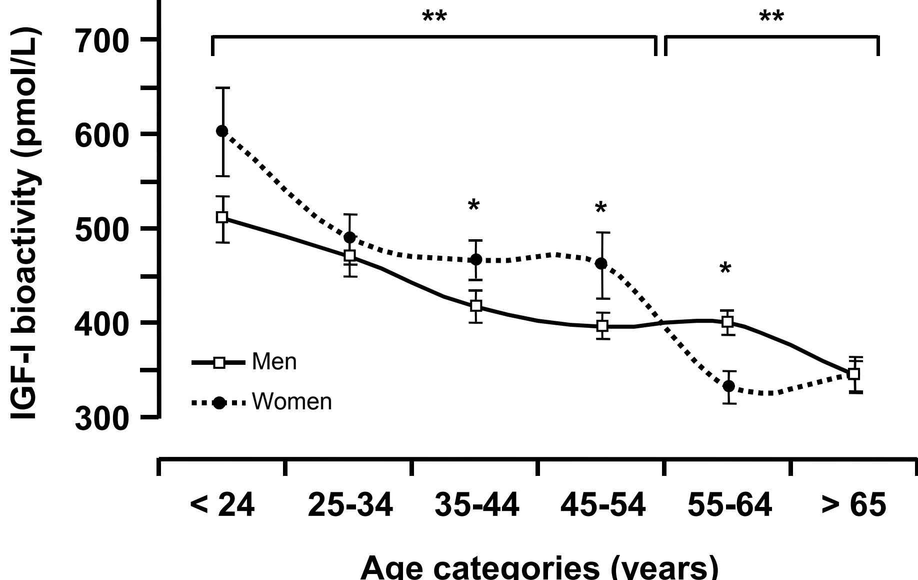

Figure 3. Mean circulating IGF-I bioactivity according to age categories and sex. Mean circulating IGF-I bioactivity levels in women (- - -) differed significantly from men (——), being higher in age groups 35-44 years (p = 0.04), and 45-54 years (p = 0.008) and lower in age group 55-64 years (p = 0.009). Between 50 and 60 years of age there was a drop in mean circulating IGF-I bioactivity in women. Overall, before 55 years of age circulating IGF-I bioactivity in women was significantly higher, and after this age significantly lower when compared to men. Data are presented as mean ± SEM. *Significant difference between men and women within an age category. **Significant difference between men and women before or after age 55 years.

(range)), Mediagnost: 2.2% (0.34 – 5.2), DSL: 2.3% (0.33 – 9.1), Nichols: 1.8% (0.32 – 5.88) and DPC: 1.8% (0.29 – 5.38). This calculated bioactive IGF-I fraction increased significantly with age for all five IGF-I immunoassays (β varied between 0.010% – 0.033% per year; $p < 0.001$ for all). Figure 5A-E shows the calculated bioactive fraction for all five IGF-I immunoassays per age category in men and women. IGF-I bioactivity was not related to height, weight or BMI, whereas total IGF-I levels were positively related to height (r varied between 0.17 – 0.23, $p < 0.003$ for all), and negatively to BMI (r_s varied between - 0.24 and -0.20, $p < 0.001$ for all).

No direct effects of two different concentrations of estradiol (500 and 1000 pmol/L) could be detected when added to the IGF-I reference dilutions or spiked to serum (Table 4).

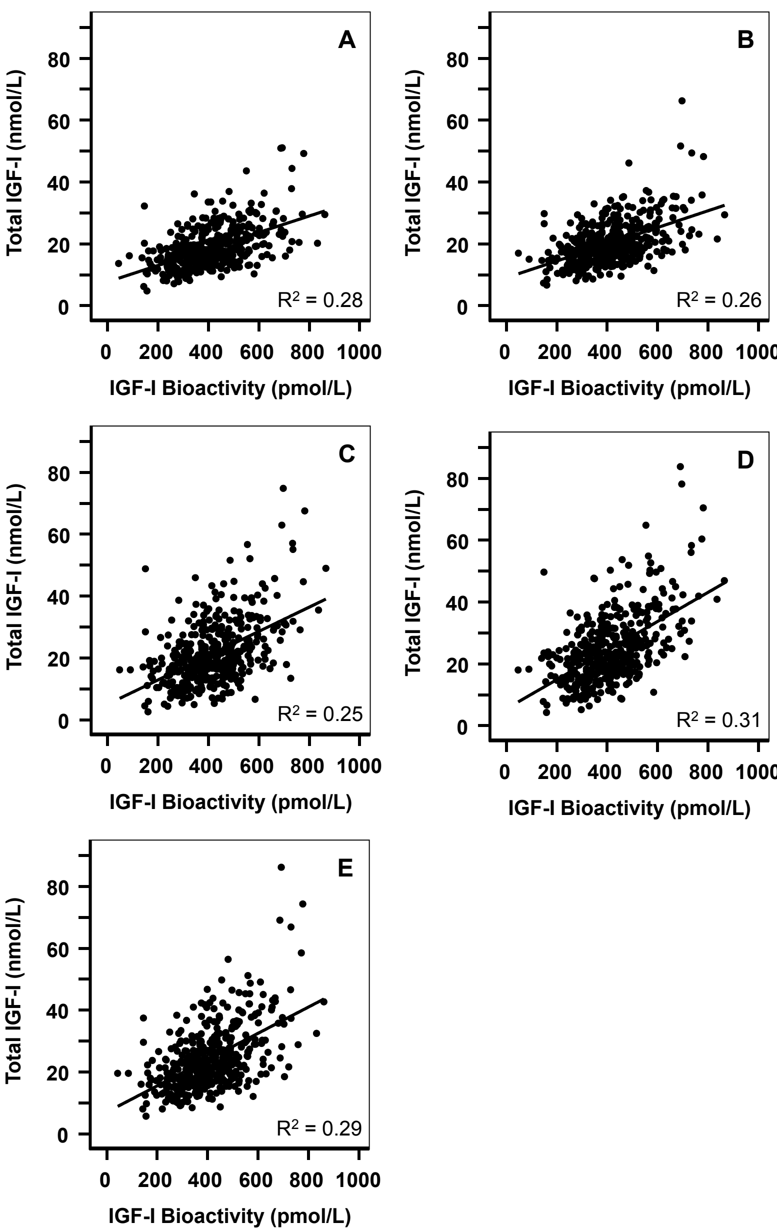

Figure 4. Relations between circulating IGF-I bioactivity measured by the IGF-I KIRA *vs.* circulating total IGF-I measured by five different IGF-I immunoassays to measure total IGF-I: (**A**) IGF-I RIA, an in-house assay at University Children's Hospital, Tuebingen, Germany; (**B**) IGF-I RIA-CT™, Mediagnost; (**C**) IGF-I-IRMA 2800 Active®, DSL; (**D**) IGF-I CIA, Nichols Advantage®; (**E**) IGF-I CIA, Immulite® DPC.

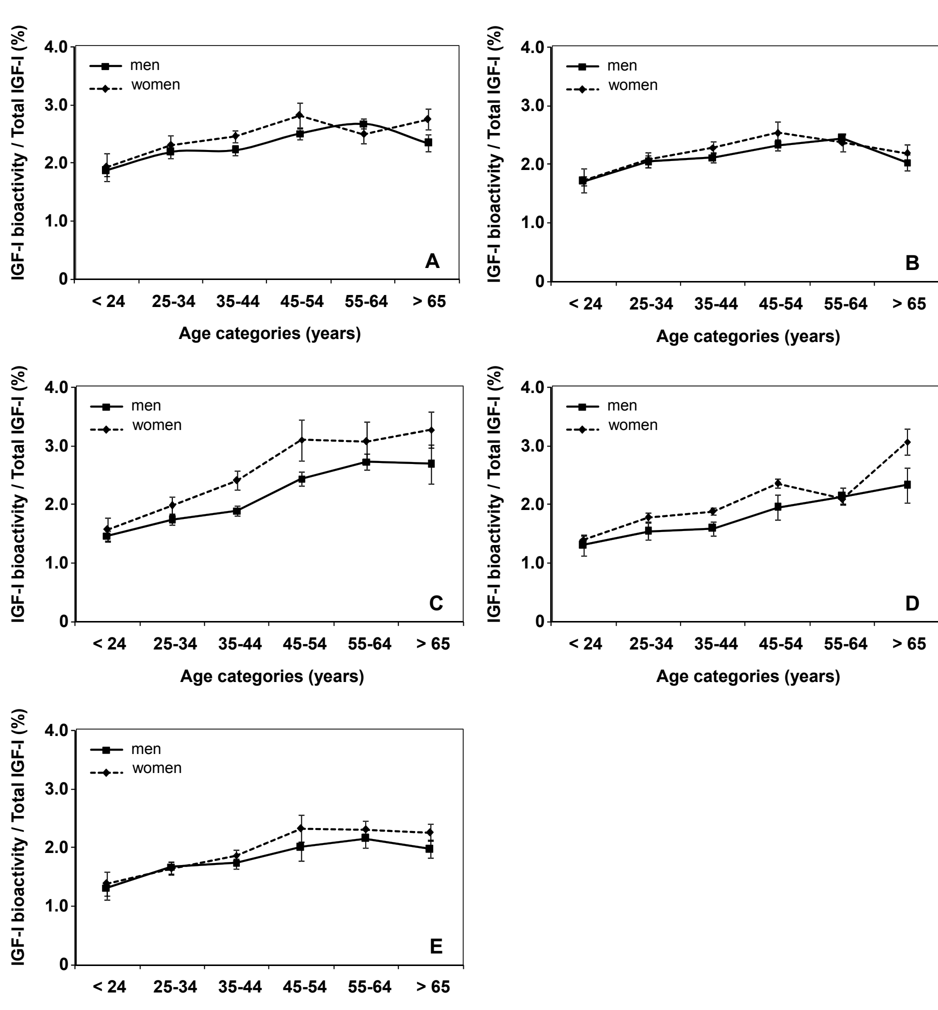

Figure 5. Mean bioactive fraction per age category (**A**) IGF-I RIA, an in-house assay at University Children's Hospital, Tuebingen, Germany; (**B**) IGF-I RIA-CT^{TM}, Mediagnost; **C**) IGF-I-IRMA 2800 Active®, DSL; **D**) IGF-I CIA, Nichols Advantage®; **E**) IGF-I CIA, Immulite® DPC. In all assays the bioactive fraction increased significantly with age in both men and women ($P < 0.001$ for all).

Table 4: IGF-I bioactivity in male serum spiked with different concentrations of estradiol

Serum sample	IGF-I Bioactivity (pmol/L) (based on control reference)	IGF-I Bioactivity (pmol/L) (based on reference with E2)
1000 pM E2	137	130
500 pM E2	138	131
No E2 added	148	140

E2 = estradiol, pM = picomolar. Next to the serum, also one series of IGF-I reference was spiked with equimolar concentrations of E2.

DISCUSSION

To our knowledge this is the first study that reports age-specific normative ranges of circulating IGF-I bioactivity in the healthy population obtained by the IGF-I KIRA. These normative values are based on measurements in more than 400 normal subjects with age ranging from 18 – 79 yrs. These age-specific normative ranges will be helpful to interpret whether IGF-I bioactivity is normal, increased and/or decreased in subjects with pathological conditions like acromegaly and GH deficiency.

Currently used IGF-I immunoassays have indeed yielded important and biologically meaningful information about the IGF-I system [20]. However, many problems have been reported when IGF-I immunoassays were used in clinical practice [13]. Attempts to resolve these problems have focused on methods of separating IGF-I from its binding proteins (IGFBPs) prior to IGF-I measurements. Although there have been many technologies developed to eliminate interference of IGFBPs, in many IGF-I immunoassays, remaining IGFBPs or BP fragments may still interfere and produce falsely increased or decreased circulating total IGF-I levels [21]. This latter effect may especially be of importance in pathophysiologic states accompanied by significant changes in IGFBP levels, such as diabetes mellitus and renal failure [21]. For example, Chestnut and Quarmby showed that while the correlation between IGF-I immunoassays was high in sera of healthy individuals, there was a lack of correlation between immunoassays when sera from individuals with diabetes were analysed [8].

IGF-I immunoassays only determine the immunoreactive properties of the IGF-I molecule, rather than its actual biological effect [22]. The separation of IGF-I from the IGFBPs prior to the IGF-I measurements ignores in fact the important modulating effects of IGFBPs on IGF-I bioavailability. However, clinicians are generally interested in the biological effects of IGF-I [22].

An important reason for using IGF-I immunoassays has been the lack of reliable IGF-I bioassays [10,16]. Previous IGF-I bioassays were based on downstream signalling events (e.g. sulphate incorporation by cartilage, cell proliferation and survival), but they often suffered from high variability and long assay duration [16,23,24]. Moreover, these IGF-I bioassays often had a lack of specificity and were labour-intensive. An ideal assay for assessing IGF-I bioactivity should be easily quantifiable, highly sensitive and based on a signal specifically transmitted by the IGF-IR [25]. In this respect, the IGF-I KIRA comes close to an ideal IGF-I bioassay as it 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 fully comparable to the traditional IGF-I immunoassays [15,16,18]. However, the KIRA is still more labour-intensive than immunoassays. In addition, we realize that IGF-I KIRA measurements were performed with serum and therefore obtained results do not necessarily reflect net IGF-I bioactivity present in the extra-vascular tissues.

Interestingly, IGF-I immunoassays that did not utilize removal of IGFBPs have been described previously in literature (5). These older assays for IGF-I were not considered useful for clinical practice, as it was assumed that IGFBP interference was a priori bad for determination of IGF-I (9). However, this opens the possibility that the results of these older IGF-I assays might correlate better with IGF-I KIRA results than do modern IGF-I immunoassays, which prior to its measurement extract IGF-I from IGFBPs.

Circulating IGF-I bioactivity showed a wide inter-individual variability among subjects in every age group. The CV of the IGF-I KIRA within the study population (a standardized measure of relative spread in data) was lower than that for total IGF-I. Since a lower magnitude of CV is considered to reflect a better reliability (precision) of measurements, this suggests that the IGF-I KIRA in this respect performs at least equal to IGF-I immunoassays.

IGF-I bioactivity was positively related to total IGF-I in all studied IGF-I immunoassays. Interestingly, for all IGF-I immunoassays the observed correlation coefficients were relatively low and comparable. Our results show that the observed relation between total IGF-I and IGF-I bioactivity is independent of the type of immunoassay that is used to determine circulating total IGF-I levels. In addition, these results suggest that the IGF-I KIRA produces new information about the IGF-I system, which differs from that obtained by IGF-I immunoassays. However, the physiological importance of this difference remains to be clarified.

Circulating IGF-I bioactivity decreased significantly with age. The decline of IGF-I bioactivity with age was less steep than that observed for circulating total IGF-I levels. As a consequence the mean calculated fraction of IGF-I bioactivity over total IGF-I increased slightly but significantly with age. Although the cross-sectional study design does not reflect the intra-individual rate of change of IGF-I bioactivity, a possible explanation for this latter observation is that IGF-I bioactivity becomes less-GH dependent with aging than total IGF-I levels. Another explanation could be that the relative increase in IGF-I bioactivity with age reflects a compensatory mechanism to overcome an age-dependent relative IGF-IR resistance. Third, also circulating IGF-II levels could

be involved. In contrast to IGF-I, circulating levels of IGF-II do not decrease after puberty but remain stable throughout life [26]. Chen et al. showed that the 293 EBNA IGF-IR cells respond not only to IGF-I but also to IGF-II, which has a cross-reactivity of 12%, to the IGF-I KIRA compared to IGF-I [15]. This opens the possibility that the relative contribution of IGF-II to the IGF-I KIRA signal increases with aging.

Remarkably, before age 55 years of age, the mean IGF-I bioactivity was significantly higher in women than in men, while after this age an inverse relationship was observed. This significant drop in IGF-I bioactivity in women between the age of 50 and 60 years was not reflected in total IGF-I levels. Although we do not have information about age of menopause in the studied women, we speculate that the observed decrease in IGF-I bioactivity after age 55 years of age is related to changes in estrogens levels [27-29]. This could explain why women had a relatively higher mean IGF-I bioactivity than men before 55 years, but also why mean IGF-I bioactivity was lower in women than in men after this age.

In conclusion, in the present study we established age-specific normative values for circulating IGF-I bioactivity levels in the healthy adult population. The IGF-I KIRA produces new information about the circulating IGF-I system that differs from that obtained by IGF-I immunoassays. Whether this information is clinically more relevant than measurement of circulating total IGF-I levels in the diagnosis and/or treatment of GH disorders is at present unclear. However, the establishment of these normative reference values for IGF-I bioactivity is the first step to answer these questions in the near future.

ACKNOWLEDGMENTS

The authors thank Professor Pierre de Meyts (Receptor Biology Laboratory, Hagedorn Research Institute, Novo Nordisk, Gentofte, Denmark) for his help to establish the IGF-I KIRA in our laboratory.

This study was in part supported by Zon-MW, Organization for Health Research and Development the Netherlands, (948-00-003). Jan Frystyk was supported by a grant from the Danish Research Council for Health and Disease.

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Chapter 3

Low Circulating IGF-I Bioactivity in Elderly Men is associated with Increased Mortality

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J Clin Endocrinol Metab. 2008 Jul;93(7): 2515-22

ABSTRACT

Background: Low IGF-I signaling activity prolongs lifespan in certain animal models, but the precise role of IGF-I in human survival remains controversial. The IGF-I kinase receptor activation assay (IGF-I KIRA) is a novel method for measuring IGF-I bioactivity in human serum. We speculated that determination of circulating IGF-I bioactivity is more informative than levels of immunoreactive IGF-I.

Objective: To study IGF-I bioactivity in relation to human survival.

Design: Prospective observational study.

Setting: A clinical research center at a university hospital.

Study participants: 376 healthy elderly men (aged 73 to 94 years).

Main outcome Measures: IGF-I bioactivity was determined by the IGF-I KIRA. Total and free IGF-I were determined by IGF-I immunoassays. Mortality was registered during follow-up (mean 82 months).

Results: During the follow-up period of 8.6 years 170 men (45%) died. Survival of subjects in the highest quartile of IGF-I bioactivity was significantly better than in the lowest quartile, both in the total study group (HR = 1.8, (95% CI: 1.2 – 2.8, p = 0.01) as well as in subgroups having a medical history of cardiovascular disease (HR = 2.4 (95% CI: 1.3 – 4.3, p = 0.003) or a high inflammatory risk profile (HR = 2.3 (95% CI: 1.2 – 4.5, p = 0.01). Significant relationships were not observed for total or free IGF-I.

Conclusion: Our study suggests that a relatively high circulating IGF-I bioactivity in elderly men is associated with extended survival and with reduced cardiovascular risk.

INTRODUCTION

Insulin-like growth factor I (IGF-I) has important anabolic and mitogenic effects, and is involved in mechanisms of function, maintenance, and repair of many tissues [1]. Involvement of IGF-I in the process of aging has been studied extensively in the last two decades. Studies in invertebrates have suggested that disruption of signalling pathways similar to the IGF-I pathway extends lifespan [2-5]. Growth hormone/IGF-I deficient mice and rats have increased longevity compared to controls [3,6,7]. Also, decreased IGF-I receptor (IGF-IR) signalling activity in female heterozygous knockout mice was shown to retard the process of aging [8]. However, the precise role played by IGF-I in human survival remains controversial [9].

IGF-I is part of a complex system consisting of two growth factors (IGF-I and IGF-II), at least six high-affinity IGF binding proteins (IGFBP-1 to -6) and several IGFBP proteases [1,10]. It is believed that only 0.5-1% of total IGF-I levels circulates in a free form, whereas about 99% of circulating IGF-I is bound by IGFBPs. Only free IGF-I is considered to interact with the IGF-IR [11].

Most of what is known about regulation of IGF-I in the circulation is based on measurements using specific IGF-I immunoassays. Since IGFBPs interfere with the accurate determination of IGF-I by immunoassay, various techniques have been developed to remove IGFBPs from samples or neutralize their influence on IGF-I immunoreactivity [11,12]. However, it is important to note that IGFBPs are important modulators of IGF-I bioactivity [1]. IGFBPs are able to alter IGF-I bioactivity without changing the extractable concentrations of total IGF-I [11].

Recently, a highly sensitive and IGF-I specific kinase receptor activation assay (IGF-I KIRA) was developed to determine IGF bioactivity in human serum [13,14]. This bioassay determines the ability of circulating IGF-I to activate the IGF-IR by quantification of intracellular receptor auto-phosphorylation upon IGF-I binding. Unlike an immunoassay, the IGF-I KIRA does not disregard modifying effects of IGFBPs and IGFBP proteases on the interaction between IGF-I and the IGF-IR (15, 16). Therefore, the IGF-I KIRA method is a new tool that could help broaden our understanding of the IGF-I system in humans, in both normal and pathological conditions.

The objective of the present study was to investigate whether IGF-I bioactivity was related to survival in a cohort of healthy elderly men. In addition, we studied the relationship between IGF-I bioactivity and cardiovascular disease (CVD) in this cohort.

RESEARCH DESIGN AND METHODS

Study Population

The design of the Zoetermeer study, a prospective cohort study conducted in clinically healthy independently living Caucasian elderly men, has been reported previously [15]. Individuals were drawn from the municipal register of Zoetermeer, the Netherlands. Inclusion criteria were male sex, age ≥ 70 years and a sufficient physical and mental status to visit the study center independently. The Medical Ethics Committee of the Erasmus MC approved the study. Out of 1567 invited men, 403 participated and gave written informed consent. At baseline, medical histories were obtained from all participants, and serum samples were collected from 376 individuals. General practitioners were contacted about the status of participants in subsequent years. Cause of death was derived from death certificates and could only be verified in a limited number of subjects. The maximum follow-up time was 8.6 years. Information on nonfatal events was not registered.

Anthropometric measurements

Height and weight were measured. Systolic (SBP) and diastolic (DBP) blood pressures were measured in duplicate. Hypertension was defined as SBP > 160 mmHg and/or DBP > 90 mmHg or antihypertensive treatment. Lean body mass and fat mass were measured using dual-energy X-ray absorptiometry (Lunar Corp., Madison, Wisconsin) [16].

Assays

At baseline, a venous blood sample was collected after an overnight fast. Serum and plasma aliquots were stored immediately after processing at -40°C. Total IGF-I levels were measured by radioimmunoassay (RIA) (Mediagnost GmbH, Tübingen, Germany) [17]. Intra-assay and inter-assay coefficients of variation (CVs) were 1.6% and 6.4%, respectively. Free IGF-I levels were measured by a non-competitive, two-site immunoradiometric assay (Beckman Coulter, Inc., Webster, Texas). Intra-assay and inter-assay CVs were 10.3% and 10.7% respectively. IGFBP-1 and IGFBP-3 levels were determined by specific RIA as previously described [17]. For IGFBP-1 and IGFBP-3, intra-assay CVs were 3.4% and 1.9%, respectively, and inter-assay CVs were 8.1% and 9.2%, respectively. Insulin was measured by a commercially available RIA (Medgenix Diagnostics, Brussels, Belgium). Intra-assay and inter-assay CVs were 8.0% and 13.7%, respectively. Immunoassays were performed soon after sample collection (in 1998) to reduce the possibility of analyte degradation.

Insulin sensitivity was calculated according to the Homeostasis Model Assessment (HOMA) model 2 (HOMA Calculator v2.2, Oxford Centre for Diabetes, Endocrinology and Metabolism). Total and high-density lipoprotein (HDL) cholesterol and triglycerides were measured using a commercially available kit [15]. Low-density lipoprotein (LDL) cholesterol levels were calculated. C-reactive protein levels (CRP) were determined with a highly sensitive method (hs-CRP) using a latex-enhanced immunoephelometric assay on a BN II analyzer (Dade Behring, Liederbach, Germany).

IGF-I Kinase receptor activation assay (IGF-I KIRA)

IGF-I bioactivity was measured using an in-house IGF-I KIRA as previously described [13]. Human embryonic renal cells (293 EBNA, Invitrogen, Germany) stably transfected with copy DNA of the human IGF-IR gene were used as read-out after stimulation with either recombinant IGF-I standards (Austral Biologicals, San Ramon, CA) or 1/10 diluted serum samples. IGF-I standards, two control samples, and serum samples from study participants were measured in duplicate on each culture plate. Intra-assay and inter-assay CVs were 6.0% and 10.9%, respectively. IGF-I KIRA measurements were performed in 2005. There are no data on the effects of long-term storage on sample IGF-I activity in the KIRA assay. However, since all samples were analyzed following an equal period of storage, any variable effect of storage time is likely removed.

Statistical Analysis

Data were analyzed using SPSS for Windows, release 12.0 (Chicago, Illinois). Only data from the 376 participants for whom serum samples were available were included in the analyses. The Shapiro-Wilk 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. Baseline characteristics are presented as means ± SD, medians with 25th and 75th percentiles ($P_{25} - P_{75}$), or numbers. Correlation coefficients (r_s) between IGF-I parameters were calculated using non-parametric tests (Spearman's correlation coefficient). Univariate general linear models were used to calculate adjusted differences in means of variables between groups (F-tests were used to evaluate significance). Linear regression was used to calculate the relation between IGF-I bioactivity and age. A multiple linear regression model was used to test equality of slopes (IGF-I bioactivity = $B_0 + B_1 \cdot \text{age} + B_2 \cdot Z + B_3 \cdot \text{age} \cdot Z + E$), where $Z = 1$ for non-survivors; $Z = 0$ for survivors and E denoting independent, identically normally distributed error

terms. IGF-I bioactivity and free IGF-I were calculated as percentage of total IGF-I according to the formula: X (pmol/L) / total IGF-I (pmol/L) x 100% (X = IGF-I bioactivity or free IGF-I level, respectively).

Cox proportional hazard models were used to analyse survival (Wald tests were used to evaluate significance of variables in the hazard model). The time-to-event variable was specified as time from baseline examination to death denoted in months. Individuals who did not have an event during the time that the subject was part of the study (N = 206) were censored. Continuous IGF-I risk factors were grouped (group 1: ≤ 25th, group 2-3: 25th – 75th and group 4: ≥ 75th percentiles) and used as categorical covariates in survival analyses. For all IGF-I parameters risk was calculated relative to the highest quartile. Within the final model, age, BMI, smoking, SBP, diabetes, LDL and HDL were considered possible confounders and were used as baseline time-constant covariates. Both crude and adjusted hazard ratios (HR) were estimated. Assumptions of proportionality of hazards were verified by graphical inspection. To control for confounding by cardiovascular disease (CVD) or inflammatory risk status, crude data were stratified and survival analysis was repeated as described previously. To correct for CVD, crude data were stratified into two groups split by the presence (N = 133) or absence (N = 242) of prevalent CVD. Presence of CVD was defined as having a medical history of MI or cerebrovascular disease, and/or being treated or having symptoms of AP, congestive heart failure or claudicatio intermittens [15]. To correct for inflammatory risk status continuous baseline hs-CRP levels were split at 3 mg/L, resulting in subjects with a low to medium inflammatory risk profile (IRP) (hs-CRP ≤ 3 mg/L, N = 238) and subjects with a high IRP (hs-CRP > 3 mg/L, N = 138) [18,19]. Survival analysis using IGF-I risk groups as categorical covariates was performed in IRP and CVD strata as described previously.

In general: cause of death was based on death certificates that could not be verified in the majority of subjects. Therefore, cause of death was not controlled for in all Cox proportional hazard models. Trends across IGF-I risk groups were based on models with linear effect of the risk factors (Armitage trend test). Two-sided P-values are reported and P-values < 0.05 were considered statistically significant.

RESULTS

Baseline characteristics of participants, categorized by survivors (N = 206, (55%)) and non-survivors (N = 170, (45%)), are shown in Table 1. Mean age

at baseline was 77.7 ± 3.5 (mean ± SD) years (range, 73 to 94). Mean time to death was 81.9 months (range, 3 to 103).

Baseline levels of IGF system parameters are shown in Table 2. Overall, IGF-I bioactivity and free IGF-I levels accounted for 2.6% (range, 0.2–5.9) and 0.6% (range, 0.2 – 2.9) of circulating total IGF-I levels, respectively ($p < 0.001$). These fractions were not significantly correlated with each other (r_s = 0.04, p = 0.48). At baseline, only mean IGFBP-1 levels differed significantly between survivors and non-survivors (Table 2). In addition, the mean calculated bioactive IGF-I fraction was significantly higher in survivors compared to non-survivors (mean ± SEM: 2.7 ± 0.06% *vs.* 2.4 ± 0.07%, adjusted for age p = 0.04), whereas the calculated mean free IGF-I fraction did not differ between these groups (p = 0.62). Also the mean total IGF-I / IGFBP3 ratio did not differ between survivors and non-survivors (adjusted for age p = 0.21).

Table 1. Baseline characteristics of survivors and non-survivors in the study cohort (N = 376)

Variable	Survivors (N = 206)		Non-Survivors (N = 170)		P-value
	Mean (SD)				
Age (year)	77.1	(3.0)	78.5	(3.9)	< 0.001
BMI (kg/m²)	25.8	(2.9)	25.2	(3.0)	0.29
Lean mass (kg)	52.3	(5.5)	51.4	(5.4)	0.21 §
Fat mass (kg)	21.3	(5.7)	21.0	(5.7)	0.84 §
SBP (mmHg)	157	(25)	156	(24)	0.85 #
DBP (mmHg)	84	(11)	83	(11)	0.49 #
Total cholesterol (mmol/L)	5.8	(1.0)	5.7	(1.1)	0.53 #
Glucose (mmol/L) *	5.5	(1.2)	5.4	(1.0)	0.82 #
	Median ($P_{25} – P_{75}$)				
Insulin (IU/L) *	8.0	(6.0 – 10.0)	8.3	(6.2 – 10.6)	0.27 #
HOMA (%S)	96	(71 – 124)	94	(75 – 124)	0.73 #
	Number (%)				
Smoking	37	(18%)	33	(19%)	0.89
Hypertension	114	(55%)	116	(68%)	0.01 #
Myocardial Infarction	24	(12%)	39	(23%)	0.002
Malignancy	18	(9%)	14	(8%)	0.91
Diabetes mellitus	14	(6.8%)	17	(10%)	0.30

All values are unadjusted. *P* values were adjusted for age. §, P values for lean mass and fat mass were additionally adjusted for height. #, §, P values for blood pressures, lipids, glucose, insulin, HbA1c and HOMA (%S), were additionally adjusted for body mass index. *, Diabetics were excluded in the analysis of means for glucose and insulin. BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, $P_{25} – P_{75}$ = range between the 25th and 75th percentile, Insulin sensitivity in percentage (%S) was calculated according to the Homeostasis Model Assessment (HOMA) model 2 (HOMA Calculator v2.2, Oxford Centre for Diabetes, Endocrinology and Metabolism).

Table 2. Means at baseline of parameters of the IGF-I system in the total study population, in survivors and in non-survivors after follow-up

Variable	Unit	Mean or geometric mean (CI 95%)						P-value*
		All subjects (N = 376)		Survivors (N = 206)		Non-Survivors (N = 170)		
IGF-I Bioactivity	pmol/L	333	(320 – 346)	344	(328 – 361)	317	(296 – 337)	0.09
Total IGF-I	nmol/L	13.3	(12.9 – 13.6)	13.3	(12.7 – 13.7)	13.2	(12.6 – 13.9)	0.72
Free IGF-I	pmol/L	76.2	(72.2 – 80.3)	77.0	(72.1 – 82.2)	75.4	(69.1 – 82.2)	0.58
IGFBP-1	nmol/L	1.1	(1.1 – 1.2)	1.1	(1.0 – 1.1)	1.2	(1.1 – 1.3)	0.03
IGFBP-3	nmol/L	90.5	(88.0 – 93.0)	93.4	(90.1 – 96.9)	86.7	(83.1 – 90.2)	0.20

For Free IGF-I and IGFBP-1 geometric means are shown and CI 95% intervals were calculated by forward-backward log-transformation. *Age adjusted P-values were calculated between survivors vs. non-survivors.

IGF-I bioactivity was significantly correlated with all studied parameters of the IGF system (Table 3) and to the total IGF-I / IGFBP-3 ratio ($r_s = 0.26$, $p < 0.001$). Furthermore, mean baseline IGF-I bioactivity was negatively related to age (slope (β) = -4.5 pmol/L/year, p = 0.01, data not shown). However, the rate of decline in IGF-I bioactivity with age did not differ between survivors (β = -3.5 pmol/L/year) and non-survivors (β = -4.1 pmol/L/year, p = 0.77). We found no significant correlations between IGF-I bioactivity and BMI, WHR, lean mass, fat mass, fasting glucose or insulin (data not shown).

Survival analyses for risk factor groups (1, 2-3 and 4) of total IGF-I, free IGF-I and IGF-I bioactivity were performed. Hazard ratios (HR) for mortality rate between groups are shown in Table 4.

Cox proportional hazard plots are shown in Figures 1A, B and C, respectively. A significant relationship was found between groups of IGF-I bioactivity and mortality (Table 4, Figure 1C). Subjects within the highest quartile of IGF-I bioactivity (group 4) had a lower mortality rate than subjects in groups with lower IGF-I bioactivity (groups 1 and 2-3). This remained significant after adjustment for age, BMI, smoking, SBP, diabetes, LDL and HDL (Table 4). For total IGF-I and free IGF-I no significant relationships were found (Table 4,

Table 3. Correlation coefficients between parameters of the IGF system in the study population (N = 376)

	IGF-I bioactivity	Total IGF-I	Free IGF-I	IGFBP-1
Total IGF-I	- 0.49**			
Free IGF-I	- 0.19**	- 0.37**		
IGFBP-1	- 0.25**	- 0.15**	- 0.10*	
IGFBP-3	- 0.34**	- 0.52**	- 0.17**	- 0.23**

Correlations are presented as Spearman coefficients.
*P < 0.05, **P < 0.01.

Table 4. Estimated hazard ratios between groups of IGF-I bioactivity, total IGF-I and free IGF-I in all subjects and after stratification for CVD + and CVD- and $IRP_{low/med}$ and IRP_{high}, respectively, during a follow-up period of 8.6 years

Group*		All Subjects			CVD -			CVD +			$IRP_{low/med}$			IRP_{high}		
		HR	CI 95%	$P_{for\ trend}$	HR	CI 95%	$P_{for\ trend}$	HR	CI 95%	$P_{for\ trend}$	HR	CI 95%	$P_{for\ trend}$	HR	CI 95%	$P_{for\ trend}$
A. Unadjusted																
IGF-I	1	1.8	1.2 – 2.8		1.6	0.9 – 2.9		2.4	1.3 – 4.3		1.4	0.8 – 2.5		2.3	1.2 – 4.5	
Bioactivity	2-3	1.2	0.8 – 1.8		1.4	0.8 – 2.5		1.1	0.6 – 1.9		1.0	0.6 – 1.6		1.9	1.0 – 3.6	
	4	Ref	…	0.01	Ref	…	0.12	Ref	…	0.01	Ref	…	0.32	Ref	…	0.01
Total	1	0.9	0.6 – 1.4		0.8	0.4 – 1.4		1.2	0.7 – 2.1		0.8	0.5 – 1.8		1.0	0.5 – 1.8	
IGF-I	2-3	0.6	0.4 – 0.9		0.7	0.4 – 1.2		0.6	0.4 – 1.1		0.6	0.3 – 0.9		0.9	0.5 – 1.6	
	4	Ref	…	0.36	Ref	…	0.29	Ref	…	0.92	Ref	…	0.12	Ref	…	0.25
Free	1	1.1	0.8 – 1.7		1.2	0.7 – 2.1		1.2	0.7 – 2.2		0.8	0.5 – 1.3		1.8	1.0 – 3.4	
IGF-I	2-3	0.7	0.5 – 1.1		0.8	0.5 – 1.3		0.8	0.5 – 1.3		0.7	0.4 – 0.9		1.1	0.6 – 2.1	
	4	Ref	…	0.93	Ref	…	0.75	Ref	…	0.76	Ref	…	0.15	Ref	…	0.25
B. Adjusted																
IGF-I	1	1.6	1.0 – 2.5		1.3	0.7 – 2.5		2.5	1.4 – 4.8		1.3	0.7 – 2.3		2.1	1.1 – 4.1	
Bioactivity	2-3	1.4	0.8 – 1.8		1.4	0.8 – 2.5		1.2	0.7 – 2.1		1.0	0.6 – 1.7		2.0	1.0 – 3.8	
	4	Ref	…	0.04	Ref	…	0.31	Ref	…	0.01	Ref	…	0.47	Ref	…	0.03
Total	1	0.9	0.6 – 1.3		0.8	0.4 – 1.4		1.1	0.6 – 1.9		0.7	0.4 – 1.3		1.1	0.6 – 2.1	
IGF-I	2-3	0.7	0.5 – 1.0		0.8	0.5 – 1.4		0.6	0.3 – 1.0		0.6	0.4 – 0.9		1.0	0.5 – 1.9	
	4	Ref	…	0.25	Ref	…	0.37	Ref	…	0.77	Ref	…	0.10	Ref	…	0.87
Free	1	1.0	0.7 – 1.6		1.3	0.7 – 2.3		1.1	0.6 – 2.0		0.9	0.5 – 1.6		1.7	0.9 – 3.4	
IGF-I	2-3	0.7	0.5 – 1.1		0.9	0.5 – 1.5		0.7	0.4 – 1.2		0.6	0.4 – 1.0		1.0	0.6 – 1.9	
	4	Ref	…	0.81	Ref	…	0.94	Ref	…	0.98	Ref	…	0.39	Ref	…	0.15

Individuals were grouped according to their baseline levels of three different IGF-I parameters (IGF-I bioactivity, Total IGF-I and Free IGF-I). Group 1: IGF-I ≤ 25th percentile, Group 2-3: IGF-I between 25th and 75th percentile, Group 4: IGF-I ≥ 75th percentile. In all models group 4 is the reference group (HR = 1.0). (A) Unadjusted models, (B) models adjusted for age, BMI, smoking, SBP, diabetes, LDL and HDL. CVD- = absence of cardiovascular disease, CVD+ = presence of cardiovascular disease, $IRP_{low/med}$ = low to medium inflammatory risk profile, IRP_{high} = high inflammatory risk profile.

Figure 1A and 1B respectively). In addition, when survival was analysed using quartiles of total IGF-I / IGFBP3 ratios no significant relationship was found (adjusted for age p for trend = 0.41, data not shown).The data were then stratified into subgroups with either positive (N = 133) or negative medical history of CVD (N = 242). Mean baseline levels of IGF-I bioactivity and all

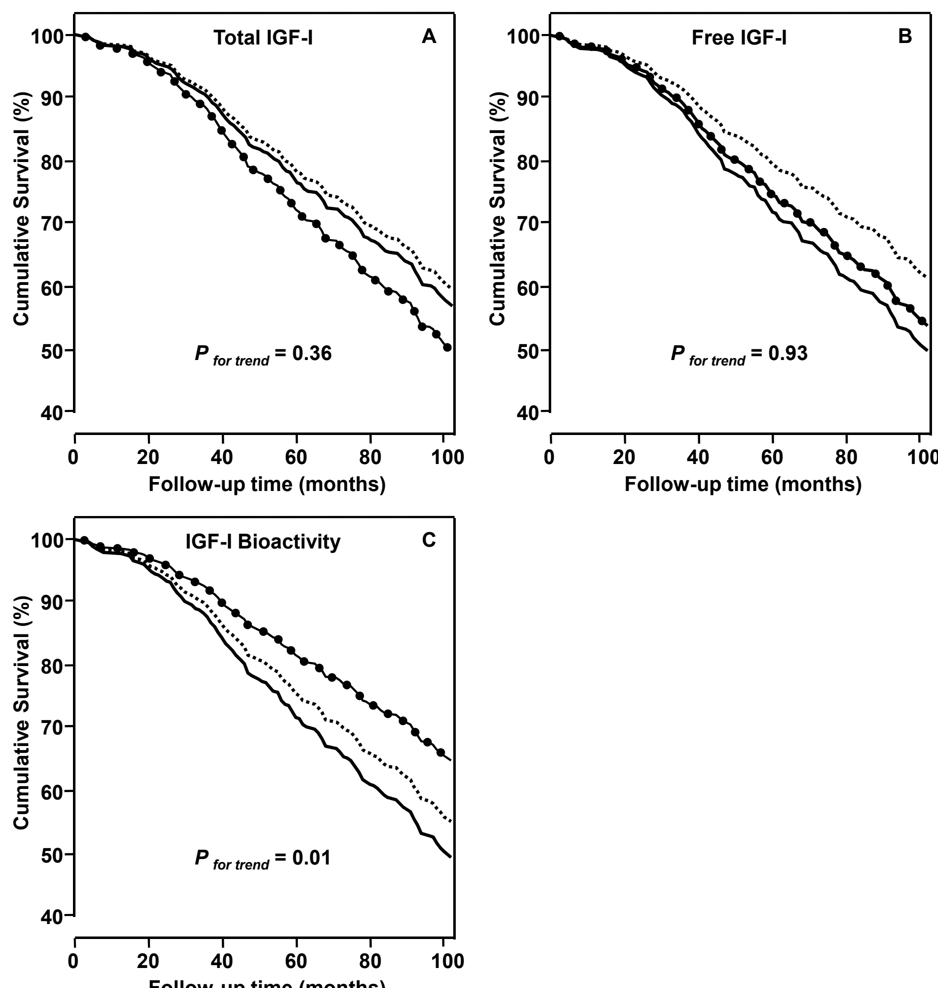

Figure 1. Cox proportional hazard plots (%) for groups of (**A**) circulating total IGF-I levels, (**B**) circulating free IGF-I levels and (**C**) IGF-I bioactivity levels. P for trend only reached statistical significance in the IGF-I bioactivity groups. Groups of IGF-I parameters: Group 1 (▬): ≤ 25th percentile; Group 2-3 (▬ ▬): between 25th and 75th percentile; Group 4 (▪●▪): ≥ 75th percentile. Trends across IGF-I bioactivity risk groups were based on Cox proportional hazard models with linear effect of the risk factor (Armitage trend test). Maximum time of follow-up was 103 months.

other measured parameters of the IGF system did not differ between these two CVD groups (data not shown).

There was a significant relationship between groups of IGF-I bioactivity and mortality in subjects with prevalent CVD (Table 4, Figure 2A).

Subjects in the lowest quartile (group 1) of IGF-I bioactivity had a significantly higher mortality rate than subjects in the highest quartile (group 4) (unadjusted p for trend = 0.003; adjusted p for trend = 0.004).

For group 1 *vs.* 2-3 the HR for mortality rate was significant (unadjusted HR = 2.3 (CI 95%: 1.4 – 3.8, p = 0.001); adjusted HR = 2.1 (CI 95%: 1.3 – 3.6, p = 0.005).

IGF-I bioactivity was significantly inversely related to hs-CRP levels (r_s = -0.17, p < 0.001, adjusted for age and BMI). Of all other measured parameters of the IGF-I system, only IGFBP-1 levels were significantly positively correlated with hs-CRP levels (r = 0.15, p = 0.04, adjusted for age and BMI).

Using hs-CRP as a marker of inflammation and mortality risk, we stratified data into a subgroup with a low to medium (N = 238) and with a high (N = 138) inflammatory risk profile (IRP). At baseline, mean IGF-I bioactivity was lower in subjects with a high IRP, than in subjects with a low to medium IRP (mean ± SEM: 312 ± 11.6 *vs.* 344 ± 7.8 pmol/L, p = 0.01, adjusted for age and BMI). Means of other parameters of the IGF-I system did not differ between IRP subgroups.

