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Corresponding Author: Dr. Joseph A.M.J.L Janssen, MD, PhD

Corresponding Author's Institution: Erasmus MC

First Author: Joseph A.M.J.L Janssen, MD, PhD

Order of Authors: Joseph A.M.J.L Janssen, MD, PhD; Aimee J Varewijck, MD, PhD; Michael P Brugts, MD, PhD

Abstract: Determination of true IGF-I bioactivity in serum and other biological fluids is still a substantial challenge. The IGF-IR Kinase Receptor Activation assay (IGF-IR KIRA assay) is a novel tool to asses IGF-IR stimulating activity (IRSA) and has opened a new era in studying the IGF system. In this paper we discuss many studies showing that measuring IRSA by the IGF-IR KIRA assay often provides fundamentally different information about the IGF system than the commonly used total IGF-I immunoassays. With the IGF-IR KIRA assay phosphorylation of tyrosine residues of the IGF-IR is used as read out to quantify IRSA in unknown (serum) samples. The IGF-IR KIRA assay gives information about net overall effects of circulating IGF-I, IGF-II, IGFBPs and IGFBP-proteases on IGF-IR activation and seems especially superior to immunoreactive total IGF-I in monitoring therapeutic interventions. Although the IRSA as measured by the IGF-IR KIRA assay probably more closely reflects true bioactive IGF-I than measurements of total IGF-I in serum, the IGF-IR KIRA assay in its current form does not give information about all the post-receptor intracellular events mediated by the IGF-IR. Interestingly, in several conditions in health and disease IRSA measured by the IGF-IR KIRA assay is considerably higher in interstitial fluid and ascites than in serum. This suggests that both the paracrine (local) and endocrine (circulating) IRSA should be measured to get a complete picture about the role of the IGF system in health and disease.

To Professor Robert J Smith, MD
Associate Editor
Growth Hormone & IGF Research

Concerns GHIR-D-19-00026-R1

Rotterdam, 26 July 2019

Dear Professor Smith,

Please find enclosed the revised version of our paper entitled: "The Insulin-like Growth Factor-I Receptor Stimulating Activity (IRSA) in Health and Disease".

Thank you for giving us the opportunity to revise our manuscript.

The revised version of our manuscript is written paying attention to the suggestions made by the referees. We added a letter detailing the changes made.

In addition, we discussed and added a paper about IRS and Dementia, which was recently published by Galle et al. (page 28, lines 648-652; reference 32).

We hope that our revised manuscript is now acceptable for publication in . On behalf of all authors, I thank you for considering our paper for publication.

Yours sincerely,

Joseph Janssen

Please address all correspondence regarding the manuscript to:
JAMJL Janssen, MD, PhD, Associate Professor of Internal Medicine
Erasmus MC

Department of Internal Medicine, Room Rg 527

Dr Molewaterplein 40
3015 GD Rotterdam
The Netherlands,
Telephone +31-10-7040704
Telephone +31-06-5003242
Email: j.a.m.j.l.janssen@erasmusmc.nl

Responses to the Reviewers of GHIR-D-19-00026- Janssen et al. -The Insulin-like Growth Factor-I Receptor Stimulating Activity (IRSA) in Health and Disease.

July 2019

Responses to the Review of GHIR-D-19-00026– Janssen et al. -The Insulin-like Growth Factor-I Receptor Stimulating Activity (IRSA) in Health and Disease.

Reviewer 1

Dr. Janssen and coworkers have written an elegant review on IRSA in health and disease. The manuscript is timely and easy to read. As stated by the authors, "the measurement of IRSA by the IGF-IR KIRA assay has opened a completely new era and is a novel tool to asses circulating IGF-I bioactivity".

Thank you for your kind words and the careful review of the manuscript.

We have completed our revisions based on the Reviewer‘s helpful comments, and below we indicate point by point the changes we made to the manuscript.

Answers to comments of Reviewer 1:

1.*First.- 6.4 IRSA and PAPP-A2 (lines 383-385): "In both families loss-of-function mutations in the PAPP-A2 gene were found which resulted in undetectable PAPP-A2 activity [17]". This is not correct. In effect, while the Spanish patients exhibit a mutation with a stop codon (hence, PAPP-A2 serum levels were undetectable), the patients of Palestinian origin (second family) exhibited low levels of PAPP-A2, but detectable low PAPP-A2 and PAPP-A2 activity. Please, modify it in the manuscript accordingly.*

A: Thank you very much for this useful addition. In the revised manuscript this was modified accordingly (see page 17, lines 399-401).

2. *Second.- When the authors analyzed the results of IRSA in anorexia nervosa and obesity, it would be of interest to indicate that measuring PAPP-A and PAPP-A2 activity could be of help to understand, their differences in circulating IRSA (measured by KIRA assay), total IGF-I and free IGF-I.*

A: Thank you very much for this suggestion. However, to our knowledge the role of PAPP-A and the IGF system has not yet been studied in anorexia nervosa (see Støving RK. MECHANISMS IN ENDOCRINOLOGY: Anorexia nervosa and endocrinology: a clinical update. Eur J Endocrinol. 2019;180 :R9-R27).
.

In the revised manuscript we added the following to the paragraph about IRSA and Anorexia Nervosa (page 18, lines 428-430):

Measuring PAPP-A and PAPP-A2 activity could be of potential value to understand differences in circulating IRSA (measured by KIRA assay), total IGF-I and free IGF-I in

anorexia nervosa. However to our knowledge the role of PAPP-A and the IGF system in anorexia nervosa has not yet been studied.

Ref 75. Støving RK. MECHANISMS IN ENDOCRINOLOGY: Anorexia nervosa and endocrinology: a clinical update. Eur J Endocrinol. 2019; 180(1):R9-R27 was added to the reference list.

Concerning a potential role of PAPP-A on the IGF system in obesity we added the following to the paragraph about IRS and Obesity (page 19, lines 441-444):

It has been hypothesized that the observed increased ability of media harvested from visceral adipose tissue (VAT) to activate the IGF-IR in vitro (measured by the KIRA assay) is secondary to an upregulated PAPP-A mediated release of IGFBP-4 complexed IGF (Gude et al. PAPP-A, IGFBP-4 and IGF-II are secreted by human adipose tissue cultures in a depot-specific manner. Eur J Endocrinol. 2016; 175: 509-519. However, it is at present unclear whether local PAPP-A translates into differences of circulating IRSA in human subjects with obesity.

3. *Third.- 6.18 IRSA, Lung Cancer and Pleural Fluid (lines 598 & 599): The authors state "In addition, PAPP-A, an IGFBP protease, that may cleave IGFBP-4 and IGFBP-5, was elevated in pleural fluid and it was speculated by the authors that IGFBP-proteases (inclusive PAPP-A)". Question: What is the evidence suggesting that PAPP-A may cleave IGFBP-5? It is generally reported that PAPP-A selectively cleaves IGFBP-4. Thank you for your comment.*

A] Overgaard et al (1) and Laursen et al. (2) have both published evidence that PAPP-A may cleave IGFBP-5. The specificity of PAPP-A and PAPP towards the six different IGFBPs was recently summarized by Claus Oxvig (see Table 1 in (3)). These three references (49, 59, and 60) were added to the revised paper (page 27, lines 624-625).

Ref 49. Laursen LS, Overgaard MT, Søe R, Boldt HB, Sottrup-Jensen L, Giudice LC, Conover CA, Oxvig C Pregnancy-associated plasma protein-A (PAPP-A) cleaves insulin-like growth factor binding protein (IGFBP)-5 independent of IGF: implications for the mechanism of IGFBP-4 proteolysis by PAPP-A. FEBS Lett. 2001; 504:36-40.

Ref 59. Overgaard MT1, Boldt HB, Laursen LS, Sottrup-Jensen L, Conover CA, Oxvig C. Pregnancy-associated plasma protein-A2 (PAPP-A2), a novel insulin-like growth factor-binding protein-5 proteinase. J Biol Chem. 2001; 276(24):21849-53

Ref 60. Oxvig C. The role of PAPP-A in the IGF system: location, location, location. J Cell Commun Signal. 2015 Jun; 9(2):177-87

4. Fourth.- 6.21 IRSA and Longevity: It is well known that patients with GH resistance (Laron Syndrome) and low or undetectable levels of total and free IGF-I are associated with longevity. Are there any results available regarding IRSA (measured by KIRA assay) in

patients with Laron syndrome? Could you, please, add a sentence in this Section related to these patients? Thank you.

A] To our knowledge to date are no data available regarding IRSA (measured by KIRA assay) in patients with Laron Syndrome. In the revised manuscript we added the following (page 15, lines 359-364):

To our best knowledge to date no data are available whether serum IRSA measured by KIRA assay is also reduced in subjects with Laron Syndrome. Using a porcine cartilage bioassay it has been found that serum IRSA in subjects with Laron Syndrome appeared to relate not directly to serum total IGF-I levels, but to the effects of the various IGFBPs on the bound IGFs (18). It was suggested that the regulation and perhaps also degradation of the IGFBPs play an important role in the regulation an availability of IGF-II in Laron Syndrome.

Ref 18. Cotterill AM1, Holly JM, Taylor AM, Davies SC, Coulson VJ, Preece MA, Wass JA, Savage MO. The insulin-like growth factor (IGF)-binding proteins and IGF bioactivity in Laron-type dwarfism. J Clin Endocrinol Metab. 1992; 74:56-63 was added to the Reference list.

We previously measured IRSA by KIRA assay in centenarians and found relatively low IRSA. Mean IRSA in 106 centenarians was 132 (107-157pmol/L) (mean (25^{th}-75^{th} percentile)), while mean IRSA in 192 centenarians' offspring and 80 offspring matched controls were 144 (119-170) pmol/L and 161 (134-187) pmol/L. In addition, centenarians showed a 2-fold higher insulin sensitivity than centenarians' offspring. Therefore we hypothesized that, despite low circulating IRSA, the post-receptor signaling pathways of the IGF-IR was up-regulated in centenarians. This information was also added to the revised manuscript (page 29, lines 661-666).

Responses to Review of GHIR-D-19-00026– Janssen et al. -The Insulin-like Growth Factor-I Receptor Stimulating Activity (IRSA) in Health and Disease.

This is an extremely well-written and comprehensive review on the IRSA and important health outcomes. The authors are commended on a timely and informative review that will be well received in the literature and should spur more interest in IGF-I physiology and measurement.

A: We appreciate your kind words and the careful review of the manuscript.

We have completed our revisions based on the Reviewer's helpful comments, and below we indicate point by point the changes we made to the manuscript.

Answers to comments of Reviewer 2:

1] Can the authors make any recommendations or statements on which commercially IGF-I assay is superior?

A] Unfortunately there are no commercially assays available to measure IRSA by KIRA. This is one of the important steps to be taken to implement wide use-spread use of the KIRA assay (see also point 6 below).
Although there are several immunoassays commercially available to measure Total and Free IGF-I, none of these latter assays generate information about modifying effects of IGBPs and IGFBP proteases on IGF-I action while it is generally accepted that bioactivity at the level of the IGF-IR is modulated by the IGFBPs and IGFBP proteases.

2] Can the authors provide a bit more explanation on the modulating effects of IGFBPs on IGF-I action?

A] We did follow this suggestion and added in the revised manuscript the following information (page 4, lines 103-106):
The IGFBPs were initially defined as serum carriers and passive inhibitors of IGF actions. However, it has been repeatedly demonstrated that IGFBPs not only inhibit IGF actions in many circumstances but they may also potentiate IGF-I actions. IGFBPs are widely expressed in almost all tissues of the body , and function as flexible endocrine and autocrine/paracrine regulators of IGF-I bioactivity.

Reference 3 (L.A. Bach, IGF-binding proteins, J Mol Endocrinol, 61 (2018) T11-T28) was added to the Reference list

3] *I recommend referring to concentration rather than levels as concentrations are measured in units.*

A] Thank you. We followed this suggestion and changed levels in concentrations through the whole paper.

4] Do the authors believe the studies with IRSA and exercise are definitive or are more studies perhaps needed?

A] Thank you for this question. We do not think that the studies about IRSA and exercise are definitive. Although the discussed studies suggest no significant changes of circulating IRSA during acute and chronic exercise , these observations, however, should not be interpreted that IRSA lacks importance or relevance in exercise. Although local IGF-I activity I has been found consistently upregulated with both acute and chronic exercises, at presence the precise and relative role of systemic versus locally produced IGF-I and physical activity is still not clear. Thus in future research samples in the body 's various compartments (blood, interstitial fluid, muscle) should be collected to measure IRSA during a variety of acute and chronic conditions of exercise.

This information was added to the revised manuscript at page 10, lines 249-253 and page 11, lines 254 -255).

5] I think the authors will be well served by also highlighting the studies that have measured free IGF-I and have shown higher relationships with health outcomes than total IGF-I. This will further reinforce their overall concept on the importance of IGF-I measurement.

A] Thank you for this suggestion. However, we decided not to follow this suggestion.

Although we agree that studies measuring free IGF-I have shown higher relationships with health outcomes than total IGF-I, neither the ultrafiltration nor the direct immunoassay to measure free IGF-I take the modifying effects of IGFBPs and IGFBP proteases on the interaction between IGF-I and the IGF-IR into account.

In addition, the relationship of free IGF-I and total IGF-I with a variety health outcomes was previously extensively reviewed by Jan Frystyk in this Journal (Frystyk J. Free insulin-like growth factors -- measurements and relationships to growth hormone secretion and glucose homeostasis. Growth Horm IGF Res. 2004 Oct;14(5):337-75

6] What are some potential next steps for the KIRA and what would need to occur to implement wide-spread use?

A] Thank you for raising this important issue. In the first version we had already briefly discussed some potential next steps for the KIRA (page 30, line 702 and page 31, lines 703-706) In the revised version of the manuscript we added the following information at page 32, lines 744-751 and page 33, lines 752-755.

Easy access and reliability of a cell line transfected with the human IGF-IR are prerequisites to implement wide-spread use of the KIRA assay. Cells transfected with the human IGF-IR frozen in microwell plates offer a potential valid alternative to fresh cells from a growing culture. When these plates can be used immediately after thawing and the frozen cells can be revitalised without passaging and washing cells, total time needed to perform the assay is considerably shortened. This strategy may further help to overcome an important bottleneck to implement wide-spread use of the KIRA assay; it removes day-to-day variation, eliminates passage effects and improves consistency of cell-based assay results. The production of these frozen cells should be standardised so that different batches are highly comparable. Freezing

and resuscitation protocols should be optimized, and the performance of these ready-to-use cells should be compared with those from continuous culture to determine whether they could be used as a replacement To further reduce costs and consistency of the KIRA the antibodies should be replaced by aptamers.

GHIR-D-19-00026R1

The Insulin-like Growth Factor-I Receptor Stimulating Activity (IRSA) in Health and Disease

Joseph A.M.J.L. Janssen[1], Aimee J. Varewijck[1], Michael P. Brugts[2]

1. Department of Internal Medicine. Division of Endocrinology, Erasmus MC, Rotterdam, The Netherlands

2. Department of Internal Medicine, Ikazia Hospital, Rotterdam, The Netherlands

The authors have no competing interests to declare

Corresponding author:

J.A.M.J.L. Janssen, MD, Ph.D.

Erasmus MC

Department of Internal Medicine

Dr. Molewaterplein 40

3015 GD Rotterdam

The Netherlands

Telephone +31-0650032421 or +31-10-7040704

Email: j.a.m.j.l.janssen@erasmusmc.nl

Abstract

Determination of true IGF-I bioactivity in serum and other biological fluids is still a substantial challenge. The IGF-IR Kinase Receptor Activation assay (IGF-IR KIRA assay) is a novel tool to asses IGF-IR stimulating activity (IRSA) and has opened a new era in studying the IGF system. In this paper we discuss many studies showing that measuring IRSA by the IGF-IR KIRA assay often provides fundamentally different information about the IGF system than the commonly used total IGF-I immunoassays. With the IGF-IR KIRA assay phosphorylation of tyrosine residues of the IGF-IR is used as read out to quantify IRSA in unknown (serum) samples. The IGF-IR KIRA assay gives information about net overall effects of circulating IGF-I, IGF-II, IGFBPs and IGFBP-proteases on IGF-IR activation and seems especially superior to immunoreactive total IGF-I in monitoring therapeutic interventions. Although the IRSA as measured by the IGF-IR KIRA assay probably more closely reflects true bioactive IGF-I than measurements of total IGF-I in serum, the IGF-IR KIRA assay in its current form does not give information about all the post-receptor intracellular events mediated by the IGF-IR. Interestingly, in several conditions in health and disease IRSA measured by the IGF-IR KIRA assay is considerably higher in interstitial fluid and ascites than in serum. This suggests that both the paracrine (local) and endocrine (circulating) IRSA should be measured to get a complete picture about the role of the IGF system in health and disease.

Keywords: IGF-I, IGF-I receptor, IGF-I Bioactivity, KIRA, Immunoassays, Endocrine, Paracrine, Health, Disease

Table of Contents

1.Introduction

Insulin-like growth factor-I (IGF-I) is tightly bound by six high affinity IGF binding proteins (IGFBP-1-6) in the circulation. In healthy subjects, approximately 95% of all circulating total IGF-I is present as a ternary complex formed by IGF-I, IGFBP-3 and acid labile subunit (ALS), making this complex quantitatively the most important, while the remaining IGF-I circulates in a free form (<1%) or as binary complexes (approximately 4–5%) [17]. It is assumed that free IGF-I is the only form of IGF-I which is able to directly stimulate the IGF-IR [42] (See Figure 1 for more details). Activation of the insulin-like growth factor-I receptor (IGF-IR) by free IGF-I stimulates multiple pathways which finally results in multiple biological effects in a variety of tissues and cells (Figure 2).

The IGFBPs were initially defined as serum carriers and passive inhibitors of IGF actions [3]. However, it has been repeatedly demonstrated that IGFBPs not only inhibit IGF actions in many circumstances, but they may also potentiate IGF-I actions [3]. IGFBPs are widely expressed in almost all tissues of the body , and function as flexible endocrine and autocrine/paracrine regulators of IGF-I bioactivity [3].

Determination of true IGF-I bioactivity in serum and other biological fluids still presents substantial challenges. After generation of highly specific antibodies for IGF-I it became possible to develop immunoassays for assessment of circulating IGF-I levels in serum and plasma [31, 69, 95]. To date total IGF-I immunoassays are clinically widely used to assess circulating IGF-I bioactivity in humans and the majority of available literature about IGF-I is based on information obtained by use of immunoassays. As a consequence, immunoassays are very often considered to be the most useful method to assess the amount of circulating IGF-I that is biologically active in the body. However, in the following paragraphs several arguments will be discussed, which will challenge this view.

A major technical problem encountered when measuring circulating IGF-I by immunoassays is interference of IGFBPs. Presence of IGFBPs in a blood sample may significantly disturb reactions between IGF-I and antibodies in the tube and this may result in spurious estimates of the total amount

of IGF-I present in that sample. Therefore, most available IGF-I immunoassays use an extraction step to remove all IGFBPs prior to the measurement in order to guarantee full accessibility to IGF-I of highly specific antibodies targeting IGF-I [16]. However, by removing all IGFBPs before measurement of IGF-I (potentially) modulating effects of IGFBPs and IGFBP proteases on IGF-IR stimulating activity (IRSA) are also eliminated [62]. Thus, as a direct consequence of the just discussed pre-analytical procedure, total IGF-I immunoassays are unable to produce any information about directly modulating effects of IGFBPs or IGFBP-proteases on IRSA.

Moreover, total IGF-I immunoassays determine the immuno-reactive properties of circulating IGF-I-like molecules, rather than the direct (stimulating) effects of these molecules on the IGF-IR [6]. In addition, total IGF-I immunoassays may recognize IGF-I isoforms that are less bioactive and able to stimulate the IGF-IR than wild type IGF-I [6]. Moreover, fragments of IGF-I that lack biological actions, may still harbor epitopes that can be recognized by antibodies targeted to IGF-I and be measured as intact IGF-I by total IGF-I immunoassays [6]. It has been further suggested that altered post-sampling integrity of IGF-I in vitro might contribute to the reported inconsistencies in circulating total IGF-I levels in literature. This latter phenomenon occurs especially under pathologic conditions [42]. Results of total IGF-I immunoassays can be further disturbed by presence of so called heterophilic antibodies in serum which may result in both falsely higher or lower total IGF-I levels [7]. Despite all these limitations total IGF-I immunoassays have become popular in the last 40 years to monitor circulating IRSA in blood samples.

2. The history of measuring IRSA by bioassays

Any intracellular point stimulated by binding of IGF-I to the IGF-I receptor may be utilized for the development of an IGF-I bioassay [66]. In the past a variety of tissues and cells have been used as target

organs in bioassays for the determination of IRSA. Salmon and Daughhaday used the incorporation of [^{35}S] sulphate into hypophysectomized rat cartilage [67]. Other bioassays used chicken embryo, weanling or fasted rats or porcine cartilage to assess incorporation of sulphate [36, 81, 93]. In the fat pad bioassay the conversion of [^{14}C] glucose to CO_2 was used to assess IRSA, while in another bioassay incorporation of tritiated thymidine into DNA of embryonic chicken fibroblasts was used [26, 61]. Although all these traditional IGF bioassays were advantageous in biological relevance, they showed certain failings in their use: lack of sensitivity, precision and specificity; in addition, high variability and long assay duration (3-6 days); comparable phenotypic responses could be the consequence of activation of an alternative receptor (e.g. the insulin receptor) [37, 66]. Results of many of these traditional bioassays were sometimes also influenced by other hormones, which were present in the measured serum samples [37]. For example, it was observed that thyroid hormone (like IGF-I) could stimulate sulphate uptake in to chicken embryo cartilage whereas cortisol was found to inhibit IGF-I-mediated effects in porcine cartilage [22, 27]. Moreover, these traditional IGF bioassays did not detect specifically the IRSA in whole serum, but rather reflected the overall stimulating activity of serum for that target tissue [37].

3. The development of the IGF-I Receptor Kinase Receptor Activation Assay (KIRA)

The IGF-IR KIRA assay was developed by Michael Sadick et al. as an alternative approach for bioassays measuring IGF-IR endpoints [66]. They showed that results obtained with an IGF-IR KIRA assay in MC-7 cells (with endogenous IGF-IR expression) correlated well with classical endpoint bioassays such as a [^{3}H]thymidine incorporation assay [66]. The principle of the IGF-IR KIRA assay is based on measurement and quantification of phosphorylated tyrosine residues of the β-subunit of the IGF-IR (Figure 3). Phosphorylation of tyrosine residues of the β-subunit of the IGF-IR normally starts the intracellular signal

cascade after binding of IGF-I to the IGF-IR [14, 66]. The IGF-IR KIRA assay utilizes two separate microtiter plates, one for ligand stimulation of intact cells, and the other for receptor capture and phosphotyrosine ELISA [66] (Figure 3). Results obtained with the IGF-IR KIRA assays are highly reproducible [66]. Since the IGF-IR KIRA assay uses a sample incubation time of 15 minutes, time is too short for stimulated cells to produce de novo IGFBPs that may interfere with IGF-I action during sample incubation [14]. The IGF-IR KIRA assay makes use of either endogenously expressed IGF-IR receptors or stably transfected IGF-IR receptors with a polypeptide flag (11). Frystyk et al. and Brugts et al. used an IGF-I KIRA assay with human embryonic cells transfected with a copy DNA of the full-length human IGF-IR [8, 14]. By this modification the IGF-IR KIRA assay became even more sensitive than that original KIRA assay described by Sadick et al. Most likely this was due to the higher expression of IGF-IRs after transfection compared to endogenously expressed IGF-IRs [14]. The standard curve of the IGF-I KIRA based on human embryonic kidney (HEK293) cells transfected with a copy DNA of the full-length human IGF-IR started at a concentration of 10 pmol/L (0.08 μg/L) IGF-I [14]. The IGF-IR KIRA assay was found to be specific: insulin, insulin analogs and proinsulin in physiological concentrations had almost no (stimulating) effect on the IGF-IR KIRA signal while IGF-II had a cross-reactivity of 12% [14]. In addition, it had a remarkable low intra- and inter-assay coefficient variation <15%) for a bioassay [8]. It has been further demonstrated that the IGF-IR KIRA is a relatively rapid and reproducible method for assessing IRSA which takes into account modifying effects of IGFBPs on the interaction between IGF-I and the IGF-IR [41].

In the next paragraphs we will give a comprehensive overview of the existing literature which illustrates the clinical significance of measuring IRSA by the IGF-IR KIRA assay.