The relationship between IGF-I bioactivity groups and mortality was significant, but only in subjects with a high IRP (Table 4, Figure 2B). Subjects in the highest quartile (group 4) had significantly better survival than subjects in the lowest quartile (group 1) (p = 0.01). This relationship remained significant after adjustment for age, BMI, smoking, SBP, diabetes, LDL and HDL (p = 0.03). Exclusion of the first year of follow-up, did not affect this relationship as the difference in mortality rate remained significant for group 1 *vs.* 4 (unadjusted HR = 2.0, 95% CI: 1.1 – 4.3, p = 0.02; adjusted HR = 2.0, 95% CI: 1.0–4.0, p < 0.05). For group 1 *vs.* 2-3 the HR for mortality rate was not significant (unadjusted HR = 1.2 (CI 95%: 0.8 – 2.0, p = 0.38; adjusted HR = 1.0 (CI 95%: 0.6 – 1.8, p = 0.88).

Lastly, IRP and CVD data were combined. At baseline, mean IGFBP-1 levels were significantly higher in subjects with a positive medical history of CVD and/or a high IRP (N = 211) compared to individuals without prevalent CVD and a low/medium IRP, p = 0.004, data not shown).

A significant trend for mortality rates was found across IGF-I bioactivity groups in subjects with a high IRP and/or a positive medical history of CVD (unadjusted p for trend = 0.003; adjusted p for trend = 0.005; Figure

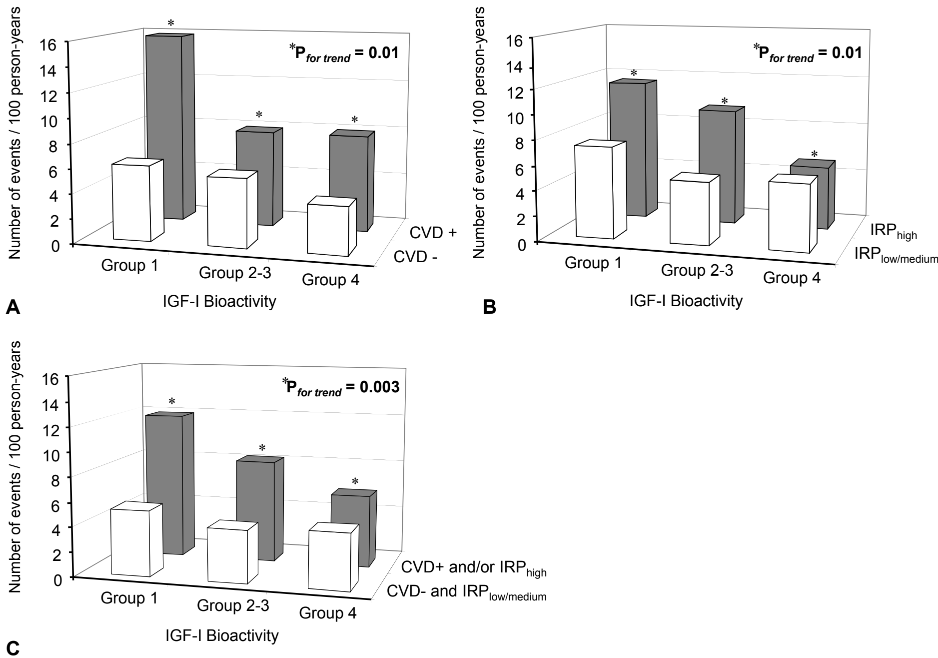

Figure 2. Crude incidence rates (number of events (deaths) per 100 person-years) are shown for groups of circulating IGF-I bioactivity levels according to strata of (**A**) absence or presence of a medical history of CVD (CVD - *vs.* CVD +, respectively), (**B**) low/medium or high inflammatory risk profile ($IRP_{low/medium}$ *vs.* IRP_{high}, respectively) and (**C**) combined subgroups of CVD and IRP (CVD+ and/or IRP_{high} *vs.* CVD- and $IRP_{low/medium}$). A significant linear trend (*) between groups of IGF-I bioactivity was only found in subjects (**A**) with prevalent CVD, (**B**) a high IRP or (**C**) prevalent CVD and/or a high IRP. Figure 2C; for group 1 *vs.* 2-3 unadjusted HR = 1.5 (95% CI: 1.0 – 2.3, $p < 0.05$) for group 1 *vs.* 4: HR = 2.3 (95% CI: 1.3 – 3.8, p = 0.002); and for group 2 *vs.* 3: HR = 1.5 (95% CI: 0.9 – 2.5, p = 0.11). HRs adjusted for age, BMI, smoking, SBP, diabetes, LDL and HDL were 1.4 (95% CI: 0.9 – 2.4, p = 0.15); 2.2 (95% CI: 1.3 – 3.8, p = 0.004); and 1.6 (95% CI: 0.9 – 2.6, p = 0.08), respectively.
Maximum time of follow-up was 103 months. Trends across IGF-I bioactivity risk groups were based on Cox proportional hazard models with linear effect of the risk factor (Armitage trend test). Groups of IGF-I bioactivity: Group 1: IGF-I bioactivity ≤ 25th percentile; Group 2-3: IGF-I bioactivity between 25th and 75th percentile; Group 4: IGF-I bioactivity ≥ 75th percentile. CVD = cardiovascular disease, IRP = inflammatory risk profile. IRP subgroups were based on hs-CRP levels: $IRP_{low/medium}$ = CRP ≤ 3 mg/L, IRP_{high} = CRP > 3 mg/L.

2C). Mortality rate was highest in group 1, the quartile with the lowest IGF-I bioactivity (for estimated HRs see legends of Figure 2C).

Neither total nor free IGF-I showed any significant relationships with mortality rate in CVD and IRP subgroups (Table 4).

DISCUSSION

This 8-year prospective study in elderly men showed that higher circulating IGF-I bioactivity is associated with better overall survival. Individuals in the lowest quartile of IGF-I bioactivity had a 1.8-fold increased mortality risk compared with individuals in the highest quartile. Interestingly, for total and free IGF-I measurements as well as for the total IGF-I / IGFBP-3 ratios we could not find such relationships.

Although men with lower IGF-I bioactivity might have died earlier or might have been excluded from the study because their physical condition (illness, frailty or other causes) prevented a visit to the research center [20], the strength of our study is its prospective design, which is likely to reduce this form of selection bias.

The IGF-I KIRA was used to measure IGF-I bioactivity [13,14], which was significantly associated with other IGF system parameters measured by immunoassay. However, none of these associations had correlation coefficients greater than 0.5. This suggests that, in comparison with immunoassays, the KIRA produces different information about circulating IGF-I.

In most IGF-I immunoassays, various techniques are used to remove IGFBPs [21]. However, one of the major functions of IGFBPs is to modulate IGF-I bioavailability. IGFBP-1 is thought to be an important direct modulator of IGF-I bioactivity [10]. In our study IGF-I bioactivity correlated better with circulating IGFBP-1 levels than with either total or free IGF-I levels, suggesting that IGFBP-1 indeed influences IGF-I bioactivity.

Of interest is the observed discrepancy between IGF-I bioactivity and free IGF-I in our study. Both parameters are believed to be informative about the fraction of circulating total IGF-I that interacts with the IGF-IR. Mean IGF-I bioactivity was significantly greater than mean free IGF-I level and correlation between these parameters was poor. An explanation could be that the IGF-I KIRA is more sensitive than free IGF-I levels in estimating the concentration of circulating IGF-I that interacts with the IGF-IR, since the KIRA is probably better at detecting the modulatory effects of IGFBPs and IGFBP proteases. In addition, as the IGF-I KIRA measures the overall ability of serum to activate the IGF-IR *in vitro*, our data do not allow us to discriminate between the relative contributions of IGF-I and IGF-II to the IGF-I KIRA signal. Therefore, IGF-II mediated effects could also have contributed to the discrepancy between the IGF-I KIRA and free IGF-I levels. From previous experiments it is known that IGF-II has a cross-reactivity of about 12% to the IGF-IR [13]. However, as has

been suggested previously, from a biological point of view it is not important whether IGF-IR activation is caused mostly by IGF-I or IGF-II [22].

In this study a relatively high circulating IGF-I bioactivity was associated with a lower mortality risk. This is in contrast to results reported in animal studies, where low circulating IGF-I levels were associated with increased survival. An explanation could be that in these animal studies effects of IGF-I on the rate of aging were studied during lifelong exposure, whereas our study only provides insight into IGF-I activity towards the end of life. In addition, insulin and IGF-I have very different functions and signalling pathways in mammals compared with their homologs in lower species (e.g. *C. elegans* and *Drosophila*). Furthermore, lifespan in humans is measured in decades as opposed to months or days in rodents, flies and nematodes [23]. Another explanation could be that catabolism and/or a systemic inflammatory response as a consequence of subclinical (undiagnosed) diseases may have induced resistance to IGF-I production. It could be that in our study low IGF bioactivity may not be a cause but rather an effect, serving as a reporter for disease or catabolism.

Humans have the potential to live for over 100 years and the precise interactions of all factors that influence aging is complex. In the Western world cardiovascular disease (CVD) and cancer are the most important determinants of human survival. Although cancer is also a common cause of death in animal models used to study longevity, CVD is not and is more specific to humans. When we stratified for the presence or absence of CVD, we found that low IGF-I bioactivity was an important independent risk factor for mortality rate in individuals with CVD. Interestingly, comparative results were found when data were stratified according to IRP (based on CRP levels), and the observed trend became even stronger when both groups were combined.

Of note, CRP is a non-specific marker of systemic inflammatory responses and has emerged as the most powerful predictor of mortality due to CVD events among eleven other biomarkers [19]. In our study CRP levels were significantly inversely related to IGF-I bioactivity and positively to IGFBP-1. Although a decline in IGF-I bioactivity, as previously discussed, could be a component of the hormonal alterations that occur in any illness or catabolic state (37), the difference in mortality rate between the highest and the lowest quartile of IGF-I bioactivity in our study remained significant when all mortalities in the first year of follow-up were excluded from the analyses. Thus, undiagnosed illness or catabolic states at baseline probably did not cause the relationship between IGF-I bioactivity and survival.

Interestingly, there are different clinical models that support the negative regulation of CRP and other inflammatory markers by GH/IGF-I administration [24,25] For example, Sesmilo et al. found low levels of CRP in patients with active acromegaly, which rose when IGF-I levels normalized after administration of a GH receptor antagonist [26]. Verma et al. found that CRP negatively influences proliferation, differentiation and survival of isolated endothelial progenitor cells *in vitro* and their ability to produce nitric oxide (NO), whereas for IGF-I opposite effects have been reported [27,28]. Altogether these findings point to the possibility of a relationship between IGF-I bioactivity, CRP and CVD. However, it remains to be seen whether reduced IGF-I bioactivity is an endocrine contributor to mortality risk or simply an epiphenomenon related to overall health/resistance to inflammation.

In conclusion, this prospective study provides evidence that low circulating IGF-I bioactivity in elderly men is associated with increased mortality, especially in those individuals in which an age-related pro-inflammatory state exists, with its attendant higher risk of mortality from CVD. Remarkably, for total and free IGF-I measurements we could not find such relationships. Compared to IGF-I immunoassays the IGF-I KIRA may offer the unique possibility of measuring the net modulating effects of IGFBPs and IGFBP proteases on IGF-IR activation by bioactive IGF-I available in human serum. In this respect, our study suggests that determination of IGF-I bioactivity may help to clarify the controversies that exist about the precise role of IGF-I in human survival.

ACKNOWLEDGMENTS

The authors thank Professor Pierre de Meyts (Receptor Biology Laboratory, Hagedorn Research Institute, Novo Nordisk, Gentofte, Denmark) for his help in establishing the IGF-I KIRA in our laboratory. In addition, the authors thank Dr. Patric Delhanty for his contribution to the manuscript. This study was in part supported by Zon-MW, Organization for Health Research and Development the Netherlands, (948-00-003).

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Chapter 4

Bioactive rather than Total IGF-I is involved in Acute Responses to Nutritional Interventions in CAPD Patients

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Nephrol Dial Transplant. 2009 Nov 9. [Epub ahead of print].

ABSTRACT

Background: Inadequate food intake plays an important role in the development of malnutrition in continuous ambulatory peritoneal dialysis (CAPD) patients.
Aim of the study: To investigate in CAPD patients whether circulating insulin-like growth factor-I (IGF-I) bioactivity may offer a more sensitive index to acute nutritional interventions than total IGF-I.
Methods: An open-label, randomized, crossover study of 2 days -with one-week interval- was performed in 12 CAPD patients in the fed state to compare a mixture of amino acids (Nutrineal 1.1%) plus glucose (AA plus G) (Physioneal 1.36 to 3.86%) dialysate versus G only as control dialysate. Fed-state conditions were created by identical liquid hourly meals. IGF-I bioactivity was measured by the kinase receptor activation assay (IGF-I KIRA); Total IGF-I was measured by immunoassay.
Results: In the fed state both after AA plus G as well as after G dialysis IGF-I bioactivity increased compared to baseline, while no changes in circulating total IGF-I levels were observed in both treatment arms. However, the increase in IGF-I bioactivity was only significant after AA plus G dialysis ($p=0.02$).
Conclusions: Our results provide evidence that in CAPD patients changes in circulating IGF-I bioactivity are associated with nutrient intake and that IGF-I bioactivity rather than total IGF-I is involved in acute responses to nutritional interventions in CAPD patients.

INTRODUCTION

Malnutrition is a major complication of continuous ambulatory peritoneal dialysis (CAPD) and is associated with increased morbidity and mortality [1]. Insulin-like growth factor-I (IGF-I) is produced in tissues throughout the body under the influence of growth hormone (GH). In addition, nutrition is one of the main regulators of circulating IGF-I [2,3]. Circulating IGF-I has been proposed as a valid sensitive marker of (mal) nutrition in subjects on CAPD, which may have advantages above serum albumin and other markers of nutritional state [4-7]. On the other hand, reduced bioavailability of IGF-I may play a role in decreasing muscle protein synthesis [8].

The GH-IGF-I axis shows major changes in chronic renal failure (CRF). Despite elevated GH levels, IGF-I secretory rate by the liver is reduced in CRF, possibly as a result of hepatic GH resistance [9]. There is also a resistance to IGF-I action, which may be the result of circulating inhibitors [8]. The amount of these circulating inhibitors increases in patients with CRF along with the decrease in glomerular filtration rate [10].

Circulating IGF-I bioactivity measured by "classic" bioassays has been reported to be low in CRF [11]. Since these "classic" bioassays suffered from high variability as well as long assay duration, IGF-I bioactivity is at present almost exclusively estimated by immunological determinations of total IGF-I. In contrast, circulating total IGF-I levels measured by immunoassays have been reported as normal or elevated in CRF. However, the results of IGF-I immunoassays may not necessarily correspond to the fraction of IGF-I being accessible to the IGF-I receptor [12].

Recently a rapid and accurate IGF-I bioassay was developed as alternative to the "classic" bioassays, named the kinase receptor activation assay (IGF-I KIRA) [12,13]. The IGF-I KIRA quantifies phosphorylation of tyrosine residues of the activated IGF-I receptor (IGF-IR) as a measure for IGF-I bioactivity in serum. In contrast to commonly used IGF-I immunoassays, the IGF-I KIRA is sensitive for modifications of IGF-IR activation by circulating Insulin-like Growth Factor Binding Proteins (IGFBPs) and IGFBP-proteases [12,14].

We hypothesized that measuring circulating IGF-I bioactivity by the IGF-I KIRA method could help to enhance our insights in the alterations of the IGF-I system in CAPD patients. Considering that a substantial portion of patients undergoing CAPD have insufficient protein and calorie intake due to anorexia, we previously evaluated in CAPD patients the metabolic effects of dialysate containing amino acids with glucose (AA plus G) and observed that this solution improved protein metabolism both in fasting [15] and in fed [16] patients on

peritoneal dialysis. From the same study we here evaluate whether circulating IGF-I bioactivity is a more sensitive index to monitor acute responses to nutritional interventions than total IGF-I. In addition, in these CAPD patients we compared basal IGF-I bioactivity and total IGF-I in relation to nutritional status.

METHODS

Study population

Twelve patients on CAPD were recruited from the Peritoneal Dialysis Unit of the Erasmus Medical Center (Erasmus MC). Inclusion criteria were: stable patients, who were on peritoneal dialysis for more than 3 months and a weekly total Kt/V>1.7 (Total Kt/V= urea clearance per week (Kt) divided by urea distribution volume (V), as delivered by peritoneal dialysis plus residual renal function). Exclusion criteria were peritonitis, other infections or inflammatory diseases in the previous 6 weeks, malignancy, and life expectancy less than 6 months. The Medical Ethics Committee of the Erasmus MC approved the study and informed consent was obtained from all patients.

Study design

The study design has been published in detail [16]. Briefly, an open-label, randomized, crossover study of 2 days with one-week interval was performed.

On the 2 study days, all patients came at 08.00 a.m. into the hospital and stayed at the Department of Nephrology of the Erasmus MC. Dialysis was performed during daytime for 9 h, while the patients consumed a liquid diet comparable in nitrogen and energy content in their habitual diet (Figure 1). The total food volume was divided into 11 equal portions (Figure 1). The first two meal portions were given at half-hourly intervals, and the remaining nine portions were given at hourly interval thereafter (Figure 1).

For dialysis either dialysate that contained a mixture of amino acids and glucose (AA plus G) (Nutrineal 1.1% plus Physioneal 1.36% to 3.86%; Baxter BV, Utrecht, the Netherlands) or dialysate that contained glucose only (G) was used. The AA plus G dialysate was obtained by mixing one bag of 2.5 L of Nutrineal 1.1% (containing 27.5 g of AA) and four bags of 2.5 L of Physioneal 1.36% to 3.86% G (see [16] for more details). The night before the study days, all patients underwent dialysis with Extraneal. Because metabolic steady-state conditions are not achieved with a standard CAPD scheme, six daytime dialysate exchanges on the 2 study days were performed using an automatic cycler (HomeChoice, Baxter B.V.).

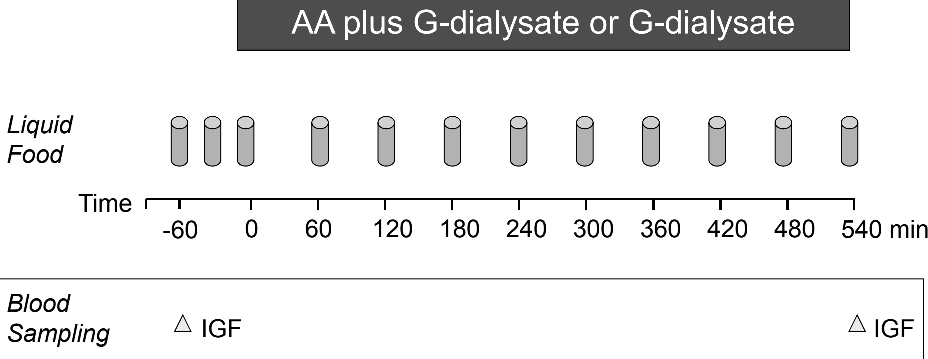

Figure 1. Schematic diagram of the study-day protocol.

Portions of the liquid food, the first two portions given half-hourly and thereafter hourly.

Time points of blood sampling for IGF-I

Blood Measurements

At 8.20 a.m. two catheters were inserted into superficial veins of both arms. Baseline blood samples were collected before starting the oral feeding. Blood samples for IGF-I parameters were taken before the first dialysate exchange (fasting state) and at the end of the study (Figure 1). Circulating IGF-I bioactivity was measured using an in-house IGF-I kinase receptor activation assay as previously described [17]. This latter assay is based on another principle than IGF-I immunoassays [13,17]. Briefly, IGF-I binding to the IGF-I receptor (IGF-IR) results in autophosphorylation of tyrosine residues located within the intracellular kinase domain, being the first step in the intracellular signal cascade. The IGF-I KIRA uses human embryonic renal cells stably transfected with cDNA of the human IGF-IR gene (293 EBNA IGF-IR) and quantifies phosphorylation of tyrosine residues of the IGF-IR to assess IGF-I bioactivity. Serum total IGF-I was measured by a solid-phase, enzyme-labeled chemiluminescent immunometric assay (CLIA) Immulite 2000 IGF-I supplied by Siemens Medical Solutions Diagnostics, Los Angeles, USA.

Both IGF-I bioactivity and total IGF-I levels were compared with the age-specific normative range values for IGF-I. Reference values for IGF-I bioactivity and total IGF-I have been published in previous reports [17,18]. Both for IGF-I bioactivity and total IGF-I individual Z-scores were calculated using the following formula: Z-score = (x–average x/S.D.) where x is the actual IGF-I bioactivity or IGF-I level, average x is the mean IGF-I bioactivity or IGF-I level at that age, and S.D. is standard deviation for the mean at that age.

Insulin was measured by a chemiluminescent immunometric assay (Immulite 2000 Insulin; Siemens Medical Solutions Diagnostics, Los Angeles, USA). Amino acids were measured by ion-exchange chromatography on a Biochrome 20 amino acid analyzer with ninhydrin detection (Biochrome, Cambridge, UK). Serum glucose, creatinine, albumin, prealbumin, and transferrin were performed by standard laboratory methods. Residual renal function was derived as the mean of renal urea and creatinine clearance (ml/min) and derived from PD adequest 2.0 software Baxter.

Other measurements

Body mass index (BMI) was defined as weight divided by the square of height (kg/m^2). Subjective global assessment (SGA) as described by Detsky et al. is based on clinical parameters [19]. Patients were classified according to their nutritional status into three categories: A: good nutritional status; B: moderate malnutrition; C: severe malnutrition. The Protein Equivalent of Nitrogen Appearance normalized (nPNA) was evaluated by the urea kinetic model (PD Adequest 2.0 software, Baxter) [16].

Effects on protein metabolism were studied using whole-body protein turnover (WBPT). Previously we performed an open-label randomized crossover study on 2 days with 6 days interval comparing the effect of AA plus G dialysate with dialysate containing only G [16]. For the present study we used data on WBPT, which were all derived from our previously performed study [16].

Statistical analysis

Data are expressed as medians with ranges unless otherwise indicated.

All variables were tested using the Shapiro-Wilk- test for normality. When no normality of distribution was found, data were log-transformed. One-way ANOVA was used to test differences between subjects with different SGA classifications. The Wilcoxon Signed Rank test was used to compare outcomes in IGF-I parameters and other variables between before and after dialysis. The paired T- test was used to compare differences between the two treatment regimens (AA plus G versus G dialysis). 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 P-value of 0.05 or less was considered statistically significant. Data were analyzed using SPSS 15 for Windows (SPSS Inc., Chicago, IL).

RESULTS

In Table 1 the baseline characteristics of the study population are presented. According to the SGA classification, 4 out of 12 patients were moderately malnourished. The mean BMI of these latter 4 patients tended to be lower and the time on peritoneal dialysis was longer than that of patients with a good nutritional status, but these differences were not statistically significant (Table 1). At baseline albumin, prealbumin and transferring levels did not differ between subjects with different SGA classifications.

In subjects with a good nutritional status (SGA A) Z-scores for circulating total IGF-I were in the upper half of the normal range (Table 2; Figure 2). Z-scores for total IGF-I exceeded the normal range (i.e Z-score >2.0 SD) in patients with moderate malnutrition (SGA B) (Table 2). The differences in total IGF-I between both groups were not statistically significant (Table 2; Figure 2).

Mean circulating IGF-I bioactivity did not differ between patients with SGA A and SGA B (Table 2; Figure 2). Z-scores for IGF-I bioactivity were in the lower half of the normal range (Table 2; Figure 2). Three subjects (two classified as SGA B) had IGF-I bioactivity outside and below the normal range (i.e. Z- score <2.0 SD) (Table 2). IGF-I bioactivity was not significantly related to total IGF-I ($r = 0.50$, $p = 0.10$).

Table 1. Characteristics of 12 patients (4 females) in the basal state stratified for nutritional status by SGA

	SGA A (n=8; 3F)		SGA B (n=4; 1F)		P-value
	Median	Range	Median	Range	
Age (yrs)	57.5	(35 – 63)	53.7	(33 – 70)	0.85
BMI (kg/m^2)	26.8	(23.7 – 32.6)	22.9	(21.4 – 32.8)	0.37
Residual renal function (mL/min/1.73 m^2)	6	(4 – 12)	6	(4 – 11)	0.77
Time on PD (months)	8	(4 – 72)	18	(4 – 28)	0.92
Kt/V	2.6	(2.0 – 3.1)	2.0	(1.9 – 2.7)	0.08
Albumin (g/L)	39	(35 – 42)	38	(36 – 40)	0.71
Prealbumin (g/L)	0.36	(0.28 – 0.54)	0.38	(0.27 – 0.43)	0.87
Transferrin (g/L)	2.25	(1.90 – 2.70)	2.15	(2.10 – 2.60)	0.99
nPNA (g/kg/day)	0.95	(0.56 – 1.37)	0.84	(0.69 – 0.97)	0.40
Protein intake (g/kg/day)	0.99	(0.68 – 1.38)	0.83	(0.59 – 0.93)	0.14
Energy intake (kcal/kg/day)	22.8	(16.0 – 26.6)	20.1	(11.1 – 32.1)	0.71

SGA: subjective global assessment; SGA A: good nutritional status; SGA B: moderate malnutrition; F = female; BMI: body mass index; PD: peritoneal dialysis; nPNA: normalized protein equivalent of nitrogen appearance (PD Adequest 2.0 software, Baxter)

Table 2. IGF-I parameters of the 12 patients in the basal state

	SGA A (n=8; 3F)		SGA B (n=4; 1F)		P-value
	Median	Range	Median	Range	
Total IGFI (nmol/L)	26.9	(11.2 – 48.2)	31.0	(18.1 – 33.0)	0.54
Z-score	1.75	(-1.30 – 4.50)	2.35	(-0.60 – 4.00)	0.36
IGF-I Bioactivity (pmol/L)	216	(136 – 358)	211	(167 – 218)	0.41
Z-score	-1.57	(-2.31 to -0.44)	-1.78	(-2.18 to -1.55)	0.34

SGA: subjective global assessment; SGA A: good nutritional status; SGA B: moderate malnutrition

Both IGF-I bioactivity and total IGF-I levels did not significantly decrease with age (IGF-I bioactivity: r = -0.16, p = 0.63; total IGF-I levels: r = 0.30, p = 0.34).

Total IGF-I levels and IGF-I bioactivity were inversely related to creatinine clearance, although these latter relationships missed statistical significance (r = -0.58, p = 0.06 and r = -0.48, p = 0.11, respectively).

In the fed state both after AA plus G and after G dialysis IGF-I bioactivity increased compared to baseline (Table 3; Figure 3). However, the increase in IGF-I bioactivity was only significant after AA plus G dialysis (p=0.02). Compared to baseline no changes in circulating total IGF-I levels were observed in both treatment arms (Table 3; Figure 3).

In the basal state serum bicarbonate, creatinine, urea, glucose and insulin levels did not statistically differ between both treatment arms (data not shown). After dialysis insulin and glucose levels increased significantly compared with

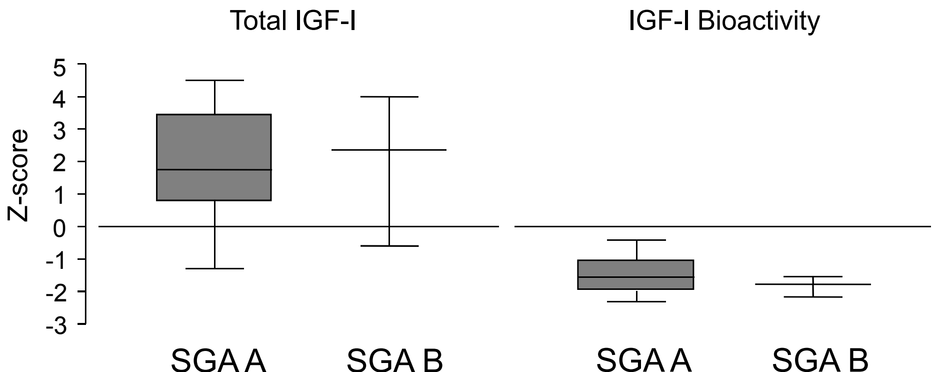

Figure 2. Top: Z-scores for total IGF-I after stratification for SGA. Bottom: Z-scores for IGF-I bioactivity after stratification for SGA. The line in the middle represents the 50th percentile of data. The boxes extend from the 25th percentile to the 75th percentile values; the whiskers extend from the 5th to the 95th percentile values of each group.

Table 3. IGF-I Parameters after AA plus G dialysate (A) and after G dialysate (B), respectively

A] After AA plus G Dialysate

	IGF-I Bioactivity (pmol/L)			Total IGF-I (nmol/L)		
	Basal	At the end of dialysis	Change* (%)	Basal	At the end of dialysis	Change* (%)
Median	211	251**	+34	30	26	-5
Range	(136 – 358)	(191 – 571)	(-33 – +59)	(11 – 48)	(10 – 43)	(-15 – +21)

B] After G Dialysate

	IGF-I Bioactivity (pmol/L)			Total IGF-I (nmol/L)		
	Basal	At the end of dialysis	Change* (%)	Basal	At the end of dialysis	Change* (%)
Median	226	251	+16	25	26	+1.5
Range	(151 – 371)	(177 – 533)	(-52 – +52)	(10 – 42)	(13 – 42)	(-13 – +23)

AA plus G dialysate = combined amino acids plus glucose dialysis, G dialysate = glucose dialysis.

*Changes at the end of dialysis compared to baseline (= 100%)

**P = 0.02; Paired t-test.

baseline in both treatment arms (Table 4). After AA plus G dialysis plasma essential and non-essential amino acid levels significantly increased compared to G dialysis (data not shown). Protein loss and excretion of urea in dialysate did not differ with either type of dialysate (data not shown). Results of whole-body protein turnover after AA plus G dialysate and after G dialysate from this study have previously been published [16]. After AA plus G dialysate

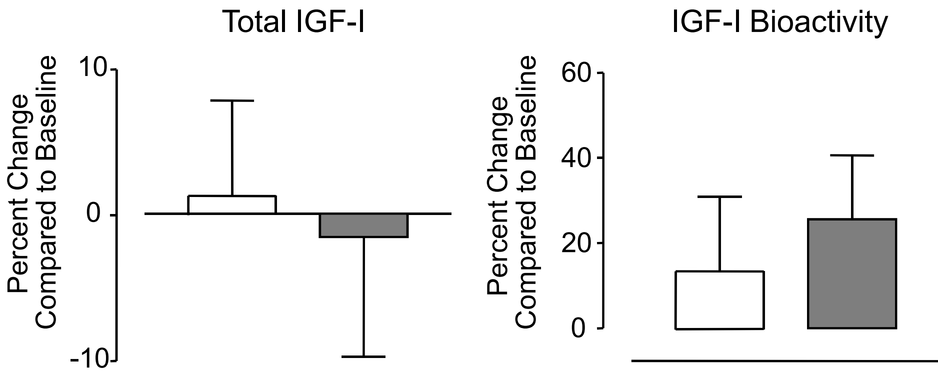

Figure 3. Top: Changes in circulating total IGF-I levels in the fed state after G and after AA and G dialysate, respectively (Basal state = 100%). Bottom: Changes in circulating IGF-I bioactivity in the fed state compared to baseline after G and after AA and G dialysate, respectively (Basal state = 100%).

Table 4. Glucose and insulin [median (range)] in the basal state and at the end of 9h dialysis with AA plus G or G dialysate, respectively

	Baseline AA plus G dialysis	After AA plus G dialysis	Baseline G dialysis	After G dialysis
Glucose (mmol/L)	4.4 (3.6 – 6.8)	5.7* (3.6 – 12.7)	4.2 (2.1 – 6.7)	5.3* (3.9 – 11.1)
Insulin (pmol/L)	106 (46 – 444)	478* (111 – 1268)	130 (35 – 288)	454* (172 – 950)

AA plus G dialysate = combined amino acids plus glucose dialysis, G dialysate = glucose dialysis.
*P<0.05 versus baseline.

leucine flux, leucine oxidation, protein intake and protein synthesis significantly increased compared with G dialysate ($p = 0.001$, $p < 0.001$, $p < 0.001$ and $p=0.039$, respectively). There were no significant differences in protein breakdown after AA plus G dialysate and G dialysate, respectively ($p = 0.346$). In addition, overall net protein balance was not significantly different between both treatment arms ($p = 0.347$).

Both after AA plus G and after G dialysis IGF-I bioactivity was significantly related to leucine flux, leucine oxidation and protein intake, but not to protein synthesis, protein breakdown and overall net protein balance (Table 5). Moreover, IGF-I bioactivity was not related to insulin levels at the end of dialysis (AA plus G: $r = 0.24$, $p = 0.47$; G: $r = 0.38$, $p = 0.25$).

Table 5. Age-adjusted correlation coefficients between IGF-I and parameters of whole-body protein turnover after AA plus G dialysate (A), and after G dialysate (B), respectively

	IGF-I Bioactivity	Total IGF-I
A] After AA plus G Dialysate		
Flux	0.61*	0.23
Oxidation	0.71*	0.30
Intake	0.62*	0.10
Synthesis	0.37	0.10
Breakdown	0.35	0.33
Net Protein balance	0.14	-0.32
B] After G Dialysate		
Flux	0.67*	0.44
Oxidation	0.72*	0.40
Intake	0.64*	0.03
Synthesis	0.46	0.41
Breakdown	0.23	0.64*
Net Protein balance	0.20	-0.49

AA plus G dialysate = combined amino acids plus glucose dialysis, G dialysate = glucose dialysis.
*P<0.05.

There were no significant relationships between total IGF-I levels and WBPT parameters after AA plus G dialysis (Table 5). After G dialysis total IGF-I levels were significantly related to protein breakdown, but not other components of WBPT (Table 5).

Neither basal IGF-I bioactivity nor total IGF-I levels were related to nPNA (IGF-I bioactivity: r = 0.37, p = 0.27; total IGF-I: r = 0.52, p = 0.09).

DISCUSSION

The major finding of the present study in CAPD patients is that in the fed state both after AA plus G and after G dialysis circulating IGF-I bioactivity increased. This increase in IGF-I bioactivity occurred while no changes in circulating total IGF-I levels were observed. Our observation suggests that IGF-I bioactivity rather than total IGF-I is involved in acute responses to nutritional interventions in CAPD patients.

Only after AA plus G dialysate the rise in IGF-I bioactivity was significantly. The lack of a significant rise in IGF-I bioactivity after G dialysis may be due to the small number of patients. Alternatively, it cannot be ruled out that the larger amount of AA accounts for a significant rise of IGF-I bioactivity.

Previously it has been suggested that nutrients influence synthesis and action of IGF-I and its binding proteins (IGFBPs) at multiple levels [3]. Both protein and energy are needed for IGF-I gene expression [2,20]. Although protein is a very strong factor in changing hepatic IGF-I gene expression, a period of 9 hrs is likely too short to induce measurable changes in circulating IGF-I levels [20]. Therefore the rise in IGF-I bioactivity at the end of the dialysis period probably reflects diet-induced changes in circulating IGFBP levels as nutrition is also a major regulator of the circulating IGFBP levels [2]. Dietary manipulations may change the circulating levels of IGFBPs more rapidly and thereby IGF-I bioactivity [2]. Although it has been found that circulating IGFBP-3 concentrations are relatively constant throughout the day, IGFBP-1 is markedly and rapidly suppressed by nutrient intake and this effect is mediated primarily by increased insulin, glucose and probably amino acid concentrations [21-25]. Insulin may also selectively stimulate the transport of IGFBP-1 to the extravascular space [26]. Both mechanisms might be involved in the changes of IGF-I bioactivity. However, the design of our study was not sufficient to disentangle which components of nutritional interventions were affecting the increase of IGF-I bioactivity.

A weakness of our study could be that we did not measure the six IGFBP levels. Because IGFBPs control IGF-I bioavailability they are believed to exert both stimulatory and inhibitory effects on IGF-I actions [27]. In addition, IGF-I bioactivity may be further controlled by posttranslational modifications of IGFBPs (e.g. partial proteolytic degradation by specific IGFBP proteases and selective dephosphorylation) [27]. Thus the interpretation of the overall effects of these six IGFBPs on IGF-I activity is very difficult, especially in CRF which is characterized by substantial changes in the circulating IGF system.. In this respect the IGF-I KIRA assay may have an important advantage over measuring IGFBPs: it gives an overall and integrated picture by capturing the complex interactions between IGF-I, IGFBPs, proteases, and the IGF-I receptor, since the IGF-I KIRA directly measures IGF-I receptor activation in vitro. Thus, the IGF-I KIRA probably provides a more accurate measure of IGF-I action *in vivo* than any other currently available model based on immunoassaysof IGFBP blood levels, since it incorporates complex interactions with binding proteins and proteases.

We observed no change in total IGF-I levels during dialysate administration in both treatment arms. In accordance with our findings, Pupim et al. also reported no changes in total IGF-I levels during intradialytic parenteral nutrition to chronic hemodialysis patients, despite a significant positive effect on protein and energy metabolism [28].

Although there was a marked rise of plasma insulin levels in both treatment arms after dialysate administration, the absence of relationship between insulin and IGF-I bioactivity does not support an insulin-mediated increase in IGF-I bioactivity.

Our results provide evidence that in CAPD patients acute changes in circulating IGF-I bioactivity are associated with nutrient intake and that in this respect IGF-I bioactivity is a more sensitive index for monitoring acute responses to nutritional interventions than total IGF-I.

In both treatment arms IGF-I bioactivity was positively related to leucine flux, leucine intake and leucine oxidation. The competing processes of amino acid deposition (synthesis for maintenance) and loss (oxidation) play an important role in maintaining protein metabolism [29]. If protein intake is not adequate, the amino acid supply is limited and oxidation is reduced to a greater extent than is synthesis [30,31]. Conversely, when amino acids are provided in excess, as probably was the case in our study, oxidation rather than synthesis is increased [30]. In other words, in this situation leucine oxidation is a more sensitive index of adequacy of amino acid intake than is protein synthesis, changing in proportion to protein intake [31]. Thus the positive relationships

between IGF-I bioactivity, leucine flux, leucine intake and leucine oxidation in our study is consistent with our finding that circulating IGF-I bioactivity can be changed by amino acid intake: thus our study provides evidence that an increase in circulating IGF-I bioactivity likely is the consequence of an increased amino acid and/or protein intake. In favor of this, it has been found that changes in nitrogen balance parallel changes in IGF-I [32]. By use of a "classic" IGF-I bioassay it has been reported that circulating IGF-I bioactivity is low in subjects on HD [11]. Our study confirms this finding for PD. Using a "classic" porcine cartilage bioassay Phillips et al. showed that circulating IGF-I bioactivity was reduced and they hypothesized that IGF-I bioactivity was depressed because of the accumulation of dialyzable inhibitors [11]. Some years later these dialyzable inhibitors were identified as IGFBPs and it was demonstrated that IGF-I activity in blood samples of CRF patients was indeed restored after removal of IGFBPs by IGF-II chromatography [33]. Thus the low IGF-I bioactivity in CAPD patients in our study may directly reflect sequestering of IGF-I by IGFBPs in the circulation.

Most CAPD patients in our study showed serum total IGF-I levels in the normal range. This observation is in line with previous studies in patients undergoing maintenance hemodialysis (HD) [10,33-35]. However, circulating total IGF-I levels exceeded even the normal range in some patients. This latter phenomenon was especially observed in patients with moderate malnutrition (SGA B). Although we did not measure IGFBP levels, previous studies have shown that serum of individuals with CRF contains increased levels of IGFBP-1, IGFBP-2, fragments of IGFBP-3, IGFBP-4 and IGFBP-6 [10,34,36]. It has been hypothesized that these IGFBPs accumulate largely because of reduced clearance: elevation of their serum levels is inversely related to glomerular filtration rate [10]. When IGFBPs are present in serum in large excess and a total IGF-I immunoassay is utilized that is subject to even modest amount of binding protein interference, the measured total IGF-I levels may be artificially high [37]. This problem may be exacerbated in patients with underlying conditions, that lower circulating IGF-I levels (like subjects with chronic renal failure and moderate malnutrition), since the decrease in IGF-I as well the increase in IGFBPs results in more unsaturated IGFBPs and intensify the interference [37]. This may explain why in our study circulating total IGF-I levels exceeded the normal range especially in CAPD patients with moderate malnutrition.

Finally, several findings in our study appeared not to be statistically significant (e.g. comparison IGF-I parameters between groups of SGA A vs. B and the correlation between insulin and IGF-I bioactivity). However, this may also have been due to the small number of patients included in our study.

In conclusion, the observed acute increase in IGF-I bioactivity in the fed state may be related to nutrition-induced changes in circulating IGFBPs. The present study suggests that IGF-I bioactivity rather than total IGF-I is involved in acute responses to nutritional interventions in CAPD patients.

ACKNOWLEDGEMENTS

The Study was supported by grants from Baxter Benelux. We thank the patients for participation and Laurens J. van Dijk, PD nurse, Adorée M. van der Wiel, dietitian, and Marja Belder, nurse, for cooperation in the study.

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Chapter 5

Heterophilic Antibodies may be a Cause of Falsely Low Total IGF-I Levels

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Eur J Endocrinol. 2009 Oct;161(4):561-5

ABSTRACT

Background: A low serum total insulin-like growth factor-I (IGF-I) is considered a diagnostic indicator of growth hormone deficiency (GHD) in the presence of hypopituitarism. Introduction of immunoradiometric (IRMA) and chemiluminescence (CLIA) IGF-I immunoassays has introduced endogenous antibodies as a new source of interference. In general this goes unnoticed and might lead to unnecessary diagnostic and therapeutic interventions.

Case: A 56-year-old man was referred with a decline in physical performance, unexplained osteopenia and weight loss of 3 kg over the past 8 months. Although clinical signs and symptoms were unremarkable, laboratory results pointed to secondary hypothyroidism and secondary hypogonadism. In addition, the serum total IGF-I level (CLIA; Diagnostics Products Corporation (DPC)) was in the low normal range. Two GH stimulation tests were performed, but these tests did not support the diagnosis GHD. Moreover, IGF-I bioactivity measured by the Kinase Receptor Activation Assay (IGF-I KIRA) was normal. Interference of heterophilic antibodies was considered. After pretreatment with specific heterophilic blocking tubes (HBT), which contain blocking reagents to eliminate heterophilic antibodies, serum free thyroxin, testosterone, and IGF-I levels turned out to be normal.

Conclusion: To our best knowledge, we here describe the first case in the literature of a patient with low serum total IGF-I levels due to interference from heterophilic antibodies in the used IGF-I immunoassay. When confronted with low IGF-I levels, which do not fit the clinical picture, interference of heterophilic antibodies should be considered in the differential diagnosis.

INTRODUCTION

In 1977 the first radioimmunoassay for insulin-like growth factor I (IGF-I) was described by Furlanetto et al. [1]. Since then, IGF-I immunoassays have been used to screen patients for the presence of growth hormone deficiency (GHD) or acromegaly. However, when IGF-I immunoassays were applicated in larger groups of patients, many problems were encountered when these assays were used for these purposes [2]. From the start IGF-I immunoassays have suffered from analytical problems including their reproducibility and accuracy for the detection of IGF-I in human serum.

Almost all IGF-I present in the circulation is bound to so-called insulin-like growth factor binding proteins (IGFBPs) [3]. As IGFBPs may interfere with specific antibody binding to IGF-I, it is considered essential for a high quality IGF-I immunoassay that binding protein interference is eliminated [2]. Therefore, in virtually all IGF-I immunoassays, techniques such as acid-ethanol extraction or saturation with IGF-II are implemented in order to remove or to neutralize interference by these IGFBPs [4]. Similarly, since variability in IGF-I measurements can lead to erroneous conclusions, it is important that antibodies utilized in IGF-I immunoassays both have high specificity and affinity for IGF-I [5].