4. IRSA and age

To date only one study has established age-specific normative values for IRSA as measured by the IGF-IR KIRA assay [8]. In a cross-sectional study circulating IRSA was measured in 400 healthy non-fasting blood donors aged 18-79 yrs. [8]. Circulating IRSA showed a wide inter-individual variability among subjects at every age. Like total IGF-I concentrations, IRSA decreased significantly with age but the decline of IRSA with age was less steep than it was observed for circulating total IGF-I concentrations [8]. Due to the cross-sectional design of this latter study no information about intra-individual changes of IRSA during aging was obtained. Nevertheless the discrepant decline with age between IRSA and total IGF-I suggests that IRSA becomes less growth hormone (GH) dependent with aging than total IGF-I concentrations [8]. Other potential explanations for this discrepant decline with age between IRSA and total IGF-I could be that the relative increase in IRSA with age compared to total IGF-I reflects a compensatory mechanism to overcome an age-dependent relative IGF-IR resistance or that the relative contribution of IGF-II to IRSA increases with age [8].

In the same study a significant drop in IRSA was observed in women aged 50-60 years which was not observed for total IGF-I [8]. Women at younger ages showed higher lRSA than males but had lower IRSA than the males after the age of 50-60 years. The decrease in estrogen levels around menopause in females might play a role in the observed drop in IRSA after the age of 50-60 years since estrogen is well known to play an important role in regulating activity of the GH/IGF-I axis [50].

IRSA as measured by the IGF-IR KIRA assay was positively related to total IGF-I but the found correlation coefficients were relatively low ($r \approx 0.50$) suggesting that IRSA as measured by the IGF-IR KIRA assay produces basically different information about the IGF-I system than IGF-I immunoassays [8]. However, the physiological importance of this difference is unclear at the moment.

In another smaller cross-sectional study of men and women aged 20-70 yrs. IRSA also tended to decrease to a lesser extent than total IGF-I with age [91]. However, in this latter study no significant

drop of IRSA around menopause was found in females, which may be related to the lower number of participants included in this latter study [91].

5. IRSA in Health

5.1 IRSA and Fasting

In a small study in which in non-obese healthy subjects effects of fasting on GH signaling and action were investigated, GH concentrations significantly increased after 37.5 h of fasting compared to levels after the overnight fast, while (immuno-reactive) total IGF-I concentrations were similar under both conditions [55]. In contrast, IRSA measured by the IGF-IR KIRA assay was significantly lower after 37.5 h of fasting compared to results following the overnight fast, whereas IGFBP-1 was significantly increased [55]. These findings are in line with Chen et al. who previously reported that after prolonged fasting reductions of total IGF-I were preceded by reductions in IRSA and free IGF-I and a simultaneous increase of IGFBP-1 concentrations [13]. Thus this time course suggests that the decline in IRSA was causally linked to the increase in IGFBP-1 [55]. In this latter study it was also found that IRSA compared to ultra-filtered free IGF-I was relatively less affected by fasting and it was hypothesized that this latter finding could be explained by the fact that in contrast to the ultrafiltration method the IGF-IR KIRA assay was able to detect the concomitant increase in IGFBP-1-complexed-IGF-I [13].

5.2. IRSA and Life style factors

When in a cohort of young women recruited from a local college campus the relationships between IRSA (measured by the IGF-IR KIRA assay) and life style factors were studied, IRSA was negatively associated with age, body fat percentage and habitual alcohol intake and positively associated with estradiol,

progesterone and selenium intake [47]. In multivariate analysis only 61% of the variation in IRSA could be attributed to circulating concentrations of immunoreactive total and free IGF-I and IGFBP-1, IGFBP-2 and IGFBP-3 [47]. It was concluded that further research is needed to better understand the biological mechanisms responsible and the consequences for the reported associations [47].

5.3 **IRSA and Exercise**

In healthy men circulating concentrations of IRSA (measured by the IGF-IR KIRA assay), and immunoreactive free IGF-I, total IGF-I and total IGF-II did not change after single 30 seconds sprints, despite an increase in GH concentrations [74]. Thus, a short sprint exercise may stimulate GH secretion but does not change IRSA nor IGF concentrations [74].

During a submaximal exercise (45 minutes of cycle ergometer at the lactate threshold) GH administration to adults with GH deficiency (GHD) induced minor changes in IGFBP-1, IGFBP-2 and IGFBP-3 without affecting IRSA, IGF-I, IGF-II or IGFBP-3 proteolysis [46]. Thus administration of GH to adults with GHD did not result in changes of IRSA during submaximal exercise [46].

After 8 weeks of resistance, aerobic and combined exercise training both circulating IRSA and immuno-reactive IGF-I remained stable in young healthy women despite a significant improvement in aerobic fitness, lean mass and upper and lower body strength [57].

Taken together, all these findings suggest no significant changes of circulating IRSA during acute and chronic exercise. However, these observations, should not be interpreted that IRSA lacks importance or relevance in exercise. Although that local IGF-I activity I has been found consistently upregulated with both acute and chronic exercises, at presence, the precise and relative role of systemic versus locally produced IGF-I and physical activity is still not clear [58]. Thus in future research samples in the body‘s

various compartments (blood, interstitial fluid, muscle) should be collected to measure IRSA during a variety of acute and chronic conditions of exercise.

5.4 **Effects of Insulin on IRSA**

During a hyperinsulinemic euglycemic clamp circulating IRSA (measured by the IGF-IR KIRA assay) acutely decreased both in controls and subjects with impaired glucose tolerance, whereas simultaneously no changes in immunoreactive total IGF-I or IGF-II were observed [1]. Similarly, IGFBP-1 concentrations significantly decreased in both groups, whereas no changes were seen in IGFBP-3, while GH and IGFBP-2 levels significantly increased [1]. The acute insulin-induced reduction of IRSA during the clamp occurred despite reduction in IGFBP-1 concentrations, and therefore reduction of IRSA during the clamp was explained by the concomitant increase of circulating IGFBP-2 concentrations, while the observed increase in GH concentrations during the clamp most likely were due to decreased negative feedback of circulating IRSA [1].

In contrast to the observed acute insulin-mediated decrease of IRSA, chronic hyperinsulinemia did not reduce circulating IRSA, which was explained by the reduction of both IGFBP-1 and IGFBP-2 concentrations during long-term exposure to high insulin levels [1].

5.5 **Effects of Glucagon on IRSA**

Intramuscular glucagon administration to lean subjects, obese subjects and patients with type 1 diabetes mellitus decreased circulating IRSA (measured by the IGF-IR KIRA assay) in all three groups despite no changes were observed in circulating immunoreactive total IGF-I and IGFBP-3 concentrations [68]. Since the reduction in IRSA occurred before the glucagon-induced surge in GH, decreased negative

feedback by IRSA provides a mechanism for the known increase in GH secretion after administration of glucagon [68]. The authors hypothesized that the decrease in IRSA after glucagon administration was related to an increase in circulating IGFBP-1 and IGFBP-2 concentrations which, in turn, most likely was mediated via a glucagon-mediated activation of the FOXO/mTOR pathway [68].

5.6 **Effects of GLP-1 on IRSA**

Short-term infusions of glucagon-like peptide-1 (GLP-1) in healthy subjects tended to increase IRSA (measured by the IGF-IR KIRA assay) and reduced IGFBP-1 concentrations [72]. Therefore it was suggested that IRSA in this study increased secondary to suppression of IGFBP-1 concentrations [72].

5.7 **IRSA in Serum vs. Interstitial Fluid**

With the suction blister technique IRSA (measured by the IGF-IR KIRA assay) was 41 % higher in interstitial fluid than IRSA in serum [24]. It was suggested that this was related to an increased enzymatic IGFBP-proteolysis and an altered composition of IGFBPs in interstitial fluid. As a consequence larger fractions of IGF-I and IGF-II were free to bind to the IGF-IR [24]. In contrast, immuno-reactive total IGF-I and IGF-II concentrations and IGF-binding proteins (IGFBPs) concentrations were approximately 50% lower in interstitial fluid than in serum [24]. Thus this study suggested that IRSA may be higher at the tissue level than in the circulation.

5.8 **Effects of Prednisolone on IRSA**

Prednisolone treatment (5mg per day during 1 week) to children with severe asthma significantly reduced IRSA (measured by the IGF-IR KIRA assay) by 12% compared to placebo, while no changes were observed for immunoreactive total IGF-I, free IGF-I, IGFBP-3, IGFBP-2 IGFBP-1 and IGFBP-1-bound IGF-I [30]. Prednisolone had no direct effects on IGF-IR phosphorylation. It was concluded that treatment with glucocorticoids induces a circulating substance that is able to inhibit IGF-IR activation in vitro without affecting circulating total or free IGF-I [30]. At present the nature of this substance is not identified [30]. Interestingly, more than twenty years ago existence of a circulating inhibitor of the IGF-IR induced by systemic glucocorticoid treatment was already hypothesized when IRSA was assessed by so called end-point bioassays that measured incorporation of radiolabeled sulfate or thymidine into cultures of porcine cartilage [53, 80].

In contrast to the findings in the study just discussed, both circulating IRSA and total IGF-I steadily increased compared to placebo when men received prednisolone in high doses (37.5 mg per day for 5 days) [63]. Although prednisolone increased circulating IRSA above placebo concentrations, this was not translated into higher levels of IRSA in interstitial fluid (collected by the suction blister technique) [63]. Thus, short-term prednisolone administration in high doses appears to exert distinct, compartment-specific effects on IRSA. The authors hypothesized that the observed increase in circulating IRSA (and total IGF-I) after prednisolone was most likely secondary to a prednisolone–mediated increase of insulin receptor resistance and IGF-IR resistance [63]. Serum obtained from participants after high dose prednisolone treatment showed reduced ability to phosphorylate IRS-1, Akt and mTOR in IGF-IR – transfected cells compared to serum after placebo, suggesting that prednisolone treatment in this high dose induced IGF-IR resistance by impeding post-IGF-IR signaling [63]. These results further support the hypothesis that glucocorticoids in high doses primarily impair anabolic actions of IGF-I by suppressing the post-receptor pathways of the IGF-IR rather than by suppressing circulating IRSA [63].

5.9 **Effects of Raloxifene and Estrogen on IRSA**

While the selective estrogen receptor modulator raloxifene and estrogen suppressed circulating immunoreactive total IGF-I equally in growth hormone deficiency (GHD) and growth hormone (GH)-replaced hypopituitary women, neither raloxifene nor estrogen affected circulating IRSA (measured by the IGF-IR KIRA assay), while reduction of the total IGF-I: IGFBP-3 ratio, considered by many people as a proxy for bioavailable IGF-I, was significantly greater during raloxifene treatment [5]. Treatment with GH significantly increased IRSA but this effect was attenuated by co-treatment with raloxifene and estrogen [5]. In addition, proportion of IRSA to total IGF-I was unaffected by any of these treatments [5]. Since during GH therapy of hypopituitary women co-treatment with raloxifene led to a smaller gain in lean body mass than GH co-treatment with estrogen, the authors concluded that the observed different effects on lean body mass between raloxifene and estrogen treatments could not be explained by differences in IRSA [5].

6. IRSA in Disease States

6.1 **IRSA and GHD**

Before start of GH treatment IRSA (measured by the IGF-IR KIRA assay) in adult patients with proven GHD was more frequently below the normal range (<-2 SD) than immunoreactive total IGF-I concentrations (81.9 vs. 61.7%, respectively) and this was especially observed in patients older than 40 years of age [88]. After start of GH treatment both IRSA and total IGF-I significantly increased but changes in IRSA did not parallel changes in total IGF-I [89]. After 12 months of GH treatment total IGF-I normalized in 81% of patients, whereas in only 50% of patients the IRSA was in the normal reference

range [89]. In addition, IRSA remained below normal in more than 40% of patients in whom total IGF-I had normalized [89]. Interestingly, the increase of the IGF-I/IGFBP3 ratio (which has been suggested to reflect an estimate of bioavailable IGF-I) after 12 months GH treatment was almost similar to the reported increase of IRSA [89].

IRSA was found to be positively related to QOL as assessed by the disease-specific Question on Life Satisfaction Hypopituitarism (QLS-H) module, whereas total IGF-I was not. These findings suggest that IRSA may be a more sensitive marker for changes in QOL during GH treatment of adult patients with GHD [87]. An interesting follow-up study would be to assess the use of IRSA for GH dose titration during GH treatment of adult patients with GHD.

Seventy-two hours after administration of a single high dose of the GH receptor blocker pegvisomant to untreated patients with GHD circulating IRSA (measured by the IGF-IR KIRA assay) significantly decreased by 14% and immunoreactive total IGF-I by 23 % compared to baseline whereas basal GH levels increased, and IGF-II, IGFBP-1, IGFBP-2 and IGFBP-3 concentrations did not alter [94]. Nonetheless, a strong positive correlation between pegvisomant concentrations and circulating IGFBP-1 and IGFBP-2 concentrations was observed, suggesting that the modulatory effects of pegvisomant on IRSA were mediated in a dose-dependent manner by concomitant increasing concentrations of IGFBP-1 and IGFBP-2 [70, 94].

To our best knowledge to date no data are available whether serum IRSA measured by KIRA assay is also reduced in subjects with Laron Syndrome. Using a porcine cartilage bioassay it has been found that serum IRSA in subjects with Laron Syndrome appeared to relate not directly to serum total IGF-I levels, but to the effects of the various IGFBPs on the bound IGFs [18]. It was suggested that the regulation and perhaps also degradation of the IGFBPs play an important role in the regulation an availability of IGF-II in Laron Syndrome [18].

6.2 **IRSA and IUGR**

Cord blood immunoreactive total IGF-I and total IGF-II, and IRSA (measured by the IGF-IR KIRA assay), were lower in neonates born with intrauterine growth restriction (IUGR) than in neonates born appropriate for gestational age (AGA) [79]. IGFBP-1 concentrations were higher in IUGR neonates than in AGA neonates [79]. As IGFBP-1 is an important regulator of IRSA this may partly explain why levels of IRSA were suppressed in IUGR neonates: higher IGFBP-1 concentrations may sequester circulating IGF-I and thereby reduce IRSA [79].

6.3 **IRSA and Acromegaly**

In a small study of newly diagnosed patients with active acromegaly (based on clinical presentation, unsuppressed GH levels during an OGTT, and elevated age-matched immune-reactive total IGF-I) IRSA (measured by the IGF-IR KIRA assay) was within the reference range in a considerable number of patients [90]. In this study, the R^2 value was 0.70 suggesting that 30% of the variation in IRSA could not be explained by levels of total IGF-I, demonstrating that IRSA is only partly dependent on total IGF-I [90]. In addition, the mean percentage of IRSA over total IGF-I was 0.81% in subjects with active acromegaly indicating that the IGF-IR KIRA assay provided fundamentally different information about the circulating IGF-I system than IGF immunoassays [90]. Age-adjusted soluble Klotho concentrations were significantly related to IRSA and it was hypothesized that elevated soluble Klotho levels may directly have reduced IRSA. Moreover, in this study IRSA was more strongly related to physical measures of QoL than total IGF-I, suggesting that IRSA may better reflect physical limitations perceived in active acromegaly [90].
In an another study among active acromegalics circulating IRSA (measured by the IGF-IR KIRA assay) decreased significantly during treatment with pegvisomant as well as with combination treatment with a somatostatin analog and pegvisomant. However, there were no significant differences in the changes of

IRSA between both treatment regimens [45]. Moreover, immunoreactive total and free IGF-I showed comparable results as obtained by IRSA [45].

6.4 **IRSA and PAPP-A2**

The metalloproteinase pregnancy-associated plasma protein A2 (PAPP-A2) has been hypothesized to increase IGF-I bioactivity by specific cleavage of IGFBP-3 and IGFBP-5 [2]. Recently two unrelated families have been described from whom family members presented with progressive postnatal growth failure, microcephaly, and thin long bones and decreased bone density [2]. In the blood markedly elevated circulating concentrations of immunoreactive total IGF-I, IGF-II, IGFBP-3, IGFBP-5 and ALS were measured. Size-exclusion chromatography showed a significant increase of IGF-I bound in its ternary complex [19]. Spontaneous GH secretion was also markedly elevated [2]. In both families loss-of-function mutations in the PAPP-A2 gene were found which resulted in one family in severely lowered and in the other family in undetectable PAPP-A2 activity [19]. When circulating IRSA was measured by the IGF-IR KIRA assay, IRSA was low and therefore it was hypothesized that low IRSA was responsible for the observed poor growth [2]. In favor of this latter hypothesis, short-term treatment with recombinant human IGF-I (rhIGF-I) increased IRSA and this was accompanied by improved growth and height in young patients with these PAPP-A2 mutations [56]. In addition, during rhIGF-I treatment spontaneous GH secretion decreased while circulating total IGF-I and IGFBP-3 concentrations remained elevated [56]. The decline in spontaneous GH secretion most likely resulted from a restored negative feedback as a consequence of the rise in circulating IRSA after rhIGF-I treatment [56].

6.5 **IRSA in Turner patients**

To overcome the retarded growth of Turner patients it has been reported that very high doses of GH are needed [82]. In untreated adult patients with Turner Syndrome IRSA (measured by the IGF-IR KIRA assay) was found to be decreased [34]. This latter result was found despite the presence of normal immuno-reactive concentrations of total IGF-I, IGFBP-1, -2 and -3 and Acid Labile Subunit (ALS) [34]. However, Western ligand blots of IGFBP-1 and-2, as well as IGFBP-4 in this study population showed signs of extensive proteolysis while the IGFBP-3 ternary complex was significantly reduced [34]. It therefore was speculated that the decreased circulating IRSA, may play a role in the reduced action of GH in Turner syndrome [34].

6.6 **IRSA and Anorexia Nervosa**

In malnourished patients with anorexia nervosa circulating IRSA (measured by the IGF-IR KIRA assay), total IGF-I (immuno-reactive) and free IGF-I (ultra-filtered) were significantly decreased and IGFBP-1 concentrations were highly increased [76]. During refeeding, a significant increase in circulating IRSA, total IGF-I and free IGF-I was observed, while BMI also increased [76]. The circulating IRSA and total IGF-I showed a correlation coefficient of 0.59 suggesting that in anorexia nervosa patients 60% of variation in IRSA could not be explained by concentrations of immunoreactive total IGF-I, thus again demonstrating that IRSA is only partly dependent on total IGF-I [76].
Measuring PAPP-A and PAPP-A2 activity could be of potential value to understand differences in circulating IRSA (measured by KIRA assay), total IGF-I and free IGF-I in anorexia nervosa. However to our knowledge the role of PAPP-A and the IGF system in anorexia nervosa has not yet been studied [75].

6.7 **IRSA and Obesity**

Despite low GH secretion and decreased IGFBP-1, 24h mean circulating IRSA (measured by the IGF-IR KIRA assay) was not decreased in obese women [28]. In addition, IRSA did not correlate with BMI and IGFBP-1 [28]. Therefore it was concluded that these findings argue against elevated IRSA as the mechanism underlying reduced GH secretion in obesity by an augmented negative feedback. In another cross-sectional placebo-controlled study GH administration during 6 months to overweight/obese women resulted in an increase of both circulating immunoreactive total IGF-I and IRSA (measured by the IGF-IR KIRA assay) [21]. Interestingly in this latter study the increase in IRSA rather than the increase in total IGF-I predicted the GH-related increase in lean mass and decrease in total adipose tissue/BMI [21]. It has been hypothesized that the observed increased ability of media harvested from visceral adipose tissue (VAT) to activate the IGF-IR in vitro (measured by the KIRA assay) is secondary to an upregulated PAPP-A mediated release of IGFBP-4 complexed IGF [35]. However, it is at present unclear whether local PAPP-A translates into differences of circulating IRSA in human subjects with obesity.

6.8 **IRSA and the Metabolic Syndrome**

In a cross-sectional study embedded in a random sample of over 1000 elderly subjects from the Rotterdam Study, a prospective population-based cohort study, a progressive rise in circulating IRSA (measured by the IGF-IR KIRA assay) was found with increasing insulin resistance as long as fasting blood glucose levels were within the normal range [11]. However, as soon impaired fasting blood glucose were present, circulating IRSA peaked and reached a plateau. Finally when blood glucose concentrations further increased and individuals could be classified as having diabetes, circulating IRSA progressively decreased [11]. In addition, IRSA peaked when three criteria of the metabolic syndrome were present and then declined significantly when five criteria of the metabolic syndrome were present suggesting an inverse U-shaped relationship between IRSA and number of components of the metabolic

syndrome [11]. This latter finding contrasts with previous results reporting an inverse relationship between the (immunoreactive) total IGF-I/IGFBP-3 ratio and components of the metabolic syndrome [11, 71].

6.9 **IRSA and Type 1 Diabetes**

Irrespective of pubertal status children and adolescents with type 1 diabetes showed lower IRSA (measured by the IGF-IR KIRA assay) and immunoreactive total IGF-I, but higher IGFBP-1 than healthy controls [73]. Suppression of IRSA was relatively more pronounced than total IGF-I and this latter finding was explained by the more concomitant increase of IGFBP-1 inhibiting IGF-I actions [73].
When comparing patients with and without residual β-cell function IRSA and IGF-II as well as IGFBP-3 were significantly higher in prepubertal patients with residual β-cell function, supporting the hypothesis that the portal supply of insulin to the liver is an important regulator of the activity of the GH-IGF axis, at least in prepubertal children, since such relation was absent in pubertal patients [73].
Insulin plays an important role in the regulation of the GH-IGF-I axis. When comparing the GH-IGF-I axis response after a single dose human NPH insulin, insulin detemir and insulin glargine in type 1 diabetes patients, it was found that independent of the actual plasma glucose concentrations, IRSA (measured by the IGF-IR KIRA assay) was higher and IGFBP-1 lower after insulin detemir than after NPH insulin and glargine administration, thereby explaining the lower GH levels [51]. By contrast, immunoreactive total IGF-I, IGFBP-2 and IGFBP-3 were comparable after administration of these three different insulins [51]. Since it is thought that the combination of a reduced GH secretion and an increased IRSA may have beneficial metabolic effects in type 1 diabetes, this study suggested that in this respect insulin detemir compared to NPH insulin and glargine is superior [51].
Ma et al. showed in type 1 diabetes patients that IRSA was more sensitive to short-term changes in

insulin exposure than total IGF-I, although the physiological significance of this observation has to be determined [52]. In this latter study again a strong inverse relationship between IRSA and circulating IGFBP-1 levels was found [52]. Moreover, despite distinct glucose-lowering properties, equal doses of human insulin, insulin aspart and two biphasic aspart preparations (BIAsp50 and BIAspo70) had similar effects on IRSA [52].

Hedman et al. studying in type 1 diabetes whether the route of insulin administration affected circulating IRSA (measured by the IGF-IR KIRA assay) found that continuous intraperitoneal insulin infusion (CIPII) induced higher circulating IRSA, but also higher circulating (immune-reactive) total IGF-I and IGF-II levels and lower IGFBP-1 than subcutaneous insulin administration [38]. This again supports the hypothesis that the route if insulin administration is important for the activity of the IGF system [38]. A low endogenous circulating IRSA is likely to augment the secretion of growth hormone, which may lead to insulin resistance and finally in an increased risk of late diabetic complications [43]. The observed higher circulating IRSA after intraperitoneal insulin administration suggests that CIPII treatment in type 1 diabetes patients is better correcting alterations of the IGF system than subcutaneous insulin administration [38].

6.10 **IRSA and Type 2 Diabetes**

Varewijck et al. found that IRSA (measured by the IGF-IR KIRA assay) was borderline significantly lower in patients with type 2 diabetes on metformin than in non-diabetic controls, while immunoreactive total IGF-I concentrations were significantly lower in patients with type 2 diabetes than in non-diabetic participants [86]. After 36 weeks of insulin therapy IRSA significantly decreased in patients with type 2 diabetes, while serum total IGF-I concentrations remained unchanged during this period [86]. The observed decline in IRSA after 36 weeks insulin therapy is in line with a study discussed above, which

showed that hyperinsulinemia suppressed IRSA, whereas total serum IGF-I did not change [1]. In this latter study it was concluded that insulin decreased IRSA through differential modulation of IGFBPs: insulin suppressed IGFBP-4 and IGFBP-1 and increased IGFBP-2 concentrations [1].

Prior to bariatric surgery in severe obese type 2 diabetic patients IRSA (measured by the IGF-IR KIRA assay) was significantly elevated, while total IGF-I was not increased [12]. After bariatric surgery IRSA only slightly increased at 3 months and was unchanged at 12 months, while simultaneously there were no changes in total IGF-I and total IGF-II [12]. In addition, IGFBP-1 significantly increased and IGFBP-3 significantly decreased and these changes continued up to 12 months [12]. The biological importance of these findings is unclear at the moment.

6.11 **Effects of Intensive Insulin Therapy on IRSA in the Intensive Care**

Upon admission at the intensive care unit immunoreactive total IGF-I concentrations and IRSA (measured by the IGF-IR KIRA assay) were lower and GH concentrations were elevated in critically ill children compared with a healthy reference population [33]. In this respect there were no differences between children randomized to conventional insulin therapy (CIT) and to intensive insulin therapy (IIT) [33]. At day 3 of admission ITT decreased IRSA compared to CIT, while total IGF-I concentrations were similar when comparing both treatment arms [33]. In addition, compared to CIT, at day 3 of admission IGFBP-3 and ALS concentrations were decreased and IGFBP-1 concentrations were increased in the IIT group [33]. According the authors the decreased IRSA in the IIT group may point to aggravated GH resistance [33]. A second possible explanation for the decreased circulating IRSA may be that the IIT suppressed endogenous portal insulin levels stronger than CIT and this may have led a to decreased hepatic IGF-I production, which has resulted in a reduced IRSA [33]. The long-term functional

consequences of ITT on the changes in the IGF-I system are unclear at present and should be further investigated.

6.12 **Effects of Insulin on IRSA in Very Low Birth Weight Infants**

In a small randomized controlled study intravenous insulin administration to very low birth weight infants throughout the first week of life improved glucose control and increased IRSA (measured by the IGF-IR KIRA assay) compared with standard care [4]. There were trends toward faster growth in leg length and increased weight gain in the infants treated with insulin (and higher IRSA) compared with the standard care group [4]. During the 7-day study period, there were no significant differences in circulating (immuno-reactive) total IGF-I levels between the infants treated with insulin and those receiving standard care [4]. Therefore it was concluded that early insulin therapy increased IRSA and improved blood glucose control and this could be contributing to less morbidity among very low birth weight infants [4].

6.13 **IRSA and Cushing Disease**

Untreated Cushing disease was characterized by normal circulating IRSA (measured by the IGF-IR KIRA assay) and immunoreactive total IGF-I concentrations [83]. Treatment of patients with an active Cushing Disease with a low dose of the somatostatin analog pasireotide (which binds with high affinity to somatostatin receptors subtypes 1-3 and 5) during 28 days reduced cortisol production and normalized urinary free cortisol in 29% [83]. During treatment of pasireotide Z-scores for IRSA and total IGF-I decreased significantly to values < -2 SD in 43% and 35%, respectively, suggesting the induction of growth hormone deficiency de novo [83].

546

547 *6.14* **IRSA and Graves Ophthalmopathy**

548 In subjects diagnosed with Graves Ophthalmopathy values for IRSA (measured by the IGF-IR KIRA assay)
549 were found to be low normal (Z-score: -1.5 SD ± 0.1 SD) whereas immunoreactive total IGF-I was normal
550 (Z-score: 0.6 ± 0.2 SD) [84]. In line with these findings it was reported more than twenty years ago that
551 IRSA was markedly reduced in thyrotoxicosis when IRSA was assessed by measuring incorporation of
552 radiolabeled sulfate into cultures of porcine cartilage [54].

553

554 6.15 **IRSA and Kidney Disease**

555 Patients with end-stage renal disease showed elevated GH, high normal circulating immunoreactive
556 total IGF-I and subnormal IRSA (measured by the IGF-IR KIRA assay) compared to controls [29]. After 7
557 days treatment with recombinant GH IRSA tended to be lower in patients with end-stage renal disease
558 than controls while total IGF-I increased to the same extent as controls [29]. The authors suggested that
559 the observed changes in IRSA (but not in total IGF-I) indicated that hepatic sensitivity to GH was reduced
560 by 50 % in patients with end-stage renal disease and that in patients with end-stage renal disease
561 changes in total IGF-I during treatment with GH are not reflecting changes in endogenous activity of IGF-
562 I [29].
563 In another study Ivarsen et al. found that directly after hemodialysis there were marked reductions in
564 IRSA (measured by the IGF-IR KIRA) and ultrafiltered free IGF-I in non-diabetic patients with end-stage
565 renal disease while there were only marginal reductions in immunoreactive total IGF-I and total IGF–II
566 [40]. They hypothesized that the decrease in IRSA was a consequence of an increase in IGFBP-1,
567 sequestering free IGF-I, and thereby reducing IRSA [40]. In accordance with this view the increase in

IGFBP-1 was accompanied by a parallel increased complex formation between IGF-I and IGFBP-1 [40]. In addition, Ivarsen et al. suggested that catabolism induced by hemodialysis may be (in part) reflected by the observed reductions in IRSA [40]. When a meal was served to patients on maintenance hemodialysis before hemodialysis, this resulted in a 20% maximum increase of IRSA at 120 min during hemodialysis, whereas total IGF-I concentrations showed a maximum increase of 5% at 180 min [64]. In another study by the same group, a baseline meal was offered at the day of hemodialysis [65]. In this latter study the expected postprandial increase in IRSA after a baseline meal was absent on all four study days. IRSA only increased above baseline when a second meal was offered at the day of hemodialysis [65]. In addition, immunoreactive total IGF-I did not significantly change and remained fairly constant on all four study days [65]. The increase in IRSA after the second meal on the day of hemodialysis suggested a beneficial effect of frequent meals for patients on maintenance hemodialysis [65].

Brugts et al. studied patients with end stage renal disease treated on continuous ambulatory peritoneal dialysis (CAPD) and found that circulating IRSA (measured by the IGF-IR KIRA assay) increased both after administration of a dialysate with a mixture of amino acids plus glucose or a dialysate that contained only glucose while no changes in circulating immunoreactive total IGF-I concentrations were observed (Figure 5) [9]. Therefore they concluded that circulating IRSA rather than total IGF-I is involved in acute responses to nutritional interventions in patients with end stage renal disease treated on CAPD [9].

6.16 **IRSA and Liver Cirrhosis**

Circulating IRSA (measured by the IGF-IR KIRA assay), immunoreactive total IGF-I and total IGF-II were reduced in patients with alcoholic liver cirrhosis compared to controls, whereas IGFBP-1, IGFBP-2 and the soluble IGF-II receptor were elevated [44]. Interestingly, the IRSA was fourfold elevated in ascites as

compared with serum while in contrast, all other IGF-I-related peptides but pro-IGF-II in ascites were reduced as compared with serum [44]. Thus this study suggested that in contrast to immunoreactive total IGF-I concentrations, IRSA can be higher in fluids from an extravascular compartment than in serum. However, the pathophysiological significance of these findings remains to be clarified [44].

In another study circulating IRSA (measured by the IGF-IR KIRA assay) significantly decreased in patients with liver cirrhosis after an oral glucose tolerance test (OGTT) and the same tendency was observed in healthy subjects [15]. This reduction of IRSA in patients with liver cirrhosis occurred despite unchanged concentrations of (immunoreactive) total IGF-I and free IGF-I [15]. It was hypothesized that the reduction of IRSA in patients with liver cirrhosis during an OGTT was related to higher concentrations of IGFBP-1 and a faster disappearance of IGFBP-1 bound IGF-I [15].

Treatment of patients with liver cirrhosis with a transjugular intrahepatic porto-systemic shunt (TIPS) may induce anabolism [39]. Holland-Fischer et al. found that the body cell mass of patients with liver cirrhosis increased after TIPS [39]. However, circulating concentrations of IRSA (measured by the IGF-IR KIRA assay), immunoreactive total IGF-I, total IGF-II and IGF-binding proteins did not change, suggesting that other mechanisms than activity of the IGF system are involved in the anabolic effects of TIPS [39].

6.17 **IRSA and Hepatocellular Carcinoma (HCC)**

Circulating IRSA (measured by the IGF-IR KIRA assay) in patients with hepatocellular carcinoma was twice as high as found in patients with liver cirrhosis [23]. However, IRSA concentrations in both groups were markedly below the concentrations observed for healthy controls [23]. Similar patterns as found for circulating IRSA were observed for circulating immunoreactive total IGF-I, IGF-II and IGFBP-3 whereas pro-IGF-II and the IGF-I to IGFBP-3 ratio showed less pronounced but nevertheless significant differences [23]. Changes in tumor burden after treatment did not affect IRSA or IGF-II [23]. It was concluded that

614 the observed differences in parameters of the IGF system between patients with hepatocellular
615 carcinoma, liver cirrhosis and healthy subjects were mainly explained by variations in liver status [23].
616 Therefore the authors questioned the clinical utility of measuring circulating IGF variables as markers of
617 hepatocellular carcinoma [23].

618

619 *6.18* IRSA**, Lung Cancer and Pleural Fluid**

620 When IRSA (measured by the IGF-IR KIRA assay) and other members of the IGF family were compared in
621 pleural fluid and in blood from patients with lung cancer and nonmalignant lung disease, it was found
622 that IRSA was threefold higher in pleural fluid than in corresponding serum samples, regardless of
623 etiology [25]. In contrast immunoreactive total IGF-I concentrations did not differ between blood and
624 pleural fluid [25]. In addition, PAPP-A, an IGFBP protease that may cleave IGFBP-4 and IGFBP-5 [49, 59,
625 60], was elevated in pleural fluid and it was speculated by the authors that IGFBP-proteases (inclusive
626 PAPP-A) were involved in the observed increase of IRSA in pleural fluid [25]. This study suggested that
627 local factors at the tissue level may have major effects on IRSA and that local IRSA may substantially
628 differ from that measured in the circulation [25].

629

630 *6.19* **IRSA, Ovarian Carcinoma and Ascites**

631 As discussed above, PAPP-A may stimulate IGF-I action through proteolysis of IGFBP-4 and in
632 experimental animal models it has been found that PAPP-A may accelerate ovarian tumor growth by
633 releasing IGF-I [78]. IRSA (measured by the IGF-IR KIRA assay) in ascites of patients with ovarian cancer
634 was 31% higher than in serum [78]. In contrast, concentrations of immunoreactive total IGF-I were
635 similar in serum and ascites, while levels of IGF-II and IGFBP-3 were decreased in ascites compared to

serum [78]. Since it was found that ascites contained a 46-fold higher concentrations of PAPP-A than serum and also IGFBP-4, it was hypothesized that PAPP-A in ascites may function to increase IGF-I actions [78]. In favor of this latter possibility it was found that ascites contained less intact IGFBP-4 than plasma and higher concentrations of proteolytically cleaved IGFBP-4 than intact IGFBP-4 [78].

6.20 **IRSA and Dementia**

Within the Rotterdam Study higher levels of circulating IRSA (measured by the IGF-IR KIRA assay) were associated with a higher prevalence and a higher incidence of dementia suggesting that IRSA increases in response to neuropathological changes that occur in dementia [20]. Similar associations were found for Alzheimer's disease and in persons without diabetes mellitus [20]. Unfortunately in this latter study no circulating immunoreactive total IGF-I concentrations were measured. As a consequence it was impossible to compare whether in this respect there existed discrepancies between IRSA and total IGF-I. Interestingly, we found in another study evidence of an interaction between ApoE-ε4 and tertiles of IRSA [32]. IRSA in the median and top tertiles was related to increased dementia incidence in hetero- and homozygotes of the ApoE-ε4 allele, but did not show any association with dementia risk in people without the ApoE-ε4 allele shedding a new light on the association between IGF-I signaling and the neuropathology of dementia [32].

6.21 **IRSA and Longevity**

IRSA as measured by the IGF-IR KIRA assay, immunoreactive total IGF-I and the IGF-I/IGFBP-3 ratio were all three significantly lower in centenarians' offspring compared to offspring matched-controls [92]. In addition, IRSA in centenarians' offspring was inversely related to insulin sensitivity [92]. Interestingly, it

was further found that in contrast to circulating total IGF-I concentrations, mean IRSA was comparable between centenarians and their offspring [92]. However, further studies are needed to understand the precise role of IRSA in the modulation of the human aging process [92].

We also measured IRSA by KIRA assay in centenarians and found relatively low IRSA [92]. Mean IRSA in 106 centenarians was 132 (107-157) pmol/L (mean (25th-75th percentile)), while mean IRSA in 192 centenarians' offspring and 80 offspring matched controls were 144 (119-170) pmol/L and 161 (134-187) pmol/L [92]. In addition, centenarians showed a 2-fold higher insulin sensitivity than centenarians' offspring [92]. Therefore we hypothesized that, despite low circulating IRSA, the post-receptor signaling pathways of the IGF-IR were up-regulated in centenarians [92].

6.22 IRSA and Mortality

In a prospective observational of more than 400 healthy elderly men (aged 73-94 yrs.) IRSA (measured by the IGF-IR KIRA assay) accounted for 2.5 % (range 0.2-5.9%) of circulating (immunoreactive) total IGF-I concentrations whereas free IGF-I (measured by immunoassay) accounted for 0.7% [10]. These findings suggested that IRSA was most likely reflecting stimulation of the IGF-IR by free IGF-I and free IGF-II and IGFs dissociated from the IGFBPs during incubation of serum samples [10]. Survival of these elderly men in the highest quartile of IRSA was significantly better than in the lowest quartile, both in the total study group (Figure 6) as well as in subgroups having a medical history of cardiovascular disease or a high inflammatory risk profile [10]. Such relationships were not observed for immunoreactive total or free IGF-I [10]. Thus this study suggested that a relatively high circulating IRSA in elderly men of 73-94 yrs. may be associated with extended survival and with reduced cardiovascular risk [10].

7. Discussion and conclusions

The main reason for using immunoassays as an estimate of circulating IGF-I bioactivity has long been the lack of reliable IGF-I bioassays [66]. The measurement of IRSA by the IGF-IR KIRA assay has opened a completely new era and is a novel tool to asses circulating IGF-I bioactivity under conditions that mimic the in vivo cellular environment as good as possible. In this review many studies are discussed showing that measuring IRSA often produces other information in detecting changes of the IGF system than the commonly used IGF-I immunoassays. In contrast to IGF-I immunoassays the IGF-IR KIRA assay is sensitive for modifications of IGF-IR activation by circulating IGFBPs and IGFBP-proteases [14, 48].

This review shows that in many conditions in health and disease results for IRSA (measured by the IGF-IR KIRA assay) and circulating total immunoreactive IGF-I concentrations (measured by immunoassays) are discordant (Table 1) . These discrepancies are probably directly related to fundamental differences that exists between both methods. With the IGF-IR KIRA assay all serum factors, which directly or indirectly may phosphorylate tyrosine residues of the IGF-IR, become detectable. Moreover, the IGF-IR KIRA assay is sensitive to modulating effects of the IGFBPs on the interactions between IGF-I and the IGF-IR [14]. Thus the IGF-IR KIRA assay may give information about net overall effects of IGF-I, IGF-II, IGFBPs and IGFBP-proteases on IGF-IR activation. In contrast, levels of immunoreactive total IGF-I are mainly determined by IGF-I bound to the IGFBPs. However, it is unclear which part of this latter fraction is involved in the activation of the IGF-IR since as discussed above, only IGF-I in the free state is able to stimulate directly the IGF-IR [42]. Thus discrepancies between IRSA (measured by the IGF-IR KIRA assay) and circulating total immunoreactive IGF-I concentrations (as measured by immunoassays) could be due to stimulating effects of biological factors on the IGF-IR that may be detected by the IGF-IR KIRA assay but are not recognized by total IGF-I immunoassays. Moreover, antibodies in IGF-I immunoassays may also recognize IGF-I molecules or fragments that are biologically inactive and unable to stimulate the IGF-IR. Therefore Zapf et al. previously suggested that results obtained by IGF-I immunoassays

should always be confirmed by IGF bioassays before conclusive statements of measured IGF-I levels on physiological or pathophysiological issues are made (87).

This review suggests that measurement of circulating IRSA (by the IGF-IR KIRA assay) is especially superior to immunoreactive total IGF-I to monitor therapeutic interventions. Although the IRSA being measured by the IGF-IR KIRA assay probably more closely reflects true bioactive IGF-I than the measurement of total IGF-I in serum by immunoassays, it is good to realize that that the IGF-IR KIRA assay does not capture all the post-receptor intracellular events mediated by the IGF-IR. The IGF-IR KIRA assay only quantifies IGF-IR phosphorylation of tyrosine residues. Nevertheless, it has been found that the IGF-IR KIRA assay shows excellent correlations with the more classical endpoint bioassays [66]. Thus the IGF-IR KIRA assay in its present version does not provide information about the further intracellular propagation and IGF-IR–mediated signal more downstream [14, 66]. On the other hand, this may be a strength of the IGF-IR KIRA assay: in so called-endpoint bioassays it is often impossible to disentangle the relative contribution of IGF-IR mediated effects to a certain end result since there is extensive cross-talk at the post-receptor level between different intracellular signaling networks which are activated by other ligands than IGF-I [77]. Moreover, many biological responses are complex and depend often on a cascade of cross-talk and post-receptor events and stimulation of the IGF-IR may activate different signaling pathways intracellularly upon receptor binding in a concentration-dependent manner (Figure 2).

Although the signal measured by the IGF-IR KIRA assay is readily direct and specific, the IGF-IR has up to 6 key tyrosine residues of which of some the role in vivo is not yet fully clear [85]. In addition, the antibody used to capture the tyrosine residues may not well recognize all residues equally well because of dependence of affinity on flanking sequence and proximity of other sites [85]. Thus the IGF-IR KIRA bioassay only provides a crude, albeit convenient, measure of kinase activation.

Interestingly, as discussed above, IRSA in samples from the interstitial fluid obtained by the suction

728 blister method was almost 50% higher than in matched serum samples [24]. Moreover, IRSA was higher
729 in ascites than in serum in patients with liver cirrhosis and in patients with ovarian cancer [15, 44]. In
730 addition, IRSA was also higher in pleural fluid than in serum in patients with lung disease [25, 78]. Thus
731 the amount of IRSA present in interstitial fluid and the extravascular tissues may not only substantially
732 differ from that measured in the circulation but that the amount of local IRSA seems not directly related
733 to circulating IRSA. These findings suggest that it is necessary to collect information about both the
734 paracrine (local) and endocrine (circulating) IRSA to obtain an overall impression of the role of the IGF
735 system in health and disease.
736 An important limitation of the IGF-IR KIRA assay in its present form is that it is more labor intensive and
737 more expensive than total IGF-I immunoassays. It measures only the amount of IGF-I (and other ligands)
738 that that can interact with the IGF-I receptor and activate its tyrosine kinases during a short time of
739 incubation (15 minutes). For the future the IGF-IR KIRA assay should be further miniaturized and
740 automated to run many samples in a relatively short time. In addition, there should be a single
741 universally accepted standard for the IGF-IR KIRA assay for calibration and large pools of reference
742 serum samples should become available to monitor the (dis)concordance in results between different
743 laboratories using an IGF-IR KIRA assay.
744 Easy access and reliability of a cell line transfected with the human IGF-IR are prerequisites to
745 implement wide-spread use of the KIRA assay. Cells transfected with the human IGF-IR frozen in
746 microwell plates offer a potential valid alternative to fresh cells from a growing culture. When these
747 plates can be used immediately after thawing and the frozen cells can be revitalized without passaging
748 and washing cells, total time needed to perform the assay is considerably shortened. This strategy may
749 further help to overcome an important bottleneck to implement wide-spread use of the KIRA assay; it
750 removes day-to-day variation, eliminates passage effects and improves consistency of cell-based assay
751 results. The production of these frozen cells should be standardized so that different batches are highly

comparable. Freezing and resuscitation protocols should be optimized, and the performance of these ready-to-use cells should be compared with those from continuous culture to determine whether they could be used as a replacement. To further reduce costs and consistency of the KIRA the antibodies should be replaced by aptamers.

In conclusion, the IGF-IR KIRA assay is a novel tool that has opened a new era. When studying changes of the GH-IGF-I axis in health and disease the IGF-IR KIRA assay provides in many conditions different information about the IGF system than the commonly used total IGF-I immunoassays. The IGF-IR KIRA assay probably more closely reflects true bioactive IGF-I compared to measurements of total IGF-I in serum by immunoassays. In health and disease IRSA measured by the IGF-IR KIRA assay was considerably higher in samples from interstitial fluid and ascites than in serum, suggesting that both local and circulating IRSA should be measured in order to get a more complete view of the role of the IGF system.

Duality of Interest

Dr. Joseph A.M.J.L, Janssen, who is co-authoring this paper, also serves as Associate Editor of Growth Hormone and IGF Research. However, this has not influenced on the handling of the paper, which has been subjected to the Journal's usual procedures. Thus, the peer review process has been handled independently of Dr. Joseph A.M.J.L. Janssen, who has been blinded to the review process. The authors otherwise have nothing to disclose.

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Author Contributions

JAMJLJ, AJV and MB designed the study;
All authors contributed to data interpretation, discussion of the paper; all authors prepared and all edited the manuscript.

Disclosure Statement

The authors have nothing to disclose

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Legends

Figure 1.

Schematic overview of the IGF system. The IGF system is composed of two Insulin-like Growth Factors IGF-I (yellow) and IGF-II (blue), 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. The IGF-IR can bind IGF-I with high affinity but also IGF-II with 10-fold lower affinity. In the Figure also insulin (red) 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 considerably lower avidity (displayed by the differences in thickness of the arrows). Interestingly, in the body the IGF-I and IR may form hybrids which are preferentially activated by IGF-I.

Figure 2

Model of the IGF-IR signaling pathway. Binding of IGF-I (or IGF-II) to the IGF-IR results in auto phosphorylation of tyrosine residues located within the intracellular kinase domain of the IGF-IR, being the first step in the intracellular signaling cascade. This starts the activation of multiple complex intracellular pathways (including the RAS-ERK-MAPK pathway and the PI3K/AKT pathway) and results in multiple biological effects in a variety of tissues and cells in the body.

Figure 3

Schematic overview of procedures the IGF-I Kinase Receptor Activation Assay (IGF-IR KIRA).The time to perform the assay is 4-days. On day 1 human embryonic kidney (EBNA) 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 0.1% human serum albumin (HSA). A second 96 well plate is coated with a human IGF-IR capture antibody. 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 the human IGF-IR capture antibody on day 2. On day 4 the 96 well plate is washed and a second antibody is added, which specifically recognizes phosphorylated residues located at the kinase domains of the IGF-IR. This latter antibody contains a europium (EUR) label so that phosphorylation of the tyrosine residues of IGF-I receptor can be quantified in a time-resolved fluorometer.

Figure 4. Age distribution of serum total IGF-I levels (upper panel) and IRSA (lower panel) in 94 patients diagnosed with growth hormone deficiency (GHD) (black dots). The shaded area depicts the 95% confidence interval in normal subjects per decade of age. IRSA in adult patients with proven GHD was more frequently below the normal range (<-2 SD) than total IGF-I concentrations. From: IGF-I Bioactivity Better Reflects Growth Hormone Deficiency than Total IGF-I. J Clin Endocrinol Metab. 2011; 96(7):2248-2254. doi:10.1210/jc.2011-0051. J Clin Endocrinol Metab | Copyright © 2011 by The Endocrine Society.

Figure 5

Top: Changes in circulating total (immunoreactive) IGF-I concentrations in the fed state after a dialysate that only contained glucose (G) and after a dialysate containing aminoacids and glucose (AA and G), respectively (basal state = 100%). Bottom: Changes in circulating IRSA in the fed state compared to baseline after G and after AA and G dialysate, respectively (basal state = 100%). Circulating IRSA increased both after administration of a dialysate with a mixture of amino acids plus glucose or a dialysate that contained only glucose while no changes in circulating total (immunoreactive) IGF-I

concentrations were observed. From: Bioactive rather than total IGF-I is involved in acute responses to nutritional interventions in CAPD patients Nephrol Dial Transplant. 2009; 25(3):940-946. doi:10.1093/ndt/gfp576.Nephrol Dial Transplant | © The Author 2009. Published by Oxford University Press on behalf of ERA-EDTA.

Figure 6

Cox proportional hazard plots (percent) showing Cumulative Survival for groups of circulating (immunoreactive) total IGF-I concentrations (A), circulating (immunoreactive) free IGF-I concentrations (B) and IRSA concentrations as measured by the IGF-IR KIRA assay(C). P for trend reached statistical significance only in the IRSA group as measured by the IGF-IR KIRA assay. Groups of IRSA are shown as follows: group 1 (—), ≤25th percentile; group 2–3 (– – –), between 25th and 75th percentile; and 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. See also text. From: Low Circulating Insulin-Like Growth Factor I Bioactivity in Elderly Men Is Associated with Increased Mortality. J Clin Endocrinol Metab. 2008; 93(7):2515-2522. doi:10.1210/jc.2007-1633. J Clin Endocrinol Metab | Copyright © 2008 by The Endocrine Society.