Measurement of circulating total IGF-I levels is considered highly sensitive and specific for the diagnosis of acromegaly [6-8](6-8). A low circulating IGF-I level in an adult with additional pituitary hormonal deficiencies, is considered highly specific for GH deficiency. However, even when the most reliable IGF-I immunoassays are used, only in 57% of severe GHD patients (defined as a peak GH of less than 2.5 ng/mL in insulin tolerance testing) circulating IGF-I levels are below the 95% confidence interval of the normal range in a healthy population [5].

The introduction of immunoradiometric (IRMAs) and chemiluminescence (CLIAs) IGF-I immunoassays has provided enhanced speed and sensitivity for IGF-I measurement and made it possible to conduct convenient total IGF-I determination without binding-protein interference [5]. As a consequence, the clinical use of IRMAs and CLIAs resulted in a reduction of the number of patients who require provocative GH testing. However, these new assay technologies may have introduced a new clinical problem when using IGF-I for the diagnosis and evaluation of patients with GHD. We here illustrate this by a case history.

METHODS

This study was performed after obtaining patient 's written informed consent for use of his blood samples for further investigations.

Serum total IGF-I was measured by a solid-phase, enzyme-labeled chemiluminescent immunometric assay (CLIA) Immulite 2000 IGF-I supplied by Siemens Medical Solutions Diagnostics (Los Angeles, USA). In this assay a murine anti-IGF-I coated to a solid phase (bead; capture antibody) and a polyclonal rabbit anti-IGF-I conjugated to alkaline phosphatase (detection antibody) are used. According the manufacturer IGFBP interferences are circumvented by an on-board predilutional and acidification step (pH<3.1) to separate IGF-I and IGFBP-3. Once the sample is neutralized again (i.e. restoring pH to 7), IGFBP binding sites are blocked by adding an excess IGF-II in order to prevent reaggregation of IGF-I and IGFBP-3. The within-assay coefficient of variation of the Immulite 2000 IGF-I varies between 3-6%. The level of IGF-I was expressed as nanomoles per liter (nM) and was compared with the age-specific normative range values for IGF-I. Reference values for IGF-I have been published in a previous report [9].

Immulite 2500 assays were used to measure the following hormonal parameters: Free thyroxin (Free T4) (by a solid-phase, chemiluminescent, competitive analog immunoassay), Thyroid Stimulating Hormone (TSH) (by a solid-phase, two site chemiluminescent, immunometric assay), Follicle Stimulating Hormone (FSH) (by a solid-phase, two site chemiluminescent, immunometric assay), Luteinizing Hormone (LH) (by a solid-phase, two site chemiluminescent, immunometric assay), testosterone (by a solid-phase, competitive chemiluminescent enzyme immunoassay), cortisol (by a solid-phase, two site competitive chemiluminescent enzyme immunoassay) and compared with the respective normative range values for these hormones. All blood samples assayed but cortisol were re-measured after pretreatment with specific heterophilic blocking tubes (HBT) (Scantibodies Laboratory Inc, Santee, Ca). The HBT contains a unique blocking reagent composed of specific binders, which inactivate heterophilic antibodies, and allows for the rapid and simple elimination of false positive heterophilic interference in plasma or serum for sandwich immunoassays. Each tube contains enough reagent to inactivate the heterophilic antibodies in 500 µL of sample. The reagent is in the form of a lyophilized pellet at the bottom of the tube. One tube was used for each sample. After pipetting each sample into the bottom of a tube, and mixing the sample with the reagent, each sample was incubated for one hour in HBT at room temperature (18º - 28º C) according the manufacturers' instructions (www.scantibodies.com).After pretreatment with

HBT blood samples were considered free from heterophilic antibody interference. GH was measured by the Immulite 2500 using a solid-phase, two sites chemiluminescent, immunometric assay.

IGF-I Kinase Receptor Activation Assay (IGF-I KIRA)

Circulating IGF-I bioactivity was measured using an in-house IGF-I kinase receptor activation assay (IGF-I KIRA) as was previously described [10,11]. The methodology of this latter assay is based on another principle than that of IGF-I immunoassays. Briefly, IGF-I binding to the IGF-I receptor (IGF-IR) results in autophosphorylation of tyrosine residues located within the intracellular kinase domain, being the first step in the intracellular signal cascade. The IGF-I KIRA uses human embryonic renal cells stably transfected with cDNA of the human IGF-IR gene (293 EBNA IGF-IR) and quantifies phosphorylation of tyrosine residues of the IGF-IR to assess IGF-I bioactivity. Since the IGF-I KIRA is based on a totally different method than IGF-I immunoassays, it has no susceptibility to interference by heterophilic antibodies.

CASE HISTORY

A 56-year-old male patient was referred to the outpatient clinic with a medical history of a decline in physical performance, forgetfulness, unexplained osteopenia and loss of weight (about 3 kg over the past 8 months). The patient had no history of previous exposure to animal proteins, but in his childhood he had lived on a farm and was exposed to diverse animal species. Although laboratory results pointed to secondary hypothyroidism and secondary hypogonadism (Table 1), these diagnoses were not clearly supported by clinical

Table 1. Results of hormonal parameters comparing the original non pretreatment assay results with the results obtained after pretreatment with HBT*

	Normal Range	Primary Result	After HBT*
Free T4 (pmol/L)	9–23	<3.9	20.0
TSH (U/L)	0.35–3.50	0.50	0.35
Testosterone (nmol/L)	12–33	<0.70	22.0
LH (U/L)	0.8–7.6	1.3	1.5
FSH (U/L)	0.7–11.1	3.0	1.9
Total IGF-I (nmol/L)	8–24	9.5	16.1
IGF-I KIRA (pmol/L)	191–566	374	372
Cortisol (nmol/L)	> 500	650	ND

*After using Heterophilic Blocking Tubes (HBT, Scantibodies Laboratory Inc, USA); ND=not determined

signs and symptoms. In addition, the serum total IGF-I level was within the lower normal range and serum cortisol level excluded adrenal insufficiency (Table 1). Two GH stimulation tests were performed, but their results did not support GHD (after growth hormone-releasing hormone (GHRH) stimulation: peak serum GH: 6.4 µg/L (normal > 5.0 µg/L); after arginine plus GHRH: peak serum GH 17.3 µg/L (normal > 16.5. µg/L)). Magnetic resonance imaging (MRI) of the brain demonstrated a normal pituitary gland with no abnormalities.

Interference with heterophilic antibodies was considered as explanation for the anomalous laboratory results. After pretreatment with specific HBT, serum free T4 and testosterone turned out to be normal (Table 1). Circulating IGF-I bioactivity measured by the Kinase Receptor Activation Assay (IGF-I KIRA) prior to HBT treatment was within the normal range. Therefore we also considered interference of heterophilic antibodies as explanation for the low normal total IGF-I levels. After pretreatment with HBT, total IGF-I level also turned out to be normal (Table 1). When we repeated the IGF-I KIRA measurement in HBT treated serum, there was no evidence for interference of heterophilic antibodies in the IGF-I bioassay (see Table 1).

DISCUSSION

Since there was an obvious mismatch in our patient between clinical findings and laboratory results for free T4 and testosterone, we suspected that the values of both hormones were falsely low due to interference with heterophilic antibodies (Figure 1). Our suspicion was confirmed after pretreatment of the blood samples with HBT, since levels were in the normal range afterwards.

The IGF-I KIRA was not susceptible to interference by heterophilic antibodies. As IGF-I bioactivity measured by the IGF-I KIRA proved to be around the median of the normal range, interference of heterophilic antibodies was also considered as an explanation for the low normal serum total IGF-I levels as measured in the Immulite assay. Indeed, when serum of our patient was pretreated with HBT, the total IGF-I level also turned out to be around the median of the age-specific normal range.

Our results demonstrate that the Immulite assays for total IGF-I, free T4 and testosterone are susceptible to interference by heterophilic antibodies. Although in the manufacturer 's manual of these assays it is mentioned that this type of interference may cause anomalous results, to our knowledge this is the first report in literature that describes this phenomenon for an IGF-I immunoassay.

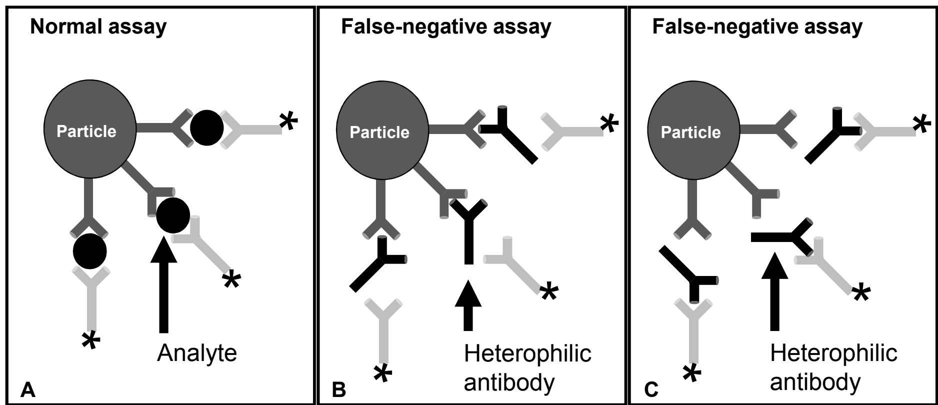

Figure 1. Schematic overview of immunoassay disturbances by heterophilic antibodies. (**A**) Normal immunoassay. In most reports about heterophilic antibody interference in immunoassays an overestimation of the analyte is described. However, false-negative assay results have been reported. Two mechanisms have been described: (**B**) binding and blocking the solid-phase capture antibody (dark grey), or (**C**) binding and blocking the labeled antibody reagent (light grey) by the heterophilic antibody (black). The mechanism being responsible for the false-negative assay results obtained in our patient is unknown.

Interference by circulating endogenous antibodies in immunoassays is as old as the technique itself and is a potential problem for all assays employing immunometric assay methods [12]. Assays using either polyclonal or monoclonal antibodies may be affected and assays previously reported to be affected by heterophilic antibody interference include those for TSH, T3, T4, PSA, testosterone and LH [13-16]. Interference from circulating antibodies is specific for an individual patient, and these proteins have the potential to interfere in an unpredictable way with some (but not necessarily all) immunoassay tests used [17]. In accordance with this latter possibility, we did not find any indication for heterophilic antibody interference in the serum TSH, LH and FSH measurements of our patient.

Most papers about heterophilic antibody interference in immunometric assays report an overestimation of the analyte, leading to lower concentrations of the analyte after blocking treatments [18]. In these false positive assay outcomes, heterophilic antibodies bind both the solid phase capture antibody as well as the labeled antibody reagent, linking them together. So it appears as if the analyte in this sample is present and causing the complex. False negative assay results like that in our patient have also been reported in literature. Two mechanisms have been described: binding and blocking the solid-phase capture antibody, or binding and blocking the labeled antibody

reagent. However, it is unknown which mechanism was responsible for the falsely negative results in our patient.

Circulating anti-animal antibodies can arise from iatrogenic and noniatrogenic causes [19]. Administration of mouse antibodies for therapeutic and imaging purposes may lead in many patients to the formation of human anti-mouse antibodies (HAMAs) [20,21]. Such antibodies have strong affinity for mouse IgG and are a major cause of immunoassay interference [20].

Kaplan and Levinson distinguish specific HAMAs from heterophilic antibodies. [22,23]. Heterophilic antibodies are human antibodies of low avidity that show broad activity against immunoglobulins from several species. These antibodies can arise as a result of occupational exposure of farm workers, from activities such as keeping pets, ingesting animal antigens in food (like cow 's milk), vaccination, infection, or even blood transfusion [19,24,25] and may persist for several years [26]. The published estimates of the presence of heterophilic antibodies in the normal population vary between 1–80% [13,19].

Non-linear sample dilution has been recognized as a method to identify samples with interference [12,27]. However, this is not universally successful. If sample dilution happens to result in apparent elevation in the same range as the undiluted sample, interference may not be detected [28].

There are several methods to reduce the effects of heterophilic antibodies. Addition of serum of non-immune animal species will reduce the effect of heterophilic antibodies in many cases [13]. This method works best when the species of the human anti-animal antibody is matched with non-immune animal serum [13]. Therefore it is routine practice for assay manufacturers to incorporate serum of non-immune animal species in the reagents of most methods. Bovine serum has been shown to be more efficient than murine serum [29]. It has been suggested that heterophilic antibodies in most patients are bovine immunoglobulins arising from the ingestion of meat and milk [13].

Polymerized IgG, chemical aggregation and heat aggregation are other methods that have been shown to improve the ability of non-specific antibodies to reduce interference [13,28].

Several blocking reagents are commercially available [18]. In our study we have used HBT, which contains lyophilized specific binders to inactivate animal antibodies [19]. A difference between values for the treated and untreated specimens is interpreted as evidence for heterophilic antibody interference. It is known that this method is not appropriate for every immunoassay. However, after pretreatment with HBT serum total IGF-I concentration in our patient turned to be like IGF-I bioactivity around the median of the normal range,

suggesting that HBT tubes is a suitable technique to eliminate heterophilic antibody interference in the Immulite 2000 IGF-I assay.

In conclusion, the Immulite total IGF-I assay method, in its present form, is susceptible to interference by heterophilic antibodies. Physicians should be aware of this phenomenon because results of circulating total IGF-I levels are often used as a basis for further diagnostic and therapeutic decisions.

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Chapter 6

IGF-I Bioactivity in an Elderly Population: Relation to Insulin Sensitivity, Insulin Levels and the Metabolic Syndrome

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Accepted for publication: Diabetes, 2009

ABSTRACT

Background: There is a complex relationship between Insulin-like Growth Factor-I (IGF-I), IGF-binding proteins, growth hormone, and insulin. The IGF-I kinase receptor activation assay (IGF-I KIRA) is a novel method for measuring IGF-I bioactivity in human serum. We speculated that determination of IGF-I bioactivity might broaden our understanding of the IGF-I system in subjects with the metabolic syndrome.

Objective: To investigate whether IGF-I bioactivity was related to insulin sensitivity and the metabolic syndrome.

Research Design and Methods: Cross-sectional study embedded in a random sample (1036 elderly subjects) of a prospective population-based cohort study. IGF-I bioactivity was determined by the IGF-I KIRA. Categories of glucose (in)tolerance were defined by the 2003 ADA criteria. Insulin sensitivity was assessed by the homeostatic model assessment (HOMA2). The ATP-III definition of the metabolic syndrome was used.

Results: In subjects with normal fasting glucose (NFG) and impaired fasting glucose (IFG) IGF-I bioactivity progressively rose with increasing insulin resistance (IR) and peaked at fasting glucose levels just below 7.0 mmol/L. In subjects with diabetes, however, mean IGF-I bioactivity dropped and was significantly lower compared to subjects with NFG and IFG.

Mean IGF-I bioactivity peaked when 3 criteria of the metabolic syndrome were present and then declined significantly when 5 criteria of the metabolic syndrome were present.

Conclusions: We observed that IGF-I bioactivity was related to insulin sensitivity, insulin levels, and the metabolic syndrome. Our study suggests that there exists a 'Starling curve for IGF-I bioactivity'. This observation contrasts with previous results obtained by measuring circulating total IGF-I concentrations.

INTRODUCTION

A complex relationship exists between insulin-like growth factor-I (IGF-I), IGF-binding proteins (IGFBPs), growth hormone (GH), and insulin. GH exerts potent effects on intermediary metabolism, amongst which is the ability to antagonize insulin actions [1]. IGF-I directly inhibits insulin secretion from pancreatic beta cells while it increases insulin sensitivity by GH-dependent and –independent mechanisms [2].

The relationships are even more complex: insulin appears to be necessary for normal liver GH responsiveness, probably by maintaining liver GH receptor levels [3]. However, chronic hyperinsulinemia reduces GH receptor expression and signaling in the liver [4,5].

Unlike insulin, the majority of circulating IGF-I is bound to six IGFBPs which are important in determining IGF-I availability and activity [6].

Recently, the IGF-I specific kinase receptor activation assay (IGF-I KIRA) was developed to determine circulating IGF bioactivity [7,8]. This bioassay determines IGF-I bioactivity by quantification of intracellular receptor autophosphorylation upon IGF-I binding. Unlike IGF-I immunoassays, the IGF-I KIRA takes into account the modifying effects of IGFBPs on the interaction between IGF-I and the IGF-I receptor (IGF-IR) [9]. The aim of the present study was to investigate whether IGF-I bioactivity was related to insulin sensitivity, insulin levels, and the metabolic syndrome (MS) in an elderly population-based cohort.

METHODS AND MEASUREMENTS

The Rotterdam study is a prospective population-based cohort study investigating incidence and risk factors of cardiovascular, neurodegenerative, locomotor, and ophthalmologic diseases in elderly people. Objectives and methods have been described in detail elsewhere [10]. Baseline measurements took place in 1993. Follow-up examinations were done in 1993–94, 1997–99, and 2002–04. For the present study blood samples were drawn and examinations were conducted at the third survey (1997-1999). The medical ethics committee of the Erasmus Medical Center approved the study and written informed consent was obtained from all participants.

Only for participants who were not known to have diabetes, fasting blood samples were obtained in the morning after an overnight fast. Fasting plasma glucose (FPG) was determined using the Hexokinase method (Boehringer

Mannheim, Mannheim, Germany). The ADA-2003 criteria were used to classify categories of glucose tolerance: normal fasting glucose (NFG; FPG <5.6 mmol/l), impaired fasting glucose (IFG; FPG 5-6–7.0 mmol/l) and diabetes (DM) (FPG ≥7 mmol/l) [11]. Serum total cholesterol and HDL-C were determined using an automatic enzymatic procedure (Boehringer Mannheim). Serum triglycerides were analyzed by Triglyceride GPO-PAP (Roche Diagnostics, Mannheim Germany). Insulin was measured with an electrochemiluminescence immunoassay (Roche Diagnostics; intra- and interassay CV<3.7%; the manufacturer indicates cross-reactivity of 20% with des-31,32-proinsulin). Sitting blood pressure was measured using a random-zero sphygmomanometer and the mean of two readings was used for analyses. Height and weight were measured while wearing indoor clothes without shoes.

Circulating IGF-I bioactivity was measured using an in-house IGF-I kinase receptor activation assay (IGF-I KIRA) (intra-assay CV: 5.2%; interassay CV: 12.2%) [8,12]. IGF-I bioactivity was measured in 1050 blood samples, randomly selected from 3792 individuals, who had participated in the third survey. IGF-I bioactivity was successfully measured in 1036 out of 1050 subjects. Fourteen subjects were excluded from analysis since the measurement of IGF-I bioactivity did not repeatedly pass defined acceptance criteria (intra-assay CV <10%).

The updated Homeostasis Assessment Model (HOMA-2) was used to assess insulin sensitivity (%S), HOMA-IR (insulin resistance), which is the reciprocal of %S, and beta cell function (%B) from pairs of fasting glucose and insulin levels [13]. The Adult Treatment Panel III (ATP-III) criteria were used to diagnose the MS [14].

STATISTICAL ANALYSIS

Characteristics of the study population are presented as mean with 95% CI. Statistical analyses were confined to those subjects in which IGF-I bioactivity was determined. Linear regression analyses were used to assess relationships of IGF-I with age. Pearson's correlation coefficients were calculated to assess (age-adjusted) associations between variables. Univariate analyses of covariance were used to compare variables stratified by deciles of HOMA-IR, adjusted for age, gender, BMI and anti-diabetic therapy when appropriate. Analyses in which values were logarithmically transformed yielded similar results to those with untransformed data. Because interpretation of logarithmic data is difficult, non-transformed data are presented with P-values obtained after logarithmic transformation of data. In 904 out of 1036 subjects (87%) all

parameters of the MS were available; 132 subjects had one or more missing values and were therefore excluded from statistical analyses concerning the MS.

A two-sided probability of P-value <0.05 was considered statistically significant. All analyses were performed with SPSS for Windows v.15 (SPSS Inc., 2001, Chicago, IL).

RESULTS

Table 1 shows clinical characteristics of the study population. Based on 2003 ADA criteria 697 subjects (69.7%) had NFG, 165 subjects (16.3%) had IFG and 153 subjects (15.1%) had DM. Sixty-two subjects (6%) were known with DM of which 19 (1.8%) were treated with only diet, 39 (3.8%) with oral antidiabetics, 3 (0.3%) were on insulin therapy and 1 (0.1%) subject was treated with oral anti-diabetics and insulin.

Mean IGF-I bioactivity was significantly lower in men than in women (men: 168.9 ± 2.5 pmol/L (mean ± SE) vs. women: 187.0 ± 3.1 pmol/L ($p < 0.001$,

Table 1. Baseline characteristics of the study population (1036 subjects; 451 males)

	Mean	(95% CI)
Age (yrs)	72.4	(62.8 – 86.3)
Height (m)	1.66	(1.52 – 1.82)
Weight (kg)	74.6	(55.6 – 97.2)
BMI (kg/m^2)	26.9	(21.5 – 33.9)
Waist (cm)	93.8	(75.2 – 111.8)
Men	97.5	(82.4 – 112.8)
Women	90.8	(72.8 – 110.0)
Systolic BP (mm Hg)	143.9	(112.0 – 182.0)
Diastolic BP (mm Hg)	75.3	(57.2 – 95.9)
Fasting glucose (mmol/L)	6.0	(4.7 – 8.7)
Fasting insulin (pmol/L)	176.7	(28.0 – 212.3)
HOMA-S (%)*	77.3	(22.6 – 161.0)
HOMA-IR*	1.88	(0.60 – 4.40)
HOMA-B (%)*	100.4	(45.7 – 174.9)
Total Cholesterol (mmol/L)	5.81	(4.28 – 7.45)
HDL-cholesterol (mmol/L)	1.38	(0.87 – 2.06)
Triglycerides (mmol/L)	1.48	(0.70 – 2.75)
IGF-I bioactivity (pmol/L)	180.3	(105.0 – 277.3)

BMI=body mass index; BP = blood pressure; HDL = high-density lipoprotein.
*HOMA = Homeostasis Assessment Model (HOMA-2). This model was used to estimate insulin sensitivity (HOMA-S (%)), insulin resistance (HOMA-IR) and beta cell function (HOMA-B (%)).

after adjustment for age and BMI). IGF-I bioactivity decreased significantly with age in men but not in women (men: β = -0.590 pmol/L/yr, p = 0.011; women: β= +0.647 pmol/L/yr, p = 0.119).

There was no association between IGF-I bioactivity and fasting insulin (r=0.049, p = 0.206). However, when analysis was confined to subjects with NFG, IGF-I bioactivity was significantly and positively related to fasting insulin (r=0.096, (age-adjusted) p=0.036). IGF-I bioactivity did not differ between diabetic subjects with and without oral antidiabetics and/or insulin therapy (169 (± SE 8.7) pmol/L vs.179 (± 1.8) pmol/L, respectively; (p = 0.47)).

After adjustment for age, overall IGF-I bioactivity was inversely related to insulin sensitivity (r = -0.113, p = 0.003), while the relationship between IGF-I bioactivity and B-cell function was borderline significant (r = 0.065, p = 0.088). When subjects were stratified to deciles of insulin resistance (HOMA-IR), mean FPG and insulin levels progressively rose per increasing HOMA-IR decile (Figure 1 A-B). Mean FPG levels in the 10th decile (7.52 ± 0.13 mmol/L) met diagnostic criteria for DM. For mean IGF-I bioactivity there was a progressive increase with increasing IR up to and including the 9th decile ($P_{for\ trend}$

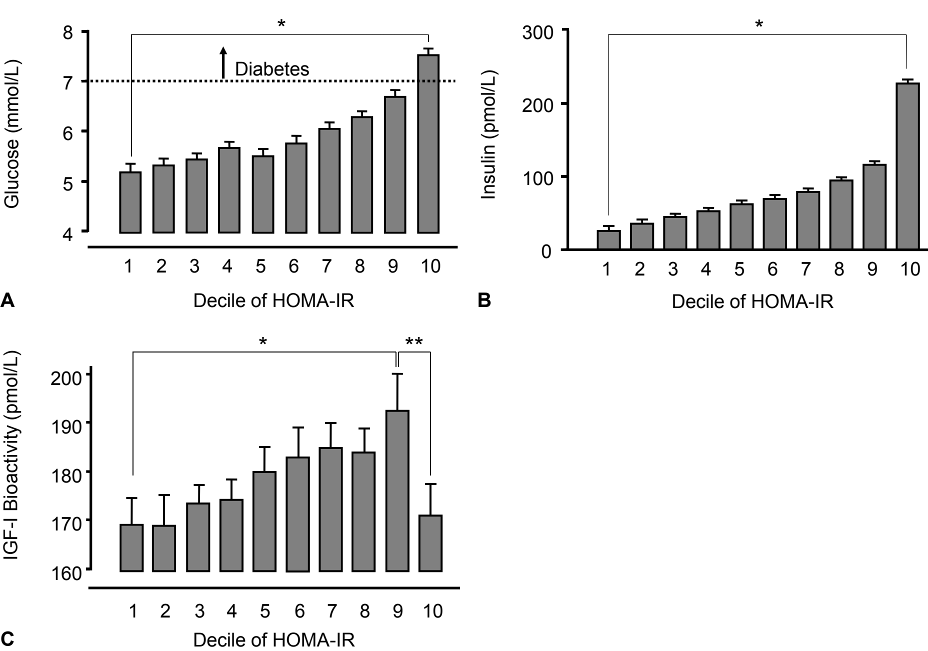

Figure 1. Mean (± SEM) fasting glucose (**A**), mean (± SEM) fasting insulin (**B**) and mean IGF-I bioactivity (± SEM) (**C**) stratified per decile of HOMA-IR. Statistical differences between deciles were calculated after adjustment for age, BMI and gender.
(**A**) * $P_{for\ trend} < 0.001$; (**B**) * $P_{for\ trend} < 0.001$; (**C**) * $P_{for\ trend} < 0.001$ and ** P = 0.005.

= 0.003) (Figure 1C). However, IGF-I bioactivity significantly dropped in the 10th decile compared to the 9th decile (Figure 1C, p = 0.005).

When analyses were repeated after excluding diabetics on medical therapy, the outcome was unaffected: IGF-I bioactivity rose significantly up to and including the 9th decile of HOMA-IR ($P_{for\ trend}$ = 0.006) and it decreased significantly in the 10th decile (9th vs. 10th decile: p = 0.010).

Mean HOMA-IR increased significantly per increasing number of components of the MS ($P_{for\ trend}$ < 0.001, adjusted for age and gender, Figure 2A). As expected, mean FPG levels rose progressively with an increasing number of components of the MS (Figure 2B). Mean fasting insulin levels were significantly higher in subjects with 5 components of the MS than in subjects with 4 or less components of the MS (Figure 2C). Up to a point, IGF-I bioactivity was also directly related to number of components of the MS, peaking when three components were present, and the diagnosis of the MS could be made.

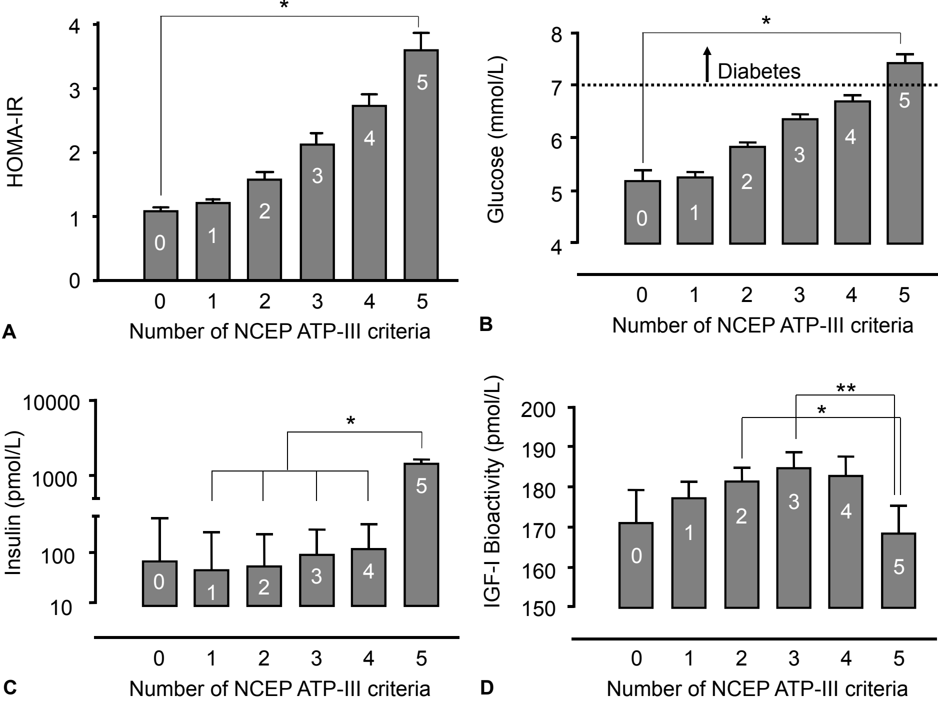

Figure 2. Relationship between the number of components of the metabolic syndrome and HOMA-IR (**A**), mean (± SEM) fasting glucose (**B**), mean (± SEM) fasting insulin (**C**) and mean (± SEM) IGF-I bioactivity (**D**).

(**A**)* $P_{for\ trend}$ < 0.001; (**B**) * $P_{for\ trend}$ < 0.001; (**C**) * Bar 5 vs. Bar 1,2,3 or 4: P < 0.005; (**D**) * P = 0.03 and ** P = 0.04.

However, in subjects with more than 3 components of the MS IGF-I bioactivity decreased significantly (mean IGF-I bioactivity in subjects with 3 vs. 5 components: p = 0.04, adjusted for age and gender) (Figure 2D). When the latter analysis was restricted to subjects with FPG levels <7.0 mmol/L an identical pattern was observed (data not shown).

DISCUSSION

The main finding of our study was that circulating IGF-I bioactivity progressively rose with increasing severity of insulin resistance and hyperinsulinemia. At FPG levels just below 7.0 mmol/L, mean IGF-I bioactivity reached a plateau. However, in subjects with DM mean IGF-I bioactivity was significantly lower than in subjects with NFG and IFG.

Strong evidence exists that insulin is essential for GH stimulation of hepatic IGF-I production [15]. Insulin deficiency results in a decreased liver GH receptor expression, which can be reversed by insulin administration [3,16]. On the other hand, patients with type 2 DM often exhibit reduced circulating total IGF-I levels. One explanation for reduced circulating total IGF-I levels in type 2 DM is that chronic hyperinsulinemia induces GH receptor resistance [4]. Under experimental conditions, hyperinsulinemia reduces not only GH receptor expression but also GH signaling at both receptor and postreceptor levels [4,5]. Although the direction of cause and effect may be difficult to assess in a cross-sectional study, this opens the possibility that the observed decline in IGF-I bioactivity in diabetic subjects was related to hyperinsulinemia-induced hepatic GH resistance.

Circulating IGFBP-1 has an inverse relationship with insulin [17]. Circulating IGFBP-1 rise and fall in response to hepatic portal blood insulin [17]. It has been suggested that low IGFBP-1 concentrations in insulin-resistant/hyperinsulinaemic states increase IGF-I bioactivity in an attempt to stimulate glucose uptake in muscle and other insulin-resistant cells. From the other hand: data in animals have showed that increased levels of IGFBP-1 can both inhibit IGF-I action and induce hyperglycaemia [17].

In a recent prospective study IGFBP-1 levels initially decreased when glucose tolerance deteriorated, but actually increased during follow-up in those individuals, who developed diabetes, which probably reflects "resistance" to portal blood insulin [18]. Although we did not measure IGFBP-1 levels in our study, the decline in mean IGF-I bioactivity in subjects with FG levels > 7.0

mmol/L may be thus also directly related to increasing inhibitory effects of elevated IGFBP-1 levels on IGF-I bioactivity.

Several epidemiological studies have linked low circulating total IGF-I and increased (total) IGF-I/IGFBP-1 ratios to an increased risk of the MS [19-23]. An increased total IGF-I/IGFBP-1 ratio has been proposed as a surrogate marker of increased IGF-I bioactivity [24]. This is probably a compensatory mechanism to maintain normal glucose tolerance, since raised IGF-I bioactivity will improve insulin sensitivity [25]. High IGF-I bioactivity may suppress GH secretion as part of a negative feedback system and thereby induce lower total IGF-I levels [23]. This may explain why low total IGF-I levels have been previously reported to be associated with an increased risk of the MS [22].

Our study also suggests that during further progression of the MS IGF-I bioactivity declines. As individuals with the MS are chronically exposed to high circulating insulin levels, the rise in IGF-I bioactivity (see Figure 2D) in subjects with 1-3 components of the MS, may be due to an insulin-mediated suppression of IGFBP-1 levels, as discussed before. However, in subjects with more than 3 components of the MS, IGF-I bioactivity significantly declined (Figure 2D). The lower IGF-I bioactivity in these latter individuals may be a direct consequence of the development of hepatic insulin resistance (manifested by a relative increase of IGFBP-1) and hyperinsulinemia-induced GH resistance, as discussed above.

A limitation of our study is that no comparisons were made between circulating IGF-I bioactivity and total IGF-I measurements. However, we previously found a weak correlation between circulating total IGF-I levels and IGF-I bioactivity suggesting that the IGF-I KIRA produces information about the IGF-I system, which essentially differs from that obtained by IGF-I immunoassays [12]. Moreover, no information is yet available on whether 10-years sample storage at -80 degrees affects measurements of IGF-I bioactivity. However, because all samples were analyzed after an equal period of storage, any variable effect of storage time is likely removed.

Another point is that we did not perform an OGTT. Therefore, it is not clear how many subjects had impaired glucose tolerance or even diabetes that was not assessed by fasting glucose alone. In addition, HOMA-IR will not always reflect insulin sensitivity accurately in subjects with differing beta-cell function.

In conclusion, our study suggests that an individual's insulin sensitivity and circulating insulin levels directly modulate IGF-I bioactivity. When IR becomes apparent, but glucose levels are still within the normal range, there is an initial rise in circulating IGF-I bioactivity. However, when glucose tolerance rises

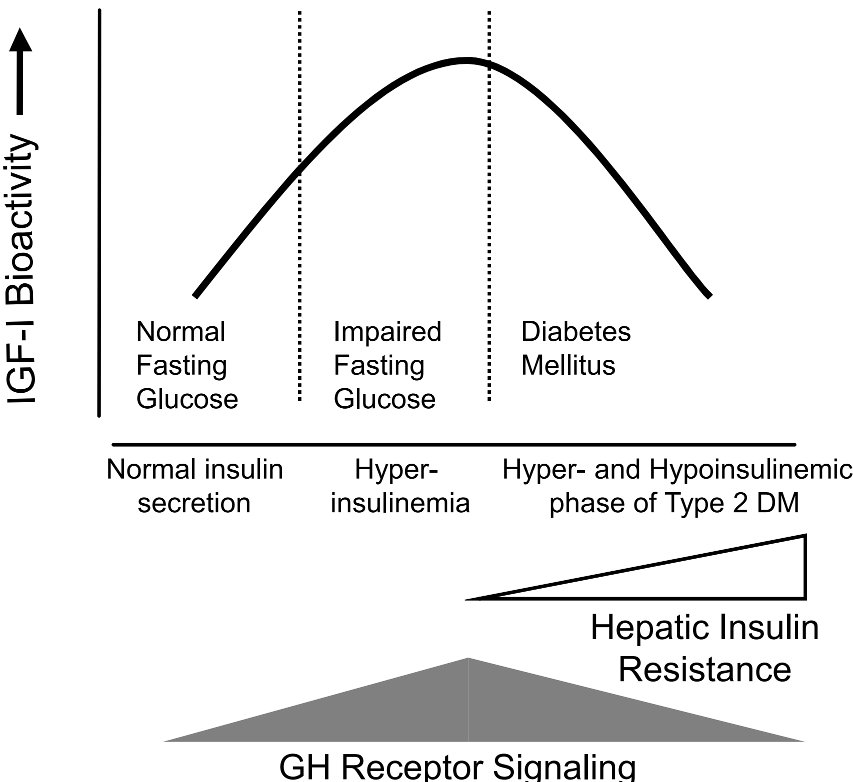

Figure 3. The 'Starling curve for IGF-I Bioactivity'. When glucose levels are within the normal range and insulin resistance increases, there is an initial compensatory hyperinsulinemia and rise in circulating IGF-I bioactivity. When glucose tolerance has increased to the point where impaired fasting glucose levels occur, IGF-I bioactivity reaches a plateau. When blood glucose levels rise further into a range indicating overt type 2 DM, GH sensitivity progressively decreases while hepatic insulin resistance progressively increases. Both mechanisms contribute to a progressive reduction of IGF-I bioactivity.

to the point where IFG occurs, IGF-I bioactivity reaches a plateau. Finally, when blood glucose levels have risen into overt type 2 DM, circulating IGF-I bioactivity progressively declines. IGF-I bioactivity seems also related to the MS. Our study suggests that there exists a 'Starling curve for IGF-I bioactivity' (Figure 3).

ACKNOWLEDGEMENTS

The authors thank Dr. Patric Delhanty for his contribution to the manuscript. This study was in part supported by Zon-MW, Organization for Health Research and Development the Netherlands, (948-00-003). Zon-MW had no involvement in the study design, in the collection, analysis, and interpretation of data.

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Chapter 7

IGF-I Gene Polymorphisms and Circulating Total IGF-I but not IGF-I Bioactivity predict Risk on Myocardial Infarction/Angina Pectoris in Men: Findings from the Rotterdam Study

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Article in preparation

ABSTRACT

Background: IGF-I may be involved in the pathogenesis of CVD. We speculated that determination of circulating IGF-I bioactivity is more informative than levels of immunoreactive total IGF-I.
Objective: We examined the associations between comon genetic variations in the IGF-I gene in relation to levels of total and bioactive IGF-I and to risk on MI/AP.
Design and Measures: Analyses included 5970 subjects of the Rotterdam study, successfully genotyped for 18 SNPs of the IGF-I gene at baseline. Total IGF-I was measured in 430 subjects at baseline and IGF-I bioactivity was measured in 1036 subjects at the third survey (1997-1998). The IGF-I gene was characterized into 6 haplotype blocks.
Results: Four hundred sixty eight subjects developed MI/AP during follow-up. Genetic variations in haplotype block 1 and block 2 were significantly related to total IGF-I in men and women. Genetic variations in haplotype blocks 3-6 of the IGF-I gene were related to circulating IGF-I bioactivity in men but not in women. Block 1 and block 2 of the IGF-I gene were also significantly associated with an increased risk for MI/AP in men but not in women. All these relationships remained significant after further adjustments. No relationships were observed between blocks 3-6 of the IGF-I gene and MI/AP.
Conclusions: We observed a gender-specific impact of genetic variations in the IGF-I gene on circulating total IGF-I levels and risk on MI/AP. Although gender-specific genetic variations of the IGF-I gene were also present for levels of circulating IGF-I bioactivity, these variations did not appear to substantially influence the risk on MI/AP.

INTRODUCTION

Twin studies have shown that at least 40% of the interindividual variability in total IGF-I levels is genetically determined [1,2]. Second, IGF-I may be involved in the pathogenesis of cardiovascular disease (CVD) [2-4]. In humans, low serum IGF-I has been associated with coronary artery disease (CAD) in both cross-sectional and prospective studies, but results are characterized by inconsistency [5-9]. The possible association between total IGF-I levels and risk of CVD may be not real, but due to measurement errors or other (unknown) confounders. In addition these associations may be the result of reverse causation, meaning that the relation between levels of total IGF-I and CVD could be the result of latent disease rather than IGF-I itself. Observational epidemiologic studies cannot help to resolve a definite answer on the role of IGF-I in the pathogenesis of CVD. However, 'Mendelian Randomization'- the assortment of genes from parents to offspring that occurs during gamete formation and conception'-is an epidemiological approach that is less susceptible to reverse causality and confounding [10]. This approach provides a natural experiment for testing causal associations between circulating IGF-I levels and CVD risk [11]. When a polymorphism of the IGF-I gene is related to different levels of circulating IGF-I, and when low levels of circulating IGF-I are indeed a causal risk for cardiovascular disease, then one would expect that individuals with an IGF-I genotype associated with low IGF-I levels have a higher risk to develop CVD.

The IGF-I gene contains a microsatellite polymorphism approximately 1 kb upstream from the IGF-I gene transcription site. This IGF-I gene polymorphism may directly influence IGF-I production. Previously we reported within the framework of the Rotterdam study that this IGF-I gene promoter polymorphism was associated with circulating total IGF-I levels [12] and that subjects with a myocardial infarction (MI) who are variant carriers of the IGF-I promoter polymorphism had an increased risk of mortality [13].

Insulin-like growth factor binding proteins (IGFBPs) may also modify IGF-I activity. So far, most knowledge about IGF-I is based on measurements of circulating total IGF-I levels by using specific immunoassays. Recently, a highly sensitive and IGF-I specific kinase receptor activation assay (IGF-I KIRA) was developed to determine IGF bioactivity in human serum [14,15]. Unlike IGF-I immunoassays, the IGF-I KIRA takes into account the modifying effects of (IGFBPs) on the interaction between IGF-I and the IGF-I –receptor. The IGF-I KIRA might be a new tool that could help broaden our understanding of the IGF-I system in humans, both in normal and pathological conditions like CVD.

Within the frame work of the Rotterdam study we studied whether IGF-I gene polymorphisms/haplotypes were related to circulating to risk on CVD, and whether these IGF-I gene polymorphisms/haplotypes were functionally related to circulating total IGF-I levels and/or IGF-I bioactivity

METHODS

The Rotterdam study is an ongoing prospective population-based cohort study investigating incidence and risk factors of cardiovascular, neurodegenerative, locomotor, and ophthalmological diseases in elderly people. The objectives and methods have been described in detail elsewhere [16]. The cohort includes 7.983 men and women aged 55 years and over (78% of the eligible population) living in a well-defined suburb of the city of Rotterdam, the Netherlands. The baseline measurements took place until 1993. Information was collected on age, gender, present health status and medical history and blood was drawn for DNA. Follow-up examinations were done in 1993–94, 1997–99, and 2002–04.

The medical ethics committee of the Erasmus Medical Center approved the study and written informed consent was obtained from all participants.

MEASUREMENTS

At the baseline examination, information concerning medical history, medication use, and smoking behavior was obtained. Height and weight were measured and body mass index (BMI) (in kg/m2) was calculated. Diabetes mellitus was defined as the use of glucose-lowering medication and/or random serum glucose level 11.1 mmol/L or over. Total serum cholesterol and high-density lipoprotein (HDL) cholesterol were determined with an automated enzymatic procedure. Blood pressure was measured, with the subject in sitting position, at the right upper arm with the use of a random zero sphygmomanometer. The same methods were used for collection of follow-up data.

Mean follow-up time since the start of the Rottterdam Study was 6.43 yrs (range 5.14-10.21) for participants, who were investigated at the third survey of the Rotterdam study (1997-1999). Information on angina pectoris (AP) was obtained at the third survey by using a Dutch version of the Rose Questionnaire [17].

IGF-I bioactivity was measured in blood samples drawn at the third survey of the Rotterdam study. Because of logistic and financial restrictions, measurement of IGF-I bioactivity was confined to about 1000 subjects. For the measurement of IGF-I bioactivity 1050 subjects were at randomly selected from 3792 individuals, who had participated in the third survey of the Rotterdam study and from whom blood was drawn. Fourteen subjects were excluded from analysis since the measurement of IGF-I bioactivity did not repeatedly pass defined acceptance criteria (intra-assay variation <10%). IGF-I bioactivity was finally reported for 1036 subjects. At baseline total IGF-I had been measured in a subsample from the first baseline cohort comprising of 430 subjects as was previously reported [12,18]. For the present study these IGF-I measurements were used in the analyses.

Total IGF-I immunoassay and IGF-I Kinase Receptor Activation Assay (IGF-I KIRA)

Total IGF-I was determined by a commercially available radioimmunoassay (RIA; Medgenix Diagnostics, Brussels, Belgium, with intra-assay and interassay variation of 6.1% and 9.9%) as previously stated (18). Circulating IGF-I bioactivity was measured using an in-house IGF-I kinase receptor activation assay as was previously described [14,15]. This assay uses human embryonic renal cells stably transfected with cDNA of the human IGF-IR gene (293 EBNA IGF-IR). After 24 h of culture in serum containing medium, cells were starved for 24 h in medium with 0.1% albumin. Thereafter, cells were stimulated at 37°C with either recombinant IGF-I standards (Austral Biologicals, San Ramon, CA) or 10-fold diluted serum samples for 15 minutes and then lysed. Crude lysates were transferred to a sandwich assay. For capture a monoclonal antibody directed against the human IGF-IR (MAD1, Novozymes Gropep, Adelaide, Australia) was used. As tracer a europium-labeled monoclonal anti-phosphotyrosine antibody (PY20, Perkin-Elmer Life Sciences) was used. Contents were read in a time-resolved fluorometer (Victor^2 multilabel counter, Perkin-Elmer, Groningen, The Netherlands). Assays were performed in 48 well plates. IGF-I standards, 2 control samples, and unknown serum samples were included in duplicate on every plate. Intra-assay CV was 5.2%. The inter-assay CVs were respectively 8.2% and 12.2% for the two control samples, which averaged (mean ± SD) 184.7 ± 15.1 pmol/L and 1039.6 ± 126.5 pmol/L (N = 121 plates), respectively. Circulating IGF-I bioactivity is expressed as pmol/L (to convert IGF-I bioactivity into mg/L, values have to should be divided by 131). Serum samples used in the IGF-I KIRA were kept at -80°C and had not been thawed before use. IGF-I KIRA measurements were performed 9-11 years after initial collection of sera.

Follow-up procedure for cardiovascular disease

Follow-up started at the baseline examination and for the present study lasted until January 1st, 2006. Information on fatal and non-fatal cardiovascular endpoints was obtained from general practitioners and letters and discharge reports from medical

specialists [19]. Reported events were coded according to the International Classification of Diseases, 10th edition (ICD-10) [20]. We defined incident CHD as MI, coronary artery bypass grafting (CABG), percutaneous transluminal coronary angioplasty (PTCA) and cardiac death. In identifying MIs, all available information, which included ECG, cardiac enzyme levels, and the clinical judgment of the treating specialist, was used.

SNP selection and genotyping for genetic characterization

Information on SNPs that were eventually used to cover genetic variance at the human IGF-I gene locus are shown in the supplementary Table S1. We screened publicly available databases (such as dbSNP) and initially collected data on 8 SNPs for the insulin-like growth factor-I gene. Tagging SNPs covered the promoter regions as well as the 3' UTR region of the IGF-I gene. In the promoter region we selected for our study the following SNPs: rs7965399, rs35765, rs35767 and rs17882264; and in the 3'UTR region: rs1520220, rs6220, rs6214 and rs6219. For five out of the eight selected SNPs statistically significant associations with total IGF-I has been reported in the literature [21-23].

Subsequently, microarray genotyping became available and was performed in the whole original Rotterdam study cohort with proper quality DNA samples (n=6449) using the Infinium II HumanHap550K Genotyping BeadChip® version 3 (Illumina, San Diego, USA). In total, 5974 samples met quality control inclusion criteria, as previously described [24]. We selected tagging SNPs for IGF-I based on HapMap Phase II, using the pairwise tagging mode [25]. We selected a minimal set of SNPs of the IGF-I gene, such that all alleles to be captured would be correlated at $r^2 > 0.99$ with a minor allele frequency > 5%. We surveyed genetic variation across the IGF-I gene from 20 kb upstream of transcription to 10 kb downstream.

SNP genotyping was accomplished using the GeneChip Human Mapping 500K Array Set HumanHap 550 v3.0 array (Illumina, San Diego, USA) for thirteen SNPs. TaqMan allelic discrimination assays were used for five other SNPs which were not available in Illumina platform (information on primers and probes is available in the supplementary Table (Table S2)). In total eighteen SNPs were selected for genetic characterization and these for were successfully genotyped in 5970 subjects (Table S1). Primer and probe sequences are

available on request. Individuals blinded to the individual's status performed all assays. All SNPs conformed to Hardy-Weinberg equilibrium (P<0.05).

Seven thousand and twelve participants of the Rotterdam study were previously genotyped for an IGF-I (CA repeat) promoter polymorphism [26]. Data from these prior studies were used to investigate whether there was linkage disequilibrium (LD) between this IGF-I gene promoter polymorphism and SNPs of the IGF-I gene.

Statistical analysis

Hardy-Weinberg equilibrium was tested with the chi-square test. We used t-tests (for continuous variables) and chi-square tests (for categorical variables) to compare baseline characteristics of subjects in both gender.

Total IGF-I was normal distributed. The natural log-transformed values for IGF-I bioactivity yielded a normal distribution. To investigate associations between genetic variations of IGF-I gene with circulating total IGF-I and IGF-I bioactivity, we used general linear regression models. The data were stratified by gender. All analyses were adjusted for age and gender except where otherwise mentioned. Additional adjustments were made (model 3) for systolic blood pressure (SBP), diastolic blood pressure (DBP), body mass index (BMI), diabetes mellitus (DM), total and HDL-cholesterol, prevalence of CVD, and current smoking.

Haplotype blocks for the IGF-I gene were defined using the confidence interval method of Gabriel et al. [27], which implemented in haploview [28]. LD between SNPs, as expressed by D′, was calculated in Haploview (29). (Figure 1). LD between neighboring SNPs with the IGF-I gene (CA repeat) promoter polymorphism was also calculated in Haploview [29].

We used the Haplo.Stats package running for R to estimate the IGF-I haplotypes [30] and to investigate associations of inferred haplotypes with circulating total IGF-I levels and IGF-I bioactivity [31]. This method assigns the probability for each haplotype pair in each individual and then models an individual's phenotype as a function of each inferred haplotype pair, weighted by their estimated posterior probability so as to account for haplotype ambiguity. We used the Haplo.glm function, which is based on a generalized linear model [32,33]. Regression coefficients were estimated with haplo.glm for each haplotypes per blocks. We restricted the analyses to haplotypes with an inferred frequency >5%. The haplotype (H1), which was the most frequent, was used as the reference group. Coefficients reflect differences in mean total IGF-I and back transformed natural logarithm (circulating IGF1 bioactivity) per copy relative to H1. To evaluate the associations between the

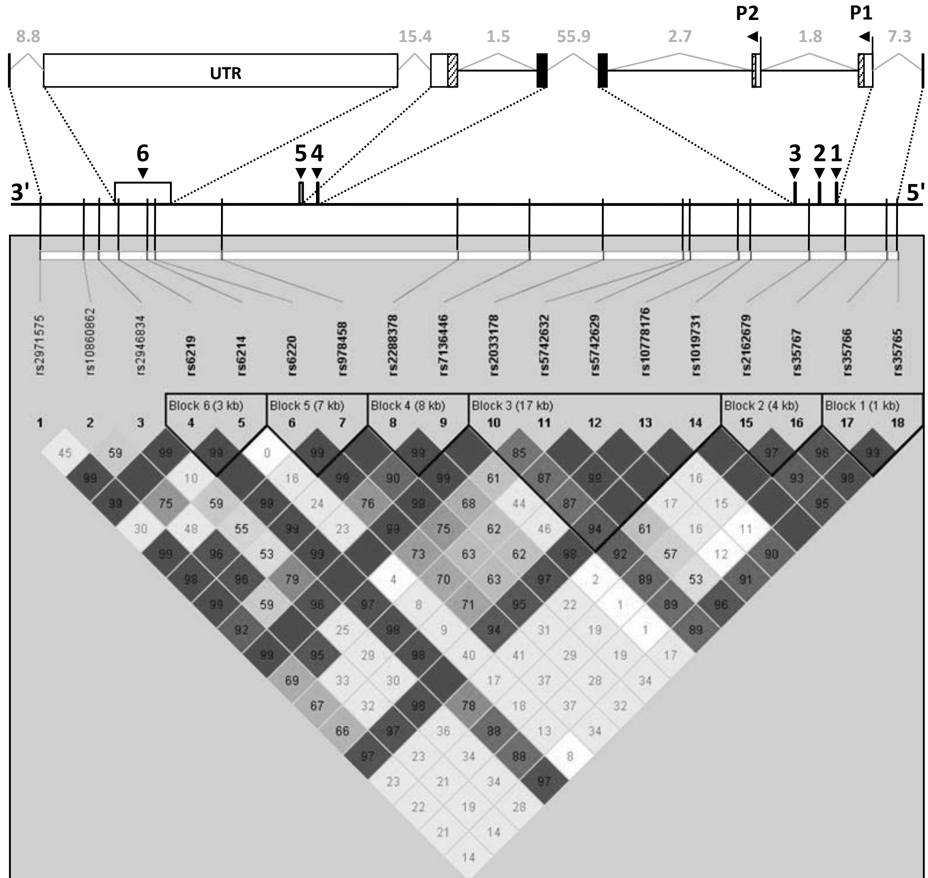

Figure 1. The IGF-I gene is shown schematically at the top to illustrate the relative positions of the 18 SNPs among the IGF-I gene**.** LD pattern of eighteen SNPs in the IGF-I gene region is shown at the bottom. Dark squares (black) represent high LD values between SNPs while the white ones low. The numbers inside the squares indicate the SNP pair wise D' values (in empty squares LD=1). Light squares show SNPs with high D' values but a low measure of confidence.

single SNPs and haplotypes with circulating total IGF-I levels and IGF-I bioactivity, we performed analysis of variance. Analysis of covariance was used to adjust for gender and age. In case of a consistent trend, we performed a linear regression analysis to quantify the association. In order to compare the mean of each haplotypes with the most common haplotypes we extracted the haplotypes from haplo.stats. To examine associations between single SNPs and haplotypes with circulating total IGF-I levels and IGF-I bioactivity, we

performed analysis of variance. Analysis of covariance was used to adjust for gender and age. In case of a consistent trend, we performed a liner regression analysis to quantify the association.

To examine whether susceptibility to MI and AP were associated with single variants in the IGF-I gene, we evaluated the relationship between IGF-I genotypes and disease risk. Odds ratios (OR) and 95% confidence intervals (95% CI) were estimated by logistic regression for the association between genotypes and risk of MI and AP.

Further, to potentially capture other unmeasured variants that may not be adequately captured by single SNPs, we evaluated the relationship between common IGF-I haplotypes and MI, and AP risk using the haplo.glm function of the program haplo Stats [34].

RESULTS

In Table 1A clinical characteristics of the study population at baseline are shown. There were more female than male participants. Mean age, BMI, total cholesterol and HDL cholesterol were significantly higher in women than men. Men had significantly higher diastolic BP and were more often smoking than women. In addition, prevalence of CVD was higher in men than in women (Table 1A). During follow-up, incidence of MI, AP and coronary heart disease was higher in men than in women while incidence of DM type 2 was not statistically different (Table 1B).

Eighteen SNPs of the IGF-I gene were selected to characterize the IGF-I gene (Figure 1). The IGF-I gene was characterized into 6 haplotype blocks.

Table 1A. General characteristics of study population of the Rotterdam study at baseline

	Total population	Male	Female	P -value
	(n=5970)	(n=2426)	(n=3544)	
Age (years)	69.4 ± 9.1	68.1 ± 8.2	70.3 ± 9.6	< 0.001
Gender (%)		2426 (40.6)	3544 (59.4)	< 0.001
Body mass index (kg/m2)	26.3 ± 4.1	25.7 ± 3.8	26.7 ± 4.1	< 0.001
Systolic blood pressure (mmHg)	139.2 ± 22.3	138.7 ± 21.8	139.6 ± 22.6	0.117
Diastolic blood pressure (mmHg)	73.7 ± 11.3	74.6 ± 11.4	73.0 ± 11.2	< 0.001
Total cholesterol (mmol/L)	6.6 ± 1.2	6.3 ± 1.2	6.8 ± 1.2	< 0.001
HDL cholesterol (mmol/L)	1.3 ± 0.4	1.2 ± 0.3	1.5 ± 0.4	< 0.001
Prevalence of cardiovascular disease (%)	886 (22.8)	444 (27.5)	442 (19.5)	< 0.001
Current smoking (%)	1343 (23.0)	717 (30.0)	626 (18.2)	< 0.001

Values are presented as means ± standard deviation or percentage.

Table 1B. Overall incidence of cardiovascular disease and type 2 diabetes during follow-up from baseline until 1st of January 2006

	Total population	Male	Female	P -value
	(n=5970)	(n=2426)	(n=3544)	
Incidence of myocardial infarction (%)	372 (6.2)	226 (9.3)	146 (4.1)	< 0.001
Incidence of angina pectoris (%)	265 (6.7)	111 (6.8)	154 (6.7)	0.894
Incidence of myocardial infarction and angina pectoris (%)	468 (11.9)	244 (14.9)	224 (9.7)	< 0.001
Incidence of coronary heart disease (%)	928 (15.5)	517(21.3)	411 (11.6)	<0.001
Incidence of type 2 diabetes (%)	562 (10.6)	246 (11.4)	316 (10.1)	0.137

Values are presented as mean ± standard deviation or percentage.

The level of LD (D') between blocks 1 and 2 of the IGF-I gene was 0.96, while the D' between blocks 4 and 5 was 0.88, both reflecting high LD. The D's of the remaining haplotypes in the IGF-I gene were between 0.37-0.68. The previously reported CA repeat in the promoter region of the IGF-I gene was mapped between rs2162679 and rs35767 in block 2 of the IGF-I gene. The D' between the CA repeat and rs2162679 and rs35767, respectively, was 0.84 and 0.86.

A. Results on total IGF-I levels

Mean total IGF-I (measured in 430 subjects) was 17.6 nmol/L (SD 7.2). Mean total IGF-I levels did not differ between men and women (men: 18.0 nmol/L (SD 7.2); women 17.2 nmol/L (SD 7.2); p = 0.13 after adjustment for age).

Single IGF-I gene SNPs and circulating total IGF-I

Regression analyses showed that total IGF-I levels were associated with IGF-I gene SNPs located in blocks 1 and 2 (overall, in men and in women). In Table 2A-C associations between single IGF-I gene SNPs and circulating total IGF-I levels are shown. We observed significant relationships between total IGF-I and the following SNPs overall (rs35766, rs2162679, rs35767, and rs10860862) (Table 2A), in men (rs35766, rs2162679, and rs35767) (Table 2B) and in women (rs35766, rs2162679, rs35767, and rs10860862) (Table 2C). Specific IGF-I SNPs accounted individually for up to 28 % change in total IGF-I levels.

Table 2A. Associations between circulating total IGF-I and IGF-I gene polymorphisms in the total population

SNP	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 1								
rs35766	TT	0.63	18.5 (0.5)	0.003		18.5 (0.5)	0.004	
	TC	0.29	17.2 (0.7)		-7.1	17.1 (0.7)		-7.6
	CC	0.08	14.5 (1.3)*		-21.5	14.9 (1.3)*		-19.5
rs35765	GG	0.73	18.1 (0.4)	0.151		18.2 (0.4)	0.073	
	GT	0.24	16.9 (0.8)		-6.6	16.8 (0.8)		-7.7
	TT	0.04	16.8 (2.0)		-7.4	16.4 (2.0)		-9.9
Block 2								
rs2162679	TT	0.63	18.6 (0.5)	0.001		18.6 (0.5)	0.001	
	TC	0.28	17.1 (0.7)		-8.1	17.1 (0.7)		-8.0
	CC	0.08	14.4 (1.3)*		-22.6	14.6 (1.3)*		-21.5
rs35767	CC	0.63	18.7 (0.5)	0.001		18.6 (0.5)	0.001	
	CT	0.28	17.0 (0.7)		-9.1	17.0 (0.7)*		-8.6
	TT	0.09	14.5 (1.2)*		-22.5	14.8 (1.2)*		-20.4
Block 3								
rs2033178	GG	0.82	18.1 (0.4)	0.073		18.1 (0.4)	0.042	
	GA	0.16	16.5 (0.9)		-8.8	16.5 (0.9)		-8.8
	AA	0.01	15.2 (3.2)		-16.0	14.2 (3.2)		-21.5
rs5742632	AA	0.61	17.3 (0.5)	0.121		17.3 (0.5)	0.159	
	AG	0.33	18.8 (0.7)		8.7	18.7 (0.6)		8.1
	GG	0.06	18.1 (1.6)		4.6	18.1 (1.5)		4.6
rs5742629	TT	0.53	17.7 (0.5)	0.855		17.7 (0.5)	0.845	
	TC	0.39	17.9 (0.6)		1.1	17.9 (0.6)		1.1
	CC	0.08	17.7 (1.3)		0.0	17.9 (1.3)		1.1
rs10778176	CC	0.54	17.7 (0.5)	0.841		17.7 (0.5)	0.830	
	CT	0.38	18.0 (0.6)		1.7	17.9 (0.6)		1.1
	TT	0.08	17.7 (1.3)		0.0	18.0 (1.3)		1.7
rs1019731	CC	0.78	17.8 (0.4)	0.781		17.8 (0.4)	0.839	
	CA	0.21	17.9 (0.8)		0.6	17.7 (0.8)		-0.6
	AA	0.01	19.6 (4.2)		10.1	19.9 (4.1)		11.8
Block 4								
rs2288378	CC	0.56	17.4 (0.5)	0.328		17.5 (0.5)	0.507	
	CT	0.38	18.3 (0.6)		5.2	18.2 (0.6)		4.0
	TT	0.06	18.2 (1.5)		4.6	17.9 (1.5)		2.3
rs7136446	TT	0.38	17.2 (0.6)	0.271		17.3 (0.6)	0.325	
	CT	0.46	18.1 (0.6)		5.2	18.1 (0.5)		4.6
	CC	0.16	18.3 (0.9)		6.4	18.1 (0.9)		4.6

P for trend is presented for single SNPs; The most frequent genotype was used as the reference when comparing total IGF-I levels between genotypes. Model 1: without adjustment. Model 2: after adjustment for gender and age. *P ≤ 0.05 compared to the reference.

Table 2A. Continued

SNP	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 5								
rs6220	AA	0.50	17.6 (0.5)	0.685		17.7 (0.5)	0.918	
	AG	0.41	17.9 (0.6)		1.7	17.8 (0.6)		0.6
	GG	0.09	18.0 (1.2)		2.7	17.8 (1.2)		0.6
rs978458	CC	0.55	17.5 (0.5)	0.378		17.7 (0.5)	0.588	
	CT	0.38	18.2 (0.6)		4.0	18.1 (0.6)		2.3
	TT	0.07	18.1 (1.4)		3.4	18.0 (1.4)		1.7
Block 6								
rs6219	CC	0.82	17.5 (0.4)	0.374		17.6 (0.4)	0.468	
	CT	0.16	19.8 (0.9)		13.1	19.7 (0.9)		11.9
	TT	0.02	12.4 (2.9)		-29.1	11.7 (2.9)*		-33.5
rs6214	CC	0.34	18.0 (0.7)	0.827		17.8 (0.6)	0.623	
	CT	0.46	17.4 (0.6)		-3.3	17.5 (0.5)		-1.7
	TT	0.20	18.5 (0.8)		2.8	18.5 (0.8)		3.9
Others								
rs2971575	AA	0.35	17.5 (0.6)	0.398		17.6 (0.6)	0.482	
	AG	0.46	17.8 (0.6)		1.7	17.7 (0.5)		0.6
	GG	0.18	18.4 (0.9)		5.1	18.4 (0.9)		4.5
rs10860862	GG	0.56	18.5 (0.5)	<u>0.002</u>		18.4 (0.5)	<u>0.012</u>	
	GT	0.35	17.7 (0.6)		-4.3	17.7 (0.6)		-3.8
	TT	0.09	14.1 (1.2)*		-23.8	14.7 (1.2) *		-20.1
rs2946834	GG	0.45	17.6 (0.6)	0.824		17.7 (0.6)	0.722	
	GA	0.44	18.3 (0.6)		4.0	18.3 (0.6)		3.4
	AA	0.12	16.6 (1.1)		-5.7	16.6 (1.1)		-6.2

P for trend is presented for single SNPs; The most frequent genotype was used as the reference when comparing total IGF-I levels between genotypes. Model 1: without adjustment. Model 2: after adjustment for gender and age. *P ≤ 0.05 compared to the reference.

Table 2B. Associations between circulating total IGF-I and IGF-I gene polymorphisms in men

SNP	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 1								
rs35766	TT	0.67	18.7 (0.7)	<u>0.043</u>		18.7 (0.7)	<u>0.004</u>	
	TC	0.26	17.7 (1.1)		-5.3	17.6 (1.1)		-5.9
	CC	0.07	13.9 (2.1)*		-25.7	15.0 (2.1)		-19.8
rs35765	GG	0.75	18.5 (0.6)	0.280		18.5 (0.6)	0.073	
	GT	0.24	16.9 (1.1)		-8.6	17.0 (1.1)		-8.1
	TT	0.01	18.8 (5.0)		1.6	18.0 (4.9)		-2.7

Table 2B. Continued

SNP	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 2								
rs2162679	TT	0.66	19.0 (0.7)	0.019		18.9 (0.7)	0.001	
	TC	0.27	17.3 (1.0)		-8.9	17.3 (1.0)		-8.5
	CC	0.07	14.2 (2.1)*		-25.3	15.1 (2.1)		-20.1
rs35767	CC	0.66	18.9 (0.7)	0.014		18.8 (0.7)	0.001	
	CT	0.27	17.3 (1.1)		-8.5	17.2 (1.1)		-8.5
	TT	0.07	13.7 (2.0)*		-27.5	14.8 (2.1)		-21.3
Block 3								
rs2033178	GG	0.84	18.6 (0.6)	0.127		18.5 (0.6)	0.042	
	GA	0.15	15.8 (1.4)		-15.1	15.9 (1.4)		-14.0
	AA	0.01	18.8 (5.0)		1.1	18.0 (4.9)		-2.7
rs5742632	AA	0.63	17.57 (0.7)	0.064		17.6 (0.7)	0.159	
	AG	0.31	19.3 (1.0)		9.8	19.0 (1.0)		8.0
	GG	0.06	20.4 (2.2)		16.1	20.5 (2.2)		16.5
rs5742629	TT	0.54	18.1 (0.7)	0.701		18.2 (0.7)	0.845	
	TC	0.37	17.9 (0.9)		-1.1	17.8 (0.9)		-2.2
	CC	0.09	19.4 (1.8)		7.2	19.5 (1.8)		7.1
rs10778176	CC	0.55	18.0 (0.7)	0.608		18.1 (0.7)	0.830	
	CT	0.36	18.0 (0.9)		0.0	17.9 (0.9)		-1.1
	TT	0.09	19.4 (1.8)		7.8	19.5 (1.8)		7.7
rs1019731	CC	0.76	18.3 (0.6)	0.684		18.3 (0.6)	0.839	
	CA	0.24	17.6 (1.1)		-3.8	17.6 (1.1)		-3..8
	AA	0.01	21.1 (7.1)		15.3	20.8 (7.0)		13.7
Block 4								
rs2288378	CC	0.56	17.9 (0.7)	0.362		18.0 (0.7)	0.507	
	CT	0.39	18.1 (0.9)		1.1	18.0 (0.9)		0.0
	TT	0.05	21.3 (2.4)		19.1	20.9 (2.3)		16.1
rs7136446	TT	0.37	18.2 (0.9)	0.783		18.1 (0.9)	0.325	
	CT	0.45	17.8 (0.8)		-2.2	18.0 (0.8)		-0.6
	CC	0.18	18.9 (1.3)		3.8	18.7 (1.3)		3.3
Block 5								
rs6220	AA	0.51	18.2 (0.8)	0.671		18.2 (0.8)	0.918	
	AG	0.41	17.7 (0.9)		-2.7	17.6 (0.8)		-3.3
	GG	0.08	20.1 (2.0)		10.4	20.2 (1.9)		11.0
rs978458	CC	0.56	17.9 (0.7)	0.439		18.0 (0.7)	0.588	
	CT	0.38	18.2 (0.9)		1.7	18.1 (0.9)		0.6
	TT	0.06	20.3 (2.2)		13.4	20.2 (2.2)		12.2

Table 2B. Continued

SNP	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 6								
rs6219	CC	0.80	18.0 (0.6)	0.977		18.0 (0.6)	0.468	
	CT	0.18	19.5 (1.3)		8.3	19.3 (1.3)		7.2
	TT	0.02	11.7 (4.1)		-35.0	11.0 (4.0)		-38.9
rs6214	CC	0.33	17.9 (1.0)	0.443		17.6 (0.9)	0.623	
	CT	0.48	17.9 (0.8)		0.0	18.0 (0.8)		2.3
	TT	0.19	19.3 (1.3)		7.8	19.5 (1.3)		10.8
Others								
rs2971575	AA	0.35	18.9 (0.9)	0.746		18.8 (0.9)	0.482	
	AG	0.46	17.4 (0.8)		-7.9	17.4 (0.8)		-7.4
	GG	0.19	18.8 (1.3)		-0.5	18.8 (1.2)		0.0
rs10860862	GG	0.56	18.4 (0.7)	0.254		18.3 (0.7)	<u>0.012</u>	
	GT	0.37	18.6 (0.9)		1.1	18.5 (0.9)		1.1
	TT	0.07	14.3 (2.0)		-22.3	15.3 (2.1)		-16.4
rs2946834	GG	0.43	18.8 (0.8)	0.099		18.8 (0.8)	0.722	
	GA	0.45	18.2 (0.8)		-3.2	18.2 (0.8)		-3.2
	AA	0.13	15.6 (1.5)		-17.0	15.7 (1.5)		-16.5

P for trend is presented for single SNPs; The most frequent genotype was used as the reference when comparing total IGF-I levels between genotypes. Model 1: without adjustment. Model 2: after adjustment for gender and age. *P ≤ 0.05 compared to the reference.

Table 2C. Associations between circulating total IGF-I and IGF-I gene polymorphisms in women

SNP	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 1								
rs35766	TT	0.6	18.3 (0.7)	<u>0.037</u>		18.3 (0.7)	<u>0.024</u>	
	TC	0.31	16.8 (0.9)		-8.2	16.7 (0.9)		-8.7
	CC	0.09	14.9 (1.7)		-18.6	14.8 (1.6)*		-19.1
rs35765	GG	0.71	17.8 (0.6)	0.363		17.9 (0.6)	0.188	
	GT	0.24	16.9 (1.1)		-5.1	16.6 (1.0)		-7.3
	TT	0.05	16.4 (2.2)		-7.9	15.9 (2.2)		-11.2
Block 2								
rs2162679	TT	0.61	18.3 (0.7)	<u>0.029</u>		18.3 (0.6)	<u>0.013</u>	
	TC	0.29	16.9 (1.0)		-7.7	17.0 (0.9)		-7.1
	CC	0.10	14.6 (1.6)*		-20.2	14.1 (1.6)*		-23.0
rs35767	CC	0.61	18.4 (0.7)	<u>0.023</u>		18.5 (0.6)	<u>0.012</u>	
	CT	0.29	16.8 (1.0)		-8.7	16.8 (0.9)		-9.2
	TT	0.11	14.9 (1.6)*		-19.0	14.7 (1.5)*		-20.5
Block 3								
rs2033178	GG	0.81	17.7 (0.6)	0.306		17.8 (0.6)	0.185	
	GA	0.17	17.0 (1.2)		-4.0	16.8 (1.2)		-5.6
	AA	0.01	12.8 (4.3)		-27.7	11.7 (4.2)		-34.3

Table 2C. Continued

SNP	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
rs5742632	AA	0.60	17.1 (0.7)	0.654		17.1 (0.7)	0.622	
	AG	0.34	18.4 (0.9)		7.6	18.4 (0.9)		7.6
	GG	0.05	16.0 (2.2)		-6.4	16.0 (2.2)		-6.4
rs5742629	TT	0.52	17.4 (0.7)	0.928		17.4 (0.7)	0.996	
	TC	0.40	18.0 (0.8)		3.4	17.9 (0.8)		2.9
	CC	0.08	16.1 (1.8)		-7.5	16.5 (1.8)		-5.2
rs10778176	CC	0.53	17.4 (0.7)	0.853		17.4 (0.7)	0.924	
	CT	0.39	17.9 (0.8)		2.9	17.8 (0.8)		2.3
	TT	0.08	16.1 (1.8)		-7.5	16.5 (1.8)		-5.2
rs1019731	CC	0.80	17.3 (0.6)	0.488		17.4 (0.6)	0.559	
	CA	0.19	18.2 (1.2)		5.2	18.0 (1.2)		3.4
	AA	0.01	18.9 (5.2)		9.2	19.4 (5.1)		11.5
Block 4								
rs2288378	CC	0.56	17.1 (0.7)	0.604		17.2 (0.7)	0.800	
	CT	0.37	18.4 (0.9)		7.6	18.4 (0.8)		7.0
	TT	0.07	16.3 (2.0)		-4.7	15.8 (1.9)		-8.1
rs7136446	TT	0.39	16.4 (0.8)	0.232		16.6 (0.8)	0.310	
	CT	0.46	18.4 (0.8)		12.2	18.3 (0.7)		10.2
	CC	0.15	17.6 (1.3)		7.3	17.5 (1.3)		5.4
Block 5								
rs6220	AA	0.49	17.1 (0.7)	0.832		17.2 (0.7)	0.920	
	AG	0.41	18.0 (0.8)		5.3	17.9 (0.8)		4.1
	GG	0.1	16.5 (1.6)		-3.5	16.2 (1.6)		-5.8
rs978458	CC	0.54	17.17 (0.7)	0.589		17.2 (0.7)	0.823	
	CT	0.38	18.38 (0.8)		7.0	18.1 (0.8)		5.2
	TT	0.08	16.9 (1.8)		-1.6	16.6 (1.8)		-3.5
Block 6								
rs6219	CC	0.84	17.1 (0.6)	0.251		17.1 (0.6)	0.270	
	CT	0.14	20.2 (1.4)*		18.1	20.2 (1.3)*		18.1
	TT	0.01	13.0 (4.2)		-24.0	12.3 (4.1)		-28.1
rs6214	CC	0.34	18.1 (0.9)	0.729		17.9 (0.9)	0.807	
	CT	0.45	16.9 (0.8)		-6.6	17.1 (0.8)		-4.5
	TT	0.21	17.9 (1.1)		-1.1	17.7 (1.1)		-1.1
Others								
rs2971575	AA	0.36	16.4 (0.9)	0.162		16.6 (0.9)	0.262	
	AG	0.47	18.1 (0.8)		10.4	18.0 (0.7)		8.4
	GG	0.18	18.2 (1.2)		11.0	18.0 (1.2)		8.4
rs10860862	GG	0.56	18.6 (0.7)	<u>0.004</u>		18.4 (0.7)	<u>0.010</u>	
	GT	0.33	16.8 (0.9)		-9.7	17.0 (0.9)		-7.6
	TT	0.11	14.0 (1.5)*		-24.7	14.3 (1.5)*		-22.3
rs2946834	GG	0.46	16.6 (0.8)	0.261		16.8 (0.8)	0.376	
	GA	0.43	18.5 (0.8)		11.4	18.3 (0.8)		8.9
	AA	0.11	17.4 (1.5)		4.8	17.4 (1.5)		3.6

P for trend is presented for single SNPs; The most frequent genotype was used as the reference when comparing total IGF-I levels between genotypes. Model 1: without adjustment. Model 2: after adjustment for gender and age. *P ≤ 0.05 compared to the reference.

IGF-I gene haplotype blocks and total IGF-I

In the whole study population and in women haplotypes in block 1 and 2 of the IGF-I gene were significantly related to total IGF-I (Table 3A-C). These significant relationships remained significant after adjustments for age (Table 3A and C). In men haplotypes in block 1 and 2 of the IGF-I gene were also significantly related to total IGF-I, but only when unadjusted. After adjustment for age, haplotypes in block 1 showed borderline significance with total IGF-I levels in men (Table 3B). No relationships were observed between blocks 3-6 of the IGF-I gene and total IGF-I (Table3A-C).

Table 3A. Associations between circulating total IGF-I and IGF-I gene haplotypes in the total population

Haplotype	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 1								
H1	TG	0.78	18.3 (0.3)	0.000		18.2 (0.3)	0.001	
H2	CT	0.15	16.9 (0.7)		-7.7	16.7 (0.7)		-8.2
H3	CG	0.07	14.8 (1.0)*		-19.1	15.4 (1.0)		-15.4
Block 2								
H1	TC	0.76	18.3 (0.3)	0.002		18.3 (0.3)	0.005	
H2	CT	0.22	16.0 (0.6)*		-12.6	16.1 (0.6)*		-12.0
H3	<0.05	0.02	18.1 (2.0)		-1.1	18.9 (1.9)		3.3
Block 3								
H1	GATCC	0.52	18.0 (0.4)	0.646		18.0 (0.4)	0.626	
H2	AATCC	0.09	16.4 (0.9)		-8.9	16.2 (0.8)		-10.0
H3	GATCA	0.11	18.0 (0.8)		0.0	17.9 (0.8)		-0.6
H4	GGCTC	0.22	18.6 (0.6)		3.3	18.5 (0.6)		2.8
H5	<0.05	0.01	15.2 (1.1)		-15.6	15.5 (1.1)		-13.9
Block 4								
H1	CT	0.61	17.6 (0.3)	0.255		17.6 (0.3)	0.375	
H2	CC	0.14	18.0 (0.7)		2.3	18.1 (0.7)		2.8
H3	TC	0.25	18.2 (0.5)		3.4	18.1 (0.5)		2.8
Block 5								
H1	AC	0.70	17.8 (0.3)	0.592		17.8 (0.3)	0.425	
H2	GT	0.26	18.2 (0.5)		2.2	18.1 (0.5)		1.7
H3	<0.05	0.04	15.3 (1.4)		-14.0	15.3 (1.4)		-14.0
Block 6								
H1	CC	0.47	17.6 (0.4)	0.315		17.5 (0.4)	0.304	
H2	CT	0.43	17.9 (0.4)		1.7	18.0 (0.4)		2.9
H3	TC	0.10	18.6 (0.9)		5.7	18.4 (0.8)		5.1

P for trend is presented for single haplotypes. The most frequent haplotype (H1) was used as the reference when comparing total IGF-I levels between haplotypes. Model 1: without adjustment. Model 2: after adjustment for gender and age. *P ≤ 0.05 compared to the reference.

Table 3B. Associations between circulating total IGF-I and IGF-I gene haplotypes in men

Haplotype	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 1								
H1	TG	0.80	18.6 (0.4)	0.015		18.5 (0.4)	0.052	
H2	CT	0.13	17.1 (1.1)		-8.1	17.1 (1.0)		-7.6
H3	CG	0.07	15.1 (1.5)*		-18.8	16.0 (1.5)		-13.5
Block 2								
H1	TC	0.76	18.6 (0.4)	0.033		18.5 (0.4)	0.086	
H2	CT	0.20	16.2 (0.9)*		-12.9	16.5 (0.9)*		-10.8
H3	<0.05	0.02	18.3 (2.9)		-1.6	18.8 (2.8)		1.6
Block 3								
H1	GATCC	0.52	18.4 (0.5)	0.614		18.5 (0.5)	0.600	
H2	AATCC	0.10	16.2 (1.3)		-12.0	16.2 (1.3)		-12.4
H3	GATCA	0.13	17.7 (1.1)		-3.8	17.7 (1.1)		-4.3
H4	GGCTC	0.22	19.4 (0.8)		5.4	19.2 (0.8)		3.8
H5	<0.05	0.01	14.2 (1.6)		-22.8	14.8 (1.6)*		-20.0
Block 4								
H1	CT	0.60	18.1 (0.5)	0.536		18.1 (0.5)	0.594	
H2	CC	0.16	17.5 (1.0)		-3.3	17.7 (1.0)		-2.2
H3	TC	0.25	18.7 (0.8)		3.3	18.6 (0.8)		2.8
Block 5								
H1	AC	0.71	18.1 (0.5)	0.812		18.1 (0.4)	0.734	
H2	GT	0.25	18.7 (0.8)		3.3	18.6 (0.8)		2.8
H3	<0.05	0.04	16.0 (1.9)		-11.6	16.0 (1.9)		-11.6
Block 6								
H1	CC	0.48	17.8 (0.6)	0.547		17.8 (0.6)	0.528	
H2	CT	0.43	18.5 (0.6)		3.9	18.6 (0.6)		4.5
H3	TC	0.11	18.2 (1.2)		2.2	17.9 (1.2)		0.6

P for trend is presented for single haplotypes. The most frequent haplotype (H1) was used as the reference when comparing total IGF-I levels between haplotypes. Model 1: without adjustment. Model 2: after adjustment for age. *$P \leq 0.05$ compared to the reference.

Table 3C. Associations between circulating total IGF-I and IGF-I gene haplotypes in women

Haplotype	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 1								
H1	TG	0.75	18.0 (0.4)	0.009		18.0 (0.4)	0.009	
H2	CT	0.17	16.7 (0.9)		-7.2	16.4 (0.9)		-8.9
H3	CG	0.07	14.6 (1.3)*		-18.9	15.0 (1.3)*		-16.7
Block 2								
H1	TC	0.74	18.0 (0.4)	0.033		18.1 (0.4)	0.028	
H2	CT	0.24	15.9 (0.7)*		-11.7	15.7 (0.7)*		-13.3
H3	Rare	0.02	17.9 (2.8)		-0.6	19.1 (2.7)		5.5

Table 3C continued

Haplotype	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 3								
H1	GATCC	0.52	17.6 (0.5)	0.864		17.6 (0.5)	0.850	
H2	AATCC	0.10	16.5 (1.2)		-6.3	16.2 (1.1)		-8.0
H3	GATCA	0.10	18.2 (1.1)		3.4	18.1 (1.1)		2.8
H4	GGCTC	0.22	17.9 (0.8)		1.7	17.9 (0.8)		1.7
H5	<0.05	0.01	15.9 (1.5)		-9.7	16.0 (1.5)		-9.1
Block 4								
H1	CT	0.62	17.2 (0.5)	0.342		17.2 (0.5)	0.477	
H2	CC	0.13	18.5 (1.0)		7.6	18.6 (1.0)		8.1
H3	TC	0.25	17.8 (0.7)		3.5	17.7 (0.7)		2.9
Block 5								
H1	AC	0.70	17.5 (0.4)	0.618		17.6 (0.4)	0.442	
H2	GT	0.27	17.8 (0.7)		1.7	17.6 (0.7)		0.0
H3	<0.05	0.03	14.6 (2.0)		-16.6	14.7 (2.0)		-16.5
Block 6								
H1	CC	0.48	17.4 (0.5)	0.439		17.4 (0.5)	0.415	
H2	CT	0.44	17.4 (0.6)		0.0	17.4 (0.5)		0.0
H3	TC	0.09	18.9 (1.2)		8.6	18.8 (1.2)		8.0

P for trend is presented for single haplotypes. The most frequent haplotype (H1) was used as the reference when comparing total IGF-I levels between haplotypes. Model 1: without adjustment. Model 2: after adjustment for age. *P ≤ 0.05 compared to the reference.

B. Results on bioactive IGF-I levels

Characteristics of participants in which IGF-I bioactivity was measured

IGF-I bioactivity was measured in 1036 subjects randomly selected from the participants of the third survey of the Rotterdam study. Mean IGF-I bioactivity was 180 pmol/L (SD: 56). Mean IGF-I bioactivity was significantly lower in men than in women (men: 170 pmol/L (SD 47); women: 187 pmol/L (SD 62); age adjusted $p < 0.001$). In Table 4A clinical characteristics of these participants are shown. Women were slightly older than men. BMI, total cholesterol and HDL cholesterol were significantly higher in women than in men, whilediastolic blood pressure and prevalence of cardiovascular disease was higher in men than in women. Incidence of MI, coronary heart disease and the combination of MI and AP was higher in men than in women, while the incidence of diabetes did not differ between the two genders (Table 4B).

Single IGF-I gene SNPs, and circulating IGF-I Bioactivity

We did not observe significant relationships between IGF-I bioactivity and SNPs that were associated with total IGF-I (Table 5A-C). However several

Table 4A. General characteristics of all subjects in which IGF-I boactivity was measured at the third round of the Rotterdam study

Characteristics	Total population	Male	Female	P-value
Total number	1036	451	585	
Age (years)	72.4 ± 7.3	71.8 ± 6.8	72.8 ± 7.7	0.03
Gender (%)		451 (43.5)	585 (56.5)	< 0.001
Body mass index (kg/m2)	26.9 ± 3.9	26.4 ± 3.2	27.3 ± 4.4	0.001
Systolic blood pressure (mmHg)	143.9 ± 21.7	145.7 ± 22.0	142.5 ± 21.3	0.02
Diastolic blood pressure (mmHg)	75.3 ± 11.7	76.8 ± 11.7	74.1 ± 11.5	< 0.001
Total cholesterol (mmol/L)	5.8 ± 1.0	5.5 ± 0.9	6.1 ± 1.0	< 0.001
HDL cholesterol (mmol/L)	1.4 ± 0.4	1.2 ± 0.3	1.5 ± 0.4	< 0.001
Prevalence of cardiovascular disease (%)	173 (23.2)	86 (27.3)	87 (20.2)	0.02
Current smoking (%)	290 (29.0)	208 (48.4)	82 (14.4)	< 0.001

Values are presented as mean ± standard deviation or percentage.

Table 4B. Incidence of cardiovascular disease and diabetes during follow-up of all subjects in which IGF-I bioactivity was measured at the third round of the Rotterdam study.

Characteristics	Total population	Male	Female	P-value
Total number	1036	451	585	
Incidence of myocardial infarction (%)	62 (6.0)	43 (9.5)	19 (3.2)	< 0.001
Incidence of angina pectoris (%)	62 (6.0)	29 (6.4)	33 (5.6)	0.123
Incidence of myocardial infarction and angina pectoris (%)	118 (11.6)	68 (15.2)	50 (8.8)	0.001
Incidence of coronary heart disease and angina pectoris (%)	167 (16.4)	96 (21.5)	71 (12.5)	< 0.001
Incidence of type 2 diabetes (%)	208 (20.1)	103 (22.9)	105 (18.0)	0.05

Values are presented as mean ± standard deviation or percentage

SNPs in the IGF-I gene located in blocks 3-6 were associated with circulating IGF-I bioactivity overall and in men (overall: rs5742632, rs2288378 and rs 978458; Table 5A); in men: rs5742632, 5742629, rs10778176 rs2288378, rs6220, rs978458 and rs6219; Table 5B), while in women no associations were found between IGF-I bioactivity and IGF-I gene SNPs (Table 5C). Specific IGF-I SNPs accounted individually for up to 30 % change in IGF-I bioactivity.

IGF-I gene haplotype blocks and IGF-I bioactivity

In the whole study population haplotypes in blocks 3 (H4), block 4 (H3) and block 5 (H2) were associated with higher circulating IGF-I bioactivity when compared to the reference (H1), but no significant trend was found (Table 6A). In men, blocks 3 (H4), block 4 (H3), block 5 (H2) and block 6 (H3) were related to significantly higher circulating IGF-I bioactivity than the respective reference haplotypes (H1) (Table 6B) and a significant trend was found in blocks 3, 4 and 5. In women no significant relationships were observed between haplotypes and IGF-I bioactivity (Table 6C).