Table 1

Table 1. Overview of reported discordant results of circulating IRSA and immunoreactive Total IGF-I concentrations in Health (A) and Disease States (B) (see text for details)

A] In Health

	IRSA	Immunoreactive Total IGF-I	Reference
Fasting	↓	≈	25
During a hyperinsulinemic euglycemic clamp	↓	≈	31
During administration of glucagon	↓	≈	32
After short-term administration of 5 mg Prednisolone/day	↓	≈	35
After administration of raloxifene	≈	↓	39
After administration of estrogen	≈	↓	39

B] In Disease States

	IRSA	Immunoreactive Total IGF-I	Reference
Untreated GH deficiency	↓↓	↓	40
During GH therapy of adult GH deficiency	↑	↑↑	41
Untreated active Acromegaly	↑	↑↑	46
PAPP-A2 mutation	↓	↑↑	48, 49
Untreated Turner Syndrome	↓	≈	52
Type 1 diabetes	↓↓	↓	58
Untreated Type 2 Diabetes	≈↓	↓↓	63
Insulin-treated Type 2 diabetes	↓	≈	63
Severe obesity and Type 2 diabetes	↑	≈	64
Intensive vs. Conventional insulin therapy on the IC	↓	≈	65
Insulin therapy to Very Low Birth Weight infants	↑	≈	66
Graves Ophthalmopathy	↓	≈	68
Endstage Renal Disease after hemodialysis	↓↓	≈↓	70, 71
Endstage Renal Disease after administration of dialysate	↑	≈	74

↓ Decreased

≈ Unchanged

↑ Increased

Figure 1

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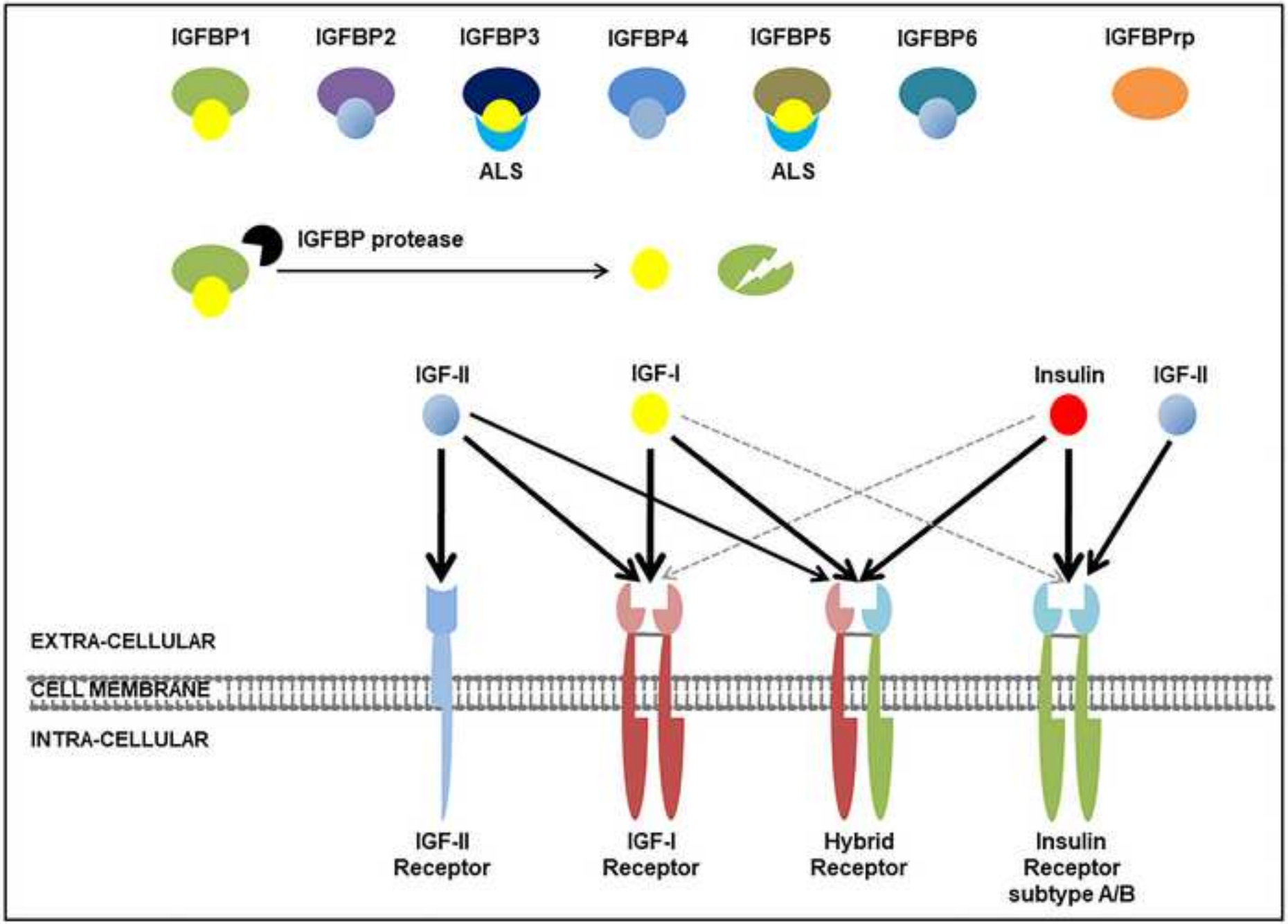

Figure 2

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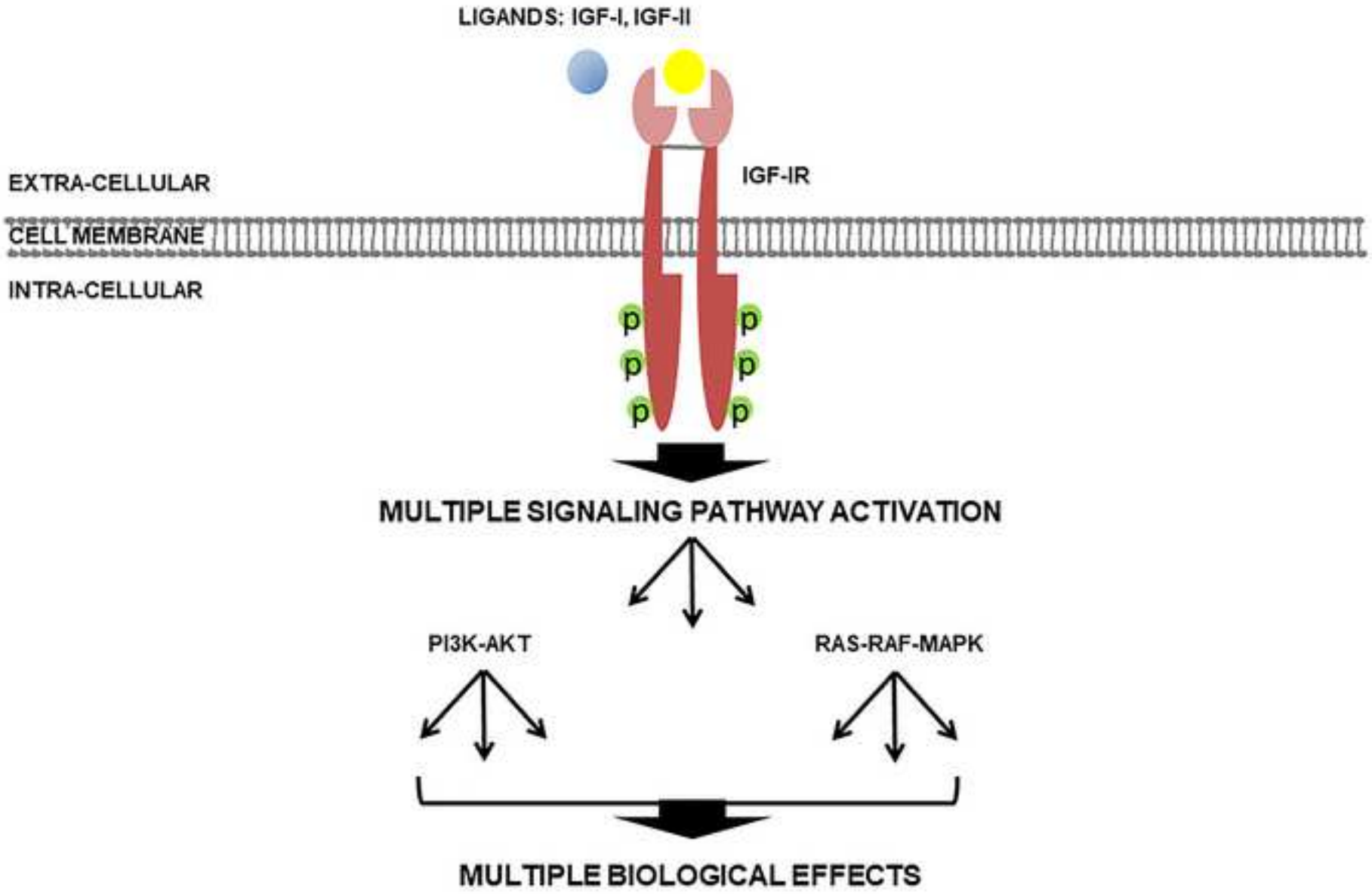

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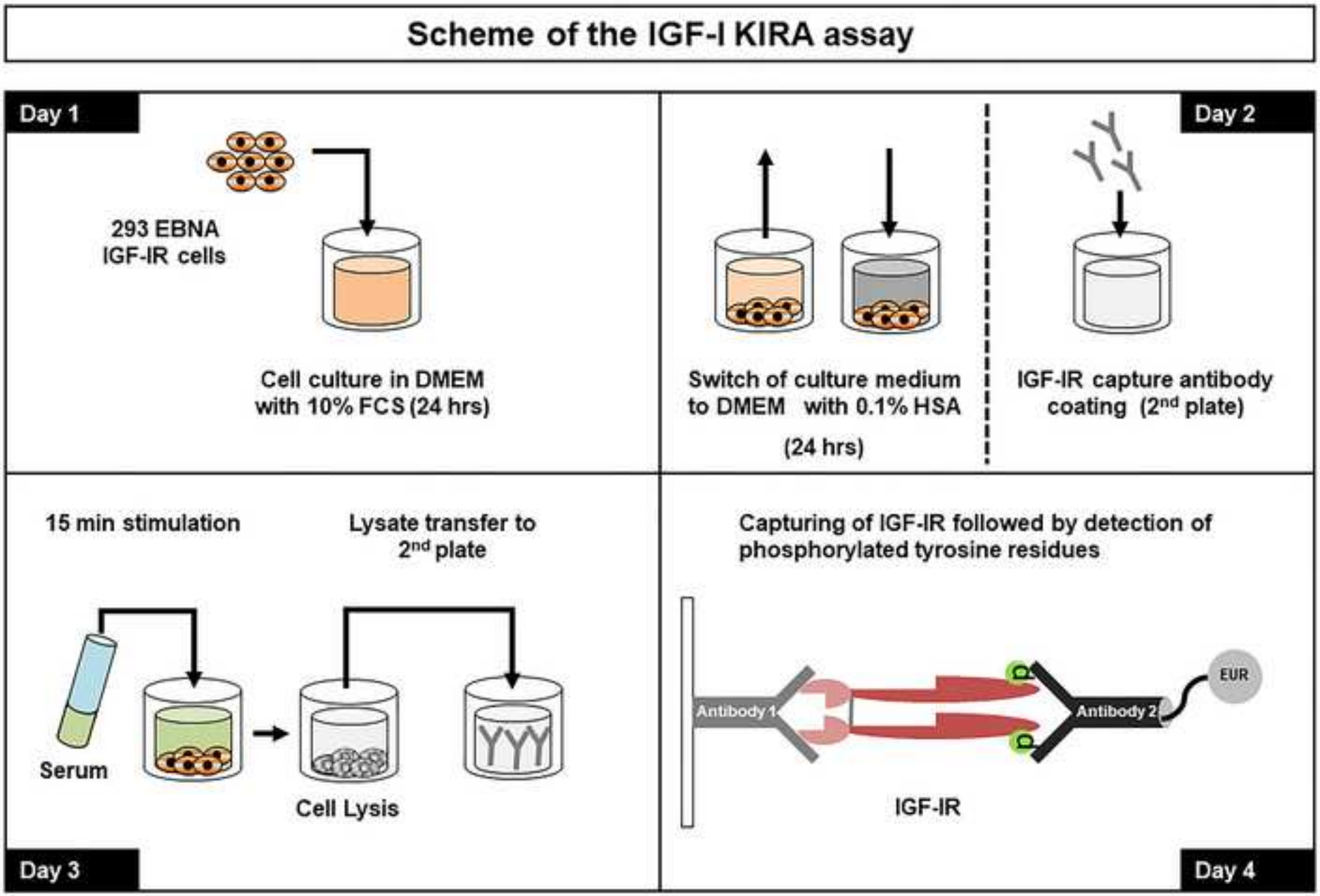

Figure 4

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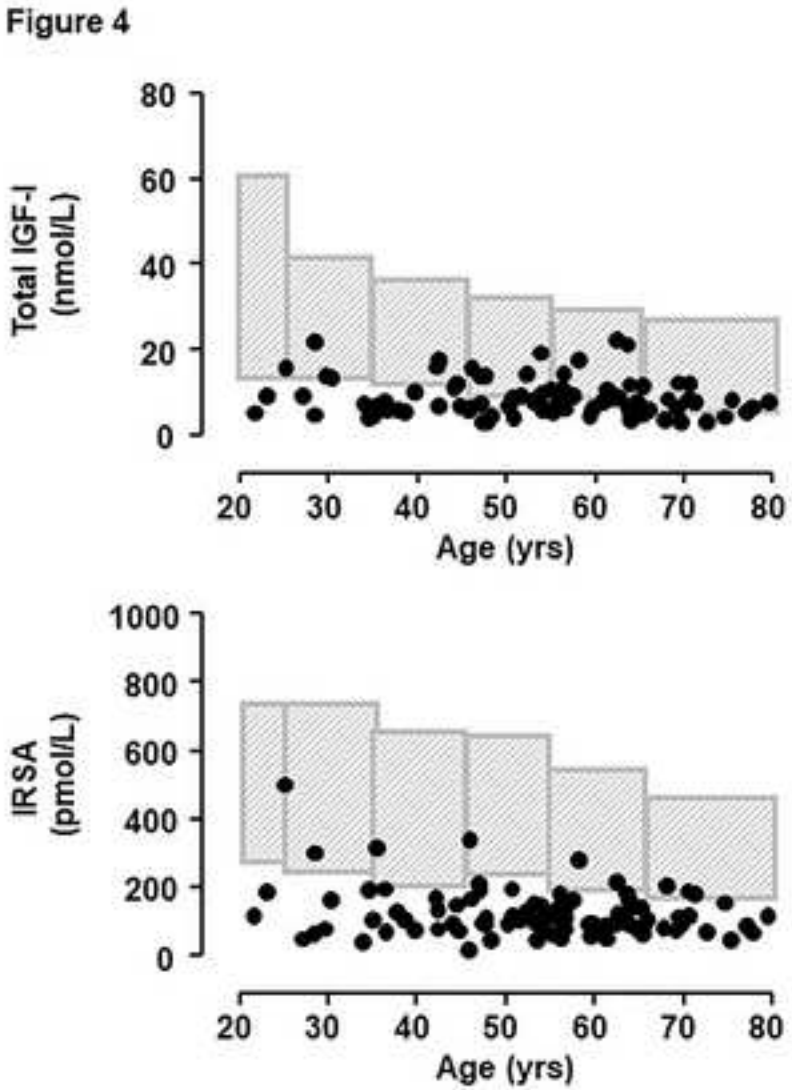

Figure 5

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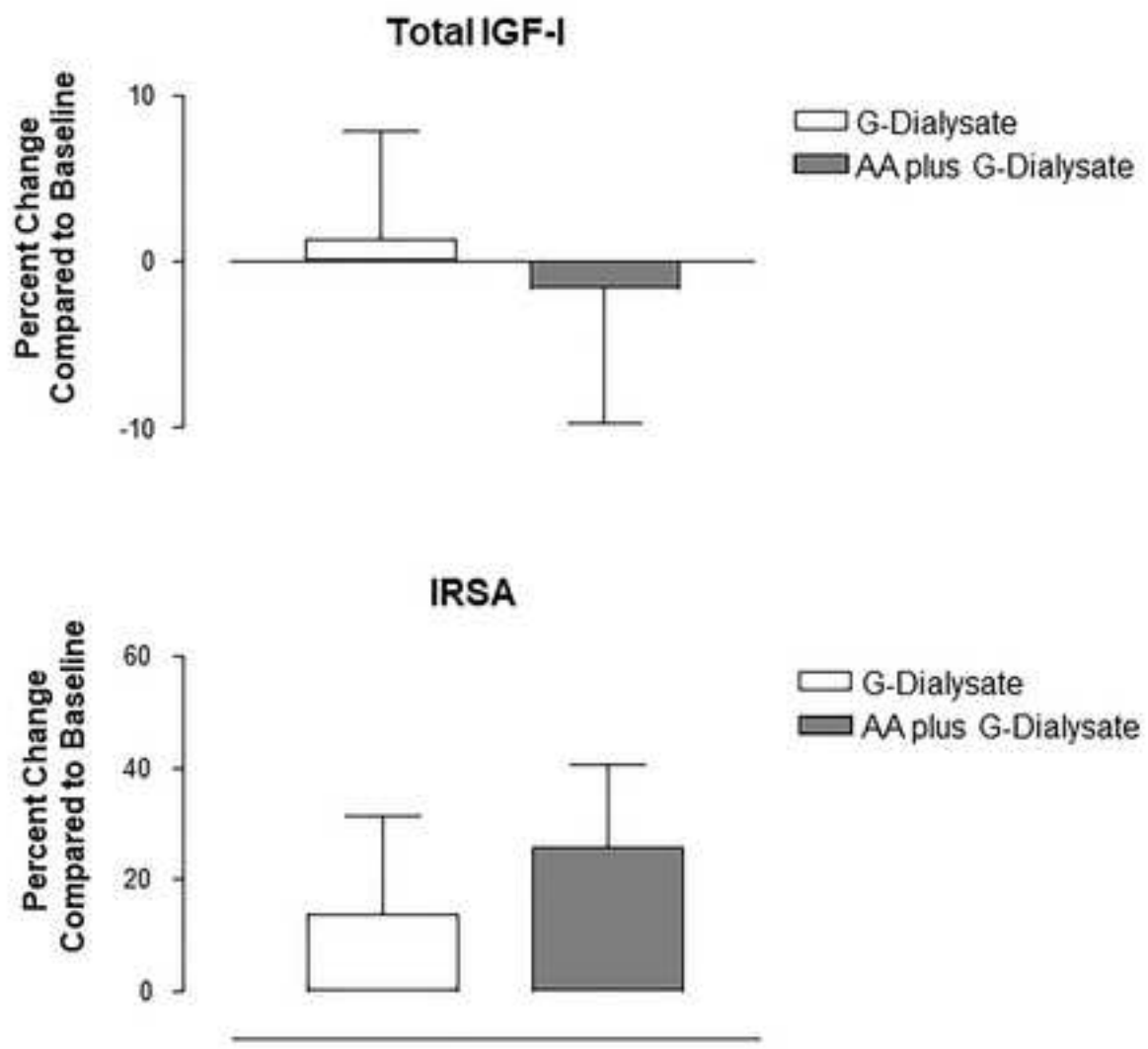

Figure 6A

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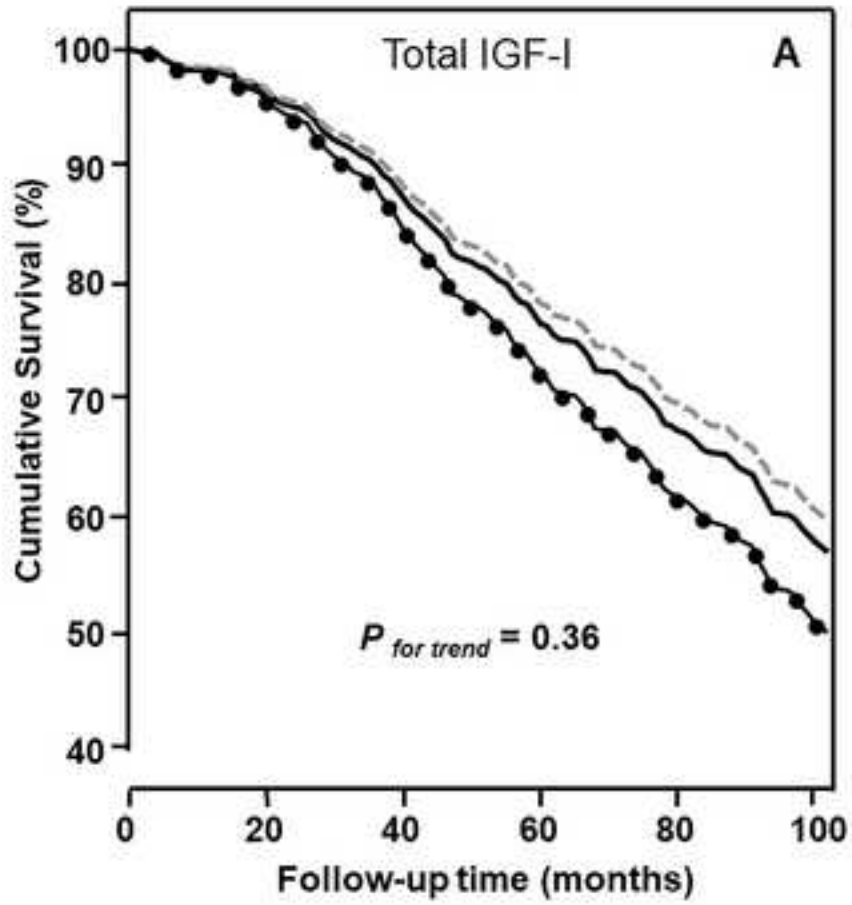

Figure 6B

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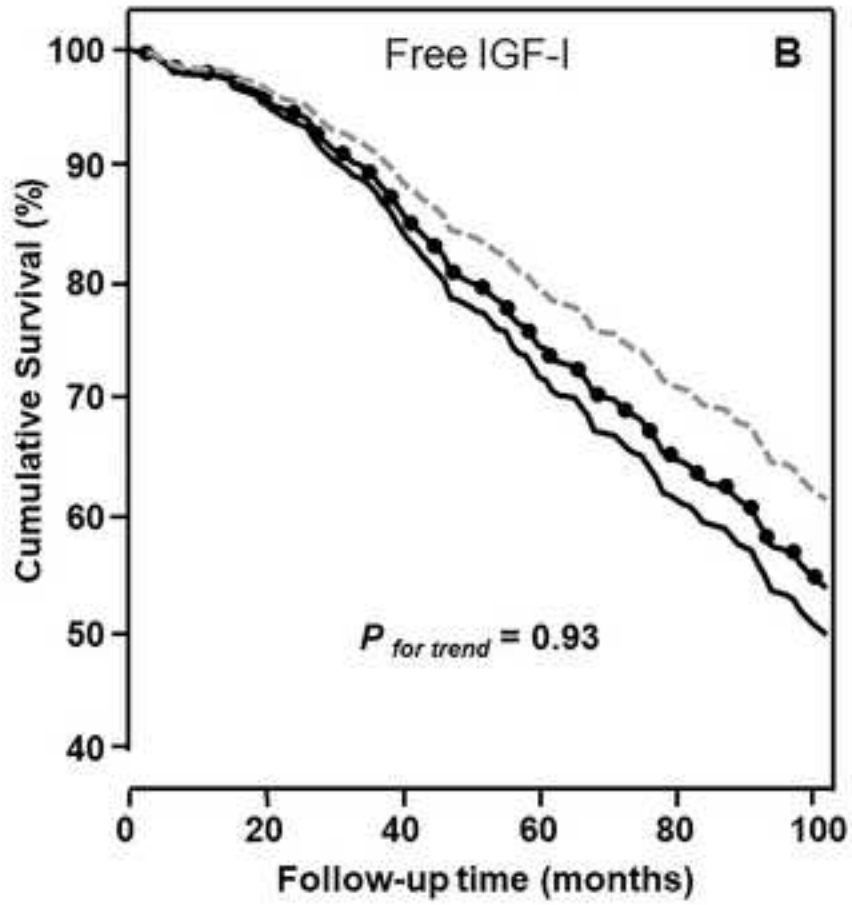

Figure 6C

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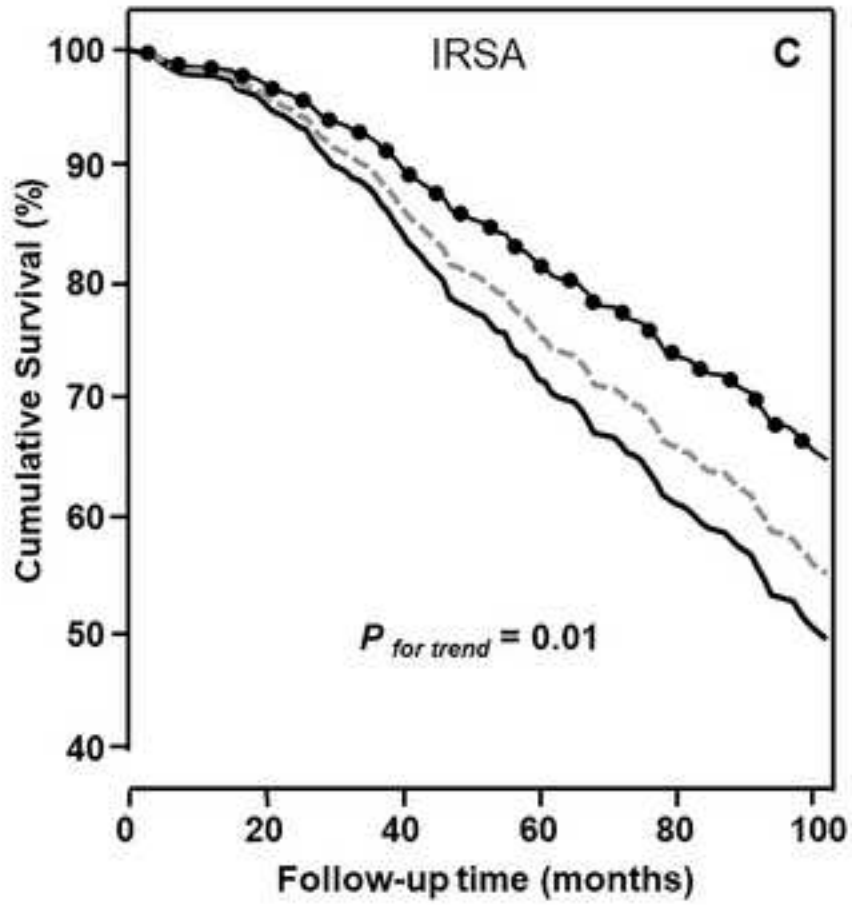


***Highlights (for review)**

Highlights

- Measuring IRSA by the IGF-IR KIRA assay often provides fundamentally different information about the IGF system than the commonly used total IGF-I immunoassays.
- The IGF-IR KIRA assay seems especially superior to immunoreactive total IGF-I in monitoring therapeutic interventions.
- Insulin-like Growth Factor-I Receptor Stimulating Activity measured by the IGF-IR KIRA assay has been found to be considerably higher in interstitial fluid and ascites than in serum.
- Both the paracrine (local) and endocrine (circulating) IRSA should be measured to get a complete picture about the role of the IGF system in health and disease.