Table 5A. Associations between circulating IGF-I bioactivity and IGF-I gene polymorphisms in the total population

SNP	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 1								
rs35766	TT	0.70	181.3 (2.2)	0.170		174.9 (2.1)	0.103	
	TC	0.27	164.0 (3.1)		-9.5	167.3 (3.2)*		-4.3
	CC	0.02	181.3 (12.0)		0.0	174.0 (11.4)		-0.5
rs35765	GG	0.78	181.3 (2.1)	0.347		173.8 (2.0)	0.275	
	GT	0.21	164.0 (3.6)		-9.5	169.2 (3.7)		-2.6
	TT	0.01	164.0 (14.7)		-9.5	169.7 (15.0)		-2.4
Block 2								
rs2162679	TT	0.70	174.6 (2.1)	0.313		174.5 (2.1)	0.232	
	TC	0.27	168.0 (3.2)		-3.8	168.2 (3.2)		-3.6
	CC	0.03	175.0 (10.8)		0.2	176.4 (10.8)		1.1
rs35767	CC	0.70	173.8 (2.1)	0.191		174.9 (2.1)	0.110	
	CT	0.27	169.6 (3.3)		-2.4	166.7 (3.2)*		-4.7
	TT	0.02	171.2 (11.1)		-1.5	176.4 (11.4)		0.9
Block 3								
rs2033178	GG	0.86	169.5 (1.8)	0.251		171.9 (1.8)	0.223	
	GA	0.13	177.5 (4.9)		4.7	177.9 (4.9)		3.5
	AA	0.00	171.9 (25.5)		1.4	182.7 (26.9)		6.3
rs5742632	AA	0.60	169.3 (2.2)	<u>0.008</u>		168.7 (2.2)	<u>0.007</u>	
	AG	0.35	177.8 (3.0)*		5.0	179.1 (3.0)*		6.2
	GG	0.05	173.0 (7.5)		2.2	178.4 (7.7)		5.7
rs5742629	TT	0.54	172.7 (2.4)	0.119		169.4 (2.3)	0.113	
	TC	0.39	173.0 (2.8)*		0.2	177.7 (2.8)*		4.9
	CC	0.07	174.0 (6.4)		0.8	171.4 (6.3)		1.2
rs10778176	CC	0.55	174.3 (2.4)	0.074		169.4 (2.3)	0.075	
	CT	0.38	168.4 (2.7)*		-3.4	177.9 (2.8)*		5.0
	TT	0.07	178.3 (6.8)		2.3	172.4 (6.5)		1.8
rs1019731	CC	0.74	174.5 (2.0)	0.910		172.6 (2.0)	0.787	
	CA	0.24	167.3 (3.4)		-4.1	173.1 (3.5)		0.3
	AA	0.02	178.4 (13.2)		2.2	176.4 (13.0)		2.2
Block 4								
rs2288378	CC	0.57	172.0 (2.3)	<u>0.017</u>		169.7 (2.2)	<u>0.013</u>	
	CT	0.37	177.4 (2.9)		3.1	175.4 (2.9)*		3.4
	TT	0.06	186.0 (7.7)*		8.1	187.5 (7.7)		10.5
rs7136446	TT	0.36	168.7 (2.8)	0.281		170.7 (2.8)	0.222	
	CT	0.48	178.8 (2.6)		6.0	172.8 (2.5)		1.2
	CC	0.15	178.9 (4.6)		6.0	177.5 (4.5)		4.0
Block 5								
rs6220	AA	0.52	169.9 (2.4)	0.053		170.2 (2.4)	<u>0.045</u>	
	AG	0.41	175.0 (2.8)		3.0	175.2 (2.7)		2.9
	GG	0.07	187.8 (7.0)		10.5	182.4 (6.8)		7.2

Table 5A. Continued

SNP	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
rs978458	CC	0.54	171.1 (2.3)	0.015		169.0 (2.3)	0.011	
	CT	0.39	172.7 (2.8)		0.9	176.4 (2.8)*		4.4
	TT	0.06	177.2 (7.1)		3.6	183.1 (7.2)		8.3
Block 6								
rs6219	CC	0.82	170.3 (1.9)	0.152		171.6 (1.9)	0.143	
	CT	0.17	175.1 (4.2)		2.8	176.8 (4.2)		3.0
	TT	0.01	182.3 (17.1)		7.0	188.7 (17.5)		10.0
rs6214	CC	0.37	169.3 (2.8)	0.325		175.6 (2.9)	0.299	
	CT	0.47	176.0 (2.6)		4.0	171.1 (2.5)		-2.6
	TT	0.16	183.6 (4.6)		8.4	171.4 (4.2)		-2.4
Others								
rs2971575	AA	0.34	171.6 (3.0)	0.692		172.4 (3.0)	0.642	
	AG	0.48	173.9 (2.5)		1.3	172.1 (2.5)		-0.2
	GG	0.18	173.3 (4.1)		1.0	175.2 (4.1)		1.6
rs10860862	GG	0.67	171.6 (2.1)	0.107		175.6 (2.1)	0.062	
	GT	0.29	176.6 (3.3)*		2.9	166.3 (3.0)*		-5.3
	TT	0.04	189.8 (10.1)		10.6	175.0 (9.2)		-0.3
rs2946834	GG	0.47	175.5 (2.6)	0.602		171.4 (2.5)	0.532	
	GA	0.43	171.1 (2.6)		-2.5	174.0 (2.6)		1.5
	AA	0.10	171.5 (5.4)		-2.3	173.6 (5.4)		1.3

P for trend is presented for single SNPs; The most frequent genotype was used as the reference when comparing total IGF-I levels between genotypes. Model 1: without adjustment. Model 2: after adjustment for gender and age. *P ≤ 0.05 compared to the reference.

Table 5B. Associations between circulating IGF-I bioactivity and IGF-I gene polymorphisms in men

SNP	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 1								
rs35766	TT	0.73	166.7 (2.8)	0.304		167.0 (2.8)	0.204	
	TC	0.25	159.1 (4.5)		-4.6	158.4 (4.5)		-5.2
	CC	0.02	171.4 (17.0)		2.8	169.0 (16.6)		1.2
rs35765	GG	0.79	165.9 (2.7)	0.522		166.2 (2.6)	0.376	
	GT	0.20	162.1 (5.2)		-2.3	160.9 (5.1)		-3.2
	TT	0.01	162.1 (22.7)		-2.3	161.7 (22.5)		-2.7
Block 2								
rs2162679	TT	0.72	166.1 (2.8)	0.539		166.2 (2.8)	0.448	
	TC	0.26	161.1 (4.5)		-3.0	160.9 (4.4)		-3.2
	CC	0.02	171.4 (17.0)		3.2	169.0 (16.7)		1.7
rs35767	CC	0.73	165.6 (2.8)	0.468		165.8 (2.8)	0.375	
	CT	0.25	160.0 (4.6)		-3.4	159.7 (4.6)		-3.7
	TT	0.02	170.6 (18.1)		3.0	168.5 (17.8)		1.6

Table 5B. Continued

SNP	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 3								
rs2033178	GG	0.85	163.4 (2.5)	0.133		163.5 (2.5)	0.149	
	GA	0.14	173.7 (6.5)		6.3	173.3 (6.5)		6.0
	AA	0.00	173.0 (48.5)		5.9	173.8 (48.4)		6.3
rs5742632	AA	0.59	158.4 (2.9)	0.001		158.2 (2.9)	0.001	
	AG	0.36	174.3 (4.1)*		10.0	174.7 (4.1)*		10.4
	GG	0.05	178.6 (11.4)		12.8	177.7 (11.3)		12.3
rs5742629	TT	0.53	159.0 (3.1)	0.031		158.7 (3.0)	0.025	
	TC	0.40	173.2 (3.8)*		8.9	173.5 (3.8)*		9.3
	CC	0.07	163.7 (8.9)		3.0	163.5 (8.8)		3.0
rs10778176	CC	0.54	158.8 (3.0)	0.021		158.7 (3.0)	0.018	
	CT	0.39	173.6 (3.9)*		9.3	173.8 (3.9)*		9.5
	TT	0.06	164.6 (9.2)		3.7	164.5 (9.1)		3.7
rs1019731	CC	0.73	164.6 (2.7)	0.945		164.5 (2.7)	0.979	
	CA	0.25	166.3 (4.8)		1.0	166.5 (4.7)		1.2
	AA	0.03	159.6 (14.2)		-3.0	157.6 (13.9)		-4.2
Block 4								
rs2288378	CC	0.55	158.0 (3.0)	0.001		158.2 (3.0)	0.001	
	CT	0.39	172.5 (3.9)*		9.2	172.1 (3.8)*		8.8
	TT	0.06	182.6 (10.8)*		15.6	182.9 (10.7)*		15.6
rs7136446	TT	0.35	159.6 (3.9)	0.192		159.3 (3.8)	0.147	
	CT	0.49	168.1 (3.4)		5.3	168.0 (3.4)		5.5
	CC	0.16	166.5 (5.9)		4.3	167.3 (5.9)		5.0
Block 5								
rs6220	AA	0.51	158.3 (3.1)	0.003		158.4 (3.1)	0.003	
	AG	0.41	172.1 (3.8)		8.7	171.9 (3.8)*		8.52
	GG	0.07	176.3 (9.2)*		11.4	177.0 (9.1)*		11.7
rs978458	CC	0.53	157.3 (3.0)	0.000		157.3 (3.0)	0.000	
	CT	0.42	172.8 (3.8)*		9.9	172.8 (3.7)*		9.9
	TT	0.06	180.5 (10.4)*		14.7	180.5 (10.3)*		14.8
Block 6								
rs6219	CC	0.81	162.2 (2.5)	0.008		162.2 (2.5)	0.008	
	CT	0.18	175.0 (5.8)*		7.9	175.4 (5.8)*		8.1
	TT	0.01	212.9 (29.7)*		31.3	207.3 (28.7)		27.8
rs6214	CC	0.38	166.8 (3.9)	0.214		166.3 (3.8)	0.277	
	CT	0.47	166.2 (3.4)		-0.4	166.3 (3.4)		0.0
	TT	0.15	156.5 (5.7)		-6.2	157.0 (5.6)		-5.6
Others								
rs2971575	AA	0.33	159.8 (3.9)	0.504		159.5 (3.9)	0.405	
	AG	0.48	169.7 (3.5)		6.2	169.5 (3.4)		6.3
	GG	0.18	161.7 (5.3)		1.2	162.6 (5.3)		1.9

Table 5B. Continued

SNP	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
rs10860862	GG	0.69	167.0 (3.1)	0.228		167.3 (3.1)	0.301	
	GT	0.29	159.1 (4.4)		-4.7	158.9 (4.6)		-4.7
	TT	0.02	177.9 (9.1)		6.5	177.5 (9.3)		6.5
rs2946834	GG	0.45	163.3 (3.5)	0.736		162.9 (3.4)	0.582	
	GA	0.44	166.9 (3.6)		2.2	167.2 (3.6)		2.6
	AA	0.11	163.3 (7.1)		0.0	164.0 (7.1)		0.7

P for trend is presented for single SNPs; The most frequent genotype was used as the reference when comparing total IGF-I levels between genotypes. Model 1: without adjustment. Model 2: after adjustment for gender and age. *P ≤ 0.05 compared to the reference.

Table 5C. Associations between circulating IGF-I bioactivity and IGF-I gene polymorphisms in women

SNP	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 1								
rs35766	TT	0.68	181.7 (3.0)	0.218		181.6 (3.0)	0.230	
	TC	0.29	174.3 (4.4)		-4.1	174.3 (4.4)		-4.0
	CC	0.02	177.5 (15.6)		-2.3	178.4 (15.6)		-1.8
rs35765	GG	0.77	180.6 (2.8)	0.392		180.5 (2.8)	0.403	
	GT	0.22	175.2 (5.2)		-3.0	175.2 (5.2)		-2.9
	TT	0.01	176.6 (20.3)		-2.2	178.2 (20.5)		-1.3
Block 2								
rs2162679	TT	0.69	181.4 (3.0)	0.313		181.5 (3.0)	0.333	
	TC	0.28	174.1 (4.5)		-4.0	174.0 (4.5)		-4.1
	CC	0.03	182.0 (14.3)		0.3	183.3 (14.4)		1.0
rs35767	CC	0.68	182.5 (3.1)	0.154		182.4 (3.1)	0.170	
	CT	0.29	172.5 (4.5)		-5.5	172.4 (4.5)		-5.5
	TT	0.03	182.4 (14.9)		-0.1	183.8 (15.0)		0.8
Block 3								
rs2033178	GG	0.87	179.1 (2.6)	0.723		179.1 (2.6)	0.680	
	GA	0.12	180.9 (7.1)		1.0	181.3 (7.1)		1.2
	AA	0.01	190.5 (33.5)		6.4	191.1 (33.6)		6.7
rs5742632	AA	0.60	177.2 (3.2)	0.416		177.2 (3.2)	0.403	
	AG	0.34	182.7 (4.3)		3.1	182.7 (4.3)		-4.8
	GG	0.06	179.2 (10.5)		1.1	179.3 (10.5)		7.5
rs5742629	TT	0.55	178.3 (3.3)	0.764		178.2 (3.3)	0.747	
	TC	0.38	181.3 (4.1)		1.7	181.5 (4.1)		1.9
	CC	0.08	177.8 (8.8)		-0.3	177.7 (8.8)		-0.3
rs10778176	CC	0.55	178.1 (3.3)	0.664		178.0 (3.3)	0.643	
	CT	0.38	181.3 (4.1)		1.8	181.5 (4.1)		2.0
	TT	0.07	179.0 (9.1)		0.5	178.9 (9.1)		0.5

Table 5C. Continued

SNP	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
rs1019731	CC	0.75	179.2 (2.9)	0.762		179.3 (2.9)	0.777	
	CA	0.24	178.7 (5.1)		-0.3	178.6 (5.1)		-0.4
	AA	0.01	201.1 (25.0)		12.2	201.5 (25.0)		12.4
Block 4								
rs2288378	CC	0.59	179.5 (3.2)	0.656		179.3 (3.2)	0.615	
	CT	0.35	177.2 (4.1)		-1.3	177.3 (4.1)		-1.1
	TT	0.06	191.8 (10.8)		6.9	192.3 (10.8)		7.3
rs7136446	TT	0.38	180.1 (4.0)	0.609		179.8 (4.0)	0.571	
	CT	0.47	176.6 (3.6)		-1.9	176.8 (3.6)		-1.7
	CC	0.15	187.0 (6.7)		3.8	187.2 (6.7)		4.1
Block 5								
rs6220	AA	0.53	180.0 (3.4)	0.852		179.8 (3.4)	0.805	
	AG	0.40	177.6 (3.9)		-1.3	177.7 (3.9)		-1.2
	GG	0.07	187.3 (9.8)		4.1	187.5 (9.8)		4.3
rs978458	CC	0.56	178.9 (3.3)	0.657		178.8 (3.3)	0.615	
	CT	0.37	179.0 (4.0)		0.1	178.9 (4.0)		0.1
	TT	0.07	185.8 (10.0)		3.9	186.4 (10.0)		4.3
Block 6								
rs6219	CC	0.82	179.5 (2.7)	0.789		179.5 (2.7)	0.859	
	CT	0.17	178.0 (6.0)		-0.8	178.4 (6.0)		-0.6
	TT	0.01	175.8 (21.8)		-2.1	177.0 (22.0)		-1.4
rs6214	CC	0.37	182.9 (4.2)	0.726		182.9 (4.1)	0.692	
	CT	0.47	175.1 (3.5)		-4.3	175.4 (3.5)		-4.1
	TT	0.17	183.6 (6.2)		0.4	183.1 (6.2)		0.1
Others								
rs2971575	AA	0.34	183.0 (4.3)	0.964		182.9 (4.3)	0.934	
	AG	0.48	174.2 (3.5)		-4.8	174.2 (3.4)		-4.8
	GG	0.18	187.0 (6.2)		2.2	187.2 (6.2)		2.4
rs10860862	GG	0.66	182.7 (3.1)	0.119		182.7 (3.1)	0.129	
	GT	0.30	172.4 (4.4)		-5.6	172.4 (4.4)		-5.6
	TT	0.05	178.1 (11.3)		-2.5	178.6 (11.3)		-2.2
rs2946834	GG	0.48	178.2 (3.6)	0.589		178.0 (3.6)	0.524	
	GA	0.43	179.9 (3.8)		1.0	180.0 (3.8)		1.1
	AA	0.10	182.7 (8.1)		2.5	183.3 (8.1)		3.0

P for trend is presented for single SNPs; The most frequent genotype was used as the reference when comparing total IGF-I levels between genotypes. Model 1: without adjustment. Model 2: after adjustment for gender and age. *P ≤ 0.05 compared to the reference.

Table 6A. Associations between circulating IGF-I bioactivity and IGF-I gene haplotypes in the total population

Haplotype	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 1								
H1	TG	0.84	173.5 (1.3)	0.178		173.6 (1.3)	0.104	
H2	CT	0.12	169.4 (3.5)		-2.4	168.8 (3.5)		-2.8
H3	<0.05	0.04	167.9 (5.8)		-3.2	166.7 (5.7)		-4.0
Block 2								
H1	TC	0.83	173.5 (1.3)	0.238		173.6 (1.3)	0.165	
H2	CT	0.16	169.3 (3.0)		-2.4	168.8 (3.0)		-2.8
H3	<0.05	0.01	170.7 (11.6)		-1.6	170.2 (11.5)		-2.0
Block 3								
H1	GATCC	0.53	170.5 (1.7)	0.138		170.4 (1.6)	0.112	
H2	AATCC	0.07	177.5 (4.8)		4.1	177.7 (4.7)		4.3
H3	GATCA	0.14	173.1 (3.3)		1.5	173.6 (3.3)		1.9
H4	GGCTC	0.23	178.7 (2.6)*		4.8	178.8 (2.6)*		4.9
H5	<0.05	0.04	161.2 (5.7)		-5.5	161.1 (5.7)		-5.5
Block 4								
H1	CT	0.61	171.7 (1.6)	0.069		171.6 (1.5)	0.050	
H2	CC	0.15	168.8 (3.1)		-1.7	169.0 (3.0)		-1.5
H3	TC	0.24	178.0 (2.6)*		3.7	178.2 (2.5)*		3.8
Block 5								
H1	AC	0.72	171.3 (1.4)	0.193		171.2 (1.4)	0.175	
H2	GT	0.25	177.9 (2.5)*		3.9	178.0 (2.5)*		4.0
H3	<0.05	0.03	164.3 (7.2)		-4.1	164.2 (7.1)		-4.1
Block 6								
H1	CC	0.51	172.9 (1.7)	0.591		172.9 (1.7)	0.596	
H2	CT	0.39	171.3 (1.9)		-0.9	171.2 (1.9)		-1.0
H3	TC	0.09	178.1 (4.0)		3.0	178.2 (4.0)		3.1

P for trend is presented for single haplotypes. The most frequent haplotype (H1) was used as the reference when comparing total IGF-I levels between haplotypes. Model 1: without adjustment. Model 2: after adjustment for gender and age. *P ≤ 0.05 compared to the reference.

Table 6B. Associations between circulating IGF-I bioactivity with IGF-I gene haplotypes in men

Haplotype	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 1								
H1	TG	0.86	165.6 (1.8)	0.305		165.7 (1.8)	0.220	
H2	CT	0.11	161.2 (4.9)		-2.7	160.1 (4.8)		-3.4
H3	<0.05	0.03	159.4 (8.6)		-3.7	159.0 (8.5)		-4.0
Block 2								
H1	TC	0.84	165.3 (1.8)	0.712		165.5 (1.8)	0.572	
H2	CT	0.14	161.3 (4.3)		-2.4	160.8 (4.2)		-2.8
H3	Rare	0.01	178.7 (17.7)		8.1	176.4 (17.4)		6.6

Table 6B continued

Haplotype	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 3								
H1	GATCC	0.52	160.7 (2.2)	0.038		160.8 (2.2)	0.032	
H2	AATCC	0.07	172.7 (6.4)		7.5	172.1 (6.4)		7.0
H3	GATCA	0.14	165.1 (4.3)		2.7	165.0 (4.2)		2.6
H4	GGCTC	0.23	174.5 (3.6)*		8.6	174.5 (3.6)*		8.5
H5	<0.05	0.04	150.2 (7.6)		-6.5	151.3 (7.6)		-5.9
Block 4								
H1	CT	0.59	163.1 (2.1)	0.014		162.9 (2.1)	0.012	
H2	CC	0.16	156.3 (4.0)		-4.2	157.3 (4.0)		-3.4
H3	TC	0.25	174.7 (3.5)*		7.1	174.5 (3.4)*		7.1
Block 5								
H1	AC	0.71	161.8 (1.9)	0.024		161.7 (1.9)	0.019	
H2	GT	0.26	174.6 (3.4)*		7.9	174.5 (3.4)*		7.9
H3	<0.05	0.03	154.7 (9.6)		-4.4	156.5 (9.7)		-3.2
Block 6								
H1	CC	0.51	164.3 (2.3)	0.140		164.0 (2.3)	0.113	
H2	CT	0.39	162.3 (2.6)		-1.2	162.6 (2.6)		-0.9
H3	TC	0.10	178.6 (5.7)*		8.7	178.4 (5.6)*		8.8

P for trend is presented for single haplotypes. The most frequent haplotype (H1) was used as the reference when comparing total IGF-I levels between haplotypes. Model 1: without adjustment. Model 2: after adjustment for age. *P ≤ 0.05 compared to the reference.

Table 6C. Associations between circulating IGF-I bioactivity with IGF-I gene haplotypes in women

Haplotype	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 1								
H1	TG	0.83	180.3 (1.9)	0.218		180.4 (1.9)	0.231	
H2	CT	0.12	175.5 (4.9)		-2.7	175.6 (4.9)		-2.7
H3	<0.05	0.05	173.0 (7.7)		-4.0	173.1 (7.7)		-4.0
Block 2								
H1	TC	0.82	180.5 (1.9)	0.149		180.4 (1.9)	0.163	
H2	CT	0.17	175.1 (4.2)		-3.0	175.2 (4.2)		-2.9
H3	<0.05	0.01	165.2 (15.1)		-8.5	165.5 (15.1)		-8.3
Block 3								
H1	GATCC	0.54	178.3 (2.4)	0.728		178.2 (2.4)	0.713	
H2	AATCC	0.07	181.7 (6.8)		1.9	182.2 (6.8)		2.2
H3	GATCA	0.13	180.7 (4.9)		1.3	180.5 (4.9)		1.3
H4	GGCTC	0.23	182.1 (3.7)		2.1	182.2 (3.7)		2.2
H5	<0.05	0.04	170.2 (8.3)		-4.5	170.0 (8.3)		-4.6
Block 4								
H1	CT	0.62	178.7 (2.2)	0.604		178.6 (2.2)	0.560	
H2	CC	0.15	180.0 (4.6)		0.7	180.0 (4.6)		0.8
H3	TC	0.24	180.8 (3.6)		1.2	180.9 (3.6)		1.3

Table 6C continued

Haplotype	Frequency		Model 1			Model 2		
			Mean (SE)	P	% Change	Mean (SE)	P	% Change
Block 5								
H1	AC	0.72	179.2 (2.1)	0.917		179.1 (2.0)	0.955	
H2	GT	0.25	180.6 (3.5)		0.8	180.7 (3.5)		0.9
H3	<0.05	0.03	172.0 (10.2)		-4.0	171.9 (10.2)		-4.0
Block 6								
H1	CC	0.51	180.3 (2.5)	0.590		180.4 (2.5)	0.624	
H2	CT	0.40	178.6 (2.8)		-0.9	178.6 (2.8)		-1.0
H3	TC	0.10	177.7 (5.6)		-1.4	178.2 (5.6)		-1.2

P for trend is presented for single haplotypes. The most frequent haplotype (H1) was used as the reference when comparing total IGF-I levels between haplotypes. Model 1: without adjustment. Model 2: after adjustment for age. *P ≤ 0.05 compared to the reference.

C. IGF-I gene SNPs, IGF-I gene haplotype blocks, and myocardial infarction

In the whole study population and in men, but not in women several SNPs of the IGF-I gene were significantly associated with risk of MI/AP. One SNP (rs35766) located in block 1 and two SNPs located in block 2 (rs2162679 and rs35767) were significantly related to an increased risk of MI/AP (Table 7A-C). In addition, only in men SNPs rs35765 (block 1) and rs10860862 (others) were also associated with risk of MI/AP (Table 7B). After adjustments for age, BMI, systolic and diastolic BP, total and HDL-cholesterol, incidence of DM type 2, prevalence of CVD and current smoking these relationships remained significant for all these SNPs in men, and for rs35766 and 35767 in the total study population (Table 7B and 7A respectively).

In the whole study population and in men there was a significantly increased risk for MI/AP for carriers of haplotype 2 in both block 1 and block 2, odds ratios were 1.23 and 1.22 in the whole study population and 1.40 and 1.46 in men, respectively (Table 8A and 8B). These relationships remained significant in men after adjustments for age, BMI, systolic and diastolic BP, total and HDL-cholesterol, incidence of DM type 2, prevalence of CVD and current smoking, while overall these relationships became borderline significant after these adjustments (Tabel 8B and 8A: models 2 and 3). In women no such relationships were found (Table 8C).

None of the IGF-I SNPs or haplotypes of the IGF-I gene that were associated with circulating IGF-I bioactivity were significantly associated with an increased risk on MI and/or AP (Table 7A-C and Table 8A-C). No significant relationships were found between SNPs/haplotypes of the IGF-I gene and coronary heart disease (data not shown).

Table 7A. Risk of myocardial infarction and angina pectoris across IGF-I gene polymorphisms in the total population

SNP	Frequency			Model 1		Model 2		Model 3	
		Control	Case	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Block 1									
rs35766	TT	0.71	0.66	1 (Ref.)	<u>0.012</u>	1 (Ref.)	<u>0.011</u>	1 (Ref.)	<u>0.032</u>
	TC	0.26	0.30	1.20 (0.97-1.49)		1.20 (0.97-1.49)		1.17 (0.94-1.46)	
	CC	0.03	0.04	1.75 (1.06-2.88)*		1.81 (1.09-2.99)*		1.69 (1.00-2.86)	
rs35765	GG	0.78	0.74	1 (Ref.)	0.078	1 (Ref.)	0.076	1 (Ref.)	0.162
	GT	0.21	0.23	1.19 (0.95-1.50)		1.18 (0.94-1.49)		1.16 (0.91-1.48)	
	TT	0.02	0.02	1.41 (0.73-2.70)		1.50 (0.78-2.90)		1.31 (0.65-2.66)	
Block 2									
rs2162679	TT	0.71	0.67	1 (Ref.)	0.050	1 (Ref.)	0.050	1 (Ref.)	0.111
	TC	0.26	0.29	1.18 (0.95-1.46)		1.16 (0.94-1.44)		1.14 (0.92-1.43)	
	CC	0.03	0.04	1.46 (0.87-2.45)		1.55 (0.92-2.61)		1.42 (0.82-2.44)	
rs35767	CC	0.71	0.66	1 (Ref.)	<u>0.016</u>	1 (Ref.)	<u>0.015</u>	1 (Ref.)	<u>0.041</u>
	CT	0.26	0.29	1.20 (0.96-1.49)		1.18 (0.95-1.47)		1.16 (0.92-1.45)	
	TT	0.03	0.04	1.71 (1.04-2.82)*		1.82 (1.10-3.01)*		1.69 (1.00-2.87)*	
Block 3									
rs2033178	GG	0.87	0.85	1 (Ref.)	0.149	1 (Ref.)	0.189	1 (Ref.)	0.363
	GA	0.12	0.15	1.26 (0.95-1.65)		1.23 (0.93-1.62)		1.21 (0.91-1.61)	
	AA	0.005	0.004	0.90 (0.21-3.90)		0.97 (0.22-4.25)		0.70 (0.15-3.23)	
rs5742632	AA	0.60	0.60	1 (Ref.)	0.614	1 (Ref.)	0.678	1 (Ref.)	0.632
	AG	0.35	0.35	1.00 (0.82-1.23)		1.01 (0.82-1.24)		0.99 (0.80-1.23)	
	GG	0.05	0.04	0.82 (0.51-1.31)		0.83 (0.52-1.33)		0.84 (0.52-1.36)	
rs5742629	TT	0.54	0.55	1 (Ref.)	0.738	1 (Ref.)	0.815	1 (Ref.)	0.768
	TC	0.39	0.39	0.99 (0.81-1.21)		1.00 (0.82-1.23)		1.00 (0.81-1.23)	
	CC	0.07	0.07	0.92 (0.62-1.37)		0.93 (0.63-1.39)		0.91 (0.60-1.38)	
rs10778176	CC	0.55	0.55	1 (Ref.)	0.749	1 (Ref.)	0.848	1 (Ref.)	0.772
	CT	0.38	0.39	1.01 (0.82-1.24)		1.03 (0.84-1.26)		1.02 (0.83-1.26)	
	TT	0.07	0.06	0.89 (0.59-1.33)		0.90 (0.59-1.35)		0.87 (0.57-1.34)	
rs1019731	CC	0.73	0.73	1 (Ref.)	0.947	1 (Ref.)	0.870	1 (Ref.)	0.799
	CA	0.24	0.25	1.03 (0.82-1.29)		1.03 (0.82-1.29)		1.07 (0.85-1.34)	
	AA	0.02	0.02	0.81 (0.39-1.68)		0.77 (0.37-1.61)		0.85 (0.40-1.82)	
Block 4									
rs2288378	CC	0.57	0.57	1 (Ref.)	0.883	1 (Ref.)	0.821	1 (Ref.)	0.997
	CT	0.37	0.38	1.05 (0.85-1.28)		1.04 (0.85-1.28)		1.02 (0.83-1.26)	
	TT	0.06	0.05	0.95 (0.61-1.47)		0.98 (0.63-1.53)		0.95 (0.60-1.51)	
rs7136446	TT	0.37	0.36	1 (Ref.)	0.605	1 (Ref.)	0.599	1 (Ref.)	0.274
	CT	0.47	0.50	1.09 (0.88-1.34)		1.07 (0.87-1.33)		1.00 (0.80-1.24)	
	CC	0.17	0.14	0.87 (0.64-1.17)		0.87 (0.64-1.18)		0.81 (0.59-1.11)	

Table 7A. Continued

SNP	Frequency			Model 1		Model 2		Model 3	
		Control	Case	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Block 5									
rs6220	AA	0.52	0.50	1 (Ref.)	0.760	1 (Ref.)	0.748	1 (Ref.)	0.940
	AG	0.4	0.43	1.10 (0.90-1.34)		1.10 (0.90-1.35)		1.08 (0.88-1.33)	
	GG	0.08	0.07	0.93 (0.63-1.38)		0.93 (0.63-1.38)		0.89 (0.59-1.35)	
rs978458	CC	0.55	0.54	1 (Ref.)	0.679	1 (Ref.)	0.670	1 (Ref.)	0.867
	CT	0.38	0.41	1.09 (0.89-1.33)		1.08 (0.88-1.32)		1.06 (0.86-1.30)	
	TT	0.06	0.06	0.95 (0.63-1.45)		0.98 (0.64-1.49)		0.94 (0.61-1.46)	
Block 6									
rs6219	CC	0.81	0.82	1 (Ref.)	0.658	1 (Ref.)	0.732	1 (Ref.)	0.951
	CT	0.17	0.18	1.00 (0.78-1.30)		1.01 (0.78-1.30)		1.03 (0.79-1.35)	
	TT	0.01	0.01	0.54 (0.17-1.75)		0.58 (0.18-1.89)		0.67 (0.20-2.22)	
rs6214	CC	0.34	0.32	1 (Ref.)	0.833	1 (Ref.)	0.810	1 (Ref.)	0.956
	CT	0.50	0.53	1.12 (0.90-1.39)		1.11 (0.89-1.38)		1.13 (0.90-1.41)	
	TT	0.16	0.15	0.99 (0.73-1.33)		0.99 (0.74-1.34)		0.93 (0.68-1.27)	
Others									
rs2971575	AA	0.33	0.33	1 (Ref.)	0.510	1 (Ref.)	0.483	1 (Ref.)	0.234
	AG	0.48	0.51	1.08 (0.87-1.34)		1.07 (0.86-1.33)		1.00 (0.80-1.25)	
	GG	0.19	0.16	0.87 (0.65-1.16)		0.86 (0.65-1.16)		0.81 (0.60-1.10)	
rs10860862	GG	0.66	0.64	1 (Ref.)	0.468	1 (Ref.)	0.368	1 (Ref.)	0.266
	GT	0.31	0.33	1.12 (0.91-1.37)		1.12 (0.91-1.38)		1.18 (0.95-1.46)	
	TT	0.04	0.03	0.97 (0.57-1.66)		1.04 (0.61-1.77)		1.00 (0.58-1.74)	
rs2946834	GG	0.48	0.46	1 (Ref.)	0.713	1 (Ref.)	0.634	1 (Ref.)	0.377
	GA	0.41	0.46	1.19 (0.97-1.46)		1.17 (0.95-1.43)		1.11 (0.90-1.37)	
	AA	0.11	0.08	0.74 (0.52-1.06)		0.74 (0.51-1.06)		0.70 (0.48-1.02)	

P for trend is presented for single SNPs; The most frequent genotype was used as the reference when comparing incidence of myocardial infarction and angina pectoris between genotypes. Model 1: without adjustment; Model 2: after adjustment for gender and age; Model 3: adjusted for gender, age and cardiovascular risk factors. *P ≤ 0.05 compared to the reference.

Table 7B. Risk of myocardial infarction and angina pectoris across IGF-I gene polymorphisms in men

SNP	Frequency			Model 1		Model 2		Model 3	
		Control	Case	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Block 1									
rs35766	TT	0.71	0.62	1 (Ref.)	0.004	1 (Ref.)	0.003	1 (Ref.)	0.005
	TC	0.27	0.34	1.44 (1.07-1.93)*		1.46 (1.08-1.96)*		1.45 (1.07-1.96)*	
	CC	0.02	0.04	1.97 (0.95-4.09)		2.06 (0.99-4.28)*		1.99 (0.93-4.27)	
rs35765	GG	0.77	0.70	1 (Ref.)	0.025	1 (Ref.)	0.020	1 (Ref.)	0.029
	GT	0.22	0.27	1.36 (0.99-1.85)		1.38 (1.01-1.88)*		1.36 (0.99-1.89)	
	TT	0.01	0.02	1.87 (0.74-4.73)		1.92 (0.76-4.87)		1.92 (0.71-5.16)	

Table 7B. Continued

SNP	Frequency			Model 1		Model 2		Model 3	
		Control	Case	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Block 2									
rs2162679	TT	0.71	0.62	1 (Ref.)	0.006	1 (Ref.)	0.005	1 (Ref.)	0.011
	TC	0.27	0.34	1.40 (1.05-1.88)*		1.40 (1.05-1.89)*		1.38 (1.01-1.87)*	
	CC	0.02	0.04	2.02 (0.97-4.19)		2.10 (1.01-4.39)*		2.00 (0.93-4.30)	
rs35767	CC	0.71	0.61	1 (Ref.)	0.002	1 (Ref.)	0.002	1 (Ref.)	0.004
	CT	0.27	0.35	1.50 (1.12-2.02)*		1.50 (1.12-2.02)*		1.47 (1.08-2.00)*	
	TT	0.02	0.04	2.09 (1.00-4.35)*		2.15 (1.03-4.51)*		2.08 (0.96-4.50)	
Block 3									
rs2033178	GG	0.86	0.84	1 (Ref.)	0.291	1 (Ref.)	0.283	1 (Ref.)	0.210
	GA	0.14	0.16	1.20 (0.82-1.74)		1.20 (0.82-1.75)		1.23 (0.83-1.82)	
	AA	0.002	0.00	1.96 (0.20-18.89)		2.17 (0.22-21.11)		3.44 (0.34-34.50)	
rs5742632	AA	0.60	0.61	1 (Ref.)	0.526	1 (Ref.)	0.476	1 (Ref.)	0.361
	AG	0.34	0.36	1.04 (0.78-1.39)		1.04 (0.78-1.38)		0.98 (0.73-1.32)	
	GG	0.05	0.03	0.60 (0.28-1.27)		0.58 (0.27-1.23)		0.59 (0.28-1.28)	
rs5742629	TT	0.55	0.55	1 (Ref.)	0.931	1 (Ref.)	0.907	1 (Ref.)	0.706
	TC	0.38	0.39	1.01 (0.76-1.35)		1.01 (0.76-1.35)		0.98 (0.73-1.32)	
	CC	0.07	0.07	0.94 (0.54-1.65)		0.93 (0.53-1.64)		0.86 (0.47-1.58)	
rs10778176	CC	0.56	0.55	1 (Ref.)	0.972	1 (Ref.)	0.989	1 (Ref.)	0.763
	CT	0.37	0.38	1.03 (0.77-1.37)		1.03 (0.77-1.37)		0.99 (0.74-1.33)	
	TT	0.07	0.07	0.97 (0.55-1.69)		0.96 (0.54-1.68)		0.88 (0.48-1.62)	
rs1019731	CC	0.73	0.72	1 (Ref.)	0.998	1 (Ref.)	0.916	1 (Ref.)	0.786
	CA	0.25	0.26	1.04 (0.76-1.42)		1.02 (0.75-1.40)		1.09 (0.79-1.51)	
	AA	0.02	0.02	0.85 (0.33-2.19)		0.81 (0.31-2.12)		0.86 (0.32-2.30)	
Block 4									
rs2288378	CC	0.57	0.54	1 (Ref.)	0.742	1 (Ref.)	0.771	1 (Ref.)	0.850
	CT	0.38	0.42	1.18 (0.89-1.56)		1.17 (0.88-1.54)		1.14 (0.85-1.53)	
	TT	0.05	0.04	0.74 (0.36-1.52)		0.75 (0.36-1.53)		0.73 (0.34-1.59)	
rs7136446	TT	0.35	0.34	1 (Ref.)	0.837	1 (Ref.)	0.813	1 (Ref.)	0.533
	CT	0.49	0.52	1.08 (0.80-1.46)		1.08 (0.80-1.46)		0.97 (0.71-1.33)	
	CC	0.16	0.14	0.91 (0.59-1.39)		0.90 (0.59-1.38)		0.86 (0.55-1.33)	
Block 5									
rs6220	AA	0.52	0.48	1 (Ref.)	0.566	1 (Ref.)	0.600	1 (Ref.)	0.722
	AG	0.40	0.45	1.22 (0.92-1.62)		1.21 (0.91-1.61)		1.19 (0.89-1.60)	
	GG	0.08	0.07	0.90 (0.51-1.58)		0.90 (0.51-1.57)		0.84 (0.46-1.55)	
rs978458	CC	0.55	0.51	1 (Ref.)	0.702	1 (Ref.)	0.781	1 (Ref.)	0.902
	CT	0.39	0.45	1.23 (0.93-1.63)		1.21 (0.91-1.60)		1.17 (0.87-1.57)	
	TT	0.06	0.04	0.71 (0.36-1.41)		0.71 (0.36-1.40)		0.69 (0.33-1.42)	
Block 6									
rs6219	CC	0.82	0.83	1 (Ref.)	0.457	1 (Ref.)	0.411	1 (Ref.)	0.342
	CT	0.17	0.16	0.92 (0.64-1.33)		0.91 (0.63-1.31)		0.87 (0.59-1.28)	
	TT	0.01	0.00	0.43 (0.06-3.30)		0.42 (0.05-3.23)		0.46 (0.06-3.57)	

Table 7B. Continued

SNP	Frequency			Model 1		Model 2		Model 3	
		Control	Case	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
rs6214	CC	0.34	0.30	1 (Ref.)	0.104	1 (Ref.)	0.112	1 (Ref.)	0.320
	CT	0.51	0.51	1.13 (0.83-1.54)		1.12 (0.82-1.53)		1.08 (0.78-1.49)	
	TT	0.15	0.19	1.41 (0.94-2.10)		1.41 (0.94-2.10)		1.25 (0.82-1.89)	
Others									
rs2971575	AA	0.32	0.31	1 (Ref.)	0.963		0.914	1 (Ref.)	0.654
	AG	0.49	0.51	1.07 (0.78-1.45)		1.06 (0.77-1.44)		0.98 (0.71-1.35)	
	GG	0.19	0.18	0.97 (0.65-1.45)		0.96 (0.64-1.44)		0.90 (0.59-1.38)	
rs10860862	GG	0.67	0.61	1 (Ref.)	0.025	1 (Ref.)	0.020	1 (Ref.)	0.037
	GT	0.3	0.35	1.29 (0.96-1.72)		1.29 (0.97-1.73)		1.30 (0.96-1.76)	
	TT	0.03	0.05	1.83 (0.92-3.66)		1.95 (0.97-3.90)		1.72 (0.83-3.55)	
rs2946834	GG	0.46	0.44	1 (Ref.)	0.792	1 (Ref.)	0.735	1 (Ref.)	0.504
	GA	0.43	0.47	1.13 (0.85-1.50)		1.11 (0.84-1.48)		1.04 (0.78-1.40)	
	AA	0.11	0.09	0.79 (0.48-1.30)		0.78 (0.47-1.29)		0.75 (0.45-1.25)	

P for trend is presented for single SNPs; The most frequent genotype was used as the reference when comparing incidence of myocardial infarction and angina pectoris between genotypes. Model 1: without adjustment; Model 2: after adjustment for gender and age; Model 3: adjusted for gender, age and cardiovascular risk factors. *P ≤ 0.05 compared to the reference.

Table 7C. Risk of myocardial infarction and angina pectoris across IGF-I gene polymorphisms in women.

SNP	Frequency			Model 1		Model 2		Model 3	
		Control	Case	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Block 1									
rs35766	TT	0.71	0.71	1 (Ref.)	0.552	1 (Ref.)	0.540	1 (Ref.)	0.762
	TC	0.26	0.25	0.96 (0.70-1.33)		0.97 (0.70-1.33)		0.95 (0.68-1.32)	
	CC	0.03	0.04	1.61 (0.81-3.22)		1.61 (0.81-3.23)		1.44 (0.69-3.03)	
rs35765	GG	0.78	0.78	1 (Ref.)	0.949	1 (Ref.)	0.876	1 (Ref.)	0.823
	GT	0.20	0.19	0.98 (0.69-1.39)		0.99 (0.70-1.41)		0.98 (0.68-1.41)	
	TT	0.02	0.02	1.13 (0.44-2.92)		1.20 (0.47-3.08)		0.88 (0.30-2.54)	
Block 2									
rs2162679	TT	0.71	0.72	1 (Ref.)	0.944	1 (Ref.)	0.848	1 (Ref.)	0.775
	TC	0.26	0.25	0.95 (0.69-1.30)		0.94 (0.68-1.30)		0.94 (0.67-1.31)	
	CC	0.03	0.04	1.13 (0.53-2.40)		1.17 (0.55-2.48)		0.99 (0.44-2.24)	
rs35767	CC	0.71	0.72	1 (Ref.)	0.830	1 (Ref.)	0.805	1 (Ref.)	0.995
	CT	0.26	0.24	0.90 (0.65-1.25)		0.90 (0.65-1.25)		0.89 (0.64-1.26)	
	TT	0.03	0.05	1.51 (0.76-3.00)		1.56 (0.78-3.12)		1.37 (0.65-2.87)	
Block 3									
rs2033178	GG	0.88	0.86	1 (Ref.)	0.432	1 (Ref.)	0.433	1 (Ref.)	0.825
	GA	0.11	0.13	1.25 (0.83-1.88)		1.26 (0.84-1.90)		1.17 (0.76-1.79)	
	AA	0.01	0.004	0.68 (0.09-5.20)		0.63 (0.08-4.88)		0.43 (0.05-3.45)	
rs5742632	AA	0.59	0.60	1 (Ref.)	0.998	1 (Ref.)	0.902	1 (Ref.)	0.816
	AG	0.35	0.34	0.97 (0.73-1.31)		0.98 (0.73-1.32)		1.00 (0.74-1.35)	
	GG	0.05	0.06	1.06 (0.58-1.93)		1.10 (0.60-2.02)		1.13 (0.61-2.09)	

Table 7C. Continued

SNP	Frequency			Model 1		Model 2		Model 3	
		Control	Case	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
rs5742629	TT	0.54	0.54	1 (Ref.)	0.759	1 (Ref.)	0.827	1 (Ref.)	0.960
	TC	0.39	0.39	0.98 (0.73-1.30)		0.99 (0.74-1.32)		1.02 (0.76-1.38)	
	CC	0.07	0.07	0.91 (0.52-1.61)		0.93 (0.53-1.63)		0.98 (0.55-1.75)	
rs10778176	CC	0.54	0.54	1 (Ref.)	0.696	1 (Ref.)	0.771	1 (Ref.)	0.983
	CT	0.39	0.40	1.01 (0.76-1.35)		1.02 (0.77-1.37)		1.06 (0.78-1.42)	
	TT	0.07	0.06	0.82 (0.45-1.49)		0.83 (0.46-1.52)		0.89 (0.48-1.63)	
rs1019731	CC	0.74	0.74	1 (Ref.)	0.829	1 (Ref.)	0.894	1 (Ref.)	0.989
	CA	0.24	0.25	1.02 (0.74-1.40)		1.03 (0.75-1.42)		1.03 (0.74-1.44)	
	AA	0.02	0.01	0.69 (0.21-2.27)		0.70 (0.21-2.30)		0.79 (0.24-2.62)	
Block 4									
rs2288378	CC	0.58	0.59	1 (Ref.)	0.881	1 (Ref.)	0.982	1 (Ref.)	0.916
	CT	0.37	0.34	0.90 (0.67-1.21)		0.92 (0.68-1.24)		0.90 (0.66-1.23)	
	TT	0.06	0.07	1.15 (0.65-2.02)		1.19 (0.67-2.09)		1.15 (0.65-2.06)	
rs7136446	TT	0.38	0.38	1 (Ref.)	0.508	1 (Ref.)	0.609	1 (Ref.)	0.388
	CT	0.45	0.48	1.05 (0.78-1.41)		1.07 (0.79-1.45)		1.01 (0.74-1.38)	
	CC	0.17	0.14	0.82 (0.53-1.26)		0.84 (0.55-1.29)		0.78 (0.50-1.22)	
Block 5									
rs6220	AA	0.52	0.53	1 (Ref.)	0.838	1 (Ref.)	0.939	1 (Ref.)	0.940
	AG	0.40	0.40	0.98 (0.73-1.31)		1.00 (0.75-1.34)		0.99 (0.73-1.33)	
	GG	0.07	0.07	0.95 (0.55-1.65)		0.97 (0.56-1.68)		0.99 (0.56-1.74)	
rs978458	CC	0.56	0.56	1 (Ref.)	0.873	1 (Ref.)	0.753	1 (Ref.)	0.853
	CT	0.38	0.36	0.94 (0.70-1.26)		0.96 (0.71-1.29)		0.94 (0.70-1.28)	
	TT	0.06	0.08	1.19 (0.70-2.04)		1.23 (0.72-2.12)		1.19 (0.69-2.07)	
Block 6									
rs6219	CC	0.81	0.80	1 (Ref.)	0.855	1 (Ref.)	0.750	1 (Ref.)	0.391
	CT	0.17	0.19	1.10 (0.77-1.57)		1.12 (0.78-1.60)		1.21 (0.84-1.75)	
	TT	0.01	0.01	0.67 (0.16-2.84)		0.71 (0.17-3.03)		0.93 (0.22-4.01)	
rs6214	CC	0.34	0.35	1 (Ref.)	0.169	1 (Ref.)	0.207	1 (Ref.)	0.244
	CT	0.49	0.54	1.09 (0.81-1.47)		1.11 (0.82-1.50)		1.18 (0.87-1.62)	
	TT	0.17	0.11	0.63 (0.39-1.01)		0.64 (0.40-1.03)		0.63 (0.38-1.02)	
Others									
rs2971575	AA	0.34	0.350	1 (Ref.)	0.319	1 (Ref.)	0.375	1 (Ref.)	0.280
	AG	0.46	0.5	1.07 (0.79-1.45)		1.08 (0.80-1.47)		1.02 (0.74-1.39)	
	GG	0.19	0.15	0.76 (0.49-1.16)		0.77 (0.50-1.18)		0.75 (0.48-1.16)	
rs10860862	GG	0.65	0.670	1 (Ref.)	0.309	1 (Ref.)	0.314	1 (Ref.)	0.562
	GT	0.31	0.31	0.97 (0.72-1.31)		0.97 (0.72-1.31)		1.06 (0.78-1.44)	
	TT	0.04	0.02	0.51 (0.20-1.28)		0.51 (0.20-1.28)		0.52 (0.20-1.31)	
rs2946834	GG	0.49	0.47	1 (Ref.)	0.683	1 (Ref.)	0.734	1 (Ref.)	0.684
	GA	0.39	0.46	1.21 (0.91-1.61)		1.22 (0.92-1.63)		1.18 (0.88-1.60)	
	AA	0.12	0.08	0.68 (0.40-1.16)		0.69 (0.40-1.17)		0.69 (0.39-1.19)	

P for trend is presented for single SNPs; The most frequent genotype was used as the reference when comparing incidence of myocardial infarction and angina pectoris between genotypes. Model 1: without adjustment; Model 2: after adjustment for gender and age; Model 3: adjusted for gender, age and cardiovascular risk factors. *P ≤ 0.05 compared to the reference.

Table 8A. Risk of myocardial infarction and angina pectoris across IGF-I gene haplotypes in the total population

Haplotype		Frequency		Model 1		Model 2		Model 3	
		Control	Case	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Block 1									
H1	TG	0.84	0.81	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	CT	0.12	0.14	1.23 (1.01-1.49)	0.038	1.23 (1.01-1.50)	0.037	1.19 (0.97-1.46)	0.091
H3	<0.05	0.04	0.05	1.32 (0.95-1.85)	0.101	1.33 (0.95-1.86)	0.100	1.30 (0.92-1.84)	0.130
Block 2									
H1	TC	0.84	0.81	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	CT	0.15	0.18	1.22 (1.02-1.46)	0.026	1.23 (1.02-1.46)	0.026	1.19 (0.99-1.43)	0.060
H3	<0.05	0.01	0.01	1.04 (0.52-2.08)	0.915	1.07 (0.53-2.15)	0.861	1.01 (0.50-2.06)	0.973
Block 3									
H1	GATCC	0.53	0.52	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	AATCC	0.06	0.08	1.22 (0.93-1.59)	0.151	1.20 (0.92-1.58)	0.185	1.14 (0.86-1.51)	0.363
H3	GATCA	0.14	0.14	1.01 (0.82-1.23)	0.943	1.00 (0.81-1.22)	0.979	1.04 (0.84-1.28)	0.743
H4	GGCTC	0.23	0.22	0.98 (0.83-1.17)	0.835	0.99 (0.83-1.17)	0.873	0.98 (0.82-1.17)	0.825
H5	<0.05	0.03	0.04	1.08 (0.75-1.55)	0.682	1.08 (0.75-1.56)	0.667	1.09 (0.75-1.60)	0.643
Block 4									
H1	CT	0.60	0.61	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	CC	0.16	0.15	0.92 (0.76-1.12)	0.418	0.92 (0.75-1.11)	0.377	0.86 (0.71-1.06)	0.155
H3	TC	0.24	0.24	0.99 (0.84-1.17)	0.934	1.00 (0.84-1.18)	0.977	0.96 (0.81-1.15)	0.683
Block 5									
H1	AC	0.72	0.71	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	GT	0.25	0.26	1.02 (0.87-1.20)	0.790	1.02 (0.87-1.20)	0.772	1.00 (0.85-1.18)	0.992
H3	<0.05	0.03	0.03	1.09 (0.73-1.65)	0.671	1.10 (0.73-1.66)	0.646	1.12 (0.73-1.72)	0.597
Block 6									
H1	AC	0.72	0.71	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	GT	0.25	0.26	1.02 (0.87-1.20)	0.790	1.02 (0.87-1.20)	0.772	1.00 (0.85-1.18)	0.992
H3	<0.05	0.03	0.03	1.09 (0.73-1.65)	0.671	1.10 (0.73-1.66)	0.646	1.12 (0.73-1.72)	0.597

P for trend is presented for single haplotypes. The most frequent haplotype (H1) was used as the reference when comparing total IGF-I levels between haplotypes. Model 1: without adjustment. Model 2: after adjustment for gender and age. Model 3: after adjustment for gender, age and cardiovascular risk factors.

Table 8B. Risk of myocardial infarction and angina pectoris across IGF-I gene haplotypes in men

Haplotype		Frequency		Model 1		Model 2		Model 3	
		Control	Case	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Block 1									
H1	TG	0.84	0.79	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	CT	0.12	0.16	1.40 (1.07-1.84)	0.014	1.42 (1.09-1.87)	0.011	1.42 (1.07-1.88)	0.016
H3	Rare	0.03	0.05	1.50 (0.94-2.39)	0.089	1.52 (0.95-2.42)	0.083	1.47 (0.90-2.38)	0.122
Block 2									
H1	TC	0.84	0.78	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	CT	0.15	0.21	1.46 (1.15-1.87)	0.002	1.48 (1.15-1.89)	0.002	1.45 (1.12-1.88)	0.004
H3	Rare	0.00	0.01	0.95 (0.33-2.76)	0.927	0.97 (0.33-2.85)	0.958	0.87 (0.29-2.62)	0.808

Table 8B continued

Haplotype		Frequency		Model 1		Model 2		Model 3	
		Control	Case	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Block 3									
H1	GATCC	0.52	0.52	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	AATCC	0.07	0.08	1.23 (0.84-1.80)	0.279	1.23 (0.84-1.81)	0.284	1.27 (0.85-1.88)	0.242
H3	GATCA	0.15	0.14	1.02 (0.77-1.36)	0.871	1.01 (0.76-1.34)	0.963	1.06 (0.79-1.41)	0.714
H4	GGCTC	0.22	0.21	0.96 (0.75-1.24)	0.764	0.95 (0.74-1.22)	0.695	0.93 (0.72-1.21)	0.607
H5	Rare	0.03	0.04	1.34 (0.83-2.15)	0.229	1.37 (0.85-2.20)	0.197	1.38 (0.83-2.29)	0.220
Block 4									
H1	CT	0.59	0.60	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	CC	0.17	0.16	0.94 (0.72-1.22)	0.632	0.94 (0.72-1.22)	0.629	0.88 (0.67-1.17)	0.377
H3	TC	0.24	0.25	1.01 (0.80-1.28)	0.921	1.01 (0.79-1.28)	0.952	0.98 (0.76-1.26)	0.891
Block 5									
H1	AC	0.72	0.71	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	GT	0.25	0.26	1.05 (0.84-1.32)	0.660	1.04 (0.83-1.30)	0.728	1.02 (0.80-1.29)	0.871
H3	<0.05	0.03	0.03	1.12 (0.64-1.95)	0.692	1.16 (0.67-2.03)	0.597	1.14 (0.63-2.06)	0.654
Block 6									
H1	CC	0.49	0.47	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	CT	0.41	0.45	1.17 (0.95-1.44)	0.142	1.16 (0.94-1.43)	0.158	1.09 (0.88-1.35)	0.447
H3	TC	0.10	0.09	0.94 (0.66-1.34)	0.746	0.93 (0.65-1.33)	0.682	0.87 (0.60-1.27)	0.475

P for trend is presented for single haplotypes. The most frequent haplotype (H1) was used as the reference when comparing total IGF-I levels between haplotypes. Model 1: without adjustment. Model 2: after adjustment for gender and age. Model 3: after adjustment for gender, age and cardiovascular risk factors.

Table 8C. Risk of myocardial infarction and angina pectoris across IGF-I gene haplotypes in women

Haplotype		Frequency		Model 1		Model 2		Model 3	
		Control	Case	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Block 1									
H1	TG	0.84	0.83	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	CT	0.12	0.12	1.05 (0.78-1.40)	0.759	1.06 (0.79-1.42)	0.704	1.00 (0.73-1.35)	0.984
H3	<0.05	0.04	0.05	1.20 (0.74-1.94)	0.463	1.17 (0.72-1.89)	0.530	1.20 (0.73-1.96)	0.474
Block 2									
H1	TC	0.83	0.83	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	CT	0.16	0.16	1.00 (0.77-1.31)	0.976	1.01 (0.77-1.31)	0.966	0.97 (0.74-1.28)	0.842
H3	<0.05	0.01	0.01	1.13 (0.45-2.83)	0.795	1.14 (0.45-2.86)	0.786	1.14 (0.45-2.87)	0.783
Block 3									
H1	GATCC	0.53	0.53	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	AATCC	0.06	0.07	1.16 (0.79-1.71)	0.444	1.17 (0.79-1.72)	0.431	1.06 (0.71-1.58)	0.784
H3	GATCA	0.14	0.14	0.98 (0.73-1.31)	0.868	0.99 (0.73-1.33)	0.942	1.01 (0.74-1.37)	0.965
H4	GGCTC	0.23	0.23	1.00 (0.79-1.28)	0.988	1.02 (0.80-1.30)	0.881	1.03 (0.80-1.32)	0.807
H5	<0.05	0.03	0.03	0.82 (0.46-1.46)	0.494	0.81 (0.45-1.44)	0.472	0.89 (0.50-1.61)	0.710
Block 4									
H1	CT	0.61	0.62	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	CC	0.15	0.14	0.89 (0.67-1.18)	0.414	0.89 (0.67-1.19)	0.441	0.84 (0.63-1.13)	0.260
H3	TC	0.24	0.24	0.97 (0.77-1.22)	0.779	0.99 (0.78-1.25)	0.919	0.96 (0.75-1.22)	0.736

Table 8C continued

Haplotype		Frequency		Model 1		Model 2		Model 3	
		Control	Case	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Block 5									
H1	CC	0.49	0.52	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	CT	0.41	0.38	0.86 (0.70-1.06)	0.167	0.88 (0.71-1.08)	0.221	0.90 (0.72-1.11)	0.326
H3	TC	0.10	0.10	0.97 (0.70-1.34)	0.839	0.99 (0.72-1.38)	0.975	1.10 (0.78-1.54)	0.593
Block 6									
H1	CC	0.49	0.52	1 (Ref.)		1 (Ref.)		1 (Ref.)	
H2	CT	0.41	0.38	0.86 (0.70-1.06)	0.167	0.88 (0.71-1.08)	0.221	0.90 (0.72-1.11)	0.326
H3	TC	0.10	0.10	0.97 (0.70-1.34)	0.839	0.99 (0.72-1.38)	0.975	1.10 (0.78-1.54)	0.593

P for trend is presented for single haplotypes. The most frequent haplotype (H1) was used as the reference when comparing total IGF-I levels between haplotypes. Model 1: without adjustment. Model 2: after adjustment for gender and age. Model 3: after adjustment for gender, age and cardiovascular risk factors.

DISCUSSION

The main finding in our study was that in men several SNPs/ haplotypes of the IGF-I gene were related to circulating total IGF-I levels ànd to MI/AP. All these SNPs/haplotypes were present in block 1 and 2 of the IGF-I gene. Although in women the same SNPs/haplotypes of the IGF-I gene were related to circulating IGF-I levels, no associations were found with MI/AP.

We previously examined within the Rotterdam study the role of a cytosine-adenosine (CA) repeat in the promoter region of the IGF-I gene in relation to circulating IGF-I levels and MI [12]. We observed that participants who did not carry the wild-type (192-bp) allele had lower circulating total IGF-I concentrations and an increased risk of MI [12]. In addition, this IGF-I promoter polymorphism was associated with excess mortality in elderly subjects with MI [13]. However, subsequent studies failed to find any consistent functional associations between these IGF-I gene variant and circulating levels of total IGF-I [35,36]. In concurrence with previous findings our study confirms that the less common repeat length CA repeat polymorphism in the promoter region of the IGF-I gene is in LD with the minor alleles of rs35767 in haplotype block 1 [37]. In our study there was not only high linkage equilibrium between this polymorphism and rs35767 but also with rs2162679. The minor alleles of both SNPs were associated with significantly lower total IGF-I levels and with an increased risk for MI/AP during follow-up of the Rotterdam study.

The so-called Mendelian randomization method is considered a powerful approach, which is useful to overcome the effects of confounding and reverse causality in epidemiological studies (10). When a polymorphism of the IGF-I

gene is related to low levels of circulating (total) IGF-I levels, and when low levels of circulating (total) IGF-I are indeed a causal risk for MI/AP, then one would expect that individuals with IGF-I genotypes/haplotypes associated with low (total) IGF-I levels have a higher risk to develop MI/AP. We found indeed that subjects with some genetic variants (SNPs, haplotypes) in block 1 and in block 2 of the IGF-I gene - compared to subjects with the wild-type - are life-long exposed to lower total IGF-I levels as well as have an increased risk to develop MI/AP.

Recent results suggest that IGF-I is a protective factor against endothelial dysfunction, atherosclerotic plaque development, the metabolic syndrome, clinical instability, and ischemic myocardial damage [38]. Some of these effects are related to the induction of constitutive nitric oxide (NO) production by IGF-I [38]. When total IGF-I levels are decreased below a critical threshold, vascular risk factors (like hypertension, cholesterol and glucose) may fully exert their detrimental effects, through unopposed endothelial dysfunction, endothelial apoptosis, and development of unstable plaques (38.

Circulating total IGF-I levels in large part determine body reserves of IGF-I [39]. The size of these reserves will become important in conditions of reduced IGF-I synthesis and/or increased IGF-I demands. We hypothesize that with aging circulating total IGF-I levels will earlier decline below a critical threshold value in subjects with some variant forms of SNPs of the IGF-I gene. This will impair the role of IGF-I as vascular protective factor and thereby increase the risk on MI/AP.

Although circulating IGF-I in women was related to the same genetic variants of the IGF-I gene as found in men, we did not find significant associations between these gene variants and MI/AP. How to explain this important gender difference? There is evidence that pathophysiology of coronary artery disease is different between men and women [40]. In the Framingham Heart Studied it was shown that women experience coronary artery disease events approximately 10 years later than men [41]. Moreover, it is increasingly being realized that hormonal differences between men and women go beyond the obvious differences in reproductive physiology. Although generally circulating total IGF-I levels do not differ between men and women, spontaneous growth hormone (GH) secretion has been reported to be approximately twofold higher in females [42,43]. Thus, both gender-specific differences in pathophysiology of coronary artery disease as well as gender-specific differences in the regulation of the GH-IGF-I axis may have played a role in the observed gender-specific associations between IGF-I gene variants and MI/AP.

We and others previously observed a weak correlation between circulating total IGF-I levels and IGF-I bioactivity suggesting that the IGF-I KIRA produces new information about the IGF-I system, which essentially differs from that obtained by IGF-I immunoassays [14,15,44,45]. In line with this, circulating IGF-I bioactivity was not associated with the risk for MI/ AP.

Mean circulating IGF-I bioactivity was higher in women than in men, while SNPs/haplotypes in blocks 3, 4, and 5 of the IGF-I gene showed gender-specific associations with circulating IGF-I bioactivity. As above discussed, a sexually dimorphic pattern of GH secretion may have influenced the circulating concentrations and actions of IGF-I bioactivity in a gender-specific way [46]. An alternative explanation for the observed differences in IGF-I bioactivity between men and women is gender-specific effects of sex hormones on the GH-IGF-I axis [46].

Specific IGF-I SNPs and haplotypes individually accounted in generally less for changes in circulating IGF-I bioactivity than for circulating total IGF-I. This suggests that IGF-I bioactivity is less dependent on genetic factors than circulating total IGF-I. In men SNPs/haplotypes closely related to circulating IGF-I bioactivity were observed in blocks 3-6 of the IGF-I gene. Blocks 3-6 of the IGF-I gene encode transcripts involved in size variation in IGF-I mRNAs and alternative splicing forms of the IGF-I gene [47]. This opens the possibility that differential processing of the IGF-I protein by posttranscriptional and/or posttranslational events is responsible for the observed relationships between genetic variants in blocks 3-6 of the IGF-I gene and circulating IGF-I bioactivity.

A limitation of our study was that circulating IGF-I bioactivity was not measured at baseline but after the third round of the Rotterdam study. This may have introduced a prevalence bias since the "worst" cases may have died in the intervening years. In favor of this latter option, the frequency of genotypes/haplotypes of participants in the third round of the Rotterdam study was almost similar to that of the controls at baseline. Another limitation of our study was that we combined MI and AP as a measure for coronary heart disease to increase power of our study. This may have introduced gender-specific differences, since women have higher rates of functional disability and a lower prevalence of obstructive coronary heart disease, as evidenced by coronary angiogram, than men [48]. In addition, information on AP was obtained at the third survey but there was no information available about the incidence of AP in the remaining period. Thus the incidence of AP was probably underrated. There are yet no data on the effects of long-term storage on sample IGF-I bioactivity in the KIRA. However, because all samples were

analyzed after an equal period of storage, any variable effect of storage time is likely removed.

Another limitation of our study is that total IGF-I and IGF-I bioactivity were only measured in a subsample of the population. In addition, we did not measure in the study IGFBPs or other factors that can modify IGF-I levels or action.

In conclusion, we observed gender specific effects of the IGF-I gene. In men but not in women there was an impact of genetic variation in the IGF-I gene on circulating total IGF-I levels and risk on MI/AP. In addition, genetic variations of the IGF-I gene were also related to circulating IGF-I bioactivity in men but not in women. However, these latter genetic variations in the IGF-I gene did not substantially influence the risk on MI/AP in this study.

Table S1. General information of the 18 SNPs used to cover the genetic information of the human IGF-I gene

No.	SNP	Position	HWE	%Genotype	MAF	Alleles	Genotyping method
1	rs2971575	101305009	0.21	100	0.12	G:T	Illumina 550K Chip
2	rs10860862	101310202	0.21	100	0.16	T:C	Illumina 550K Chip
3	rs2946834	101311944	0.24	97.3	0.16	C:T	Illumina 550K Chip
4	rs6219	101314322	0.11	100	0.16	T:C	TaqMan*
5	rs6214	101317699	0.91	99.8	0.14	C:A	TaqMan*
6	rs6220	101318645	0.22	100	0.26	C:T	TaqMan*
7	rs978458	101326369	0.19	100	0.27	T:C	Illumina 550K Chip
8	rs2288378	101354138	0.10	99.4	0.23	A:G	Illumina 550K Chip
9	rs7136446	101362645	0.53	100	0.07	G:A	Illumina 550K Chip
10	rs2033178	101371206	0.09	99.9	0.40	T:C	Illumina 550K Chip
11	rs5742632	101380604	0.67	100	0.24	C:T	Illumina 550K Chip
12	rs5742629	101381393	0.88	100	0.26	C:T	Illumina 550K Chip
13	rs10778176	101387109	0.75	98.2	0.28	A:G	Illumina 550K Chip
14	rs1019731	101388555	0.34	100	0.41	C:T	Illumina 550K Chip
15	rs2162679	101395389	0.31	99.9	0.10	C:T	Illumina 550K Chip
16	rs35767	101399699	0.08	100	0.32	G:A	TaqMan*
17	rs35766	101404603	0.63	100	0.19	G:T	Illumina 550K Chip
18	rs35765	101405826	0.17	100	0.43	A:G	TaqMan*

HWE = Hardy-Weinberg Equilibrium, MAF = Minor allele frequency

*Sequences of designed primers and probes used in these Taqman assays are shown in Table S2.

Table S2. Primers and probes designed for Taqman assays to genotype specific IGF-I SNPs

No.	SNPs	Primer Fw (5'> 3')	Primer Rev (5'> 3')	Probes (5'> 3')
4	rs6219	GTACCCGCTGCTAAA-CACACT	TGAATAGACATTTTCA-TGATACACAGACACA	VIC-CTGAAACCTCAAGCTGTCTA FAM-CTGAAACCTCAAACTGTCTA
5	rs6214	CCTCTCAACAAAACT-TTATAGGCAGTCT	AGTGAAGGAAATAAG-TCATAGACACTCTTAGA	VIC-AGACTTAACGTGTTTTC FAM-CAGACTTAACATGTTTTC
6	rs6220	GAAAAGAAGGAATC-ATTGTGTTTTTCAAA-ATGAA	GCACTCACTGACTCTT-CTATGCA	VIC-TCTAGTAAAACCTCGTTTAAT FAM-ATCTAGTAAAACCTTGTTTAAT
16	rs35767	GGATTTCAAGCAGA-ACTGTGTTTTCA	GGTGGAAATAACCTG-GACCTTGAAT	VIC-CCTGAGAGTCATGTGGAAA FAM-CTGAGAGTCATGCGGAAA
18	rs35765	GCCAAGTCAAGTGG-TCACAAC	GCTCCTGCACTTACTG-TTTCTTG	VIC-TTCTCTTTGGAAATGAT FAM-CTCTTTGGCAATGAT

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Chapter 8

Conclusions and General Discussion

The general aim of the research presented in this thesis was to evaluate whether circulating IGF-I bioactivity measured by the IGF-I-KIRA has the potential to produce new insights in the functional properties of the circulating IGF system in relation to human aging, health and disease.

8.1 The general aim of the thesis; what lies beneath?

One of the major challenges in the field of IGF research is to clarify how IGF-I bioactivity is regulated *in vivo*. At the basis of this challenge lies the fascinating but highly complex structure of the molecular network that comprises the IGF system of which IGF-I is a major determinant. Classically the IGF system includes two growth factors (IGF-I and IGF-II), six IGF-binding proteins (IGFBP-1 to -6), nine IGFBP-related proteins (IGFBP-rPs), two cell surface receptors (IGF-IR and the IGF-II mannose-6-phosphate receptor (M-6-PR) and several IGFBP specific proteases [1,2]. When compared to other hormone systems, IGFs are thus enclosed by a large number of regulatory proteins, suggesting tight and complex regulation of their actions.

In addition to the sophisticated structure of the IGF system itself, a good insight in the regulation of IGF-I bioactivity *in vivo* is further complicated by the direct or indirect interactions between the IGF system and other hormones/factors. These interactions could have various impact on IGF-I bioactivity. One of the most striking examples is the interaction between IGFs and insulin (actions), which is in part due to the structural and functional overlap between IGFs and insulin including those of their cognate receptors. From an evolutionary point of view - IGFs and insulin can be interpreted as being complementary players in one large biological functional system.

IGF-I immunoassays: The history of the development of assays with the aim to estimate IGF-I bioactivity *in vivo* has followed a process that is highly comparable to that for other (endocrine) factors. The last thirty years the main focus in the design of IGF-I assays has been primarily on the highly specific and accurate determination of the circulating ligand concentration. This has been performed by means of sophisticated immunological methods and by use of highly specific (monoclonal) IGF-I antibodies that show little to no cross reactivity with IGF-II and (pro)insulin. Nowadays these IGF-I immunoassays are used widely in daily clinical routine as a tool to screen for, to diagnose and to evaluate treatment of GH disorders (e.g. acromegaly and GHD) [3-7].

Although the type of technique applied in IGF-I immunoassays has evolved from competitive binding strategies (IGF-I RIA) towards two antibody capture techniques (IRMA, CLIA), their underlying 'ligand based' design has not been altered. Nevertheless, it should be realized that the presence of IGFBPs in

plasma was encountered to negatively influence accuracy of IGF-I immunoassays. The last, as a result of binding competition that exists between IGF-I antibodies and IGFBPs. In order to overcome this interference in IGF-I immunoassays a set of various pre-analytical methods have been developed serving the general aim of complete IGFBP removal and/or inhibition of their IGF-I binding capacities. Indeed, from a technical point of view this has resulted in highly specific assays for measurements of total circulating IGF-I levels. However, not all sample preparation methods efficiently remove all IGFBPs or BP fragments, and there is currently no reference method for IGF-I measurement against which these IGF-I assays can be calibrated [8].

In line with the idea that the circulating ligand concentration is a direct derivate of hormone activity, results of total IGF-I immunoassays are generally interpreted as good estimates of circulating IGF-I bioactivity. However, in the paragraph that follows several arguments will be discussed why the IGF-I KIRA probably reflects IGF-I bioactivity better than (immunoreactive) total IGF-I levels: **1.** Although occurring in large quantities in the circulation (up to 100 nM), over 99% of IGF-I is bound to IGFBPs. This means that only a small amount of circulating total IGF-I is actually in a free (uncomplexed) form. Only free IGF-I is assumed to be bioactive, as being available for IGF-IR binding. In this respect, immunoassays that measure total IGF-I levels probably do not provide information about the direct bioavailability of IGF-I at the receptor level. Indeed, this given course has lead to the development of immunological methods to measure free IGF-I (in line with e.g. measurements of free estrogens/androgens and free thyroid hormones). Although the rational of measuring free IGF-I has been questioned, it has been shown that free IGF-I can give biologically meaningful information which could not be established by measurement of total IGF-I. In healthy fasting individuals Chen et al. showed that free IGF-I but not total IGF-I had a reciprocal relation with increasing GH levels, suggesting free rather than total IGF-I levels as determinant of the negative feedback loop on GH release [9]. **2.** In addition to IGF transport functions and protection from degradation, IGFBPs are important modulators of IGF system activity [10]. Both inhibitory as well as potentiating effects of IGFBPs on IGF actions have been reported. Though, as a consequence of their design the influence of IGFBPs is eliminated in IGF-I immunoassays. Therefore these assays do not detect and thus ignore the modifying effects of IGFBPs and/or proteases on IGF-I bioactivity. Thus, the concession made in IGF-I immunoassays (IGFBP removal) to guarantee high assay accuracy may lead to loss of biological information on how IGF-I bioactivity is regulated *in vivo*. **3.** Another argument aims at the possibility of the limited value of single

immunological measurements of IGF-I (or other members of the IGF system) in relation to IGF-I bioactivity. Due to the complex structure of the IGF system it is difficult to understand and define what impact individual variability in circulating levels of IGF-I and/or IGFBPs eventually have on overall IGF-I bioactivity, since this is dependent on the context of these changes in relation to other determinants of IGF bioactivity. Theoretically changes in circulating total IGF-I levels can but do not necessarily lead to variation in bioactivity, since they could also represent a compensatory mechanism in order to keep hormone bioactivity unchanged reflecting system redundancy. Thus, although in several pathological conditions (DM type 2, CVD, cancer) relative changes in the composition of the IGF system have been recognized, the degree of the actual impact of these changes on IGF-I bioactivity itself is not known. **4.** IGF-I immunoassays only determine the immunoreactive properties of the IGF-I molecule, rather than its actual biological effect. Theoretically, fragments of the IGF-I molecule that are not recognized by IGF-I antibodies could (still) be biologically active. Or vice versa, fragments of IGF-I that have no biological effects could still harbor epitopes that are bound by IGF-I antibodies and thus measured. **5.** Antibody interactions can be disturbed by presence of heterophilic antibodies in serum, resulting in falsely higher or lower total IGF-I levels. Although this phenomenon has been well described for several immunoassays (e.g. for thyroid stimulating hormone (TSH) and luteinizing hormone (LH)), for IGF-I immunoassays this has not. Here, in *chapter 5*, we described the first case in the literature in which falsely low total IGF-I levels were measured due to the presence of heterophilic antibodies. As the IGF-I KIRA was not affected by the presence of heterophilic antibodies we demonstrated that no effects on IGF-I bioactivity were present. This study therefore nicely shows the consequence of methodological differences between IGF-I immuno- and bioassay.

Of note, it must be clear to the reader of this thesis that it is not the aim of the author to undermine the use of immunoassays to determine circulating levels of IGF system parameters. Conversely, IGF-I immunoassays (in combination with others designed for IGF-II and IGFBPs) have indeed yielded important and meaningful information about the IGF system in general. Nevertheless, today's visions, ideas and knowledge about the function of the circulating IGF system in health and disease are (almost) completely dictated by information gathered by use of a single type of method (immunoassays). As is the case for almost every method, immunoassays have limitations, and within their strength a weakness may be enclosed; When using immunoassays, concessions are made to the original structure of the IGF system (as discussed

above), to guarantee full accessibility of highly specific antibodies to respective targets of interest. This could lead to both loss of information enclosed in the regulatory network of the IGF system itself and misinterpretation of data. Recognition and acceptance of this limitation however also creates a new field to study the possible added value of alternative methods to measure IGF system activity next to (and not instead of) IGF-I immunoassays and others. Last has been the general aim and drive using the IGF-I KIRA method in the studies included in this thesis.

The elegance of the IGF-I KIRA is that it is designed to detect IGF-IR activity in a biological system of human origin without interrupting the physiological structure (and thus most likely function) of the IGF system [11].

8.2 Performance and downscaling of the IGF-I KIRA

The IGF-I KIRA employs a two-plate system, one (plate 1) for cell culture and stimulation of intact human 293 EBNA cells transfected with cDNA of the human IGF-IR, followed by receptor solublization, and one (plate 2) for specific capture and analysis of IGF-IR phosphorylation status by ELISA [11,12].

Sensitivity and specificity: Compared to the IGF-I KIRA developed by Sadick et al., which uses untransfected MCF-7 cells [12], the IGF-I KIRA uses IGF-IR tranfected 293 EBNA cells and shows higher sensitivity and specificity for IGFs, especially for IGF-I. Chen et al. reported that the sensitivity of the IGF-I KIRA for IGF-I was 0.08 µg/L which equals 10.4 pmol/L. In addition the standard curve was shown to be best fitted by a linear model between 0.1 – 10 µg/L (13.0 – 1370 pmol/L). At our laboratory we were able to replicate these findings. Concentrations of recombinant IGF-I used in standard curves in the studies presented in our studies ranged from 62.5 – 1000 pmol/L. Concerning assay sensitivity, the lowest concentration used in IGF-I standards (62.5 pmol/L) gave results that easily exceeded those of non-stimulated cells, serving as non-specific background. We also found a linear relationship of the observed points within the specified concentration range of IGF-I standards, which covered over 99% of all measured serum samples in which circulating IGF-I bioactivity was obtained (Figure 1A).

Since the IGF-IR can also bind IGF-II and insulin no absolute specificity for IGF-I was to be expected. Specificity for IGF-I was reported about 10- and 200-fold higher for IGF-I than for IGF-II and insulin, respectively. Figure 1B shows the relative contribution of recombinant IGF-II to the IGF-I KIRA at equal concentrations measured at our laboratory. Insulin was also tested but showed no activity within this concentration range (data not shown).

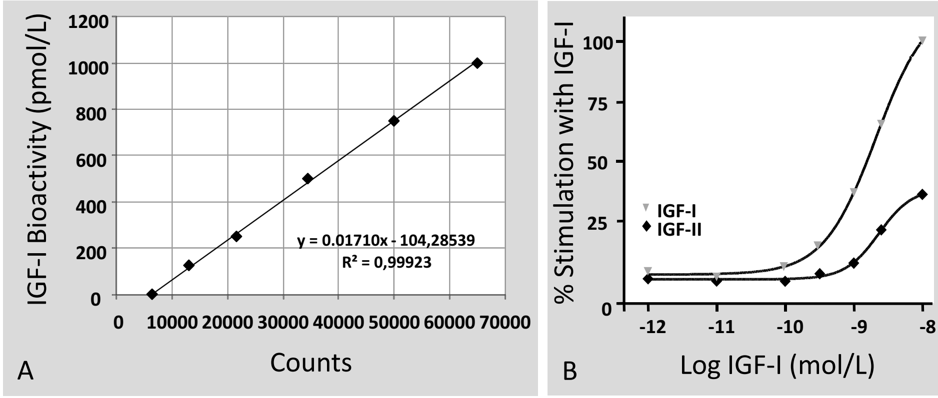

Figure 1: (**A**) IGF-I KIRA reference line as used in the studies in this thesis. Within the range of 63 – 1000 pMol/L the reference showed a lineair relationship with the number of counts. Serum levels of almost all samples measured were within this range. (**B**) Comparison of equal concentrations of recombinant IGF-I and IGF-II in the IGF-I KIRA. At a concentration of 1*10-9 Mol/L (1000 pMol/L) IGF-II reached about 11-15% of activity of IGF-I, while it reached equal activity at 1*10-8 Mol/L when compared to IGF-I at 1*10-9.

Accuracy: For the IGF-I KIRA in MCF-7 cells intra- and inter-assay coefficients of variation (respectively CV_{intra} and CV_{inter}) were studied by Sadick et al. for samples with relatively low (~1.10 ng/mL or ~151 pmol/L), mid (~5.3 ng/mL or ~693 pmol/L) and high (~22.3 ng/mL or ~ 2914 pmol/L) IGF-I bioactivity. They found CV_{intra} of 7.2%, 5.0% and 9.5% (n=24) and CV_{inter} of 10.5%, 6.5% and 11.8% (n=11) for the three samples tested respectively. Chen et al. found for the IGF-I KIRA in transfected 293 EBNA cells that CVs of IGF-I standards on 1 plate averaged 7%. Furthermore, CV_{inter} was estimated by repetitive measurements of a (non-specified) control sample assayed 34 times on 6 different days and averaged 13%. Together, these findings suggest that the IGF-I KIRA's precision is an appropriate method to measure IGF-I bioactivity in larger settings, e.g. in population based studies. One could argue, however, that the calculated CV_{intra} and CV_{inter} in these studies were based on relatively low numbers, meaning that the experiments from which these data were obtained could have been performed in a small number of cell culture passages thereby limiting between assay variability. In the last five years, however, several studies have been performed in which IGF-I bioactivity was determined using the IGF-I KIRA with acceptable sample numbers, including those presented in this thesis. From these studies the conclusion can be drawn that the originally estimated CV's also hold in larger settings. Of note, although the absolute number of counts per second obtained in the read-out system did vary between assays that were run at different time points

(e.g. different weeks), this did not influence assay accuracy. Retrospectively, we can also conclude that the (arbitrary) chosen level of acceptance (<10%) for intra-sample variation of duplicates has been reasonable. Using this cut-off value, in our hands, the IGF-I KIRA showed 92-96% efficiency in the studies described, meaning that about 4-8% of samples when measured in duplicate showed over 10% variation per run and therefore had to be measured again. For a bioassay this level of efficiency is remarkably high. Explanations for the 4-8% failure are most probably multifactorial, being both of technical origin (cell culture techniques, antibody coating/binding, plate blocking, sample storage (conditions) etc.) and of human origin (errors made in sample dilutions or sample misplacement on plates during stimulation, learning curves etc.). In *chapter 2* we described that CV of the IGF-I KIRA within the study population (a standardized measure of relative spread in data) was comparable and actually slightly lower than that for IGF-I immunoassays. Because a lower magnitude of this variability is considered to reflect better reliability (precision) of measurements, this suggests that the IGF-I KIRA in this respect performs at least as well as IGF-I immunoassays. When one considers that the IGF-I KIRA assay is actually both a bioassay and an ELISA, and that in its current form most of the methodology is done by hand, the level of precision that can be reached is quite remarkable.

Assay downscaling: In the original design of the IGF-I KIRA a 24 well cell culture plate (plate 1) was used. Chen et al. validated the IGF-I KIRA as such for this measurement of IGF-I bioactivity in human serum. However, when using a 24 well plate, this design largely limited the number of unknown serum samples that could be run per assay when all measurements, including those of standard preparations and control samples, should be performed in duplicate. Measurements of IGF-I bioactivity in this thesis were performed by use of 48 well culture plates. Using 48 well plates and starting with half of the number of plated cells results did not differ when compared to 24 well plates (Figure 2). This means that no information was lost due to downscaling of the assay as such. Next to the ability of sample number enlargement per assay, this adaptation had also positive effects on the cost effectiveness of the IGF-I KIRA. Volumes of needed materials and serum could be largely reduced. The use of 96 well plates was also evaluated, but this encountered some problems as assay sensitivity for IGF-I was less than when using 24 or 48 wells plates (data not shown). This is probably due to the lower number of cells and thus of IGF-IRs. It is in line with the finding of Chen et al, who reported the use of transfected cells probably explained that higher sensitivity could be reached than when using cells with endogenous IGF-IR expression.

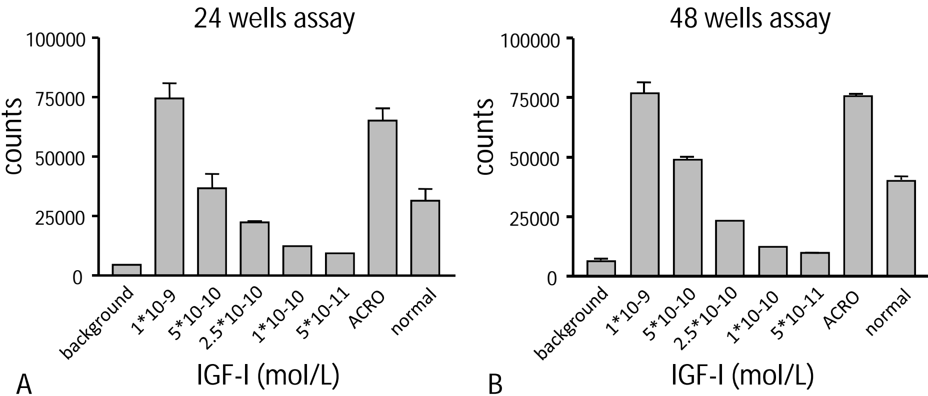

Figure 2: IGF-I KIRA outcomes for serial dilutions of recombinant IGF-I and serum samples. (**A**) outcomes using a 24 well culture plate; 400.000 cells per well were cultured on day 1 of the assay. (**B**) outcomes using a 48 well culture plate; 200.000 cells per well were cultured on day 1 of the assay. Outcomes for serum samples of a patient with acromegaly (ACRO) and of a normal control subject did not differ from each other when IGF-I bioactivity levels were calculated by use of the respective reference.

In addition, because cell stimulation in the IGF-I KIRA is done by hand, higher sample variability was found when using 96 wells plates. In addition, since the stimulation of cells in the IGF-I KIRA occurs only during 15 minutes, after which the cells are lysed, the use of 96 wells introduced difficulties in a good time management of all procedures.

8.3 IGF-I bioactivity in relation to health and IGF-I immunoassays

In *chapter 2* we have described a study with the aim to determine normal values of circulating IGF-I bioactivity. These age-specific normative ranges can be helpful in future to interpret whether IGF-I bioactivity is normal, increased and/or decreased in subjects with pathological conditions like acromegaly and GH deficiency. Circulating IGF-I bioactivity was measured in a population which consisted of 426 healthy blood donor individuals. In this population Ranke et al. earlier had determined normal values for total IGF-I levels using five different immunoassays, three IGF-I immunometric asssays and two IGF-I RIAs. Our second aim was to analyze correlation coefficients between IGF-I bioactivity and measurements of total IGF-I, in order to estimate to which degree IGF-I bioactivity measured by the IGF-I KIRA can be explained by immunoreactive measurements of total IGF-I.

Concerning assay outcomes, we found that distribution of circulating IGF-I bioactivity came close to a normal Gaussian curve which contrasted to the distribution of total IGF-I levels measured by all IGF-I immunoassays. This

means that while levels of total IGF-I are not, circulating IGF-I bioactivity is normally distributed in the healthy population. Although one might expect hormone activity to be normally distributed in the population, this was previously not demonstrated by use of immunoassays for total IGF-I. In the study presented in *chapter 3* circulating IGF-I bioactivity also showed a normal distribution in the study population of elderly men, while such distribution was absent for total and free IGF-I. Thogether these findings suggest that the IGF-I KIRA more closely resembles the physiological IGF system activity *in vivo* than do immunoreactive measurements of total and free IGF-I.

As expected correlations observed between circulating IGF-I bioactivity and total IGF-I levels reached significance ($p < 0.001$), however r^2 ranged from 0.25 to 0.31 in the study described in *chapter 2*. This means that in general 70-75% of the variation in IGF-I bioactivity could not be explained by levels of total IGF-I, being independent of the type of IGF-I immunoassay used.