Conflict of Interest Statement

The authors have nothing to disclose

Graphical Abstract (for review)

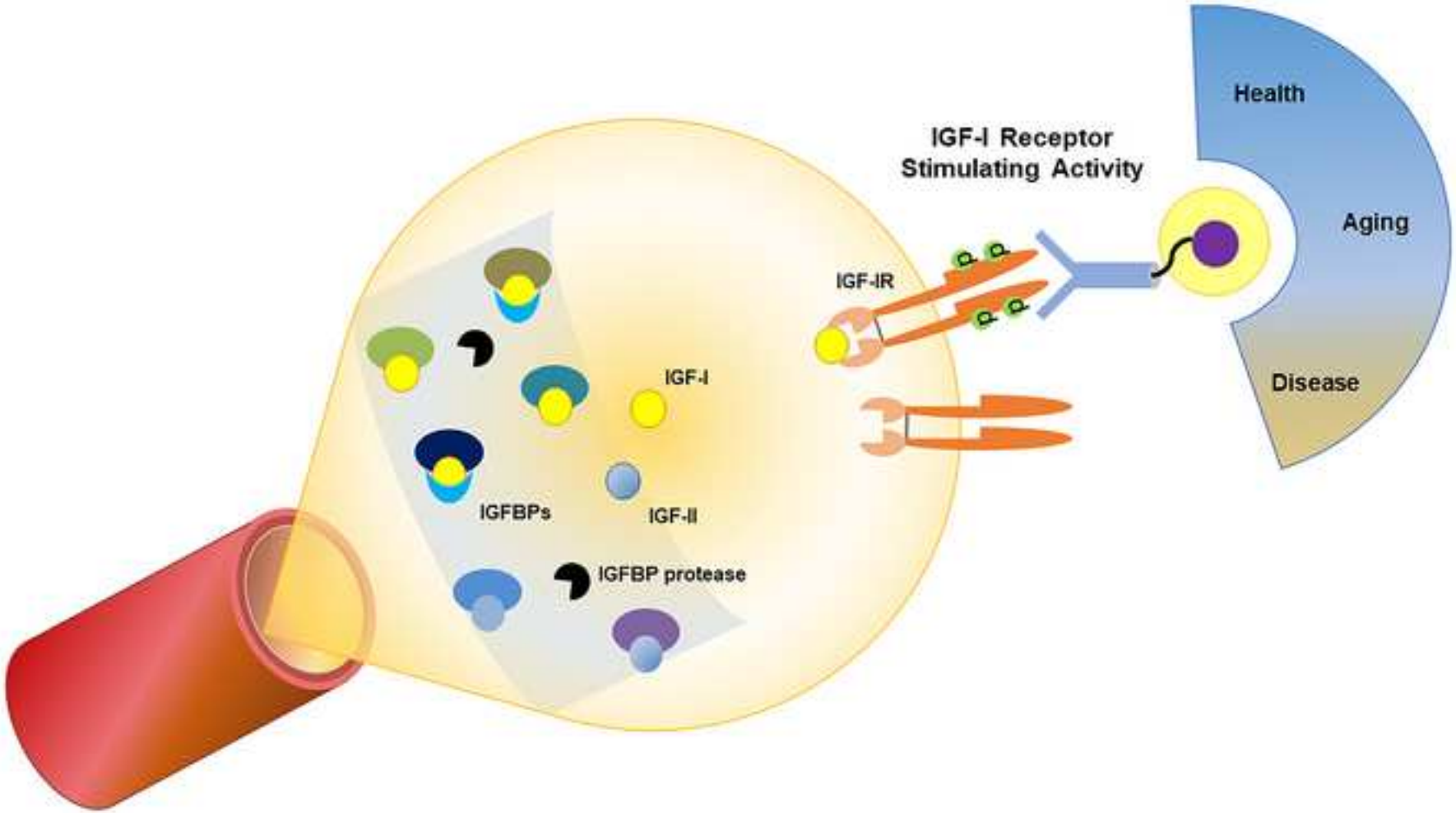


Figure 1

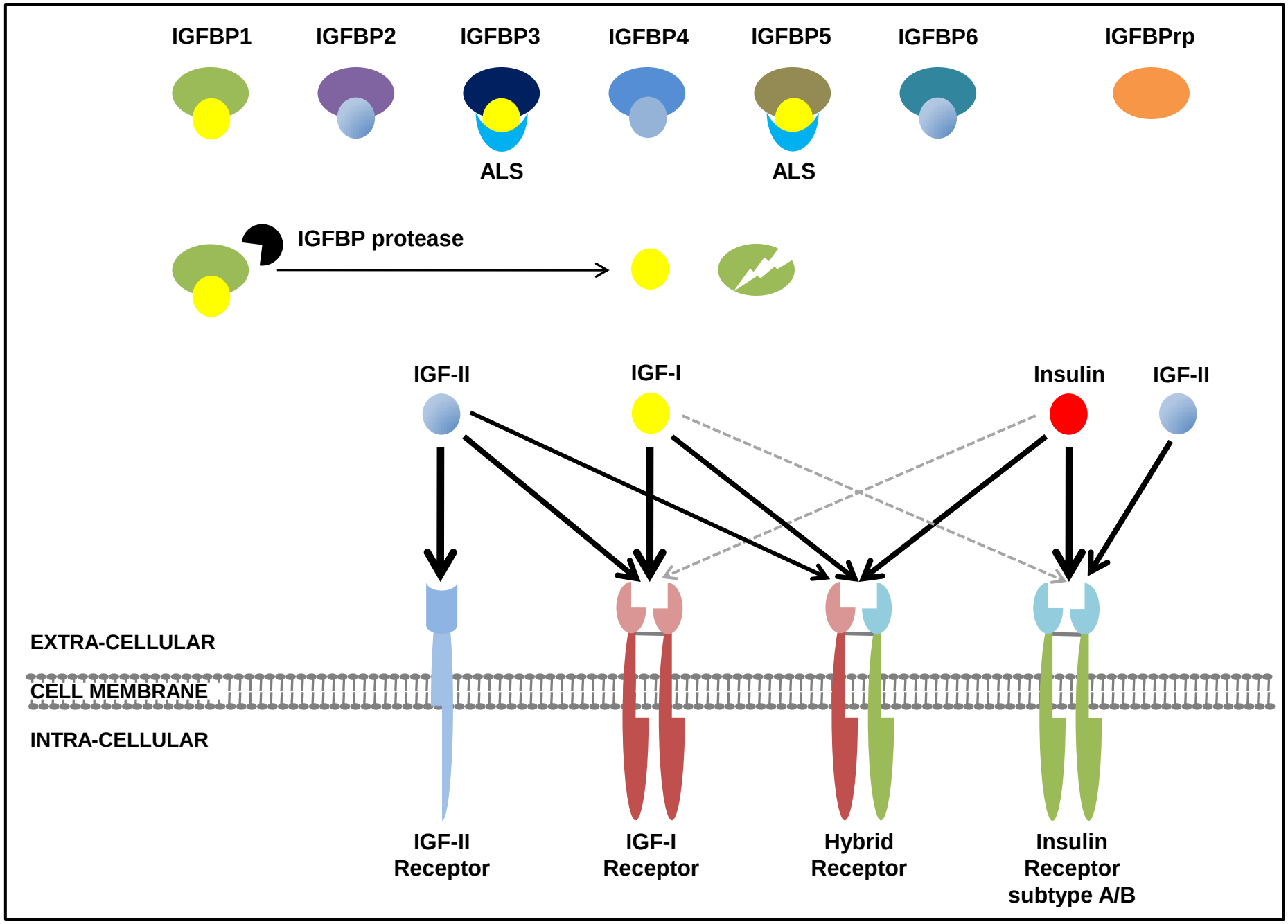

IGFBP1
IGFBP2
IGFBP3
IGFBP4
IGFBP5
IGFBP6
IGFBPrp
ALS
ALS
IGFBP protease
IGF-II
IGF-I
Insulin
IGF-II
EXTRA-CELLULAR
CELL MEMBRANE
INTRA-CELLULAR
IGF-II Receptor
IGF-I Receptor
Hybrid Receptor
Insulin Receptor subtype A/B

Figure 2

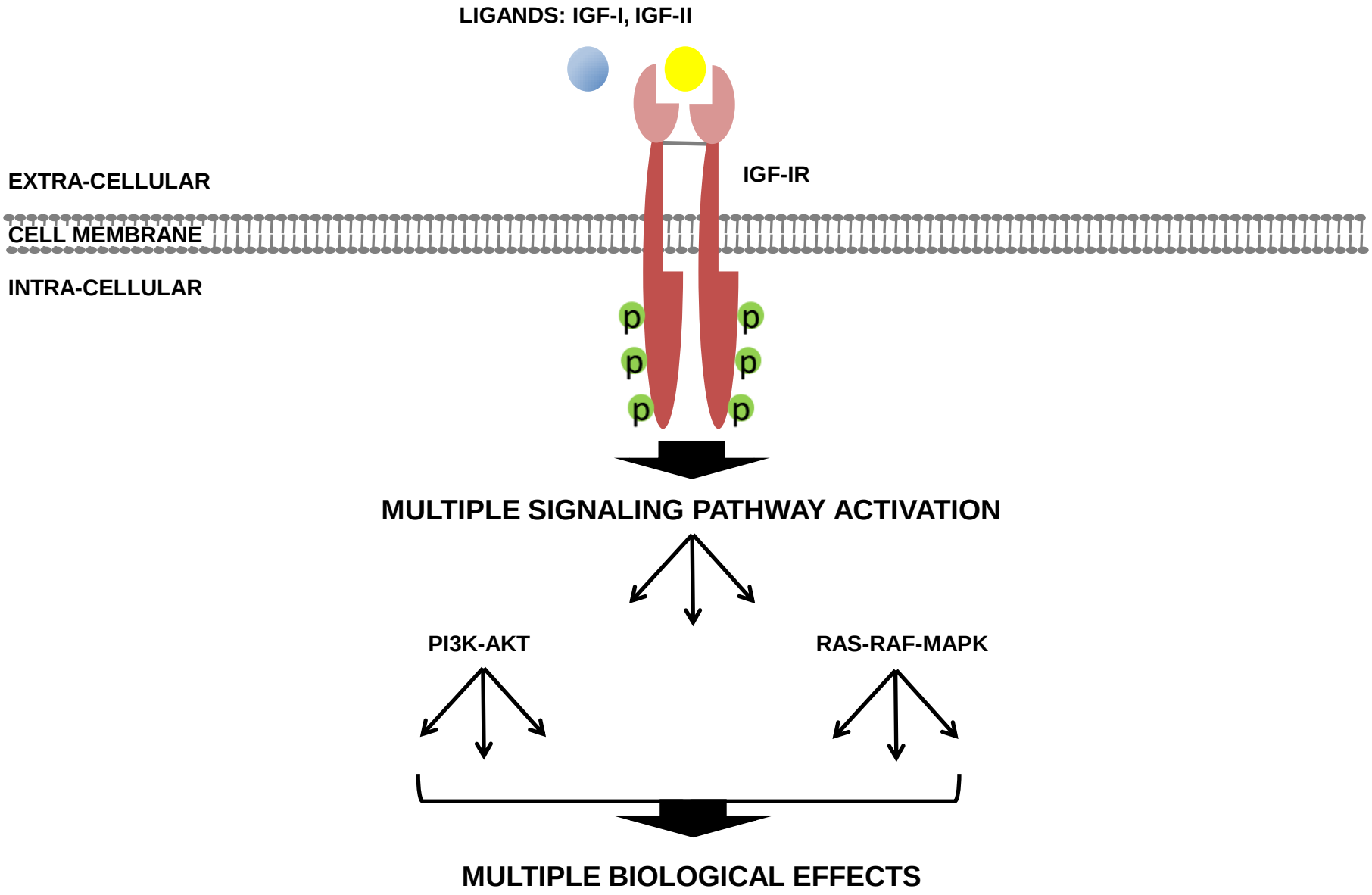
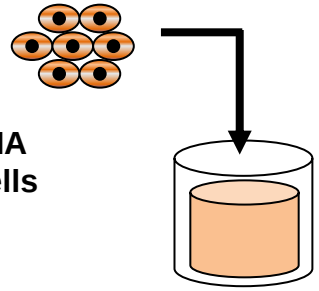

LIGANDS: IGF-I, IGF-II
EXTRA-CELLULAR
IGF-IR
CELL MEMBRANE
INTRA-CELLULAR
p
MULTIPLE SIGNALING PATHWAY ACTIVATION
PI3K-AKT
RAS-RAF-MAPK
MULTIPLE BIOLOGICAL EFFECTS

Figure 3

Scheme of the IGF-I KIRA assay

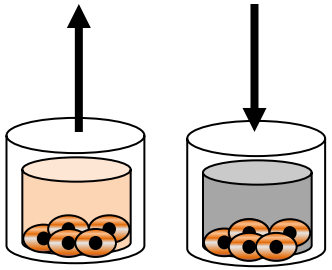
Day 1

293 EBNA
IGF-IR cells



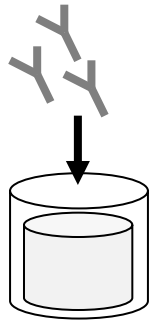
Cell culture in DMEM
with 10% FCS (24 hrs)

Day 2



Switch of culture medium
to DMEM with 0.1% HSA

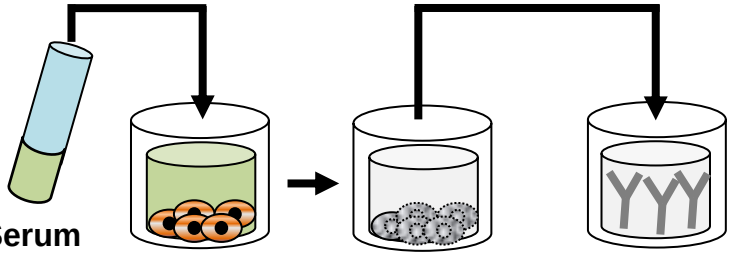
(24 hrs)



IGF-IR capture antibody
coating (2nd plate)

15 min stimulation

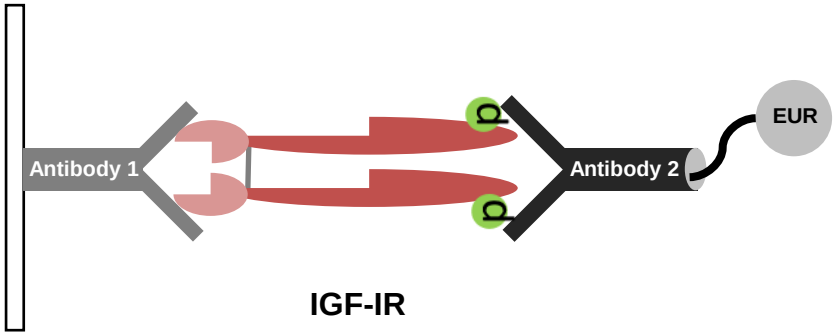
Lysate transfer to
2nd plate



Serum

Cell Lysis

Capturing of IGF-IR followed by detection of
phosphorylated tyrosine residues



IGF-IR

Day 3

Day 4

Figure 4

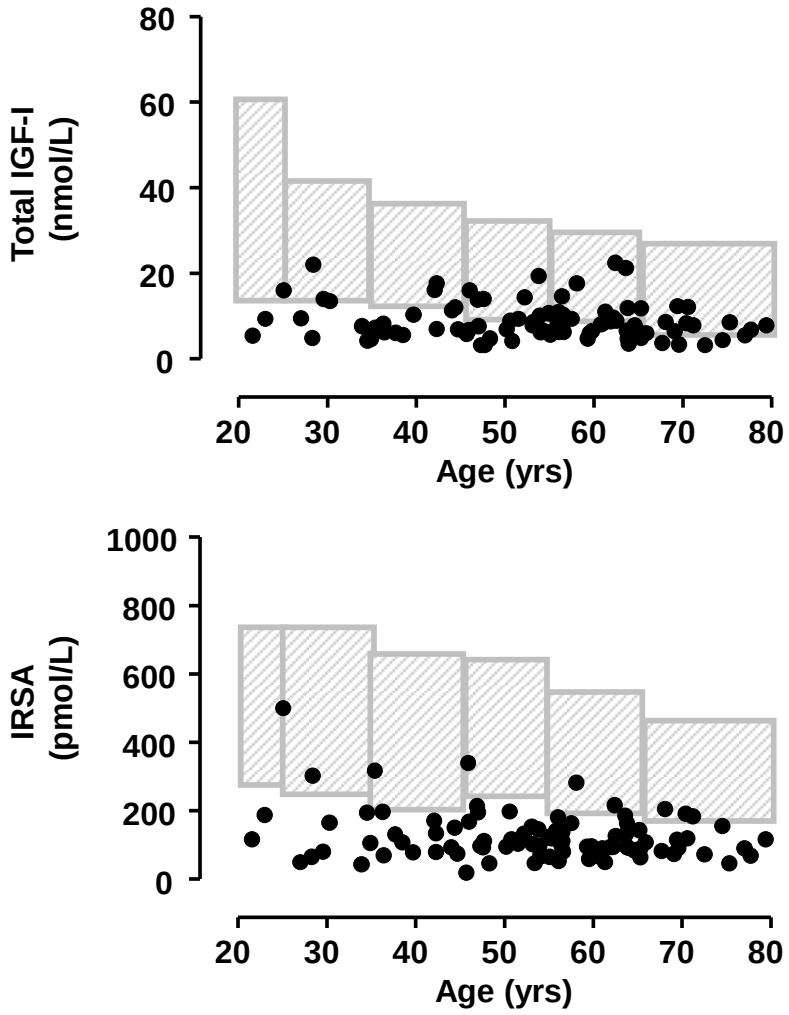

Total IGF-I
(nmol/L)
80
60
40
20
0
20
30
40
50
60
70
80
Age (yrs)
IRSA
(pmol/L)
1000
800
600
400
200
0
20
30
40
50
60
70
80
Age (yrs)

Figure 5

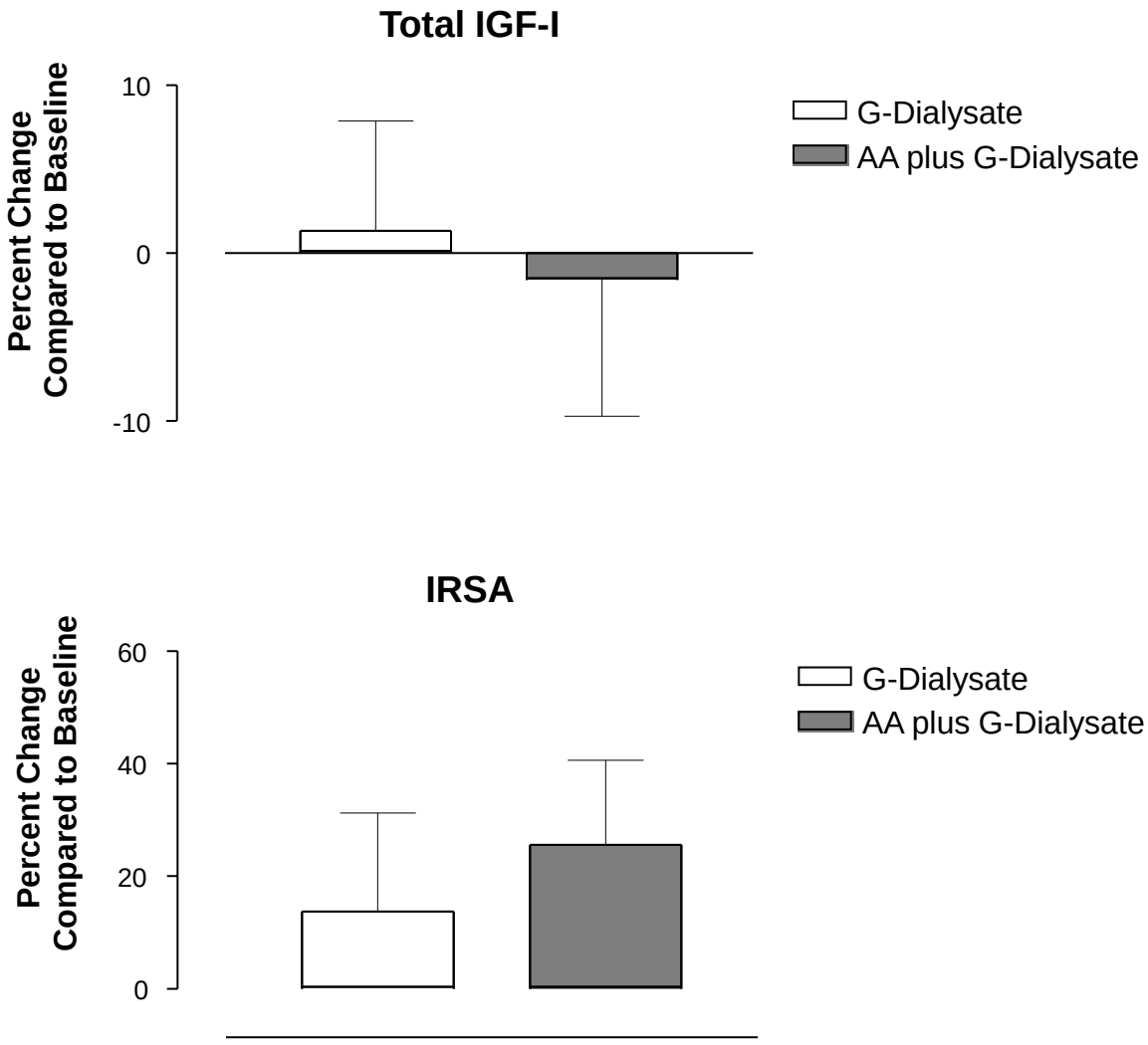

Total IGF-I
Percent Change Compared to Baseline
10
0
-10
G-Dialysate
AA plus G-Dialysate
IRSA
Percent Change Compared to Baseline
60
40
20
0
G-Dialysate
AA plus G-Dialysate

Figure 6

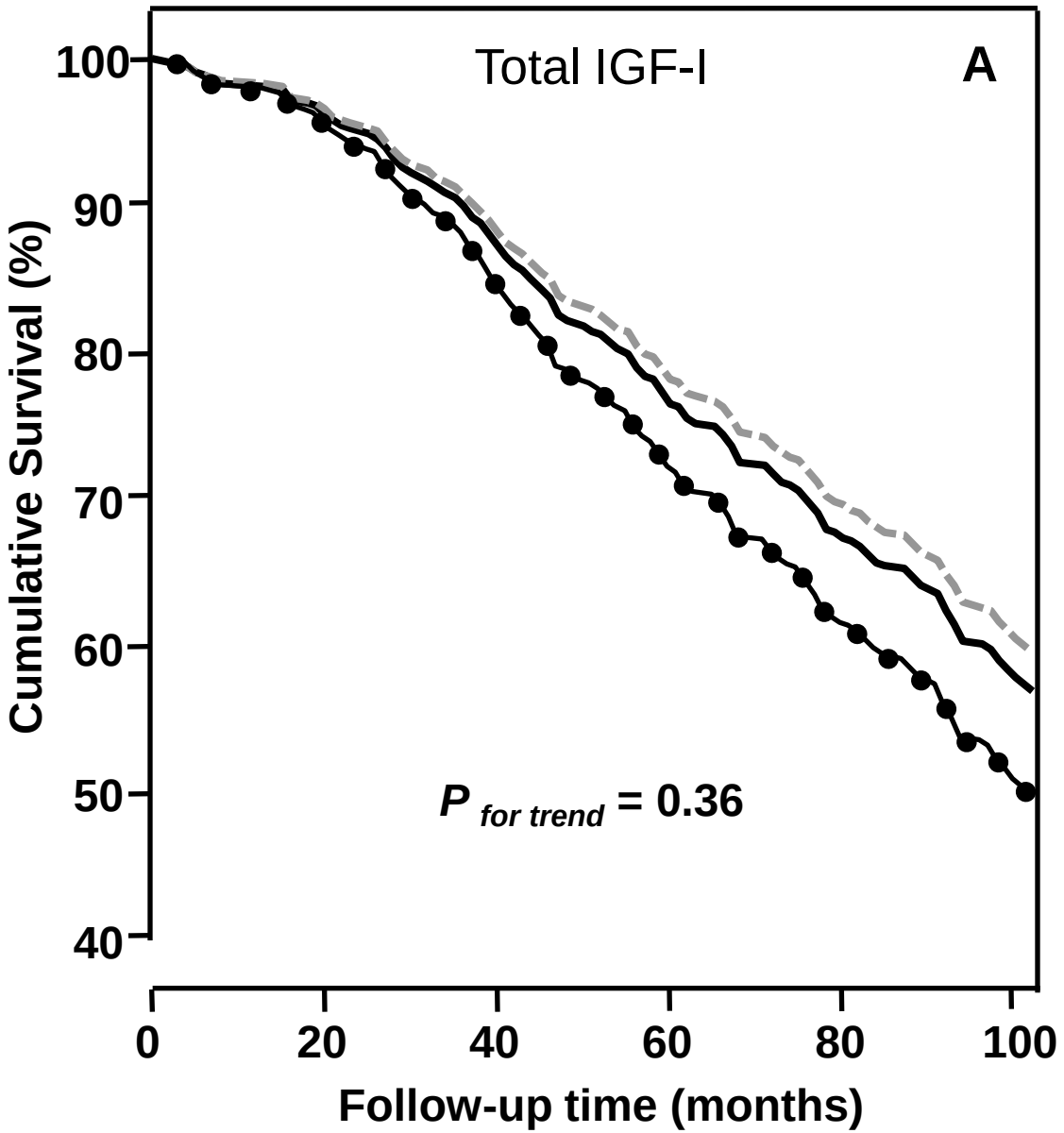


FIG. 6A

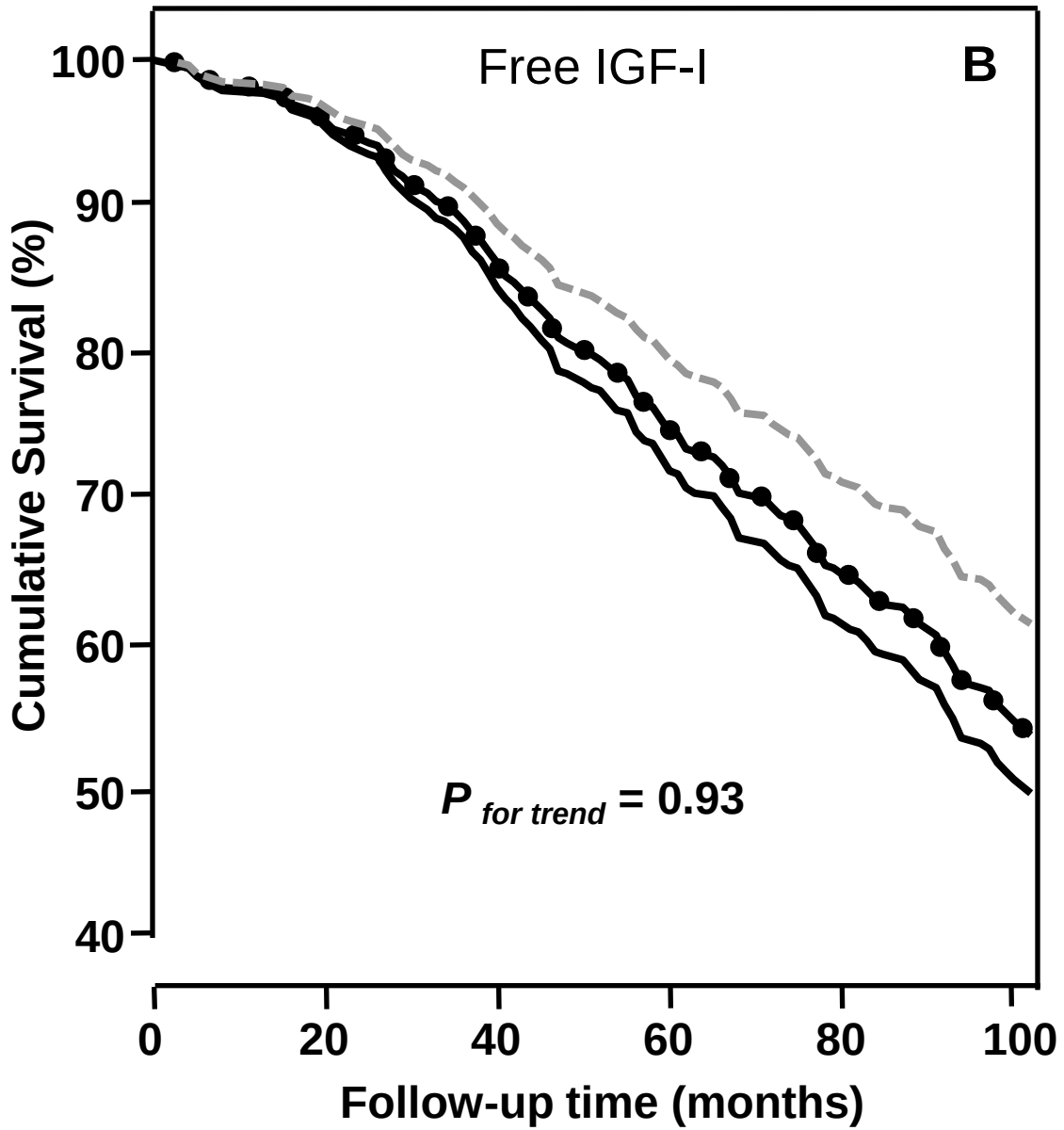

Free IGF-I
B
Cumulative Survival (%)
100
90
80
70
60
50
40
0
20
40
60
80
100
Follow-up time (months)
P for trend = 0.93

FIG. 6B

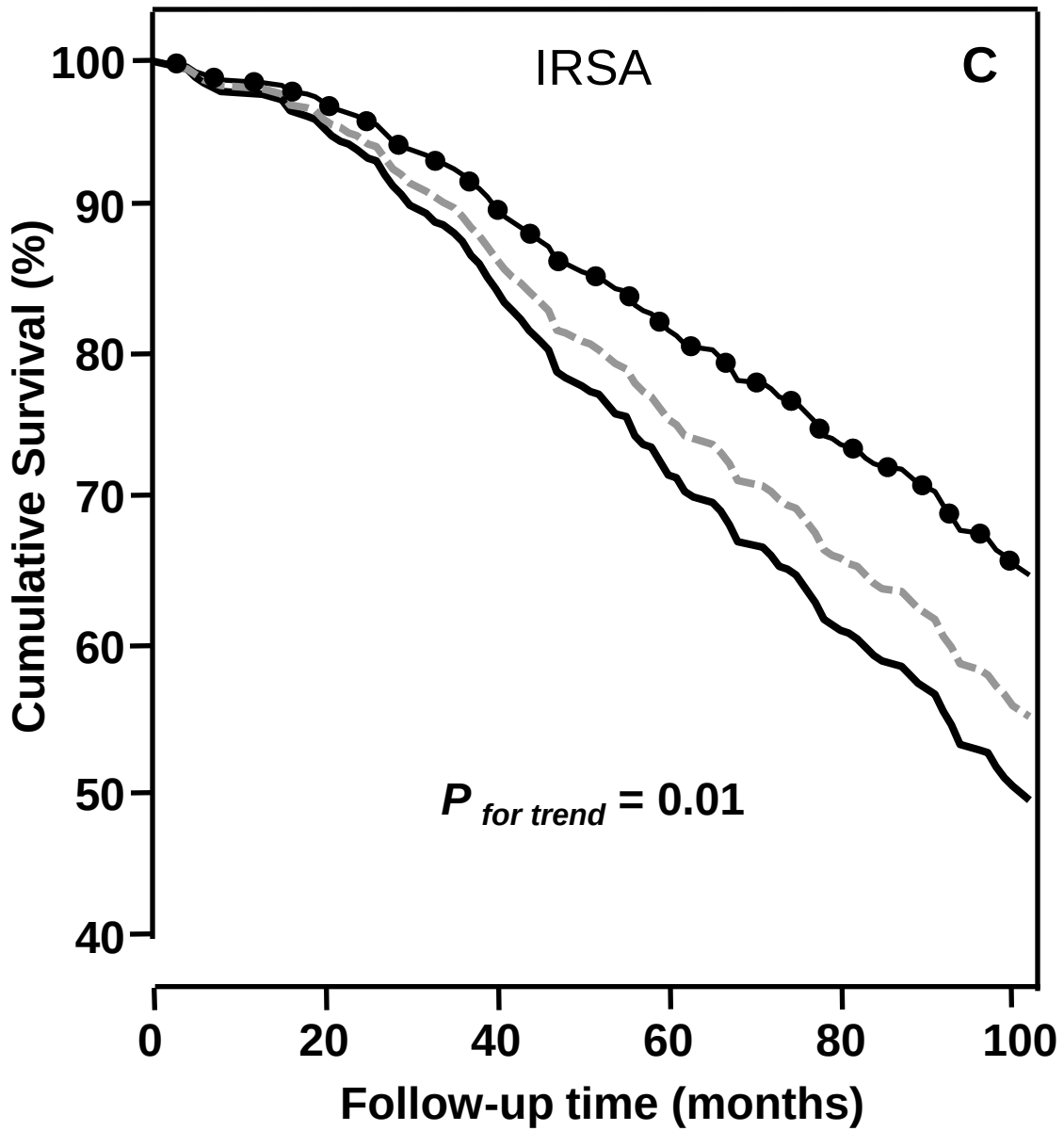

IRSA
C
100
90
80
70
60
50
40
Cumulative Survival (%)
0
20
40
60
80
100
Follow-up time (months)
P for trend = 0.01

FIG. 6C

The Insulin-like Growth Factor-I Receptor Stimulating Activity (IRSA) in Health and Disease

Joseph A.M.J.L. Janssen[1], Aimee J. Varewijck[1], Michael P. Brugts[2]

1.Department of Internal Medicine. Division of Endocrinology, Erasmus MC, Rotterdam, The Netherlands

2.Department of Internal Medicine, Ikazia Hospital, Rotterdam, The Netherlands

The authors have no competing interests to declare

Corresponding author:

J.A.M.J.L. Janssen, MD, Ph.D.

Erasmus MC

Department of Internal Medicine

Dr Molewaterplein 40

3015 GD Rotterdam

The Netherlands

Telephone +31-0650032421 or +31-10-7040704

Email: j.a.m.j.l.janssen@erasmusmc.nl

Abstract

Determination of true IGF-I bioactivity in serum and other biological fluids is still a substantial challenge. The IGF-IR Kinase Receptor Activation assay (IGF-IR KIRA assay) is a novel tool to asses IGF-IR stimulating activity (IRSA) and has opened a new era in studying the IGF system. In this paper we discuss many studies showing that measuring IRSA by the IGF-IR KIRA assay often provides fundamentally different information about the IGF system than the commonly used total IGF-I immunoassays. With the IGF-IR KIRA assay phosphorylation of tyrosine residues of the IGF-IR is used as read out to quantify IRSA in unknown (serum) samples. The IGF-IR KIRA assay gives information about net overall effects of circulating IGF-I, IGF-II, IGFBPs and IGFBP-proteases on IGF-IR activation and seems especially superior to immunoreactive total IGF-I in monitoring therapeutic interventions. Although the IRSA as measured by the IGF-IR KIRA assay probably more closely reflects true bioactive IGF-I than measurements of total IGF-I in serum, the IGF-IR KIRA assay in its current form does not give information about all the post-receptor intracellular events mediated by the IGF-IR. Interestingly, in several conditions in health and disease IRSA measured by the IGF-IR KIRA assay is considerably higher in interstitial fluid and ascites than in serum. This suggests that both the paracrine (local) and endocrine (circulating) IRSA should be measured to get a complete picture about the role of the IGF system in health and disease.

Keywords: IGF-I, IGF-I receptor, IGF-I Bioactivity, KIRA, Immunoassays, Endocrine, Paracrine, Health, Disease

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1.Introduction

Insulin-like growth factor-I (IGF-I) is tightly bound by six high affinity IGF binding proteins (IGFBP-1-6) in the circulation. In healthy subjects, approximately 95% of all circulating total IGF-I is present as a ternary complex formed by IGF-I, IGFBP-3 and acid labile subunit (ALS), making this complex quantitatively the most important, while the remaining IGF-I circulates in a free form (<1%) or as binary complexes (approximately 4–5%) [16]. It is assumed that free IGF-I is the only form of IGF-I which is able to directly stimulate the IGF-IR [38] (See Figure 1 for more details). Activation of the insulin-like growth factor-I receptor (IGF-IR) by free IGF-I stimulates multiple pathways which finally results in multiple biological effects in a variety of tissues and cells (Figure 2).

Determination of true IGF-I bioactivity in serum and other biological fluids still presents substantial challenges. After generation of highly specific antibodies for IGF-I it became possible to develop immunoassays for assessment of circulating IGF-I levels in serum and plasma [29, 61, 86]. To date total IGF-I immunoassays are clinically widely used to assess circulating IGF-I bioactivity in humans and the majority of available literature about IGF-I is based on information obtained by use of immunoassays. As a consequence, immunoassays are very often considered to be the most useful method to assess the amount of circulating IGF-I that is biologically active in the body. However, in the following paragraphs several arguments will be discussed, which will challenge this view.

A major technical problem encountered when measuring circulating IGF-I by immunoassays is interference of IGFBPs. Presence of IGFBPs in a blood sample may significantly disturb reactions between IGF-I and antibodies in the tube and this may result in spurious estimates of the total amount of IGF-I present in that sample. Therefore, most available IGF-I immunoassays use an extraction step to remove all IGFBPs prior to the measurement in order to guarantee full accessibility to IGF-I of highly specific antibodies targeting IGF-I [15]. However, by removing all IGFBPs before measurement of IGF-I (potentially) modulating effects of IGFBPs and IGFBP proteases on IGF-IR stimulating activity (IRSA) are

also eliminated [54]. Thus, as a direct consequence of the just discussed pre-analytical procedure, total IGF-I immunoassays are unable to produce any information about directly modulating effects of IGFBPs or IGFBP-proteases on IRSA.

Moreover, total IGF-I immunoassays determine the immuno-reactive properties of circulating IGF-I-like molecules, rather than the direct (stimulating) effects of these molecules on the IGF-IR [5]. In addition, total IGF-I immunoassays may recognize IGF-I isoforms that are less bioactive and able to stimulate the IGF-IR than wild type IGF-I [5]. Moreover, fragments of IGF-I that lack biological actions, may still harbor epitopes that can be recognized by antibodies targeted to IGF-I and be measured as intact IGF-I by total IGF-I immunoassays [5]. It has been further suggested that altered post-sampling integrity of IGF-I in vitro might contribute to the reported inconsistencies in circulating total IGF-I levels in literature. This latter phenomenon occurs especially under pathologic conditions [38]. Results of total IGF-I immunoassays can be further disturbed by presence of so called heterophilic antibodies in serum which may result in both falsely higher or lower total IGF-I levels [6]. Despite all these limitations total IGF-I immunoassays have become popular in the last 40 years to monitor circulating IRSA in blood samples.

2.The history of measuring IRSA by bioassays

Any intracellular point stimulated by binding of IGF-I to the IGF-I receptor may be utilized for the development of an IGF-I bioassay [58]. In the past a variety of tissues and cells have been used as target organs in bioassays for the determination of IRSA. Salmon and Daughhaday used the incorporation of [^{35}S] sulphate into hypophysectomized rat cartilage [59]. Other bioassays used chicken embryo, weanling or fasted rats or porcine cartilage to assess incorporation of sulphate [32, 72, 84]. In the fat pad bioassay the conversion of [^{14}C] glucose to CO_2 was used to assess IRSA, while in another bioassay

incorporation of tritiated thymidine into DNA of embryonic chicken fibroblasts was used [24, 53]. Although all these traditional IGF bioassays were advantageous in biological relevance, they showed certain failings in their use: lack of sensitivity, precision and specificity; in addition, high variability and long assay duration (3-6 days); comparable phenotypic responses could be the consequence of activation of an alternative receptor (e.g. the insulin receptor) [33, 58]. Results of many of these traditional bioassays were sometimes also influenced by other hormones, which were present in the measured serum samples [33]. For example, it was observed that thyroid hormone (like IGF-I) could stimulate sulphate uptake in to chicken embryo cartilage whereas cortisol was found to inhibit IGF-I-mediated effects in porcine cartilage [20, 25]. Moreover, these traditional IGF bioassays did not detect specifically the IRSA in whole serum, but rather reflected the overall stimulating activity of serum for that target tissue [33].

3.The development of the IGF-I Receptor Kinase Receptor Activation Assay (KIRA)

The IGF-IR KIRA assay was developed by Michael Sadick et al. as an alternative approach for bioassays measuring IGF-IR endpoints [58]. They showed that results obtained with an IGF-IR KIRA assay in MC-7 cells (with endogenous IGF-IR expression) correlated well with classical endpoint bioassays such as a [^{3}H]thymidine incorporation assay [58]. The principle of the IGF-IR KIRA assay is based on measurement and quantification of phosphorylated tyrosine residues of the β-subunit of the IGF-IR (Figure 3). Phosphorylation of tyrosine residues of the β-subunit of the IGF-IR normally starts the intracellular signal cascade after binding of IGF-I to the IGF-IR [13, 58]. The IGF-IR KIRA assay utilizes two separate microtiter plates, one for ligand stimulation of intact cells, and the other for receptor capture and phosphotyrosine ELISA [58] (Figure 3). Results obtained with the IGF-IR KIRA assays are highly reproducible [58]. Since the IGF-IR KIRA assay uses a sample incubation time of 15 minutes, time is too

short for stimulated cells to produce de novo IGFBPs that may interfere with IGF-I action during sample incubation [13]. The IGF-IR KIRA assay makes use of either endogenously expressed IGF-IR receptors or stably transfected IGF-IR receptors with a polypeptide flag (11). Frystyk et al. and Brugts et al. used an IGF-I KIRA assay with human embryonic cells transfected with a copy DNA of the full-length human IGF-IR [7, 13]. By this modification the IGF-IR KIRA assay became even more sensitive than that original KIRA assay described by Sadick et al. Most likely this was due to the higher expression of IGF-IRs after transfection compared to endogenously expressed IGF-IRs [13]. The standard curve of the IGF-I KIRA based on human embryonic kidney (HEK293) cells transfected with a copy DNA of the full-length human IGF-IR started at a concentration of 10 pmol/L (0.08 μg/L) IGF-I [13]. The IGF-IR KIRA assay was found to be specific: insulin, insulin analogs and proinsulin in physiological concentrations had almost no (stimulating) effect on the IGF-IR KIRA signal while IGF-II had a cross-reactivity of 12% [13]. In addition, it had a remarkable low intra- and inter-assay coefficient variation <15%) for a bioassay [7]. It has been further demonstrated that the IGF-IR KIRA is a relatively rapid and reproducible method for assessing IRSA which takes into account modifying effects of IGFBPs on the interaction between IGF-I and the IGF-IR [37].

In the next paragraphs we will give a comprehensive overview of the existing literature which illustrates the clinical significance of measuring IRSA by the IGF-IR KIRA assay.

4. IRSA and age

To date only one study has established age-specific normative values for IRSA as measured by the IGF-IR KIRA assay [7]. In a cross-sectional study circulating IRSA was measured in 400 healthy non-fasting blood donors aged 18-79 yrs. [7]. Circulating IRSA showed a wide inter-individual variability among subjects at every age. Like total IGF-I levels, IRSA decreased significantly with age but the decline of IRSA with age

was less steep than it was observed for circulating total IGF-I levels [7]. Due to the cross-sectional design of this latter study no information about intra-individual changes of IRSA during aging was obtained. Nevertheless the discrepant decline with age between IRSA and total IGF-I suggests that IRSA becomes less growth hormone (GH) dependent with aging than total IGF-I levels [7]. Other potential explanations for this discrepant decline with age between IRSA and total IGF-I could be that the relative increase in IRSA with age compared to total IGF-I reflects a compensatory mechanism to overcome an age-dependent relative IGF-IR resistance or that the relative contribution of IGF-II to IRSA increases with age [7]. In the same study a significant drop in IRSA was observed in women aged 50-60 years which was not observed for total IGF-I [7]. Women at younger ages showed higher lRSA than males but had lower IRSA than the males after the age of 50-60 years. The decrease in estrogen levels around menopause in females might play a role in the observed drop in IRSA after the age of 50-60 years since estrogen is well known to play an important role in regulating activity of the GH/IGF-I axis [45].

IRSA as measured by the IGF-IR KIRA assay was positively related to total IGF-I but the found correlation coefficients were relatively low ($r \approx 0.50$) suggesting that IRSA as measured by the IGF-IR KIRA assay produces basically different information about the IGF-I system than IGF-I immunoassays [7]. However, the physiological importance of this difference is unclear at the moment.

In another smaller cross-sectional study of men and women aged 20-70 yrs IRSA also tended to decrease to a lesser extent than total IGF-I with age [82]. However, in this latter study no significant drop of IRSA around menopause was found in females, which may be related to the lower number of participants included in this latter study [82].

5. IRSA in Health

5.1 **IRSA and Fasting**

In a small study in which in non-obese healthy subjects effects of fasting on GH signaling and action were investigated, GH levels significantly increased after 37.5 h of fasting compared to levels after the overnight fast, while (immuno-reactive) total IGF-I levels were similar under both conditions [50]. In contrast, IRSA measured by the IGF-IR KIRA assay was significantly lower after 37.5 h of fasting compared to results following the overnight fast, whereas IGFBP-1 was significantly increased [50]. These findings are in line with Chen et al. who previously reported that after prolonged fasting reductions of total IGF-I were preceded by reductions in IRSA and free IGF-I and a simultaneous increase of IGFBP-1 levels [12]. Thus this time course suggests that the decline in IRSA was causally linked to the increase in IGFBP-1 [50]. In this latter study it was also found that IRSA compared to ultra-filtered free IGF-I was relatively less affected by fasting and it was hypothesized that this latter finding could be explained by the fact that in contrast to the ultrafiltration method the IGF-IR KIRA assay was able to detect the concomitant increase in IGFBP-1-complexed-IGF-I [12].