In *chapter 3* we also observed that the correlation between bioactive and free IGF-I was significant, however it was lower than that observed for total IGF-I. Similar correlation coefficients between IGF-I bioactivity and total IGF-I and free levels have been reported by others, although some variability is to be expected due to differences in the populations studied [13]. Of interest in this setting are observations by Karl et al., who demonstrated that in healthy young women immunoreactive levels of total and free IGF-I in combination with those of IGFBP-1, 2 and 3 could explain on average only 61% of the variation observed in circulating IGF-I bioactivity in a multiple regression model [14]. Interestingly, other factors like age, sex-hormones, selenium and alcohol intake also were associated with circulating IGF-I bioactivity. From these findings the conclusion can be drawn that circulating IGF-I bioactivity differs considerably from measurements of immunoreactive IGF-I (or other IGF related parameters) and thus cannot (always) be estimated by these methods.

Another interesting finding, described in *chapter 3* is that bioactive IGF-I levels were found to be significantly higher than levels of free IGF-I and that correlation between these two measures is relatively poor. This finding is supported by others [15]. Theoretically one might expect that both measures determine that part of total IGF-I that comes available for receptor activation. Several arguments however support that the differences found could actually be realistic. First, the IGF-I KIRA not only determines the part of circulating IGF-I that is in a free form and thus able to bind the IGF-IR, but it is also sensitive to the part of IGF-I that actively (due to protease activity or IGFBP (cell-) binding) or passively (due to dissociation) comes free from IGFBPs. Second,

measures of free IGF-I do only represent the immunoreactive properties of unbound IGF-I molecules harboring the epitope required to be bound by the specific IGF-I antibody and thus not whether this molecule also has bioactive properties. In addition, as argued before not only IGF-I but also IGF-II is able to activate the IGF-IR which next to IGF-I can contributes to the IGF-I KIRA output (see also next alinea) [11].

In line with circulating total IGF-I levels we found that circulating IGF-I bioactivity declined with age in both men and women. Interestingly, we observed that the fraction of IGF-I bioactivity over total IGF-I increased with age (*chapter 2:* figure 5A-E). This observation suggests that the degree of decline of total IGF-I levels with age is not translated in proportion to that of IGF-I bioactivity. Explanations for this observation could be at the level of difference in GH dependency, adaptation of IGF-I bioactivity to increasing insulin resistance with age or the relative increase of the contribution of IGF-II to the IGF-I KIRA outcome with age as is described in *chapter 2*. A notable remark on this is however that our cross-sectional study design does not reflect the rate of individual changes in IGF-I bioactivity with aging.

Age is a major determinant of circulating total IGF-I concentrations, with a decrease in circulating total IGF-I concentrations with aging. Part of this decrement is believed to be due to the known age-related decrease in GH secretion [16]. However, circulating IGF-I becomes less and less GH-dependent with age, ultimately leading to a situation in which circulating IGF-I concentrations become more and more influenced by nutrition, liver function, sex steroid levels and insulin [17]. One also has to consider that circulating concentrations of IGF-II are generally around two- to four-fold higher than those of IGF-I in adults and IGF-II levels most likely do not decline with age [18]. As both IGFs appear to activate the IGF-IR, IGF-II can potentially elicit the same actions as IGF-I at the cellular level. These arguments could substantially affect the slope of decline with age for circulating IGF-I bioactivity. Interesting are also the findings of Frystyk et al. who reported that although absolute measures of total and bioactive IGF-I where lower, percent IGF-I bioactivity was higher in obese subjects compared to both lean and overweight subjects [19]. It is well-known that with increasing age risk of obesity and insulin resistance increases.

A finding of major interest is the suggestion that there exists a sexual dimorphism for circulating IGF-I Bioactivity (*Chapter 2*). In women we found a remarkable profile for IGF-I bioactivity levels with age, as circulating IGF-I bioactivity dropped disproportionally around the age range of 55. This finding suggests that IGF-I bioactivity is directly or indirectly positively driven

by sex-hormones (e.g. estrogens or progestagens). This suggestion should be presented with appropriate caution, however, because the cross-sectional design of the study does not allow to claim causation. Furthermore there was a lack of information in the individuals studied about the use of oral estrogens and/or menstrual status in the study population. We could not detect a direct effect of estrogens on IGF-I bioactivity; when estrogen was added at physiological circulating concentrations (in females) to male serum or to the reference this did not differ from controls (*chapter 2:* Table 4). However, a couple of studies demonstrated increased serum total IGF-I concentrations during the luteal phase of the menstrual cycle, possibly concomitant with elevated estradiol concentrations [20,21]. Second, in case of measures of IGF-I bioactivity by the IGF-I KIRA Karl et al. recently reported significant positive associations with circulating levels of estradiol and progesterone in young healthy women [14]. These findings strengthen our own suggestions, linking IGF-I bioactivity to female sex-hormones. It is not unthinkable that in men testosterone is also associated with circulating IGF-I bioactivity and that this results in a similar decline later in life. This is, however, speculative and at this moment we do not have any data to support this suggestion.

In *chapters 6* and *7* we have described results of circulating IGF-I bioactivity in elderly individuals aged 55 years and over that were included in the Rotterdam study. Interestingly, mean circulating levels of IGF-I bioactivity were again higher in women than in men. Furthermore, circulating IGF-I bioactivity decreased significantly with age in men, but it did not in women. It is well known that the growth hormone (GH)/insulin-like growth factor (IGF)-I axis exhibits gender-specific differences. One of these differences is the pattern of the pulsatile GH release by the pituitary [22,23]. These gender-specific differences may be related to multiple factors that occur during aging and that strongly influence neuro-endocrine activity of the GH-IGF-I axis. Factors like visceral fat, accompanying changes in body composition during aging, varying sex-steroid hormone concentrations and unequal physical fitness may result in gender-specific differences in the activity of the GH-IGF-I axis and thereby IGF-I bioactivity. Of interest are results of IGF-I bioactivity reported by Karl et al. that indicate factors involved in the observed sex differences in IGF-I bioactivity [14]. The authors reported that in contrast to measures of free and total IGF-I circulating IGF-I bioactivity in young women was positively associated with percent body fat, a parameter which is characterized by sexual dimorphism. It has been shown that cultured human adipocytes secrete substantial amounts of IGF-I, IGF-II and IGFBP-3 into the cell medium [24]. Thus, it is conceivable that part of the circulating IGFs and IGFBPs originate from

adipose tissues and that thus their contribution to the circulating pool might substantially differ between the sexes.

8.4 IGF-I bioactivity in relation to human longevity and cardiovascular disease

The insulin/IGF signaling pathways have been linked to aging and longevity. From studies obtained in nematodes, flies and rodents direct and indirect evidence exists that low insulin/IGF signaling may result in extended life expectancy. Findings in humans, however, are controversial and studies are limited or show indirect evidence due to ethic considerations. A number of epidemiological studies suggest that low-IGF-I activity, at least at advanced age, is not associated with longevity in humans [25]. We speculated that, due to its different methodological character when compared to IGF-I immunoassays, the IGF-I KIRA might be an interesting tool to study circulating IGF-I bioactivity in relation to human longevity.

In *chapter 3* we have described a prospective study in which we studied circulating IGF-I bioactivity next to free and total IGF-I in relation to survival in elderly men. We found that circulating IGF-I bioactivity measured by the IGF-I KIRA showed a profile in relation to survival, that differed considerably from that obtained by free and total IGF-I. We found relatively high, rather than low circulating IGF-I bioactivity to be related with longer survival in elderly men. Interestingly, in a sub-analysis in which we stratified for presence or absence of cardiovascular disease (CVD) and/or evidence for a high CVD risk profile based on CRP levels (a measure of subclinical systemic inflammation), we observed that low circulating IGF-I bioactivity was an independent risk factor for mortality especially in individuals with CVD and/or a high inflammatory risk profile.

The precise interactions of all factors (e.g. genetic diversity/ DNA damage (repair), food, environment and disease) that play a role in the process of organism aging and longevity is extremely complex. In humans this process is even more difficult to study due to our capacity to actively influence several of such factors. On one hand this capacity could create substantial bias. On the other hand, however, some of these influences might also help to gain more insight into the (pathological) processes that determine human longevity, and of the biological factors/systems that play a role in these processes. To illustrate this with an example: there exists both a well-known relationship between smoking and risk of death from CVD by atherosclerosis as well as from cancer by DNA damage. Smoking is probably not only a causative factor in these relationships but mainly acts as a modulator, accelerating two major processes that determine human survival, namely vascular inflammation/

atherosclerosis giving rise to a high risk to die from CVD and DNA damage resulting in death from cancer or more progressive aging.

When this reasoning is applied to our findings than how should we interpret our results in relation to the role of IGF signaling in relation to human longevity? We speculate that IGF-I bioactivity could substantially influence human survival by modulating / limiting the risk of death by CVD. Due to factors specified to (changes in) human lifestyle CVD is a major determinant of human survival, especially through the process of atherosclerosis (an inflammatory disease of the artery wall) [26-28]. Although results of the IGF-I KIRA may not necessarily reflect IGF-I bioactivity at the local tissue level, a notable exception to this is formed by the vascular endothelium, however. This tissue is in direct contact with the blood from which IGF-I bioactivity is determined. Indeed IGF-I has been suggested to have strong vascular protective properties [29]. A potential mechanism by which IGF-I could prevent CVD is the maintenance of normal NO bioavailability and consequent activation of anti-atherogenic mechanisms to maintain normal function of vascular endothelium cells. As an additional mechanism IGF-I also activates cell survival signaling pathways and has been shown to stimulate stem cell survival and their homing to damaged tissue, including that of endothelial progenitor cells to the heart and vascular endothelium [30-32]. Through such mechanisms, individuals with a relatively high circulating IGF-I bioactivity might be relatively protected from endothelial damage by inflammatory processes and thus have a lower risk of CVD than those with low IGF-I bioactivity. A third mechanism that could be of significance is insulin resistance. There is increasing evidence that preservation of insulin sensitivity is one of the essential conditions to maintain health and promote longevity in humans. Most striking evidence comes from longitudinal studies in centenarians [25,33]. In general, the prevalence of insulin resistance increases with age and thus has a relatively high prevalence in the elderly. It has been shown that insulin resistance is strongly associated with risk of CVD [28]. Therefore a mechanism that might explain or might have contributed to the findings in this study is the effect of circulating IGF-I bioactivity on insulin sensitivity. It could thus be that relatively high IGF-I bioactivity represent individuals that can still overcome or at least in part compensate for insulin resistance, whereas those with relative lower circulating IGF-I bioactivity have lost this ability. In this respect it should be remembered that IGFs have some biological actions, which in some aspects are indistinguishable from those of insulin. IGF-I has also been shown to be a determinant of insulin sensitivity. However, in the study described in *chapter 3* we did not determine insulin sensitivity. (Note: In *chapter 6* we presented

a study in which we focused on the relationship between IGF-I bioactivity, insulin sensitivity and the metabolic syndrome (see *paragraph 8.5 below*)).

We realize that it cannot be claimed from the study described in *chapter 3* that circulating IGF-I bioactivity is substantially involved in the process of human aging (longevity) as such. Our study stands on its own, since no previously performed other study has studied circulating IGF-I bioactivity in relation to human survival. As stated earlier, it remains to be seen whether reduced IGF-I bioactivity is an endocrine contributor to mortality risk or an epiphenomenon related to overall health/resistance to inflammation. Another aspect is that the study participants were all men. Whether this relationship is also present in women, however, is at present unknown.

Low circulating total IGF-I levels have been associated before with higher risk on CVD in longitudinal studies [34]. However in our study direct comparison between levels of immunoreactive IGF-I and IGF-I bioactivity clearly showed a different profile with human survival and risk on CVD. This suggests that bioactive IGF-I probably is more sensitive than immunoreactive parameters to study the IGF system in relation to these outcomes. Of interest in this respect is a recently published prospective study by Friedrich et al. which supports our findings. These authors showed that after 8.5 years of follow-up in 1988 men that men with low total IGF-I levels at baseline compared to men with high total IGF-I levels at baseline had an almost 2-fold higher risk of all-cause mortality (IGF-I: HR 1.9 (1.3-2.7) and CVD mortality (IGF-I: HR 1.9 (1.0 -3.71). This association was not found in 2069 women.

We believe that the findings in our study could be of major interest since for the first time they provide direct insights in the function of the circulating IGF system in relation to human survival. In addition, these findings could be of importance in the field of CVD. Hence, future studies are needed to replicate, expand and clarify these findings into further detail. This in order to answer the question whether and how circulating IGF-I bioactivity may influence the risk of death from CVD (in man).

8.5 IGF-I bioactivity as a marker of nutritional status in chronic kidney disease

Nutrition, in particular calorie and protein intake, is known to affect the IGF system [35-37]. From this perspective IGF-I could be an interesting protein to study as a marker of nutritional status. Chronic renal failure (CRF) is a disease prone for development of protein and energy malnutrition (PEM). Especially individuals with CRF on dialysis are prone to develop PEM [38]. Several factors are thought to contribute to PEM in CRF, e.g. inflammation, metabolic acidosis, uremia, protein loss and anorexia [39,40]. To compensate for PEM in

CRF patients dialysate solutions that contain glucose (G) and/or aminoacids (AA) have been studied both in the fasting and in the fed state [41,42]. In CRF patient on dialysis circulating total IGF-I has been proposed as a valid marker of (mal) nutrition in subjects on dialysis [43-45].

The IGF system and the kidney influence each other's function. IGF-I is known to increase renal glomerular filtration rate and plasma flow [46]. In addition, the GH-induced positive phosphate balance by enhanced renal absorption, is thought to be mediated entirely by IGF-I [47]. Most studies that have focused on circulating total IGF-I levels in CRF found normal or even elevated levels in subjects with CRF [48]. It is thought however that in CRF IGF-I bioavailability is actually decreased, thus opposite to what total IGF-I levels might suggest [49]. Interestingly – about three decades ago - Phillips et al. reported when using a 'classic' IGF-I bioassay that circulating IGF-I bioactivity was low in adults with CRF, and they suggested 'somatomedin inhibitory factors' to be present in the blood in excess [50,51]. When compared to healthy controls Frystyk et al. found in non-dialyzed CRF patients that mean serum free IGF-I levels were significantly decreased (-53%), whereas levels of total IGF-II, IGFBP-1 and IGFBP-2 were increased (40%, 546%, 270%, respectively) [52]. Total circulating IGF-I levels were unaffected, as were intact IGFBP-3 levels. Of note, there were however increased levels of IGFBP-3 fragments in CRF patients, suggesting higher IGFBP protease activity. Together these findings support the suggestion that impaired kidney function severely affects IGF system activity.

In *chapter 4* we described results of circulating IGF-I bioactivity and total IGF-I measurements performed in a randomized cross-over study in 12 patients who were on continuous ambulatory peritoneal dialysis (CAPD) [42]. Two different dialysis solutions were used; either enriched with glucose (G) or glucose plus amino acids (AAG). IGF-I bioactivity and total IGF-I were measured at baseline and after the respective dialysis program, about 9 hours later. Previously the metabolic effects of AAG and G dialysate were evaluated in this study. It was observed that the AAG dialysate improved protein metabolism in the fed state [42].

While mean circulating total IGF-I levels in CAPD subjects were in the upper half of the normal range with good (SGA A) nutritional status and even exceeding the normal range in 4 subjects with moderate (SGA B) nutritional status, mean circulating IGF-I bioactivity was in the lower half of the normal range. Based on the observed circulating total IGF-I levels one would expect relatively high or at least unchanged IGF-I activity in CAPD subjects, when compared to healthy controls. Our findings on circulating IGF-I bioactivity

confirm however what has been previously suggested by Philips et al. [50], namely that circulating IGF-I bioactivity is low rather than high in CRF subjects. This better fits the clinical phenotype of CRF subjects, which are generally characterized by an underfed status.

The question rises how to explain the discrepancy between measures of total and bioactive IGF-I? It could be that total IGF-I levels are falsely elevated in CRF subjects due to interference of IGFBPs in the IGF-I immunoassays. On the other hand levels of total IGF-I may be truly elevated, but as a result of hampered kidney function next to IGF-I also IGFBP clearance is sub-optimal eventually leading to a relative low bioavailability of IGF-I. Both explanations are in line with findings of Frystyk et al. described above [52]. In addition, although Frystyk et al. observed in CRF patients that there might be a higher IGFBP protease activity, our results show that this does lead to a relatively high IGF-I bioactivity in these subjects, as might be expected. An explanation could be that protease activity in these subjects is indeed increased, but that this does not result in effective release of IGFs from IGFBPs. Another explanation could be that there is no increased IGFBP protease activity *in vivo* in these patients, but that this observation is an artifact due to sample collection, storage and preparation methods *in vitro*. Since we did not measure IGFBP fragments in this study we do not know whether an altered protease activity was present in our study participants.

Circulating IGF-I bioactivity did not differ between SGA A and B. Most probably this is explained by the low number of SGA B patients, since our study was not adequately powered to detect differences between both SGA categories. In addition, SGA may not reliably identify CRF patients with an abnormal nutritional state and misclassify a large number of subjects [53].

No differences in circulating total IGF-I levels could be found after AAG nor G dialysis when compared to baseline measures. [54]. In contrast, when using the IGF-I KIRA we were able to detect acute increases in circulating IGF-I bioactivity in CRF patients on CAPD upon AAG and G dialysis. For AAG dialysis this difference reached statistically significant difference, whereas the lack of a significant rise in IGF-I bioactivity after G dialysis is probably due to the small number of patients.

The observed differences in the dynamics of circulating total and bioactive IGF-I levels in response to nutritional intake are of interest. Our study suggests that IGF-I bioactivity even in the fed state can be readily changed (reflecting system dynamics) having a causative relationship with AAG uptake. It also shows that the IGF-I KIRA is sensitive enough to observe small differences in IGF-I bioactivity which might have biological importance and also enlarges

insight in the system functionality. The changes in IGF-I bioactivity were however independent on variability in levels of total IGF-I upon nutritional intake. This means that other dymamical changes upon nutrition in the IGF system are responsible for variability in IGF-I bioactivity. We think that changes in levels and phosphorylation status of IGFBPs and/or IGFBP protease activity upon nutritional intake are most likely responsible for the changes in IGF-I bioactivity. One of the mechanisms that could be involved is the insulin driven down regulation of IGFBP-1 upon nutrition.

An advantage of our study is its randomized cross-over design, enlarging statistical power by limiting inter-individual variance. Second, implementation of measurements in time enabled us to study causative relationships. Third, the short time schedule during which this study was performed also limited intra-individual variance (e.g. day to day variance, or bias due to unknown infection or progression of state of disease). On the other hand, because of the short time period no conclusions can be drawn concerning the effects of AAG or G dialysis on IGF-I bioactivity in relation to nutritional status and kidney function over time. Prospective data spanning at least several months is essential to study this aspect in future. Nevertheless, this is the first study that provides a mechanistic insight into the function of the circulating IGF system in subjects on CAPD. Our results indicate that in CAPD patients bioactive but not total IGF-I is sensitive to acute changes in nutritional intake.

In conclusion: We believe that levels of total IGF-I may not be informative in subjects on CAPD in relation to their nutritional status nor when used as a marker of (acute) metabolic responses to nutrition. Total IGF-I levels may be falsely elevated and do not well represent the IGF system activity in these subjects. In contrast, circulating IGF-I bioactivity may be a new promising marker to study in this field of research.

8.6 IGF-I bioactivity in relation to insulin sensitivity and the metabolic syndrome

8.6.1 IGF-I bioactivity and its relation to insulin sensitivity

The IGF system, GH and insulin are thought to play complementary roles in the maintenance of glucose homeostasis. This interplay between GH, insulin and IGF-I is, however, complex. IGF-I probably plays an important role to balance the well-recognized contrasting metabolic actions of GH and insulin, which both affect IGF-I (bioactivity). Hepatic IGF-I production is driven by both GH and insulin, whereas IGF-I negatively feeds back on both pituitary GH and pancreatic insulin release. In addition, IGF-I is thought to increase peripheral insulin sensitivity by both GH-dependent and –independent

mechanisms. Furthermore, GH and insulin also regulate levels of circulating IGFBPs. GH stimulates hepatic IGFBP-3 production, whereas insulin inhibits that of IGFBP-1, and to a lesser degree of IGFBP-2 [10,55,56].

In *chapter 6* we described a cross-sectional study in 1036 elderly individuals that focused on the relationship between circulating IGF-I bioactivity, insulin sensitivity and the metabolic syndrome (MS). A finding of interest was that mean circulating levels of IGF-I bioactivity progressively rose with increasing fasting glucose and insulin levels (and thereby HOMA-IR), while it abruptly declined in individuals that met criteria for DM type 2. Although no direct causality can be concluded as a consequence of the cross-sectional study design, and also no measures of total IGF-I, free IGF-I, IGF-II and IGFBPs were performed, these findings might be of value in the field of IGF research which concerns the role played by the human IGF system in the pathophysiological process of insulin resistance towards type 2 DM. As stated before, the history of this field of research is characterized by controversial outcomes. Recently, Rajpathak et al. published a review about this subject, in which the authors claim that circulating total IGF-I levels in general have been reported normal in subjects with obesity and (pre-)diabetes [57]. However evidence is also available that in subjects with type 2 DM circulating total IGF-I levels are decreased [58,59]. An explanation for the controversy in literature concerning total IGF-I levels may be related to methodological problems to measure IGF-I by immunoassays, especially in diabetic subjects. Chestnut et al. evaluated 9 different IGF-I immunoassays and sample preparation methods using samples from healthy donors and from donors with type 1 and type 2 DM [8]. The authors reported that assays compared poorly when samples from type 1 and type 2 DM were used. Dependent on the IGF-I immunoassay used, levels of total circulating IGF-I were high, normal or decreased in subjects with type 1 or type 2 DM compared to controls. The authors suggested that interference by remaining IGFBPs or of their fragments were the cause of the differences observed between these immunoassays. These assay differences may explain some of the differences seen in the literature on IGF-I levels in diabetes.

For free IGF-I relatively high levels have been found in subjects with type 2 DM. Thus, in type 2 DM there may be a response to increase free IGF-I concentrations to compensate for reduced insulin bioactivity. Unfortunately to date very limited prospective data are available. Sandhu et al. found an inverse relationship between circulating total IGF-I levels and risk of DM type 2 [60]. However, the total follow-up time of this study (4.5 years) was relatively short in relation to the developmental process from disturbed glucose homeostasis towards DM type 2 (≥10-15 years). Also this study was hampered

by a low number of subjects that developed impaired glucose tolerance (IGT) (N=44) or DM type 2 (N=7), resulting in low statistical power. IGT and type 2 DM subjects had to be analyzed together, which might have created bias. It can thus be stated that to date suggestive but no hard evidence has been provided that links IGF-I clearly to the pathophysiological process of insulin resistance and type 2 DM.

Interesting findings come from studies in which IGFBP-1 was studied in relation to glucose homeostasis and risk of DM type 2. In white Europeans Cruickshank et al. found a U-shape curve fore mean fasting IGFBP-1 levels in individuals with respectively normal (NGT) and impaired glucose tolerance (IGT) and DM type 2, with NGT and diabetic subjects having higher fasting IGFBP-1 levels than those with IGT [61]. Fasting proinsulin levels were lower in NGT individuals and higher in diabetics. Mean circulating total IGF-I levels did not significantly differ among individuals with NGT, IGT and type 2 DM. In another longitudinal (incident case-control) study performed in Swedish white men aged 35-56 years, Lewitt et al. followed individuals for 10 years to develop IGT and/or type 2 DM [62]. All subjects had normal oral glucose tolerance tests (OGTT) at baseline. Interestingly, baseline levels of IGFBP-1 appeared to discriminate between individuals that were still NGT (highest) and those that developed IGT and type 2 DM (lowest). At 10 years of follow-up mean IGFBP-1 levels in those who developed DM type 2 rose 32%, up to the level in those that still had NGT, whereas in individuals with IGT at ten years IGFBP-1 levels did not change. Lewitt et al. concluded that low IGFBP-1 levels could be used as a predictive marker for risk of DM type 2, whereas total IGF-I levels could not.

Although not being studied in a prospective setting, our findings for circulating IGF-I bioactivity in relation to glucose homeostasis is in agreement with the prospective observations for IGFBP-1. The profile in our study observed for circulating IGF-I bioactivity in relation to glucose homeostasis and insulin sensitivity is inverse to that previously reported for IGFBP-1. This is in line with the thought that IGFBP-1 is a modulator of circulating IGF-I bioactivity. Although we did not measure IGFBP-1 levels in our study, the decline in IGF-I bioactivity in subjects with FG levels > 7.0 mmol/L may be directly related to (increasing) inhibitory effects of elevated IGFBP-1 levels on IGF-I bioactivity. Our study, however, is the first that provides data on circulating IGF-I bioactivity in relation to glucose homeostasis and insulin sensitivity/resistance.

The IGF-I KIRA is sensitive to modulations of IGFBPs on IGF-IR activation [11,13]. This opens the possibility that the observed alterations in IGFBP-1 levels in relation to glucose homeostasis are not only a marker of hepatic

insulin sensitivity [63], but most likely also affects circulating IGF-I bioactivity itself. Of note, we can only speculate on this since we did not measure IGFBP-1 in our study.

An important discriminative aspect of our study is that we did not only study circulating IGF-I bioactivity according to (crude) groups of NGT, IGT and type 2 DM, but also (and especially) according to increases of HOMA-IR. An advantage of this design is that it enlarges the insight on how IGF-I bioactivity is related to the relative degree of insulin resistance. This strategy is in agreement with the generally accepted concept that type 2 DM is an endpoint status of a probably decade's spanning pathophysiological process characterized by progressive loss of insulin sensitivity in time. By using HOMA-IR as an indicator of a time-dependent pathophysiological process, one creates a model within a cross-sectional study that to some degree shows similarity with that of a prospective design. Of course it does not inform about direct causality, but it is more informative to gain insight into the (possible) mechanistic adaptations of a biologal system in relation to these pathophysiological processes. Essential requirements for this are a large study population, which creates enough statistical power to stratify individuals according to subgroups of the pathophysiological indicator (in this case HOMA-IR) and the use of a detection method that covers the biological system. We think that we met these requirements by studying the relation between the IGF system and insulin resistance/glucose homeostasis, as IGF-I KIRA measurements were performed in more than 1000 individuals to assess circulating IGF-I bioactivity.

We believe that the suggestive rise in circulating IGF-I bioactivity with increasing HOMA-IR could be part of a compensating mechanism trying to overcome insulin resistance through insulin like actions of IGFs on glucose uptake and on peripheral insulin sensitivity. A prominent candidate for the driving force in this could be insulin itself, by simultaneously stimulating hepatic IGF-I production and suppressing IGFBP-1 and -2. In addition, insulin also appears to be necessary for normal liver responsiveness to GH [64]. On the other hand the observed increase of circulating IGF-I bioactivity with increasing HOMA-IR could also be the consequence of an compensatory adaptation to an increased IGF-IR resistance. Although this could explain the relative increase in mean IGF-I bioactivity in parallel with the increase in fasting glucose/HOMA-IR, it does however not explain the abrupt decline in IGF-I bioactivity in individuals with type 2 DM. When being independent of insulin resistance, one would expect a further (disproportional) increase in IGF-I bioactivity (or at least in IGF-I levels) in type 2 DM as is observed for

insulin. Our findings thus support the idea that insulin, the IGF system and GH are dependent on each other for optimal biological activity/performance.

The disproportional high fasting insulin levels observed in individuals that met criteria for type 2 DM (based on fasting glucose levels >7.0 mmol/L) most probably resemble the existence of hepatic insulin resistance (Hep-IR). Hep-IR has been associated with reduced hepatic insulin clearance [65], being one of the mechanisms leading to chronic hyperinsulinaemia. Chronic hyperinsulinaemia has been shown to reduce GHR expression and signaling in the liver and has also been associated with increased IGFBP-1 levels [66]. In support with this are the findings by Chen et al. in patients with liver cirrhosis. They hypothesized that the lower fasting levels of bioactive IGF-I together with the 2-fold increase in IGFBP-1 levels in these patients in coexistence with an abnormal glucose metabolism was due to liver cirrhosis-related insulin resistance [13].

The abrupt decline in circulating IGF-I bioactivity observed in diabetics in our study are consistent with the rise in IGFBP-1 levels after development of type 2 DM observed by Lewitt et al. [62], referring to the probable dependency of circulating IGF-I bioactivity on isulin actions. On the other hand, the decline of IGF-I bioactivity in type 2 DM might even deteriorate insulin resistance in the peripheral tissues by several mechanisms (e.g. impairment of: insulin sensitivity by muscle tissue [67], anti-inflammatory effects [57,68], uptake of FFA and pancreatic β-cell function and survival [69]). Although causation cannot be claimed from our study design, the results of Lewitt et al. support the idea that changes in circulating IGF-I bioactivity most probably precede those of detectable insulin resistance as a decrease in IGFBP-1 levels was already present before abnormal fasting or post OGTT glucose levels. This indicates that the IGF system plays a crucial and causative role in the development of insulin resistance and DM type 2. However, it has to be acknowledged that these hypotheses remain to be tested in prospective studies.

8.6.2 IGF-I bioactivity and its relation to the Metabolic Syndrome

The metabolic syndrome (MS) is a condition characterized by a cluster of evidence-based cardiovascular risk factors, known as MS components (**1.** abdominal obesity, **2.** hypertriglyceridemia, **3.** low-serum high-density lipoprotein (HDL) cholesterol, **4.** elevated blood pressure, **5.** glucose intolerance) and a pro-inflammatory state [70]. Presence of MS has been defined as having 3 or more components, regardless of the components that contribute to this. The MS has been strongly associated with an increased risk of CVD [71], and type 2 DM [72].

Insulin-resistance is thought to be the most important denominator of the MS [72,73] and has been called 'the cornerstone abnormality'. Based on our observations as described in *paragraph 8.5.1*, we also studied IGF-I bioactivity in relation to the MS. We found a non-linear inverse u-shaped (parabolic) relation between IGF-I bioactivity and the number of present MS components. Mean circulating IGF-I bioactivity raised gradually up to the level of three present MS components (and thus presence of MS), but it declined when all 5 MS components were present. This pattern substantially differs, however, from that reported for circulating total IGF-I levels in relation to MS components by others (discussed below).

In the last decade IGF-I has been studied and with raising interest in relation to MS. With some degree of inconsistency, circulating total IGF-I levels have been reported decreased in relation to individual components of the MS [60,74]. Two well documented cross-sectional studies on this subject have been published recently (data was derived from the Third Nutrition Examination Survey (NHANES III)) [75,76]. In almost 3400 healthy adults (aged 20-50 yrs) Sierra-Johnson et al. [76] showed that subjects in the lowest quartile of the IGF-I/IGFFBP3 ratio – which was assumed as an reliable estimate of IGF-I bioavailability - were more likely to meet the NCEP ATP-III criteria for MS than those in the 2th-4th quartile (OR (95% CI): for men 3.1 (2.0-5.1), for women 3.6 (2.4-5.5)). This was also significant for insulin resistance (2.1 (1.3-3.3) and 2.1 (1.5-3.0), respectively), arbitrarily defined as the upper quartile of the HOMA2 distribution. Interestingly, mean IGF-I/IGFBP-3 ratio decreased in a linear fashion as the number of MS components increased. Saydah et al. [75] studied these parameters in relation to MS in 5900 participants of NHANES III, also including elderly subjects and diabetics. They showed to some degree overlapping results to those reported by Sierra-Johnson et al. Independent of diabetic status, all individual MS components were associated with lower mean levels of total IGF-I and IGFBP-3, a lower IGF-I/IGFBP-3 ratio and with higher levels of insulin. Only in non-diabetic subjects mean levels of circulating total IGF-I and the IGF-I/IGFBP-3 ratio decreased significantly in a linear fashion in subjects with either 0, 1-2 or 3-5 components of MS, whereas insulin levels showed an opposite pattern. Participants with diabetes had lower mean total IGF-I and IGFBP-3 levels and a lower mean IGF-I/IGFBP-3 ratio than non-diabetic subjects, regardless of the number of MS components.

Unfortunately, no data of circulating total IGF-I and/or IGFBPs levels are available in the third survey of the Rotterdam study, and thus no direct comparisons could be made between these IGF parameters and circulating IGF-I bioactivity, nor with MS components. We do recognize this limitation of

our study. We believe, however, that the results reported in the NHANES III studies can be used instead to discuss similarities and differences that seem to exist between measurements of circulating bioactive and total IGF-I in relation to the MS. This is possible because of the relatively high number of parallels between our study and the NHANES III studies (arguments: in all studies data were derived from well-characterized longitudinal population-based studies with large sample sizes and identical models were used to define MS (NCEP ATP-III) and to calculate insulin sensitivity/resistance (HOMA2 model).

As insulin resistance is the most important denominator of the MS, it can therefore be used to validate the outcome of the MS criteria used. Using the NCEP ATP III criteria for MS we observed a disproportional rise in insulin levels in subjects that met all 5 MS criteria and a significant rise of mean HOMA-IR with increasing MS components. Similarity of these results with other studies, including those of the NHANES III studies, means that the population studied was representative for our study aim. As IGF-I bioactivity was low in subjects with 5 MS components, this confirms the suggestion that IGF-I bioavailability is relatively low in these subjects. However, we also showed that mean IGF-I bioactivity in these individuals did not significantly differ from those having 0 or 1 components but only from those having 2 or 3. In contrast, as the IGF-I/IGFBP-3 ratio had a stronger association with MS than measures of IGF-I or IGFBP-3 alone, Sierra-Johnson et al. suggested that IGF-I bioavailability decreased linearly with increasing MS components [76].

With the appropriate respect to observations done and suggestions made by others, several conclusions can be drawn from the in part conflicting results. **1.** It appears that IGF-I bioactivity has an inverse U-shaped rather than a linear relationship with the development of the MS and thus cardiovascular risk. We believe that this observed pattern fits better to what is to be expected from a biological system, namely harboring dynamic buffer capacity or system redundancy. Buffering capacity is, however, only possible within a certain range. This could explain why mean IGF-I bioactivity seems to show a linear increase rather than an decrease up to the level of 3 MS components. One of the mechanisms involved could be adaptation of IGF-I bioactivity in an attempt to overcome the degree of insulin resistance, although with the requirement that Hep-IR is still absent (a mechanism described in paragraph 8.5.1). **2.** The IGF-I/IGFBP-3 ratio is probably not a very sensitive measure as estimate of IGF-I bioavailability (an observation that we analyzed ourselves in *chapter 3*). By use of the IGF-I/IGFBP-3 ratio a parabolic pattern between MS components and IGF-I bioactivity - which we believe is more plausible when approached from a biological point of view - was not observed. An

explanation could be within the range of the study limitations recognized by the authors themselves, namely the absence of measurements of other determinants of IGF-I bioavailability (e.g. IGFBP-1 and 2). Although not much data are available, it was recently reported in a cohort of 163 patients with type 2 DM that a low level of IGFBP-2 was independently associated with an increased risk of the MS (OR 0.31 [95 % CI 0.11 - 0.90]; p = 0.03) [77], suggesting that other determinants could be of importance as well in relation to IGF-I bioactivity. Of course one could argue that we also did not measure IGFBPs. However, the elegance of the IGF-I KIRA is that it has been shown to be sensitive for IGFBPs [11] and outcomes can be interpreted as an integral of all and not a subset of factors that determine IGF-I bioactivity. This means that by use of the IGF-I/IGFBP-3 ratio probably information about IGF system functionality is missed, as data in relation to the MS were discrepant when compared to those of IGF-I bioactivity. Thus, although the possibility still exists that the IGF-I/IGFP3 ratio could be a marker to monitor risk of development of the MS, our study provides data that this ratio is too limited to provide a full mechanistic insight into the MS, as the authors proclaimed. **3.** In healthy individuals with absent components of the MS a relatively high circulating total IGF-I level seems to be present, but circulating IGF-I bioactivity seems to be relatively low. This supports the findings discussed in *chapter 2*; high immunoreactive levels of total IGF-I do not necessarily represent high IGF-I bioactivity. **4.** Low levels of circulating IGF-I bioactivity seem to be present both in the healthy state as well as when severe insulin resistance has developed. Thus, this means that a low circulating IGF-I bioactivity as such is not informative about health status, but should be interpreted in relation to insulin sensitivity and other markers of CVD.

In conclusion: It is of our belief that the strength of the study described in *chapter 6* is that it is the only study so far that provides direct measurements of circulating IGF-I bioactivity which were studied in relation to insulin sensitivity and the MS. It provides new information about the possible role and/or dynamics of the IGF system in relation to the pathophysiological process of insulin resistance and the MS. This information is in part unique as it seems to differ from information in this field gathered by immunoreactive measurements of IGF-I. From our data we conclude that IGF-I bioactivity rises in parallel to insulin resistance up to the level that Hep-IR develops, after which IGF-I bioactivity drops and insulin levels increase disproportionately. Nonetheless, we do recognize that an important limitation of our study is the cross-sectional design. This means that we cannot determine whether the

association between IGF-I bioactivity and insulin resistance is causal, and thus we can only speculate about the direction of causation. Nevertheless, we think that this study provides new insights about the relationships between the IGF-I system and insulin resistance. Prospective data are needed, however, to test our hypotheses.

8.7 IGF-I gene polymorphisms in relation to variance in total and bioactive IGF-I levels and risk of cardiovascular disease

Twin studies have revealed that total IGF-I levels are heritable, with a high degree of heritability at birth (maybe even up to 93%), but a lower degree in middle aged and elderly twin subjects ranging from about 40-60% [78-80]. However, although family-based studies suggest that genetic variation could substantially contribute to circulating levels of IGF-I and IGFBPs, analyses of associations with multiple SNPs preferentially located within the regions of their genes have been only subtle for circulating levels and even less for risk on complex diseases. Restricted to the possible contribution of genetic variability itself, it is to be expected however that not only polymorphic variation in IGF (related) genes themselves but also that at multiple other loci influence their gene transcription and thus levels (e.g. activity of transcription factors, hormones, inhibitors of transcription (silencing RNA) etcetera). Thus, although the contribution of an individual genetic variable is expected to be low, a large number of influencing variables together could be of substantial importance. On the other hand one must be aware that the influence of genetic variability on the IGF system may be substantially overruled by environmental factors such as nutritional status, life style factors and others, thereby referring to the classic '*Nature versus Nurture'* debate.

In *chapter 7* we have described a study in which we analyzed whether genetic variance at the human IGF-I gene locus was associated with circulating levels of total and bioactive IGF-I; 18 SNPs giving rise to 6 haplotype blocks were studied. In addition we studied the relationship between these IGF-I gene SNPs/haplotypes and the risk of myocardial infarction (MI) and/or angina pectoris (AP). Study participants in which circulating total (N=430) and bioactive IGF-I levels (N=1036) were determined were randomly selected from the first and third survey of Rotterdam study, respectively.

In both men and women mean levels of circulating total IGF-I were significantly associated with individual SNPs that were mainly located within haplotype blocks (1 and 2) covering the genetic variance of the promoter regions from which IGF-I gene transcription is initiated (P1 and P2) and further 5' upstream. Haplotypes in blocks 1 and 2 were also associated with mean

levels of circulating total IGF-I. Genotypes within haplotype blocks 1 and 2 that were associated with a lower mean total IGF-I level were also associated with an increased risk of myocardial MI/AP tested for the total population of the first survey of the Rotterdam study for which genotyping was performed (N=5974). However, this last association was only found in men. In individuals in which we determined circulating IGF-I bioactivity we also found significant associations, but with a different profile. Individual SNPs were significantly associated with mean levels of IGF-I bioactivity but this was only observed in men. Second these SNPs were located within haplotype blocks 3 to 6, covering genetic variance of introns 1 to 5 and of the 3' untranslated region (3'UTR). Haplotypes within blocks 3 to 5 also reached significance in men. We could not find associations for haplotypes in blocks 3-6 and risk of MI/AP.

A systematic step by step interpretation of our results probably best fits this study as several findings are of interest but they were not all observed in the same individuals (e.g. total IGF-I was measured in 430 individuals of first survey, whereas IGF-I bioactivity was measured in 1036 of the third survey of the Rotterdam study). We do recognize this study limitation. However, as no measurements of circulating IGF-I bioactivity were (nor could be) performed in the first survey of the Rotterdam study and no financial resources were available to measure total IGF-I in the third study survey we were not able to analyze these parameters together. This makes both measurements were not fully comparable and thus the results should be interpreted separately. Of interest is the association of mean total IGF-I levels and genetic variance at the locus of the IGF-I gene promoters. The total circulating level of IGF-I is a result of gene transcription initiated from exons 1 (P1) and 2 (P2) of the IGF-I gene. It is known that several transcription factors bind to the 5' UTR region of the IGF-I gene [81]. By use of a computer program designed to predict transcription factor binding sites especially at gene promoter loci (MatInspector: http://www.sigmaplot.com/products/genomatix/ matinspector.php) we found that SNPs rs35765 and rs35767 are located within predicted binding sites at the 5'UTR of the human IGF-I gene for hepatocyte nuclear transcription factor 1 alpha (HNF-1a) and the of oncogene TFE3 [82], respectively. Although no literature is available to support the last prediction, the human IGF-I gene has been shown to be trans-activated by HNF-1a in reporter gene cotransfection assays from at a locus close to P1 [83]. Interestingly, HNF-1a has also been clearly linked to the occurrence of maturity-onset diabetes of the young (MODY) in humans [84]. Although this mechanism could be involved currently no functional data is available about whether this SNP is indeed functional. Therefore, it must be clear that this possible explanation

for the associations found between individual IGF-I SNPs and genotypes within blocks 1 and 2 that cover genetic variance at the IGF-I gene promoter and mean total IGF-I levels are speculative. Another possibility is that SNPs located within haplotype blocks 1 and 2 are themselves not functional, but that they are in high linkage disequilibrium with other polymorphisms that are functional. In this respect we also looked at the linkage between the polymorphisms in blocks 1 and 2 and a VNTR CA repeat at -1 Kb of exon 1 that has been associated in the Rotterdam study with levels of total IGF-I, height and risk on DM type 2, MI, heart failure, stroke and others including risk on proteinuria in diabetics [85-89]. Linkage disequilibrium (D') between the CA repeat and rs2162679 and rs35767, respectively, was 0.84 and 0.86. Although the CA repeat has been used in several studies for years to study genetic variance at the human IGF-I locus today no functional data is available for this VNTR. It probably means that this VNTR itself is not functional and that publication bias have to be taken into consideration as an explanation for this absence of data; IGF-I promoter activation studies have been performed in other settings and with success [81].

Interestingly, SNPs located within blocks 1 and 2, as well as haplotypes within these blocks were not only associated with mean total IGF-I levels in a subset of individuals, but also with risk of MI/AP in the total study population of the Rotterdam study, however only in men. Genotypes/haplotypes associated with lower mean IGF-I levels at baseline showed higher risk at follow-up, which is in line with findings reported by others: this counts both for the link between IGF-I and CVD, as well as for the sexual dimorphism observed in this. The question rises how this sexual dimorphism should be explained? In relation to CVD IGF-I may have different functions in men and women, or being a vascular protective agent in both sexes but far more pronounced in men compared to women. In addition, the absence of associations with IGF-I gene polymorphisms and CVD in women could also be due to the fact that the highest incidence of CVD is at older age (about a decade later) than in men.