5.2. **IRSA and Life style factors**

When in a cohort of young women recruited from a local college campus the relationships between IRSA (measured by the IGF-IR KIRA assay) and life style factors were studied, IRSA was negatively associated with age, body fat percentage and habitual alcohol intake and positively associated with estradiol, progesterone and selenium intake [43]. In multivariate analysis only 61% of the variation in IRSA could be attributed to circulating concentrations of immunoreactive total and free IGF-I and IGFBP-1, IGFBP-2 and IGFBP-3 [43]. It was concluded that further research is needed to better understand the biological mechanisms responsible and the consequences for the reported associations [43].

5.3 **IRSA and Exercise**

In healthy men circulating concentrations of IRSA (measured by the IGF-IR KIRA assay), and immunoreactive free IGF-I, total IGF-I and total IGF-II did not change after single 30 seconds sprints, despite an increase in GH concentrations [66]. Thus, a short sprint exercise may stimulate GH secretion but does not change IRSA nor IGF concentrations [66].

During a submaximal exercise (45 minutes of cycle ergometer at the lactate threshold) GH administration to adults with GH deficiency (GHD) induced minor changes in IGFBP-1, IGFBP-2 and IGFBP-3 without affecting IRSA, IGF-I, IGF-II or IGFBP-3 proteolysis [42]. Thus administration of GH to adults with GHD did not result in changes of IRSA during submaximal exercise [42].

After 8 weeks of resistance, aerobic and combined exercise training both circulating IRSA and immuno-reactive IGF-I remained stable in young healthy women despite a significant improvement in aerobic fitness, lean mass and upper and lower body strength [52].

Taken together, all these findings suggest no significant changes of IRSA during acute and chronic exercise.

5.4 **Effects of Insulin on IRSA**

During an hyperinsulinemic euglycemic clamp circulating IRSA (measured by the IGF-IR KIRA assay) acutely decreased both in controls and subjects with impaired glucose tolerance, whereas simultaneously no changes in immunoreactive total IGF-I or IGF-II were observed [1]. Similarly, IGFBP-1 levels significantly decreased in both groups, whereas no changes were seen in IGFBP-3, while GH and IGFBP-2 levels significantly increased [1]. The acute insulin-induced reduction of IRSA during the clamp

occurred despite reduction in IGFBP-1 levels, and therefore reduction of IRSA during the clamp was explained by the concomitant increase of circulating IGFBP-2 concentrations, while the observed increase in GH concentrations during the clamp most likely were due to decreased negative feedback of circulating IRSA [1].

In contrast to the observed acute insulin-mediated decrease of IRSA, chronic hyperinsulinemia did not reduce circulating IRSA, which was explained by the reduction of both IGFBP-1 and IGFBP-2 levels during long-term exposure to high insulin levels [1].

5.5 **Effects of Glucagon on IRSA**

Intramuscular glucagon administration to lean subjects, obese subjects and patients with type 1 diabetes mellitus decreased circulating IRSA (measured by the IGF-IR KIRA assay) in all three groups despite no changes were observed in circulating immunoreactive total IGF-I and IGFBP-3 levels [60]. Since the reduction in IRSA occurred before the glucagon-induced surge in GH, decreased negative feedback by IRSA provides a mechanism for the known increase in GH secretion after administration of glucagon [60]. The authors hypothesized that the decrease in IRSA after glucagon administration was related to an increase in circulating IGFBP-1 and IGFBP-2 levels which, in turn, most likely was mediated via a glucagon-mediated activation of the FOXO/mTOR pathway [60].

5.6 **Effects of GLP-1 on IRSA**

Short-term infusions of glucagon-like peptide-1 (GLP-1) in healthy subjects tended to increase IRSA (measured by the IGF-IR KIRA assay) and reduced IGFBP-1 levels [64]. Therefore it was suggested that IRSA in this study increased secondary to suppression of IGFBP-1 concentrations [64].

5.7 **IRSA in Serum vs. Interstitial Fluid**

With the suction blister technique IRSA (measured by the IGF-IR KIRA assay) was 41 % higher in interstitial fluid than IRSA in serum [22]. It was suggested that this was related to an increased enzymatic IGFBP-proteolysis and an altered composition of IGFBPs in interstitial fluid. As a consequence larger fractions of IGF-I and IGF-II were free to bind to the IGF-IR [22]. In contrast, immuno-reactive total IGF-I and IGF-II levels and IGF-binding proteins (IGFBPs) levels were approximately 50% lower in interstitial fluid than in serum [22]. Thus this study suggested that IRSA may be higher at the tissue level than in the circulation.

5.8 **Effects of Prednisolone on IRSA**

Prednisolone treatment (5mg per day during 1 week) to children with severe asthma significantly reduced IRSA (measured by the IGF-IR KIRA assay) by 12% compared to placebo, while no changes were observed for immunoreactive total IGF-I, free IGF-I, IGFBP-3, IGFBP-2 IGFBP-1 and IGFBP-1-bound IGF-I [28]. Prednisolone had no direct effects on IGF-IR phosphorylation. It was concluded that treatment with glucocorticoids induces a circulating substance that is able to inhibit IGF-IR activation in vitro without affecting circulating total or free IGF-I [28]. At present the nature of this substance is not identified [28]. Interestingly, more than twenty years ago existence of a circulating inhibitor of the IGF-IR induced by systemic glucocorticoid treatment was already hypothesized when IRSA was assessed by so called end-point bioassays that measured incorporation of radiolabeled sulfate or thymidine into cultures of porcine cartilage [48, 71].

In contrast to the findings in the study just discussed, both circulating IRSA and total IGF-I steadily

increased compared to placebo when men received prednisolone in high doses (37.5 mg per day for 5 days) [55]. Although prednisolone increased circulating IRSA above placebo levels, this was not translated into higher levels of IRSA in interstitial fluid (collected by the suction blister technique) [55]. Thus, short-term prednisolone administration in high doses appears to exert distinct, compartment-specific effects on IRSA. The authors hypothesized that the observed increase in circulating IRSA (and total IGF-I) after prednisolone was most likely secondary to a prednisolone–mediated increase of insulin receptor resistance and IGF-IR resistance [55]. Serum obtained from participants after high dose prednisolone treatment showed reduced ability to phosphorylate IRS-1, Akt and mTOR in IGF-IR – transfected cells compared to serum after placebo, suggesting that prednisolone treatment in this high dose induced IGF-IR resistance by impeding post-IGF-IR signaling [55]. These results further support the hypothesis that glucocorticoids in high doses primarily impair anabolic actions of IGF-I by suppressing the post-receptor pathways of the IGF-IR rather than by suppressing circulating IRSA [55].

5.9 **Effects of Raloxifene and Estrogen on IRSA**

While the selective estrogen receptor modulator raloxifene and estrogen suppressed circulating immunoreactive total IGF-I equally in growth hormone deficiency (GHD) and growth hormone (GH)-replaced hypopituitary women, neither raloxifene nor estrogen affected circulating IRSA (measured by the IGF-IR KIRA assay), while reduction of the total IGF-I: IGFBP-3 ratio, considered by many people as a proxy for bioavailable IGF-I, was significantly greater during raloxifene treatment [4]. Treatment with GH significantly increased IRSA but this effect was attenuated by co-treatment with raloxifene and estrogen [4]. In addition, proportion of IRSA to total IGF-I was unaffected by any of these treatments [4]. Since during GH therapy of hypopituitary women co-treatment with raloxifene led to a smaller gain in lean body mass than GH co-treatment with estrogen, the authors concluded that the observed different

effects on lean body mass between raloxifene and estrogen treatments could not be explained by differences in IRSA [4].

6. IRSA in Disease States

6.1 IRSA and GHD

Before start of GH treatment IRSA (measured by the IGF-IR KIRA assay) in adult patients with proven GHD was more frequently below the normal range (<-2 SD) than immunoreactive total IGF-I levels (81.9 vs. 61.7%, respectively) and this was especially observed in patients older than 40 years of age [79]. After start of GH treatment both IRSA and total IGF-I significantly increased but changes in IRSA did not parallel changes in total IGF-I [80]. After 12 months of GH treatment total IGF-I normalized in 81% of patients, whereas in only 50% of patients the IRSA was in the normal reference range [80]. In addition, IRSA remained below normal in more than 40% of patients in whom total IGF-I had normalized [80]. Interestingly, the increase of the IGF-I/IGFBP3 ratio (which has been suggested to reflect an estimate of bioavailable IGF-I) after 12 months GH treatment was almost similar to the reported increase of IRSA [80].

IRSA was found to be positively related to QOL as assessed by the disease-specific Question on Life Satisfaction Hypopituitarism (QLS-H) module, whereas total IGF-I was not. These findings suggest that IRSA may be a more sensitive marker for changes in QOL during GH treatment of adult patients with GHD [78]. An interesting follow-up study would be to assess the use of IRSA for GH dose titration during GH treatment of adult patients with GHD.

Seventy-two hours after administration of a single high dose of the GH receptor blocker pegvisomant to

untreated patients with GHD circulating IRSA (measured by the IGF-IR KIRA assay) significantly decreased by 14% and immunoreactive total IGF-I by 23 % compared to baseline whereas basal GH levels increased, and IGF-II, IGFBP-1, IGFBP-2 and IGFBP-3 levels did not alter [85]. Nonetheless, a strong positive correlation between pegvisomant levels and circulating IGFBP-1 and IGFBP-2 levels was observed, suggesting that the modulatory effects of pegvisomant on IRSA were mediated in a dose-dependent manner by concomitant increasing levels of IGFBP-1 and IGFBP-2 [62, 85].

6.2 **IRSA and IUGR**

Cord blood immunoreactive total IGF-I and total IGF-II, and IRSA (measured by the IGF-IR KIRA assay), were lower in neonates born with intrauterine growth restriction (IUGR) than in neonates born appropriate for gestational age (AGA) [70]. IGFBP-1 concentrations were higher in IUGR neonates than in AGA neonates [70]. As IGFBP-1 is an important regulator of IRSA this may partly explain why levels of IRSA were suppressed in IUGR neonates: higher IGFBP-1 may sequester circulating IGF-I and thereby reduce IRSA [70].

6.3 **IRSA and Acromegaly**

In a small study of newly diagnosed patients with active acromegaly (based on clinical presentation, unsuppressed GH levels during an OGTT, and elevated age-matched immune-reactive total IGF-I levels) IRSA (measured by the IGF-IR KIRA assay) was within the reference range in a considerable number of patients [81]. In this study, the R^2 value was 0.70 suggesting that 30% of the variation in IRSA could not be explained by levels of total IGF-I, demonstrating that IRSA is only partly dependent on total IGF-I [81]. In addition, the mean percentage of IRSA over total IGF-I was 0.81% in subjects with active acromegaly

indicating that the IGF-IR KIRA assay provided fundamentally different information about the circulating IGF-I system than IGF immunoassays [81]. Age-adjusted soluble Klotho levels were significantly related to IRSA and it was hypothesized that elevated soluble Klotho levels may directly have reduced IRSA. Moreover, in this study IRSA was more strongly related to physical measures of QoL than total IGF-I, suggesting that IRSA may better reflect physical limitations perceived in active acromegaly [81].
In an another study among active acromegalics circulating IRSA (measured by the IGF-IR KIRA assay) decreased significantly during treatment with pegvisomant as well as with combination treatment with a somatostatin analog and pegvisomant. However, there were no significant differences in the changes of IRSA between both treatment regimens [41]. Moreover, immunoreactive total and free IGF-I showed comparable results as obtained by IRSA [41].

6.4 **IRSA and PAPP-A2**

The metalloproteinase pregnancy-associated plasma protein A2 (PAPP-A2) has been hypothesized to increase IGF-I bioactivity by specific cleavage of IGFBP-3 and IGFBP-5 [2]. Recently two unrelated families have been described from whom family members presented with progressive postnatal growth failure, microcephaly, thin long bones and decreased bone density [2]. In the blood markedly elevated circulating concentrations of immunoreactive total IGF-I, IGF-II, IGFBP-3, IGFBP-5 and ALS were measured. Size-exclusion chromatography showed a significant increase of IGF-I bound in its ternary complex [17]. Spontaneous GH secretion was also markedly elevated [2]. In both families loss-of-function mutations in the PAPP-A2 gene were found which resulted in undetectable PAPP-A2 activity [17]. When circulating IRSA was measured by the IGF-IR KIRA assay, IRSA was low and therefore it was hypothesized that low IRSA was responsible for the observed poor growth [2]. In favor of this latter hypothesis, short-term treatment with recombinant human IGF-I (rhIGF-I) increased IRSA and this was

accompanied by improved growth and height in young patients with these PAPP-A2 mutations [51]. In addition, during rhIGF-I treatment spontaneous GH secretion decreased while circulating total IGF-I and IGFBP-3 levels remained elevated [51]. The decline in spontaneous GH secretion most likely resulted from a restored negative feedback as a consequence of the rise in circulating IRSA after rhIGF-I treatment [51].

6.5 **IRSA in Turner patients**

To overcome the retarded growth of Turner patients it has been reported that very high doses of GH are needed [73]. In untreated adult patients with Turner Syndrome IRSA (measured by the IGF-IR KIRA assay) was found to be decreased [31]. This latter result was found despite the presence of normal immuno-reactive levels of total IGF-I, IGFBP-1, -2 and -3 and Acid Labile Subunit (ALS) [31]. However, Western ligand blots of IGFBP-1 and-2, as well as IGFBP-4 in this study population showed signs of extensive proteolysis while the IGFBP-3 ternary complex was significantly reduced [31]. It therefore was speculated that the decreased circulating IRSA, may play a role in the reduced action of GH in Turner syndrome [31].

6.6 **IRSA and Anorexia Nervosa**

In malnourished patients with anorexia nervosa circulating IRSA (measured by the IGF-IR KIRA assay) , total IGF-I (immuno-reactive) and free IGF-I (ultra-filtered) were significantly decreased and IGFBP-1 levels were highly increased [67]. During refeeding, a significant increase in circulating IRSA, total IGF-I and free IGF-I was observed, while BMI also increased [67]. The circulating IRSA and total IGF-I showed a correlation coefficient of 0.59 suggesting that in anorexia nervosa patients 60% of variation in IRSA

could not be explained by levels of immunoreactive total IGF-I, thus again demonstrating that IRSA is only partly dependent on total IGF-I [67].

6.7 **IRSA and Obesity**

Despite low GH secretion and decreased IGFBP-1, 24h mean circulating IRSA (measured by the IGF-IR KIRA assay) was not decreased in obese women [26]. In addition, IRSA did not correlate with BMI and IGFBP-1 [26]. Therefore it was concluded that these findings argue against elevated IRSA as the mechanism underlying reduced GH secretion in obesity by an augmented negative feedback. In another cross-sectional placebo-controlled study GH administration during 6 months to overweight/obese women resulted in an increase of both circulating immunoreactive total IGF-I and IRSA (measured by the IGF-IR KIRA assay) [19]. Interestingly in this latter study the increase in IRSA rather than the increase in total IGF-I predicted the GH-related increase in lean mass and decrease in total adipose tissue/BMI [19].

6.8 **IRSA and the Metabolic Syndrome**

In a cross-sectional study embedded in a random sample of over 1000 elderly subjects from the Rotterdam Study, a prospective population-based cohort study, a progressive rise in circulating IRSA (measured by the IGF-IR KIRA assay) was found with increasing insulin resistance as long as fasting blood glucose levels were within the normal range [10]. However, as soon impaired fasting blood glucose were present, circulating IRSA peaked and reached a plateau. Finally when blood glucose levels further increased and individuals could be classified as having diabetes, circulating IRSA progressively decreased [10]. In addition, IRSA peaked when three criteria of the metabolic syndrome were present and then declined significantly when five criteria of the metabolic syndrome were present suggesting an

inverse U-shaped relationship between IRSA and number of components of the metabolic syndrome [10]. This latter finding contrasts with previous results reporting an inverse relationship between the (immunoreactive) total IGF-I/IGFBP-3 ratio and components of the metabolic syndrome [10, 63].

6.9 **IRSA and Type 1 Diabetes**

Irrespective of pubertal status children and adolescents with type 1 diabetes showed lower IRSA (measured by the IGF-IR KIRA assay) and immunoreactive total IGF-I, but higher IGFBP-1 than healthy controls [65]. Suppression of IRSA was relatively more pronounced than total IGF-I and this latter finding was explained by the more concomitant increase of IGFBP-1 inhibiting IGF-I actions [65].

When comparing patients with and without residual β-cell function IRSA and IGF-II as well as IGFBP-3 were significantly higher in prepubertal patients with residual β-cell function, supporting the hypothesis that the portal supply of insulin to the liver is an important regulator of the activity of the GH-IGF axis, at least in prepubertal children, since such relation was absent in pubertal patients [65].

Insulin plays an important role in the regulation of the GH-IGF-I axis. When comparing the GH-IGF-I axis response after a single dose human NPH insulin, insulin detemir and insulin glargine in type 1 diabetes patients, it was found that independent of the actual plasma glucose levels, IRSA (measured by the IGF-IR KIRA assay) was higher and IGFBP-1 lower after insulin detemir than after NPH insulin and glargine administration, thereby explaining the lower GH levels [46]. By contrast, immunoreactive total IGF-I, IGFBP-2 and IGFBP-3 were comparable after administration of these three different insulins [46]. Since it is thought that the combination of a reduced GH secretion and an increased IRSA may have beneficial metabolic effects in type 1 diabetes, this study suggested that in this respect insulin detemir compared to NPH insulin and glargine is superior [46].

Ma et al. showed in type 1 diabetes patients that IRSA was more sensitive to short-term changes in

insulin exposure than total IGF-I, although the physiological significance of this observation has to be determined [47]. In this latter study again a strong inverse relationship between IRSA and circulating IGFBP-1 levels was found [47]. Moreover, despite distinct glucose-lowering properties, equal doses of human insulin, insulin aspart and two biphasic aspart preparations (BIAsp50 and BIAspo70) had similar effects on IRSA [47].

Hedman et al. studying in type 1 diabetes whether the route of insulin administration affected circulating IRSA (measured by the IGF-IR KIRA assay) found that continuous intraperitoneal insulin infusion (CIPII) induced higher circulating IRSA, but also higher circulating (immune-reactive) total IGF-I and IGF-II levels and lower IGFBP-1 than subcutaneous insulin administration [34]. This again supports the hypothesis that the route if insulin administration is important for the activity of the IGF system [34]. A low endogenous circulating IRSA is likely to augment the secretion of growth hormone, which may lead to insulin resistance and finally in an increased risk of late diabetic complications [39]. The observed higher circulating IRSA after intraperitoneal insulin administration suggests that CIPII treatment in type 1 diabetes patients is better correcting alterations of the IGF system than subcutaneous insulin administration [34].

6.10 **IRSA and Type 2 Diabetes**

Varewijck et al. found that IRSA (measured by the IGF-IR KIRA assay) was borderline significantly lower in patients with type 2 diabetes on metformin than in non-diabetic controls, while immunoreactive total IGF-I concentrations were significantly lower in patients with type 2 diabetes than in non-diabetic participants [77]. After 36 weeks of insulin therapy IRSA significantly decreased in patients with type 2 diabetes, while serum total IGF-I concentrations remained unchanged during this period [77]. The observed decline in IRSA after 36 weeks insulin therapy is in line with a study discussed above, which

showed that hyperinsulinemia suppressed IRSA, whereas total serum IGF-I did not change [1]. In this latter study it was concluded that insulin decreased IRSA through differential modulation of IGFBPs: insulin suppressed IGFBP-4 and IGFBP-1 and increased IGFBP-2 concentrations [1].

Prior to bariatric surgery in severe obese type 2 diabetic patients IRSA (measured by the IGF-IR KIRA assay) was significantly elevated, while total IGF-I was not increased [11]. After bariatric surgery IRSA only slightly increased at 3 months and was unchanged at 12 months, while simultaneously there were no changes in total IGF-I and total IGF-II [11]. In addition, IGFBP-1 significantly increased and IGFBP-3 significantly decreased and these changes continued up to 12 months [11]. The biological importance of these findings is unclear at the moment.

6.11 **Effects of Intensive Insulin Therapy on IRSA in the Intensive Care**

Upon admission at the intensive care unit immunoreactive total IGF-I levels and IRSA (measured by the IGF-IR KIRA assay) were lower and GH levels were elevated in critically ill children compared with a healthy reference population [30]. In this respect there were no differences between children randomized to conventional insulin therapy (CIT) and to intensive insulin therapy (IIT) [30]. At day 3 of admission ITT decreased IRSA compared to CIT, while total IGF-I levels were similar when comparing both treatment arms [30]. In addition, compared to CIT, at day 3 of admission IGFBP-3 and ALS levels were decreased and IGFBP-1 levels were increased in the IIT group [30]. According the authors the decreased IRSA in the IIT group may point to aggravated GH resistance [30]. A second possible explanation for the decreased circulating IRSA may be that the IIT suppressed endogenous portal insulin levels stronger than CIT and this may have led a to decreased hepatic IGF-I production, which has resulted in a reduced IRSA [30]. The long-term functional consequences of ITT on the changes in the IGF-I system are unclear at present and should be further investigated.

6.12 **Effects of Insulin on IRSA in Very Low Birth Weight Infants**

In a small randomized controlled study intravenous insulin administration to very low birth weight infants throughout the first week of life improved glucose control and increased IRSA (measured by the IGF-IR KIRA assay) compared with standard care [3]. There were trends toward faster growth in leg length and increased weight gain in the infants treated with insulin (and higher IRSA) compared with the standard care group [3]. During the 7-day study period, there were no significant differences in circulating (immuno-reactive) total IGF-I levels between the infants treated with insulin and those receiving standard care [3]. Therefore it was concluded that early insulin therapy increased IRSA and improved blood glucose control and this could be contributing to less morbidity among very low birth weight infants [3].

6.13 **IRSA and Cushing Disease**

Untreated Cushing disease was characterized by normal circulating IRSA (measured by the IGF-IR KIRA assay) and immunoreactive total IGF-I levels [74]. Treatment of patients with an active Cushing Disease with a low dose of the somatostatin analog pasireotide (which binds with high affinity to somatostatin receptors subtypes 1-3 and 5) during 28 days reduced cortisol production and normalized urinary free cortisol in 29% [74]. During treatment of pasireotide Z-scores for IRSA and total IGF-I decreased significantly to values < -2 SD in 43% and 35%, respectively, suggesting the induction of growth hormone deficiency de novo [74].

6.14 **IRSA and Graves Ophthalmopathy**

In subjects diagnosed with Graves Ophthalmopathy values for IRSA (measured by the IGF-IR KIRA assay) were found to be low normal (Z-score: -1.5 SD ± 0.1 SD) whereas immunoreactive total IGF-I was normal (Z-score: 0.6 ± 0.2 SD) [75]. In line with these findings it was reported more than twenty years ago that IRSA was markedly reduced in thyreotoxicosis when IRSA was assessed by measuring incorporation of radiolabeled sulfate into cultures of porcine cartilage [49].

6.15 **IRSA and Kidney Disease**

Patients with end-stage renal disease showed elevated GH, high normal circulating immunoreactive total IGF-I and subnormal IRSA (measured by the IGF-IR KIRA assay) compared to controls [27]. After 7 days treatment with recombinant GH IRSA tended to be lower in patients with end-stage renal disease than controls while total IGF-I increased to the same extent as controls [27]. The authors suggested that the observed changes in IRSA (but not in total IGF-I) indicated that hepatic sensitivity to GH was reduced by 50 % in patients with end-stage renal disease and that in patients with end-stage renal disease changes in total IGF-I during treatment with GH are not reflecting changes in endogenous activity of IGF-I [27].

In another study Ivarsen et al. found that directly after hemodialysis there were marked reductions in IRSA (measured by the IGF-IR KIRA) and ultrafiltered free IGF-I in non-diabetic patients with end-stage renal disease while there were only marginal reductions in immunoreactive total IGF-I and total IGF–II [36]. They hypothesized that the decrease in IRSA was a consequence of an increase in IGFBP-1, sequestering free IGF-I, and thereby reducing IRSA [36]. In accordance with this view the increase in IGFBP-1 was accompanied by a parallel increased complex formation between IGF-I and IGFBP-1 [36]. In addition, Ivarsen et al. suggested that catabolism induced by hemodialysis may be (in part) reflected by the observed reductions in IRSA [36]. When a meal was served to patients on maintenance

hemodialysis before hemodialysis, this resulted in a 20% maximum increase of IRSA at 120 min during hemodialysis, whereas total IGF-I levels showed a maximum increase of 5% at 180 min [56].