Quite surprisingly we observed that mean levels of IGF-I bioactivity were associated with SNPs and haplotypes located within other regions of the IGF-I gene than total IGF-I levels, and only in men. Although one cannot ignore the high linkage between certain SNPs, more associations reached significance than one would expect by chance only, suggesting that these findings actually have physiological meaning. Of most interest is that mean levels of IGF-I bioactivity in men were also associated with SNPs located within other functional regions of the IGF-I gene than total IGF-I, namely spanning that from exon 3 up to the 3'UTR region. This last region, including exon 5 and

exon 6 is known to be subjected to tissue specific alternative splicing giving rise to different IGF-I mRNA transcripts and IGF-I precursor peptides [90,91]. Exon 6 gives rise to the Ea-domain, Exon 5 to the Eb-domain (being both regulated by hormonal factors) and a part of both exons to the Ec domain [92,93]. This last domain is present in an isoform of IGF-I (Mechano Growth Factor), which is produced by muscle tissue upon stretch or damage [93,94]. Alignment of the Ea domain among different species shows that the sequence is highly conserved, suggesting a biological function for this region, whereas sequences for the Eb and Ec-domain are more variable [95]. SNPs in the 3'UTR region may thus be associated with differential transcription of IGF-I precursors. Today not much is known about the different IGF-I precursor peptides nor about the function of the E-domains *in vivo* as only one 'identical' mature IGF-I protein is produced from all precursors. However, our results at least indicate that there could be a link between the genetic variability at the locus of the IGF-I 3'UTR gene, IGF-I precursors and circulating IGF-I bioactivity. If this speculation holds, the observed sexual dimorphism for the associations observed might be explained by differences in hormone factors and/or muscle mass. In addition it could also be that mRNA stability of IGF-I transcripts is affected by genetic variability at the 3'UTR region, thus leading to differences in posttranslational modifications. We realize that at this moment we cannot present data to support these suggestions.

Another observation of interest comes from our search in the MatInspector database to predict whether SNPs analyzed change transcription binding sites. By use of the MatInspector programme we observed that SNP rs6220 is located within a recognized DNA binding site of FOXO proteins. This could mean that the 3' regulatory region of the human IGF-I gene might be targeted by these forkhead transcription factors. This might influence IGF-I bioactivity. Again there is at present no literature that supports this finding and therefore studies are needed to test this hypothesis.

In our study we did not observe any significant associations between SNP and/or haplotypes located within blocks 3-6 and risk of MI and/or AP. Several explanations can be considered for this; First it could be that this gene region indeed is associated with levels of circulating IGF-I bioactivity but not with risk of MI and/or AP. On the other hand we have shown in *chapter 3* that IGF-I bioactivity was strongly associated with survival in men especially with risk of death from CVD. Differences in the definition of disease outcome could be of influence (CVD in chapter 3 was differently defined than MI and/or AP in *chapter 7*). Furthermore, we also showed in the same individuals (*chapter* 6) that IGF-I bioactivity had a non-linear inverse u-shaped relationship with the

number of components of the MS, a condition characterized by a cluster of cardiovascular risk factors. It is of importance to realize that this pattern exists, as the overall distribution of individuals within this spectrum can greatly influence statistical outcomes based on linear models. This means that if the majority of individuals is at the left side of the inverse u-shaped curve of IGF-I bioactivity one might find that high levels of IGF-I bioactivity are associated with risk CVD, whereas if the majority of individuals is at the right side of the inverse u-shaped curve, this is true for low levels. Second, if individuals are equally spread one might not find any relationship with CVD risk.

A limitation of our study was that circulating IGF-I bioactivity was not measured at baseline but only after the third round of the Rotterdam study. This may have introduced a prevalence bias since the "worst" cases may have died in the intervening years between the first and the third survey. More likely, however, is that the genetic contribution to IGF-I bioactivity is far less than that of non-genetic factors, whereas total IGF-I levels are more genetically determined.

In conclusion: Although having some recognizable limitations, we do think that the study presented in chapter 7, gives new insight in the relationship between levels of circulating total and bioactive IGF-I and human genetics. It is of large interest that again we observed a sexual dimorphism. This study also contributes to the data that link the IGF-I system to risk of cardiovascular disease. Furthermore our study is the first that suggests that polymorphic variability at the locus of the IGF-I gene differentially affects levels of total and bioactive IGF-I. Where polymorphisms at the 5'UTR region probably affect total circulating IGF-I levels, those at the 3'UTR region of the human IGF-I gene might affect circulating IGF-I bioactivity (in men). Of course future studies are necessary to further investigate these findings.

8.8 General reflection and future perspectives

It is of the authors believe that the general 'Aim of the Thesis' has been met by the studies included in the thesis. First, our results indeed suggest that measurements of circulating IGF-I bioactivity by use of the IGF-I KIRA can substantially differ from those obtained by immunoreactive measurements of total IGF-I levels, even when obtained in combination with measures of IGFBPs. This has been confirmed by others and is most likely explained by the different type of methodology on which the IGF-I KIRA is based when compared to immunoassay measurements of circulating levels; Namely, a specific response produced by living cells (IGF-IR activation) upon exposure to an intact biological system. Beforehand, we therefore speculated that this

method has the potential to enlarge insight in the function and responses of the IGF system in relation to health and disease.

We have shown for the first time that the IGF-I KIRA can be easily used in relatively large population based settings. When one considers that the KIRA assay is actually both a bioassay and an ELISA, the level of assay performance that could be reached was quite remarkable. We have established normal values for circulating IGF-I bioactivity in healthy adults, which could and can be used in other studies. We observed a sexual dimorphism in circulating levels for bioactive IGF-I, with most obvious a significant drop in IGF-I bioactivity around the expected age of menopause in women. This could mean that in women sex-hormones affect IGF-I bioactivity *in vivo*, a finding of interest in relation to for instance the development of (breast) cancer.

In a prospective study circulating IGF-I bioactivity was found as an independent predictor of survival in elderly men, especially when focusing on cardiovascular risk. For an IGF-I bioassay this has not been shown previously. Next to its prospective design, strength of this study was that direct comparisons could be made with measures of total and free IGF-I, for which in this study we could not find these relationships. Although one might expect measures of free and bioactive IGF-I to be closely related – both aim to determine direct available IGF-I for IGF-IR activation – outcomes of both methods seemed to differ extensively. Probably effects of IGFBP and protease activity, involvement of IGF-II and other biological factors important for IGF-I bioactivity as well as differences in the methodology and type of population studied are involved in this.

In a cross-sectional setting we found that IGF-I bioactivity was related to glucose homeostasis and insulin sensitivity as mean levels gradually raised with increasing HOMA-IR and dropped exactly at the level when type 2 DM was present. Furthermore, mean IGF-I bioactivity showed a parabolic relationship with the number of MS components present. This type of data is new in the field of IGF research and clearly enhances insight in the function of the IGF system in relation to insulin resistance. We hope that these results in future can be studied in a prospective setting. This is obviously needed to obtain information about causality of the observed changes in circulating IGF-I bioactivity: are they cause or consequence of insulin resistance?

In the Rotterdan study we observed that levels of IGF-I bioactivity were associated with genetic variation at the IGF-I gene locus. Again we observed a sexual dimorphism, as significant associations were only observed in men. Of most interest, the polymorphisms that were associated with IGF-I bioactivity in men were located in another functional region of the IGF-I gene (3'UTR

region), than those that were associated with levels of total IGF-I (promoter region). This finding opens the possibility that IGF-I bioactivity in part is determined by genetic variability at the 3'UTR region of the IGF-I gene, a region which is known to be subject to mRNA alternative splicing and post-translational modification. This finding for IGF-I bioactivity is unique, but whether it also has physiological consequences remains to be seen.

A limitation at this moment is that the IGF-I KIRA is fully processed by hand. However it is not unthinkable that because of its design (at least) a large part of the assay is easily adapted to automation. This means that larger numbers of samples can be separately run at one time, making this assay more time- and economically-efficient.

It should be emphasized that the IGF-I KIRA is not developed to replace immunoassay measures of IGF-I (or those of other IGF system parameters). However, by using both types of techniques the IGF-I KIRA can be of interest to clarify how IGF-I bioactivity is regulated *in vivo*. At this moment the IGF-I KIRA has not been used in clinical studies (e.g. GH disorders). Future studies are thus of major interest and need to determine the additive value that the IGF-I KIRA may have in the clinical setting next to immunoassay measures.

In theory the KIRA design is applicable to all cell surface receptors harboring kinase receptor activity upon specific ligand binding. As next to insulin and IGFs also the IR and the IGF-IR share high structure homology it is of interest to design identical KIRA bioassays for insulin. By use of comparable bioassays this may help to further unravel the functional overlap and differences between IGFs and insulin. We are currently developing insulin bioassays with an identical design as the IGF-I KIRA at our laboratory. This opens the possibility to study both parameters (IGF-I and insulin bioactivity) in parallel with full comparison.

Last, when interpreting the IGF-I KIRA outcomes one should consider the following: when using this method to determine circulating IGF-I bioactivities of individuals, outcomes are plotted against a universal background, namely an artificially IGF-IR transfected cell system. This means that outcomes may not necessarily reflect the actual circulating IGF-I bioactivity of a certain individual generated through its own IGF-IRs (e.g. this opens the possibility that a relatively high IGF-I bioactivity level measured by the IGF-I KIRA is actually an indicator of a low *in vivo* IGF-IR signaling activity). Therefore an interesting future perspective may be to study whether it is possible exists to design IGF bioassays that even better reflect an individual's IGF-I bioactivity.

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Chapter 9

Summary

SUMMARY

One of the major challenges in the field of IGF research is to clarify how IGF-I bioactivity is regulated *in vivo*. The highly complex molecular network that comprises the IGF system together with its dynamic interactions with other factors lies at the basis of this interesting challenge. Classically, the IGF system includes two growth factors (IGF-I and IGF-II), six IGF-binding proteins (IGFBP-1 to -6), nine IGFBP-related proteins (IGFBP-rPs), two cell surface receptors (IGF-IR and the IGF-II mannose-6-phosphate receptor (M-6-PR) and several IGFBP specific proteases. From this it can be concluded that IGFs are enclosed by a large number of regulatory proteins and factors, suggesting tight, complex as well as dynamic regulation of their actions.

To date immunoassays are used for measurement of circulating levels of individual components of the IGF system in order to estimate IGF activity *in vivo*. Especially IGF-I immunoassays for measures of total IGF-I are used in the clinic to screen for, diagnose and evaluate treatment of GH disorders (e.g. acromegaly and GHD). It should, however, be realized that in IGF-I immunoassays large concessions are made to the structure of the IGF system. To guarantee full accessibility of highly specific antibodies for IGF-I binding IGFBPs are removed, thereby ignoring their substantial influences on IGF-I activity. Second, although the type of technique applied in IGF-I immunoassays has evolved from competitive binding strategies (IGF-I RIA) towards two antibody capture techniques (IRMA, CLIA), their underlying 'ligand based' design has not been altered. However, circulating hormone levels may not necessarily reflect IGF bioavailability (which may also be true for measures of circulating free IGF-I). Third, even if levels determined by immunoassay techniques are highly accurate it is still extremely difficult to define the impact of individual circulating levels of IGF-I and/or IGFBPs on IGF-I bioactivity. This is dependent on the context of these changes in relation to each other and to other determinants of IGF-I bioactivity. Thus, although immunoassays without any doubt have yielded important information about the IGF system, meaningful information about the IGF system may still not be detected.

In 2003 Frystyk at al. developed a highly specific and sensitive bioassay to measure circulating IGF-I bioactivity in human serum (IGF-I KIRA). This assay quantifies IGF-IR phosphorylation of living human cells upon stimulation with serum as a direct measure of circulating IGF system activity, defined as IGF-I bioactivity. Important discriminative features of the IGF-I KIRA in relation to immunoassays are that it uses a highly specific biological response as outcome, and that the IGF system from which this response is derived is

kept intact. Thus, the IGF-I KIRA may more naturally reflect IGF-I bioactivity *in vivo* as IGF-IR activation is the first and biologically most specific response of all factors that directly influence IGF-I bioactivity (it is the level at which all extracellular information is integrated into a cellular response).

In the studies included in this thesis the IGF-I KIRA was used to study IGF-I bioactivity in relation to aging, health and disease. Our general aim was to evaluate whether measures of circulating IGF-I bioactivity have the potential to produce new insights in the functional properties of the circulating IGF system.

In *chapter 2* we have described a cross-sectional study in which normal values for the IGF-I KIRA were determined. Circulating IGF-I bioactivity was measured in 426 healthy blood donor individuals. Ranke et al. had recently determined values for circulating total IGF-I levels within this population using 5 different immunoassays. We found that the distribution of circulating IGF-I bioactivity came close to a normal Gaussian curve, contrasting the distribution of total IGF-I levels. The age-specific normative ranges can be helpful in future to interpret whether IGF-I bioactivity is normal, increased and/or decreased in subjects with pathological conditions like acromegaly and GH deficiency. In women circulating IGF-I bioactivity significantly dropped around the age range of 55. This suggests that IGF-I bioactivity is in part driven by sex hormones. Correlations observed between IGF-I bioactivity and measurements of total IGF-I reached significance ($p<0.001$), however r^2 ranged from 0.25 to 0.31. This means that 70-75% of the variability in IGF-I bioactivity could not be explained by levels of total IGF-I. We observed that IGF-I bioactivity declined with age, while the fraction of IGF-I bioactivity over total IGF-I increased with age.

In *chapter 3* we have described a prospective study in which we analyzed circulating IGF-I bioactivity next to free and total IGF-I in relation to overall survival in a population of about 400 elderly men. Correlations between levels of IGF-I bioactivity and those of total and free IGF-I were significant but weak and mean IGF-I bioactivity was about 2-3 times higher than free IGF-I levels. We found that of the composed survival models only that for circulating IGF-I bioactivity reached significance. A relatively high, rather than low circulating IGF-I bioactivity was related with longer survival. In a sub-analysis we stratified for presence or absence of cardiovascular disease (CVD) and/or evidence of a high CVD risk profile based on CRP levels (a measure of subclinical systemic inflammation). We observed that low circulating IGF-I bioactivity was an independent risk factor for mortality and especially in individuals with CVD and/or a high inflammatory risk profile. We believe that these findings

could be of interest since for the first time they provide direct insights in the function of the circulating IGF system in relation to human survival.

In *chapter 4* we have described results of circulating IGF-I bioactivity and total IGF-I measurements performed in a randomized cross-over study in 12 fed patients who were on continuous ambulatory peritoneal dialysis (CAPD) as kidney replacement therapy. Two different dialysis solutions were used; either enriched with glucose (G) or glucose plus amino acids (AAG). IGF-I bioactivity and total IGF-I were measured at baseline and after the dialysis program (9 hours later). We found that the mean circulating total IGF-I levels in CAPD subjects were in the upper half of the normal range, whereas mean circulating IGF-I bioactivity levels were in the lower half of the normal range however. No differences in circulating total IGF-I levels could be found after AAG or G dialysis when compared to baseline. IGF-I bioactivity increased in CRF patients on CAPD upon both AAG and G dialysis, but only for AAG dialysis this difference reached significant difference. This means that IGF-I bioactivity even in the fed state can be readily changed (reflecting system dynamics), having a causative relationship with AAG uptake. We concluded that bioactive rather than total IGF-I is involved in acute responses to nutritional interventions in CAPD patients.

In *chapter 5* we have described a case report of a relatively healthy patient with low serum total IGF-I levels, eventually recognized to be caused by interference from heterophilic antibodies in the IGF-I immunoassay. IGF-I immunoassays are dependent on the highly specific interaction between the IGF-I protein and the IGF-I antibody. This interaction can (theoretically) be disturbed by so-called heterophilic antibodies present in serum. Such interference, well-known for other immunoassays, has however never been described for IGF-I immunoassays. Here we report the first case in the literature. Our observation implicates that in some cases heterophilic antibodies could be a pitfall in the interpretation of levels of total IGF-I especially when used in the diagnosis or treatment of GH disorders. We showed that IGF-I bioactivity in this patient was within the mid-normal range for age and sex. Using the scantibody technique with the aim to eliminate heterophilic antibody interference we observed that the total IGF-I level in this patient was within the mid-normal range. This study thus also adds to the data showing that measures of IGF-I bioactivity can substantially differ from those obtained by immunoassays.

In *chapter 6* a cross-sectional study (N=1036) in elderly individuals is described. We focused on the relationship between circulating IGF-I bioactivity, insulin resistance (HOMA-IR) and the metabolic syndrome (MS). Mean

circulating IGF-I bioactivity levels progressively rose with increasing mean fasting glucose and insulin levels (and thereby HOMA-IR), but it declined when undiagnosed type 2 DM was present. We believe that the rise in circulating IGF-I bioactivity with increasing HOMA-IR could be part of a compensating mechanism to overcome insulin resistance through insulin like actions of IGFs on glucose uptake and on peripheral insulin sensitivity. Hepatic insulin resistance could be the cause of the decline in circulating IGF-I bioactivity in diabetics. This study supports the idea that the IGF system is involved in the pathogenesis of type 2 DM. Causality cannot be obtained from this study however.

The metabolic syndrome (MS) is a condition characterized by a cluster of evidence based cardiovascular risk factors of which insulin resistance is thought to be 'the corner stone'. A non-linear (inversed U-shaped) relation between IGF-I bioactivity and the number of MS components present was observed. Circulating IGF-I bioactivity raised gradually up to the level of three present MS components (and thus the presence of MS), but it declined when all 5 MS components were present. These data contrast to the relation between total IGF-I and the total number of MS components reported by others in which a linear decreasing relationship was reported. We speculated that there could exist a Starling Curve for circulating IGF-I bioactivity.

In *chapter 7* we have described a study in which we analyzed whether genetic variance at the human IGF-I gene locus was associated with circulating levels of total and bioactive IGF-I; 18 SNPs giving rise to 6 haplotype blocks were studied. In addition we studied the relationship between these IGF-I gene SNPs/haplotypes and risk of MI and/or AP. Study participants in which circulating total (N=430) and bioactive IGF-I levels (N=1036) were determined were randomly selected from the first and third survey of Rotterdam study respectively. Mean levels of circulating total IGF-I were significantly associated with individual SNPs that were mainly located within haplotype blocks (1 and 2) covering the genetic variance of the promoter regions. Indeed, haplotypes in blocks 1 and 2 were also associated with mean levels of circulating total IGF-I. These haplotypes were also associated with risk of MI/AP, but only in men.

Individual SNPs were significantly associated with mean levels of IGF-I bioactivity but this was only observed in men. These SNPs were located within haplotype blocks 3 to 6, covering genetic variance of introns 2 to 5 and of the 3' untranslated region (3'UTR). Haplotypes within blocks 3 to 5 also reached significance in men. We did not observe associations for genotypes/haplotyes in blocks 3-6 and risk of MI/AP. We believe that this study provides new

insight in the relationship between levels of circulating total and bioactive IGF-I and human genetics. Furthermore levels of IGF-I bioactivity may be less determined by genetic variability than total circulating levels.

Overall we believe that by the use of the IGF-I KIRA to asses circulating IGF-I bioactivity we have been able to demonstrate that this technique indeed provides information about the IGF-I system, that differs from that obtained by immunoassays. Therefore the IGF-I KIRA is an interesting technique that in future can be used next to (and not instead of) immunoassays to further unravel the functions of the IGF system in health and disease.

SAMENVATTING

Een van de grote uitdagingen in het veld van onderzoek dat zich toespitst op 'insulin-like growth factors' (IGFs) is te verklaren hoe de IGF-I bioactiviteit wordt gereguleerd *in vivo*. Aan de basis van deze uitdaging ligt het complexe netwerk van het IGF systeem, alsmede de dynamische interacties die het heeft met andere factoren. Het klassieke IGF systeem bestaat uit twee groeifactoren (IGF-I en IGF-II), zes IGF bindende eiwitten (IGFBP-1 t/m -6, negen IGFBP gerelateerde eiwitten (IGFBP-rPs), twee cel receptoren (de IGF-IR en de IGF-II mannose-6-fosfaat receptor (M-6-PR) en verschillende IGFBP specifieke proteasen. Hieruit kan geconcludeerd worden dat IGFs omringd worden door een groot aantal regulatoire eiwitten en factoren. Dit suggereert zowel stringente, complexe als dynamische regulatie van de acties die door IGFs worden bewerkstelligd.

Tegenwoordig wordt gebruik gemaakt van immunoassays ter bepaling van circulerende concentraties van de individuele componenten van het IGF systeem. Met name IGF-I immunoassays, ter bepaling van de totale IGF-I spiegels, worden gebruikt in de kliniek met betrekking tot het screenen naar, het diagnosticeren van, en het evalueren van de behandeling van groeihormoon (GH) gerelateerde ziekten (bijvoorbeeld acromegalie en groeihormoon deficiëntie). Men moet zich echter realiseren dat inherent aan het gebruik van IGF-I immunoassays concessies moeten worden gedaan ten aanzien van de structuur van het IGF systeem. IGFBPs worden verwijderd uit het bloedmonster om volledige toegankelijkheid te garanderen van specifieke antilichamen voor IGF-I, met als gevolg dat de invloeden van IGFBPs op de IGF-I activiteit dus worden genegeerd. Ten tweede, ondanks dat de toegepaste technieken in IGF-I immunoassays zijn geëvolueerd van competitieve bindingsstrategieën (IGF-I RIA) naar bindingsmethoden berustend op het gebruik van twee antilichamen (IRMA, CLIA), is het algemene principe – de focus ligt op de ligand concentratie – onveranderd gebleven. Echter, de circulerende hormoon spiegels hoeven niet per se de biologische beschikbaarheid van IGFs te vertegenwoordigen (iets wat ook geldt voor de bepaling van vrij IGF-I). Ten derde, zelfs indien spiegels middels immunoassay technieken zeer accuraat bepaald zouden worden, geldt alsnog dat het bijzonder moeizaam is de individuele invloed van IGF-I en/of IGFBPs spiegels (of veranderingen daarin) op de IGF-I bioactiviteit te definiëren. Dit laatste is namelijk mede afhankelijk van de context van deze spiegels / veranderingen ten opzichte van elkaar, alsmede van andere factoren die van invloed zijn op de IGF-I bioactiviteit. Resumerend, ondanks het feit dat immunoassays zonder enige

twijfel belangrijke informatie over het IGF systeem aan het licht hebben gebracht, sluit dit geenszins de mogelijkheid uit dat waardevolle informatie over het IGF systeem nog steeds niet wordt gedetecteerd.

In 2003 is door Chen et al. een zeer specifieke en sensitieve bioassay ontwikkelt ter bepaling van de circulerende IGF-I bioactiviteit in humaan serum (IGF-I KIRA). Deze assay kwantificeert de IGF-IR fosforylering in levende cellen na stimulatie met serum als een directe afgeleide van de circulerende IGF systeem activiteit, gedefinieerd als IGF-I bioactiviteit. Belangrijke onderscheidende eigenschappen van de IGF-I KIRA in relatie tot IGF-I immunoassays zijn dat het als meetbare uitkomst gebruik maakt van een zeer specifieke biologische respons, en dat het IGF systeem, waardoor deze respons gegenereerd wordt, geheel intact blijft. Daarom zou de IGF-I KIRA een meer natuurlijke afspiegeling kunnen geven van de *in vivo* IGF-I bioactiviteit, aangezien activatie van de IGF-IR de eerste en biologisch meest specifieke respons is van alle factoren die direct de IGF-I bioactiviteit beïnvloeden (het vertegenwoordigt het niveau waarbij alle extracellulaire informatie wordt geïntegreerd en vertaald naar een intracellulaire response).

In de studies opgenomen in dit proefschrift is gebruikt gemaakt van de IGF-I KIRA ter bestudering van de IGF-I bioactiviteit in relatie veroudering, gezondheid en ziekte. Onze algemene doelstelling was te evalueren of bepalingen van de circulerende IGF-I bioactiviteit de potentie hebben nieuwe inzichten te verschaffen over de eigenschappen van het circulerende IGF systeem.

In *hoofdstuk 2* hebben wij een cross-sectionele studie beschreven waarin normaalwaarden voor de IGF-I KIRA methode zijn bepaald. De circulerende IGF-I bioactiviteit is hierbij gemeten in bloed van 426 gezonde bloeddonoren. Recent heeft Ranke et al. totale IGF-I waarden bepaald in deze populatie, waarbij gebruik is gemaakt van vijf verschillende IGF-I immunoassays. Onze bevindingen waren dat de distributie van de circulerende IGF-I bioactiviteit in de studie populatie dicht in de buurt kwam van een normale Gaussiaanse curve. Dit staat in contrast tot de distributies gevonden in de populatie voor totaal IGF-I. De leeftijdsspecifieke normaal waarden vormen een houvast bij de interpretatie van toekomstige IGF-I bioactiviteit metingen of deze normaal, verhoogd en/of verlaagd zijn bij individuen met onderliggende pathologische aandoeningen zoals acromegalie en GH deficiëntie. Bij vrouwen rond de leeftijd van 55 jaar daalde de circulerende IGF-I bioactiviteit significant. Dit suggereert dat de IGF-I bioactiviteit ten dele wordt gedreven door oestrogenen/prostagenen. Wij vonden significante correlaties tussen de IGF-I bioactiviteit en metingen van totaal IGF-I ($P<0,001$), echter de r^2 varieerde van 0.25 tot 0.31. Dit betekent dat 70-75% van de variatie in de IGF-I bioactiviteit niet

kon worden verklaard door spiegels van totaal IGF-I. Ook vonden wij dat de IGF-I bioactiviteit daalde met de leeftijd, maar dat de ratio tussen de IGF-I bioactiviteit en totaal IGF-I, uitgedrukt in percentage, steeg met de leeftijd.

In *hoofdstuk 3* hebben wij een prospectieve studie beschreven waarin de circulerende IGF-I bioactiviteit naast vrij en totaal IGF-I zijn geanalyseerd in relatie tot overleving in een populatie van nagenoeg 400 oudere mannen. Correlaties tussen de spiegels van IGF-I bioactiviteit en die van totaal en vrij IGF-I waren significant, doch zwak, en de gemiddelde IGF-I bioactiviteit was 2 tot 3 maal hoger dan die van vrij IGF-I. Wij vonden dat alleen de samengestelde overlevingsmodellen voor de IGF-I bioactiviteit significantie bereikten. Relatief hoge, in plaats van een lage IGF-I bioactiviteit, was geassocieerd met een langere overleving. In een sub-analyse hebben wij de populatie gestratificeerd voor de aan- danwel afwezigheid van hart- en vaatziekte (CVD) en/of bewijs voor een hoog CVD risico profiel gebaseerd op CRP spiegels (een maat voor subklinische sytemische inflammatie). Wij vonden dat een lage IGF-I bioactiviteit een onafhankelijke risico factor was voor mortaliteit en met name bij individuen met CVD en/of een hoog inflammatoir risico profiel. Wij veronderstellen dat deze bevindingen van belang kunnen zijn aangezien zij directe inzichten geven in de functie van het circulerend IGF syteem in relatie tot humane overleving.

In *hoofdstuk 4* hebben wij onze resultaten beschreven van de circulerende IGF-I bioactiviteit en totaal IGF-I bepaald in een gerandomiseerde cross-over studie met 12 gevoede nierpatiënten, gebruik makend van continue ambulante peritoneale dialyse (CAPD) als nier vervangende therapie. Twee verschillende dialyse oplossingen zijn gebruikt; verrijkt met alleen glucose (G) dan wel met glucose en aminozuren (AAG). De IGF-I bioaciviteit en totaal IGF-I werden voorafgaand en na het dialyse programma (9 uur later) bepaald. We vonden dat de gemiddelde circulerende totaal IGF-I spiegels zich bevonden in bovenste helft van de normaal waarden, terwijl de gemiddelde IGF-I bioactiviteit spiegels zich echter in de onderste helft van de normaalwaarden bevonden. Wij vonden geen verschillen in circulerende totaal IGF-I spiegels na AAG of G dialyse wanneer deze werden vergeleken met de waarden voorafgaand aan dialyse. De IGF-I bioactiviteit steeg in de CAPD patiënten na zowel AAG als G dialyse, echter dit bereikte alleen significantie in de AAG dialyse groep. Dit betekent dat zelfs in een gevoede toestand de IGF-I bioactiviteit snel kan worden aangepast (dit als reflectie van systemische dynamiek), daarbij een causale relatie met AAG opname hebbende. Wij concludeerden dat bioactief maar niet totaal IGF-I betrokken is bij de acute responsen op voedingsinterventies bij CAPD patiënten.

In *hoofdstuk 5* hebben wij een case report beschreven van een relatief gezonde patient met lage totaal IGF-I spiegels die uiteindelijk gebaseerd bleek op interferentie van de gebruikte IGF-I immunoassay door heterofiele antilichamen. IGF-I immunoassays zijn afhankelijk van de specifieke interactie tussen het IGF-I proteïne en het IGF-I antilichaam. Deze interactie kan theoretisch worden verstoord door in serum aanwezig zijnde heterofiele antilichamen. Een dergelijke interferentie, een bekend fenomeen in andere immunoassays, was tot op heden niet beschreven voor IGF-I immunoassays. Alhier rapporteren wij de eerste 'case' in de literatuur. Onze observatie zou kunnen impliceren dat in sommige gevallen heterofiele antilichamen een valkuil kunnen vormen bij de interpretatie van totale IGF-I spiegels, met name bij gebruik in de diagnostiek of behandeling van GH aandoeningen. Wij toonden aan dat de IGF-I bioactiviteit bij deze patiënt zich bevond in de mid-normale waarden rekening houdend met leeftijd en geslacht. Gebruik makend van de 'scantibody' techniek, met als doel de interferentie door hetrofiele antilichamen te elimineren, vonden wij dat de totale IGF-I spiegel van deze patiënt zich bevond in de mid-normale range. Deze studie vormt een aanvulling op de data die aantonen dat bepalingen van de IGF-I bioactiviteit substantieel kunnen verschillen van die verkregen met immunoassay technieken.

In *hoofdstuk 6* wordt een cross-sectionele studie beschreven, verricht in oudere individuen (N=1036). In deze studie hebben wij ons toegespitst op de relatie tussen de circulerende IGF-I bioactiviteit, insuline resistentie (HOMA-IR) en het metabool syndroom (MS). De circulerende IGF-I bioactiviteit steeg progressief, parallel aan de gemiddelde nuchtere glucose en insuline waarden (en daarmee de HOMA-IR), maar daalde wanneer type 2 DM aanwezig was. Wij veronderstellen dat de stijging van de IGF-I bioactiviteit parallel aan de toename in de HOMA-IR ten gevolge van een compensatoir mechanisme kan zijn; dit ter overwinning van de heersende insuline resistentie middels de insulineachtige effecten van IGFs op de cellulaire glucose opname en op de perifere insuline gevoeligheid. Het bestaan van hepatische insuline resistentie zou de oorzaak kunnen zijn van het verval van de circulerende IGF-I bioactiviteit bij diabetici. Deze studie ondersteunt het idee dat het IGF systeem betrokken is in de pathogenese van DM type 2. Een oorzakelijk verband kan echter niet verkregen worden uit de opzet van deze studie.

Het MS is een toestand die wordt gekarakteriseerd door een kluster van vijf bewezen cardiovasculaire risico factoren waarbij wordt verondersteld dat insuline resistentie de zogenaamde hoeksteen vormt van het syndroom. Wij vonden een parabole relatie tussen de IGF-I bioactiviteit en het aantal aanwezige componenten van het MS. De gemiddelde IGF-I bioactiviteit steeg

gradueel tot op het niveau van 3 aanwezige MS componenten, maar het daalde bij aanwezigheid van alle 5 de componenten. Deze data staat in contrast tot de relatie tussen totaal IGF-I spiegels en het aantal componenten van het MS die is beschreven door anderen, waarbij een afnemend lineair verband is gevonden. Wij speculeren dat er een Straling Curve zou kunnen bestaan voor de circulerende IGF-I bioactiviteit.

In *hoofdstuk 7* hebben wij een studie beschreven waarin de associatie tussen de genetische variatie ter plaatse van het humane IGF-I locus en circulerende spiegels van totaal en bioactief IGF-I is geanalyseerd; 18 SNPs werden bestudeerd welke tevens zijn gebruikt ter samenstelling van 6 haplotype blokken. Daarnaast hebben we de relatie bestudeerd tussen deze SNPs/haplotypen en het risico op een hart infarct en/of angina pectoris (MI/AP). Studie participanten waarbij circulerend totaal (N=430) en bioactief IGF-I (N=1036) werden bepaald werden at random geselecteerd vanuit respectievelijk de eerste en derde onderzoeksronde van de Rotterdam studie. Spiegels van circulerend totaal IGF-I waren significant geassocieerd met individuele SNPs die voornamelijk gelokaliseerd waren in haplotype blokken (1 en 2) die de genetische variatie van de promotor regionen van het IGF-I gen omvatten. Haplotypen in blok 1 en 2 waren ook geassocieerd met spiegels van totaal IGF-I. Deze haplotypen waren tevens significant geassocieerd met het risico op MI/AP, echter alleen in mannen.

Individuele SNPs waren significant geassocieerd met spiegels van de IGF-I bioactiviteit, doch dit werd alleen gevonden in mannen. Deze SNPs waren gelokaliseerd in haplotype blokken 3 tot en met 6, welke de genetische variatie omvatten van intron 2 tot en met 5 alsmede van de 3' untranslated region (3'UTR). Haplotypen in blokken 3 tot en met 5 bereikten ook significantie in mannen. We vonden geen significante associaties voor haplotypen in blokken 3 tot en met 6 en het risico op MI/AP. We veronderstellen dat deze studie nieuwe inzichten geeft in de relatie tussen spiegels van circulerend totaal en bioactief IGF-I en humane genetica. Verder zouden spiegels van bioactief IGF-I minder onder invloed van genetische variatie kunnen staan dan spiegels van circulerend totaal IGF-I.

In het algemeen vinden wij dat met behulp van de IGF-I KIRA methode, ter bepaling van de IGF-I bioactiviteit, wij in staat zijn geweest om aan te tonen dat deze techniek informatie over het IGF systeem kan verschaffen die verschilt van die verkregen met immunoassays. Dit maakt de IGF-I KIRA een interessante techniek die in de toekomst kan worden toegepast naast (en niet in plaats van) immunoassays om de functie van het IGF systeem bij gezondheid en ziekte verder te ontrafelen.

Dankwoord

DANKWOORD

Aangekomen bij het *Dankwoord* van het proefschrift wordt ik aangegrepen door tegenstrijdige emoties. Enerzijds overheerst een gevoel van vreugde - ten dele gekenmerkt door opluchting - bij het tot stand komen van het boekje als geheel. Anderzijds bestaat het gevoel van afscheid moeten nemen van een levensfase waar ik mij met volle passie voor heb ingezet, een passie die verslavend is.

Een groot aantal personen hebben een belangrijke bijdrage geleverd aan de studies opgenomen in dit proefschrift. Graag wil ik hier een ieder daarvoor bedanken, waarvan een aantal in het bijzonder.

Professor Lamberts, mijn *Promotor*, hartelijk dank voor het geheel aan geboden kansen, vertrouwen, stimuli en professionele begeleiding tijdens mijn periode als uw promovendus. Ik ben zeer vereerd dat ik mag aansluiten in de rij met promovendi die onder uw inspirerende supervisie het hebben mogen brengen tot doctor. Het was en is mij een waar genoegen.

Dr. Janssen, maar veel liever gezegd 'Joop', met woorden kan ik niet omvatten wat ik aan jou te danken heb. Van begin tot eind ben jij zeer trouw, betrokken en enthousiast gebleken in je taak als co-promoter. Jouw onovertroffen kennis en visie op het gebied van IGF en insuline werking, in combinatie met je passionele behulpzaamheid, is meer dan bewonderenswaardig en vormt de drijvende kracht van veel (nieuw) relevant wetenschappelijk onderzoek aan onze universiteit. Ik heb dan ook zeer veel van je geleerd. Tevens besef ik dat je mij veel vrijheid hebt gegeven om mezelf te kunnen ontplooien. Ik hoop dat onze samenwerking nog lang voort mag duren. Het '*Bloed',* het *'Zweet'* en de *Tranen'* waren het meer dan waard; BEDANKT Joop!

Professor van der Lely, Professor Visser en Professor van Saase, hartelijk dank voor het plaats nemen in de kleine commissie van mijn promotie.

Dr. Frystyk, dear Jan, here I would like to thank you for your support and efforts concerning the integration and development of the IGF-I KIRA at our laboratory. I am honoured that I have been able to work and publish with you, in this more than interesting field of research. I truely hope that we can continue and augment our cooperation in the near future.

Beste Aimee en Christiaan, mijn paranimfen! Mijn keuze om juist jullie te vragen voor deze taak was verreweg de minst moeilijke keuze van het gehele promotie traject.

Christiaan, geworden van collega tot geliefde vriend! Veel steun en goede herrinneringen heb ik over gehouden aan onze overlappende werk periode op het lab, waarin vooral humor en onderling respect de boventoon voerden.

Daar waar ik jouw paranimf mocht zijn voelt het goed dat jij dat nu bij mij wilt zijn! Lieve Aimee, binnen gekomen als 'mijn studente' en tevens geworden tot lieve vriendin. Zeer vastberaden was je om na je keuze onderzoek meteen terug te stromen naar de kliniek. Ik vind het geweldig echter hoe ik nu bij jouw terug zie dat de 'KIRA feeling' zich inmiddels diep heeft geworteld. We hebben reeds mooi werk samen verricht – om daarbij ook Jeanette zeker niet te vergeten (bedankt Jean!) – en ik weet zeker dat daar nog veel meer aan toegevoegd gaat worden, uiteindelijk leidend tot een prachtig proefschrift 'You go Girl!'.

Lieve collegae en vrienden van het lab Neuro-Endo, mijn thuishaven op het Erasmus MC, dank voor de mooie tijd op 'ons' lab. Beste Leo, wat heb ik veel steun aan jou gehad, op werk-gebied, maar zeker ook op persoonlijk vlak. Zonder jouw drijvende en structurerende invloed had veel KIRA werk waarschijnlijk nimmer tot stand kunnen komen. Net als Joop, heb jij mij veel vertrouwen en wetenschappelijke vrijheid gegeven, bedankt daarvoor! Marlijn, Peter en Diane, onze 'back bone' van het lab, dank voor de fijne samenwerking. Marlijn, bedankt voor je inzet tijdens de laatste fase van mijn practisch werk met de IGF-I KIRA en opzet van de insuline KIRA. Katy, bedankt voor de samenwerking, uiteindelijk heb jij mij de funesses van de bioassay bijgebracht. Ik ben het nimmer vergeten! 'Neuro-endo collegae' - Jan-Willem, Henk, Joost, Pauline, Manon, Fadime, Ramona, Davine, Roxanne en Laura – dank voor de gezelligheid en de helpende hand wanneer nodig. Veel succes in jullie verdere loopbaan. Cristina e Giovanni, grazie! Rogier, kerel, als laatste van 'het trio' nog flink aan het buffelen in het lab. Ik wens je alle goeds toe en heb er alle vertrouwen in!

Collegae van het laboratorium van de interne geneeskunde, 'de 5^{e} verdieping', bedankt voor de prettige werksfeer, de gezelligheid in het algemeen, tijdens de lab-dagen en de congressen. Piet Uitterlinden, beste Piet, dank voor je behulpzaamheid o.a. met het verzamelen van 'controles' en je verzette werk voor het heterofiele antilichamen paper. IGF-I (en Joop niet te vergeten) heeft ons aan elkaar doen verbinden; 'Immunoassay vs. Bioassay' zullen we maar zeggen, 'the story continues'. Dank voor de leuke tijd en je vaak wijze raad! Bas Karels, 'buurman', klein van postuur maar groot qua persoonlijkheid. De metroritten van en naar het werk waren altijd leuk, de gesprekken altijd goed, en de grappen altijd fantastisch! Ik zie je in de supermarkt!

Mojgan thank you so much for all your efforts and the extra hours that you spent, even when I already had left the laboratory. Chapter 7 of the thesis - together with the publications that will be written - couldn't have been adchieved without your help. I wish you and your sister all the best in Canada.

Beste collegae op de RIDE projecten, Marieke, Wendy, Nadia en Lisette, als club van 5 zijn we gestart, dank voor de gezellige tijd en de gedeelde smart. De meesten van jullie zal ik ongetwijfeld weer tegen komen in de kliniek, en wie weet wederom als directe collega. Marieke waar was ik geweest zonder jouw know how van de epidemiologie! Lisette, bedankt voor de introductie en je behulpzaamheid in het doolhof van informatiestromen die je kennelijk moet beheersen om iets te SNaPpen van polymorfismen. Zonder jouw was dat zeker niet gelukt!

Collegae van de epidemiologie, bedankt voor de prettige samenwerking. Dames van het ERGO centrum, ook jullie ben ik zeker niet vergeten (Aneke, bedankt het was een leuke ervaring als ERGO-arts in jouw team!).

Beste Collegae in het Ikazia ziekenhuis (in de ruimste zin van het woord), dank voor de prettige werksfeer, jullie collegialiteit en jullie betrokkenheid.

Lieve familie, te weten Pa, Ma, Edwin en Dewie, Frans, Mariet, Mireille en Daan, alhoewel jullie geen directe relatie hebben met het werk beschreven in dit proefschrift, is jullie indirecte bijdrage desalniettemin van groot belang geweest. Dit in de vorm van een onuitputtelijke morele steun. Jullie betrokkenheid bij mij als persoon, in combinatie met de liefde, warmte en humor die ik van jullie heb mogen (en nog steeds mag) ontvangen, vormden een belangrijk drijfveer om dit boekje tot een succes te maken. Dank hiervoor!

Lianne, mijn lieve vrouw inmiddels, wat heb ik veel te danken aan jouw onvoorwaardelijke steun, liefde, doorzettingsvermogen en inzet. Veel heb je moeten laten, en veel heb je juist alleen moeten doen, in de lange periode dat ik mij voornamelijk bezig hield met het schrijven van dit proefschrift, in afwisseling met mijn werkzaamheden in de kliniek. Dat was niet altijd makkelijk voor je, dat besef ik ter dege. Je hebt me zelfs geholpen met pipetteren op het lab toen ik qua tijdsplanning moeilijk kwam te zitten; welke echtgenoot kan dat zeggen, je bent een schat! Het proefschrift ligt er nu – en dus zeker mede namens jou! - en ik weet dat je trots op me bent! Daar waar ik stond te glunderen toen jij promoveerde, weet ik dat jij dat zult doen nu ik daar ben aangekomen. Lieve schat, BEDANKT!, ik had het niet zonder jou willen doen!

Michel

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CURRICULUM VITAE

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Opleidingen en cursussen

1997-1997	Gymnasium β *CSG Johannes Calvijn, Rotterdam*
1998-1998	Biofarmaceutische Wetenschappen (propedeuse) *Universiteit Leiden*
1998-2005	Geneeskunde *Erasmus Universiteit Rotterdam, faculteit Hoboken*
2002	Keuze onderzoek 4^{e} jaar: 'The Identification of anti-Somatostatin Receptor Subtype 2 Specific Antibodies using Phage Display Technology' *Supervisoren: Dr. K.Radocevi en Dr. P.M. van Hagen, Internist-immunoloog, Erasmus MC, Rotterdam*
2004-2005	Keuze co-schap: Afdeling Endocrinologie Erasmus MC, Rotterdam *Supervisor: Dr. R. Feelders, internist-endocrinoloog, Erasmus MC, Rotterdam*
2005	Artsexamen (cum laude)
2005-2008	Promovendus: Titel project: 'Insulin-like Growth Factor (IGF-I) Polymorphisms and IGF-I Bioactivity in Aging and Age-related Disease'. *Promotor: Prof. S.W.J. Lamberts, internist-endocrinoloog* *Co-promotor: Dr. J.A.M.J.L. Janssen, internist-endocrinoloog*
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6. **Brugts MP**, van Duijn CM, Hofland LJ, Witteman JC, Uitterlinden AG, Lamberts SWJ, Janssen JAMJL.
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Submitted (both authors contributed equally to this work)*

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Article in preparation (both authors contributed equally to this work)*

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2. 'Hormone activity tied to men's longevity'
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