In another study by the same group, a baseline meal was offered at the day of hemodialysis [57]. In this latter study the expected postprandial increase in IRSA after a baseline meal was absent on all four study days. IRSA only increased above baseline when a second meal was offered at the day of hemodialysis [57]. In addition, immunoreactive total IGF-I did not significantly change and remained fairly constant on all four study days [57]. The increase in IRSA after the second meal on the day of hemodialysis suggested a beneficial effect of frequent meals for patients on maintenance hemodialysis [57].

Brugts et al. studied patients with end stage renal disease treated on continuous ambulatory peritoneal dialysis (CAPD) and found that circulating IRSA (measured by the IGF-IR KIRA assay) increased both after administration of a dialysate with a mixture of amino acids plus glucose or a dialysate that contained only glucose while no changes in circulating immunoreactive total IGF-I levels were observed (Figure 5) [8]. Therefore they concluded that circulating IRSA rather than total IGF-I is involved in acute responses to nutritional interventions in patients with end stage renal disease treated on CAPD [8].

6.16 **IRSA and Liver Cirrhosis**

Circulating IRSA (measured by the IGF-IR KIRA assay), immunoreactive total IGF-I and total IGF-II were reduced in patients with alcoholic liver cirrhosis compared to controls, whereas IGFBP-1, IGFBP-2 and the soluble IGF-II receptor were elevated [40]. Interestingly, the IRSA was fourfold elevated in ascites as compared with serum while in contrast, all other IGF-I-related peptides but pro-IGF-II in ascites were reduced as compared with serum [40]. Thus this study suggested that in contrast to immunoreactive total IGF-I levels, IRSA can be higher in fluids from an extravascular compartment than in serum.

568 However, the pathophysiological significance of these findings remains to be clarified [40].
569 In another study circulating IRSA (measured by the IGF-IR KIRA assay) significantly decreased in patients
570 with liver cirrhosis after an oral glucose tolerance test (OGTT) and the same tendency was observed in
571 healthy subjects [14]. This reduction of IRSA in patients with liver cirrhosis occurred despite unchanged
572 concentrations of (immunoreactive) total IGF-I and free IGF-I [14]. It was hypothesized that the
573 reduction of IRSA in patients with liver cirrhosis during an OGTT was related to higher levels of IGFBP-1
574 and a faster disappearance of IGFBP-1 bound IGF-I [14].
575 Treatment of patients with liver cirrhosis with a transjugular intrahepatic porto-systemic shunt (TIPS)
576 may induce anabolism [35]. Holland-Fischer et al. found that the body cell mass of patients with liver
577 cirrhosis increased after TIPS [35]. However, circulating concentrations of IRSA (measured by the IGF-IR
578 KIRA assay), immunoreactive total IGF-I, total IGF-II and IGF-binding proteins did not change, suggesting
579 that other mechanisms than activity of the IGF system are involved in the anabolic effects of TIPS [35].

580

581 *6.17* **IRSA and Hepatocellular Carcinoma (HCC)**

582 Circulating IRSA (measured by the IGF-IR KIRA assay) in patients with hepatocellular carcinoma was
583 twice as high as found in patients with liver cirrhosis [21]. However, IRSA levels in both groups were
584 markedly below the concentrations observed for healthy controls [21]. Similar patterns as found for
585 circulating IRSA were observed for circulating immunoreactive total IGF-I, IGF-II and IGFBP-3 whereas
586 pro-IGF-II and the IGF-I to IGFBP-3 ratio showed less pronounced but nevertheless significant differences
587 [21]. Changes in tumor burden after treatment did not affect IRSA or IGF-II [21]. It was concluded that
588 the observed differences in parameters of the IGF system between patients with hepatocellular
589 carcinoma, liver cirrhosis and healthy subjects were mainly explained by variations in liver status [21].

Therefore the authors questioned the clinical utility of measuring circulating IGF variables as markers of hepatocellular carcinoma [21].

6.18 **IRSA, Lung Cancer and Pleural Fluid**

When IRSA (measured by the IGF-IR KIRA assay) and other members of the IGF family were compared in pleural fluid and in blood from patients with lung cancer and nonmalignant lung disease, it was found that IRSA was threefold higher in pleural fluid than in corresponding serum samples, regardless of etiology [23]. In contrast immunoreactive total IGF-I concentrations did not differ between blood and pleural fluid [23]. In addition, PAPP-A, an IGFBP protease, that may cleave IGFBP-4 and IGFBP-5, was elevated in pleural fluid and it was speculated by the authors that IGFBP-proteases (inclusive PAPP-A) were involved in the observed increase of IRSA in pleural fluid [23]. This study suggested that local factors at the tissue level may have major effects on IRSA and that local IRSA may substantially differ from that measured in the circulation [23].

6.19 **IRSA, Ovarian Carcinoma and Ascites**

As discussed above, PAPP-A may stimulate IGF-I action through proteolysis of IGFBP-4 and in experimental animal models it has been found that PAPP-A may accelerate ovarian tumor growth by releasing IGF-I [69]. IRSA (measured by the IGF-IR KIRA assay) in ascites of patients with ovarian cancer was 31% higher than in serum [69]. In contrast, concentrations of immunoreactive total IGF-I were similar in serum and ascites, while levels of IGF-II and IGFBP-3 were decreased in ascites compared to serum [69]. Since it was found that ascites contained a 46-fold higher concentrations of PAPP-A than serum and also IGFBP-4, it was hypothesized that PAPP-A in ascites may function to increase IGF-I

612 actions [69]. In favor of this latter possibility it was found that ascites contained less intact IGFBP-4 than
613 plasma and higher concentrations of proteolytically cleaved IGFBP-4 than intact IGFBP-4 [69].

614

615 *6.20* **IRSA and Dementia**

616 Within the Rotterdam Study higher levels of circulating IRSA (measured by the IGF-IR KIRA assay) were
617 associated with a higher prevalence and a higher incidence of dementia suggesting that IRSA increases
618 in response to neuropathological changes that occur in dementia [18]. Similar associations were found
619 for Alzheimer's disease and in persons without diabetes mellitus [18]. Unfortunately in this latter study
620 no circulating immunoreactive total IGF-I levels were measured. As a consequence it was impossible to
621 compare whether in this respect there existed discrepancies between IRSA and total IGF-I.

622

623 *6.21* **IRSA and Longevity**

624 IRSA as measured by the IGF-IR KIRA assay, immunoreactive total IGF-I and the IGF-I/IGFBP-3 ratio were
625 all three significantly lower in centenarians' offspring compared to offspring matched-controls [83]. In
626 addition, IRSA in centenarians' offspring was inversely related to insulin sensitivity [83]. Interestingly, it
627 was further found that in contrast to circulating total IGF-I levels, mean IRSA was comparable between
628 centenarians and their offspring [83]. However, further studies are needed to understand the precise
629 role of IRSA in the modulation of the human aging process [83].

630

631 *6.22* **IRSA and Mortality**

In a prospective observational of more than 400 healthy elderly men (aged 73-94 yrs.) IRSA (measured by the IGF-IR KIRA assay) accounted for 2.5 % (range 0.2-5.9%) of circulating (immunoreactive) total IGF-I levels whereas free IGF-I (measured by immunoassay) accounted for 0.7% [9]. These findings suggested that IRSA was most likely reflecting stimulation of the IGF-IR by free IGF-I and free IGF-II and IGFs dissociated from the IGFBPs during incubation of serum samples [9]. Survival of these elderly men in the highest quartile of IRSA was significantly better than in the lowest quartile, both in the total study group (Figure 6) as well as in subgroups having a medical history of cardiovascular disease or a high inflammatory risk profile [9]. Such relationships were not observed for immunoreactive total or free IGF-I [9]. Thus this study suggested that a relatively high circulating IRSA in elderly men of 73-94 yrs may be associated with extended survival and with reduced cardiovascular risk [9].

7.Discussion and conclusions

The main reason for using immunoassays as an estimate of circulating IGF-I bioactivity has long been the lack of reliable IGF-I bioassays [58]. The measurement of IRSA by the IGF-IR KIRA assay has opened a completely new era and is a novel tool to asses circulating IGF-I bioactivity under conditions that mimic the in vivo cellular environment as good as possible. In this review many studies are discussed showing that measuring IRSA often produces other information in detecting changes of the IGF system than the commonly used IGF-I immunoassays. In contrast to IGF-I immunoassays the IGF-IR KIRA assay is sensitive for modifications of IGF-IR activation by circulating IGFBPs and IGFBP-proteases [13, 44].
This review shows that in many conditions in health and disease results for IRSA (measured by the IGF-IR KIRA assay) and circulating total immunoreactive IGF-I levels (measured by immunoassays) are discordant (Table 1) . These discrepancies are probably directly related to fundamental differences that exists between both methods. With the IGF-IR KIRA assay all serum factors, which directly or indirectly

655 may phosphorylate tyrosine residues of the IGF-IR, become detectable. Moreover, the IGF-IR KIRA assay
656 is sensitive to modulating effects of the IGFBPs on the interactions between IGF-I and the IGF-IR [13].
657 Thus the IGF-IR KIRA assay may give information about net overall effects of IGF-I, IGF-II, IGFBPs and
658 IGFBP-proteases on IGF-IR activation. In contrast, levels of immunoreactive total IGF-I are mainly
659 determined by IGF-I bound to the IGFBPs. However, it is unclear which part of this latter fraction is
660 involved in the activation of the IGF-IR since as discussed above, only IGF-I in the free state is able to
661 stimulate directly the IGF-IR [38]. Thus discrepancies between IRSA (measured by the IGF-IR KIRA assay)
662 and circulating total immunoreactive IGF-I levels (as measured by immunoassays) could be due to
663 stimulating effects of biological factors on the IGF-IR that may be detected by the IGF-IR KIRA assay but
664 are not recognized by total IGF-I immunoassays. Moreover, antibodies in IGF-I immunoassays may also
665 recognize IGF-I molecules or fragments that are biologically inactive and unable to stimulate the IGF-IR.
666 Therefore Zapf et al. previously suggested that results obtained by IGF-I immunoassays should always be
667 confirmed by IGF bioassays before conclusive statements of measured IGF-I levels on physiological or
668 pathophysiological issues are made (87).
669 This review suggests that measurement of circulating IRSA (by the IGF-IR KIRA assay) is especially
670 superior to immunoreactive total IGF-I to monitor therapeutic interventions. Although the IRSA being
671 measured by the IGF-IR KIRA assay probably more closely reflects true bioactive IGF-I than the
672 measurement of total IGF-I in serum by immunoassays, it is good to realize that that the IGF-IR KIRA
673 assay does not capture all the post-receptor intracellular events mediated by the IGF-IR. The IGF-IR KIRA
674 assay only quantifies IGF-IR phosphorylation of tyrosine residues. Nevertheless, it has been found that
675 the IGF-IR KIRA assay shows excellent correlations with the more classical endpoint bioassays [58]. Thus
676 the IGF-IR KIRA assay in its present version does not provide information about the further intracellular
677 propagation and IGF-IR–mediated signal more downstream [13, 58]. On the other hand, this may be a
678 strength of the IGF-IR KIRA assay: in so called-endpoint bioassays it is often impossible to disentangle

679 the relative contribution of IGF-IR mediated effects to a certain end result since there is extensive cross-
680 talk at the post-receptor level between different intracellular signaling networks which are activated by
681 other ligands than IGF-I [68]. Moreover, many biological responses are complex and depend often on a
682 cascade of cross-talk and post-receptor events and stimulation of the IGF-IR may activate different
683 signaling pathways intracellularly upon receptor binding in a concentration-dependent manner (Figure
684 2).
685 Although the signal measured by the IGF-IR KIRA assay is readily direct and specific, the IGF-IR has up to
686 6 key tyrosine residues of which of some the role in vivo is not yet fully clear [76]. In addition, the
687 antibody used to capture the tyrosine residues may not well recognize all residues equally well because
688 of dependence of affinity on flanking sequence and proximity of other sites [76]. Thus the IGF-IR KIRA
689 bioassay only provides a crude, albeit convenient, measure of kinase activation.
690 Interestingly, as discussed above, IRSA in samples from the interstitial fluid obtained by the suction
691 blister method was almost 50% higher than in matched serum samples [22]. Moreover, IRSA was higher
692 in ascites than in serum in patients with liver cirrhosis and in patients with ovarian cancer [14, 40]. In
693 addition, IRSA was also higher in pleural fluid than in serum in patients with lung disease [23, 69]. Thus
694 the amount of IRSA present in interstitial fluid and the extravascular tissues may not only substantially
695 differ from that measured in the circulation but that the amount of local IRSA seems not directly related
696 to circulating IRSA. These findings suggest that it is necessary to collect information about both the
697 paracrine (local) and endocrine (circulating) IRSA to obtain an overall impression of the role of the IGF
698 system in health and disease.
699 An important limitation of the IGF-IR KIRA assay in its present form is that it is more labor intensive and
700 more expensive than total IGF-I immunoassays. It measures only the amount of IGF-I (and other ligands)
701 that that can interact with the IGF-I receptor and activate its tyrosine kinases during a short time of
702 incubation (15 minutes). For the future the IGF-IR KIRA assay should be further miniaturized and

automated to run many samples in a relatively short time. In addition, there should be a single universally accepted standard for the IGF-IR KIRA assay for calibration and large pools of reference serum samples should become available to monitor the (dis)concordance in results between different laboratories using an IGF-IR KIRA assay.

In conclusion, the IGF-IR KIRA assay is a novel tool that has opened a new era. When studying changes of the GH-IGF-I axis in health and disease the IGF-IR KIRA assay provides in many conditions different information about the IGF system than the commonly used total IGF-I immunoassays. The IGF-IR KIRA assay probably more closely reflects true bioactive IGF-I compared to measurements of total IGF-I in serum by immunoassays. In health and disease IRSA measured by the IGF-IR KIRA assay was considerably higher in samples from interstitial fluid and ascites than in serum, suggesting that both local and circulating IRSA should be measured in order to get a more complete view of the role of the IGF system.

Duality of Interest

Dr. Joseph A.M.J.L, Janssen, who is co-authoring this paper, also serves as Associate Editor of Growth Hormone and IGF Research. However, this has not influenced on the handling of the paper, which has been subjected to the Journal's usual procedures. Thus, the peer review process has been handled independently of Dr. Joseph A.M.J.L. Janssen, who has been blinded to the review process. The authors otherwise have nothing to disclose.

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Author Contributions

JAMJLJ, AJV and MB designed the study;
All authors contributed to data interpretation, discussion of the paper; All authors prepared and all edited the manuscript.

Disclosure Statement

The authors have nothing to disclose

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Legends

Figure 1.

Schematic overview of the IGF system. The IGF system is composed of two Insulin-like Growth Factors IGF-I (yellow) and IGF-II (blue), 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. The IGF-IR can bind IGF-I with high affinity but also IGF-II with 10-fold lower affinity. In the Figure also insulin (red) 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 considerably lower avidity (displayed by the differences in thickness of the arrows). Interestingly, in the body the IGF-I and IR may form hybrids which are preferentially activated by IGF-I.

Figure 2

Model of the IGF-IR signaling pathway. Binding of IGF-I (or IGF-II) to the IGF-IR results in autophosphorylation of tyrosine residues located within the intracellular kinase domain of the IGF-IR, being the first step in the intracellular signaling cascade. This starts the activation of multiple complex intracellular pathways (including the RAS-ERK-MAPK pathway and the PI3K/AKT pathway) and results in multiple biological effects in a variety of tissues and cells in the body.

Figure 3

Schematic overview of procedures the IGF-I Kinase Receptor Activation Assay (IGF-IR KIRA).The time to perform the assay assay is 4-days. On day 1 human embryonic kidney (EBNA) 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 0.1% human serum albumin (HSA). A second 96 well plate is coated with an human IGF-IR capture antibody. 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 the human IGF-IR capture antibody on day 2. On day 4 the 96 well plate is washed and a second antibody is added, which specifically recognizes phosphorylated residues located at the kinase domains of the IGF-IR. This latter antibody contains an europium (EUR) label so that phosphorylation of the tyrosine residues of IGF-I receptor can be quantified in a time-resolved fluorometer.

Figure 4. Age distribution of serum total IGF-I levels (upper panel) and IRSA (lower panel) in 94 patients diagnosed with growth hormone deficiency (GHD) (black dots). The shaded area depicts the 95% confidence interval in normal subjects per decade of age. IRSA in adult patients with proven GHD was more frequently below the normal range (<-2 SD) than total IGF-I levels. From: IGF-I Bioactivity Better Reflects Growth Hormone Deficiency than Total IGF-I. J Clin Endocrinol Metab. 2011;96(7):2248-2254. doi:10.1210/jc.2011-0051. J Clin Endocrinol Metab | Copyright © 2011 by The Endocrine Society.

Figure 5

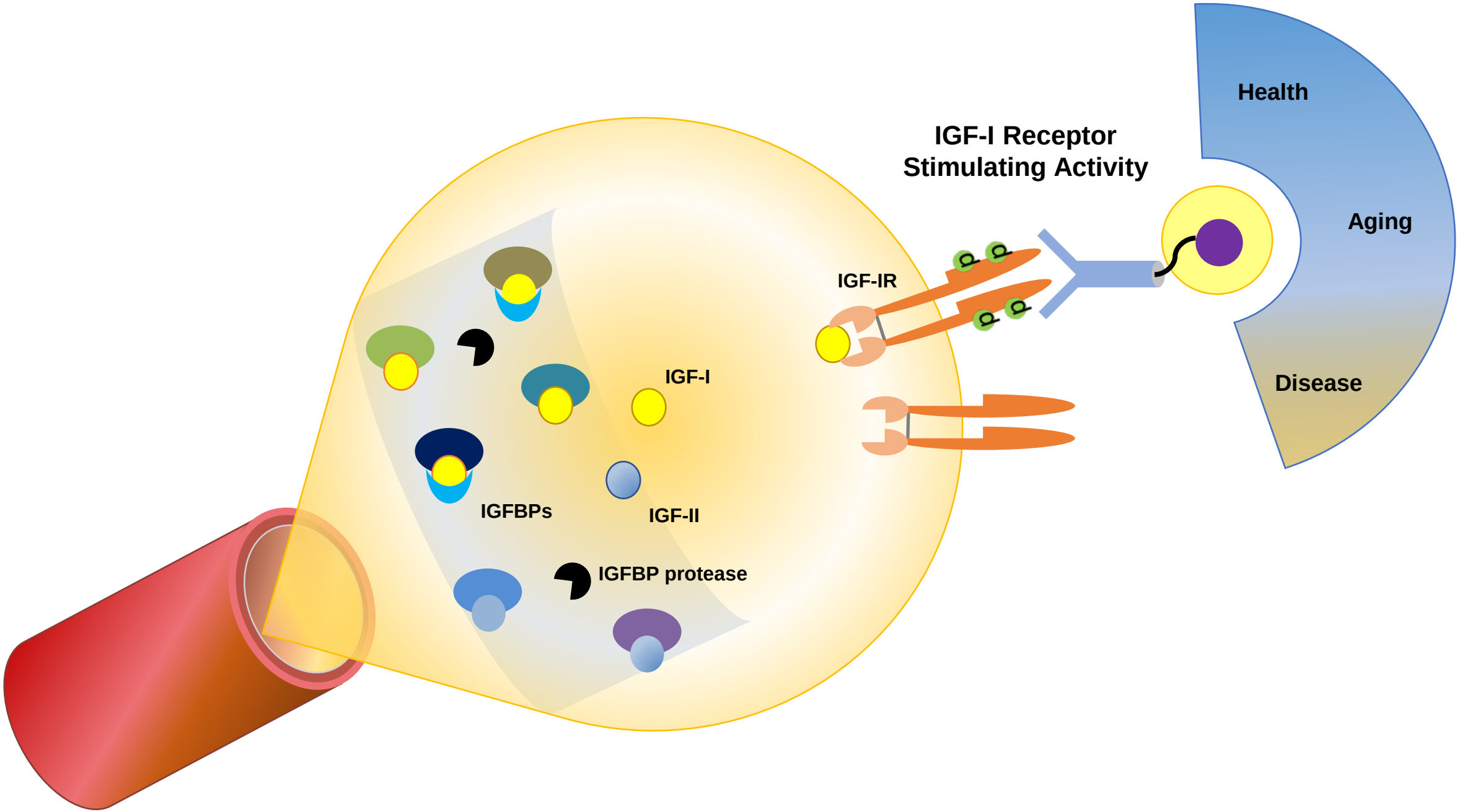
Top: Changes in circulating total (immunoreactive) IGF-I levels in the fed state after a dialysate that only contained glucose (G) and after a dialysate containing aminoacids and glucose (AA and G), respectively (basal state = 100%). Bottom: Changes in circulating IRSA in the fed state compared to baseline after G and after AA and G dialysate, respectively (basal state = 100%). Circulating IRSA increased both after administration of a dialysate with a mixture of amino acids plus glucose or a dialysate that contained only glucose while no changes in circulating total (immunoreactive) IGF-I levels were observed. From:

Bioactive rather than total IGF-I is involved in acute responses to nutritional interventions in CAPD patients Nephrol Dial Transplant. 2009;25(3):940-946. doi:10.1093/ndt/gfp576.Nephrol Dial Transplant |

Figure 6

Cox proportional hazard plots (percent) showing Cumulative Survival for groups of circulating (immunoreactive) total IGF-I levels (A), circulating (immunoreactive) free IGF-I levels (B) and IRSA levels as measured by the IGF-IR KIRA assay(C). P for trend reached statistical significance only in the IRSA group as measured by the IGF-IR KIRA assay. Groups of IRSA are shown as follows: group 1 (—), ≤25th percentile; group 2–3 (– – –), between 25th and 75th percentile; and 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. See also text. From: Low Circulating Insulin-Like Growth Factor I Bioactivity in Elderly Men Is Associated with Increased Mortality. J Clin Endocrinol Metab. 2008;93(7):2515-2522. doi:10.1210/jc.2007-1633. J Clin Endocrinol Metab |

Graphical Abstract (for review)



Cover Letter (including at least 3 Reviewers and their contact details)

To Professor Jan Frystyk

Medical Research Laboratories

Aarhus University Hospital

Norrebrogade 44

DK 8000 Aarhus C

Denmark

Rotterdam, March 2019

Dear Professor Frystyk, dear Jan

Please find enclosed a review entitled: “The Insulin-like Growth Factor-I Receptor Stimulating Activity (IRSA) in Health and Disease”.

In this review paper we give a comprehensive overview and discuss many studies showing that measuring IRSA by the IGF-IR KIRA assay often provides fundamentally different information about the IGF system than the commonly used total IGF-I immunoassays.

The IGF-IR KIRA assay seems especially superior to immunoreactive total IGF-I in monitoring therapeutic interventions. In addition, in several conditions in health and disease IRSA measured by the IGF-IR KIRA assay is considerably higher in interstitial fluid and ascites than in serum. This suggests that both the paracrine (local) and endocrine (circulating) IRSA should be measured to get a complete picture about the role of the IGF system in health and disease.

We would greatly appreciate if you would consider our manuscript as Review for publication in Growth Hormone & IGF Research

The work is not submitted for publication elsewhere until the editorial board has decided whether to publish the article.

All authors have seen and approved the final version of the manuscript being submitted.

There is no conflict of interest.

The manuscript contains 86 references, one Table and six Figures. Figures 4-6 have been published before. We have been authorized by the Endocrine Society to re-use Figures 4 and Figure 6 and by Oxford University Press on behalf of ERA-EDTA to re-use Figure 5. Color should only be used for the version on line, but not when Figures will become in print.

I propose the following 3 Reviewers

1. Professor David Clemmons.
   UNC Health Care, Chapel Hill
   david_clemmons@med.unc.edu
2. Professor Kersin Brismar
   Karolinska Institute, Department of Molecular Medicine and Surgery
   Kerstin.Brismar@ki.se
3. Professor Diego Ferone
   Univesita degli Studi di Genova, Endocrinology Unit
   ferone@unige.it
4. Professor Bu Beng Yeap
   University of Western Australia, Department of Endocrinology and Diabetes
   Bu.yeap@uwa.edu.au
5. Dr Kevin Yuen
   Barrow Pituitary Center, Phoenix
   Kevin.yuen@dignityhealth.org

As Associate Editor of Growth Hormone and IGF Research, I should be blinded to the review process

Also on behalf of the other co-authors

Joseph

J.A.M.J.L. Janssen, MD, Ph.D.

Erasmus MC

Department of Internal Medicine

Dr Molewaterplein 40, Room Rg 526

3015 GD Rotterdam

The Netherlands

Telephone +31-0650032421 or +31-10-7040704

Email: j.a.m.j.l.janssen@erasmusmc.nl