

# The insulin-like growth factor system- Effects of circulating proteases

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***To Oskar***

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# ABSTRACT

The insulin-like growth factor-I and -II (IGF-I/ -II) are peptide hormones important for growth and metabolism. They stimulate cell proliferation and differentiation and inhibit apoptosis. In addition, they have insulin-like effects on glucose and amino acid metabolism. A large quantity of IGFs is synthesized in the liver and is subsequently transported to the circulation to act as endocrine factors. In addition, IGFs produced in extra hepatic tissues may act locally as paracrine/ autocrine factors. In serum most IGF circulate in a ternary complex with IGF-binding protein-3 (IGFBP-3) and the acid labile subunit (ALS). The affinity between IGF and IGFBP-3 exceeds that between IGF and the type 1 IGF receptor demonstrating that most of the circulating IGF is unavailable for the receptor. However, a slight decrease in binding affinity within the ternary complex increases the bioavailability of IGFs. Post-translational modifications of the IGFBPs are common and proteolytic cleavage of IGFBPs represents one example. IGFBP proteolysis results in reduced IGF affinity and subsequently increased IGF bioavailability. Increased IGFBP-3 proteolysis is found in several clinical conditions associated with insulin resistance, e.g. critical illness, post-operatively and type 2 diabetes. IGFBP-3 proteolysis may partly compensate for insulin resistance by increasing IGF availability and thereby also increasing insulin-like effects. In the present licentiate thesis we have studied the mechanisms of IGFBP-3 proteolysis *in vivo* in humans. We have also attempted to evaluate the molecular consequences of IGFBP-3 proteolysis. We first studied (paper I) whether cannulation and/ or venous stasis increases serum IGFBP-3 proteolysis by activating fibrinolytic and thrombogenic enzymes. These enzymes have the capacity to cleave IGFBP-3 *in vitro*. We found that limited vascular damage and/ or reduced blood flow in response to cannulation and/ or venous stasis did not affect IGFBP-3 proteolysis. Furthermore, cannulation and/ or venous stasis did not affect the elevated levels of IGFBP-3 proteolysis after surgery. This does not exclude that extensive activation of fibrinolytic and thrombogenic enzymes by myocardial infarction, venous thrombosis or wound healing may result in elevated IGFBP-3 proteolysis. This further demonstrates that the current methods to detect serum IGFBP-3 proteolysis are valid if our sampling recommendations are followed. We then investigated (paper II) whether the cytokine interleukin-6 (IL-6) increases serum IGFBP-3 proteolysis or IGFBP-1 in healthy adults. This was investigated since IL-6 is elevated in several inflammatory diseases associated with increased IGFBP-3 proteolysis. We found that a 3-hour IL-6 infusion did not increase IGFBP-3 proteolysis up to 5 hours post-infusion as compared to saline infusion. This finding is important if we should target the mechanisms resulting in changes in the IGF-system during inflammation and insulin resistance. Based on these results we cannot exclude that higher levels of IL-6 (>0.1 ng/ ml) may immediately stimulate IGFBP-3 proteolysis or that prolonged exposure to IL-6 may affect IGFBP-3. Interestingly, we found that IGFBP-1 was increased after the end of IL-6 infusion by a mechanism independent of insulin. However, the change in IGFBP-1 in response to IL-6 did not affect total or free IGF-I. Finally, we have attempted (project report) to isolate the major IGFBP-3 proteolytic fragment in human post-operative serum in order to 1) obtain amino acid sequence data of the cleavage site to unravel the protease and 2) examine the changes in IGF affinity resulting from proteolytic cleavage. By using IGF-I affinity chromatography, we isolated a 30 kDa IGFBP-3 fragment in post-operative serum. N-terminal amino acid sequencing revealed the sequence GASS. This is identical to the N-terminal of IGFBP-3. The low recovery did not allow us to identify the C-terminal sequence or to obtain enough material for binding studies. Alternative purification strategies were explored including an attempt to raise IGFBP-3 antibodies for affinity chromatography.

In summary this licentiate thesis provides human *in vivo* data demonstrating that IGFBP-3 proteolysis is elevated after abdominal surgery but that it is not affected by venous cannulation and/ or stasis or by short-term exposure to IL-6. Furthermore, currently established methods to detect IGFBP-3 proteolysis can be used to evaluate potential inducers of IGFBP-3 proteolysis *in vivo* including fibrinolytic and thrombogenic enzymes. We also demonstrate that IL-6 is a more potent regulator of serum IGFBP-1 compared to IGFBP-3 proteolysis. However, the significance of this is unknown since the level of free IGF-I was unchanged. In addition, we suggest that the development of purification procedures to isolate and characterize IGFBP-3 fragments may be helpful in identifying the proteases that increase serum IGFBP-3 fragmentation *in vivo*. This may provide useful information about alterations in the IGF-system during inflammation and insulin resistance

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# LIST OF PAPERS

**I Lack of Insulin-like growth factor binding protein-3 protease activation by venous cannulation**

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**II Interleukin-6 infusion in humans increases IGFBP-1 while IGFBP-3 proteolysis is unchanged**

Gustafsson S, Carlsson-Skwirut C, Berg U, Ekström K, Klarlund-Pedersen B, Bang P  
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# PROJECT REPORT

**III Purification of 30 kDa IGFBP-3 fragments in human post-operative serum**

Gustafsson S, Carlsson-Skwirut C, Bang P

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# ABBREVIATIONS

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IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
ALS	Acid labile subunit
IGFR1	Type 1 IGF receptor
IGF2R	Type 2 IGF receptor
IR	Insulin receptor
IRS	Insulin receptor substrate
PI3-Kinase	Phosphatidylinositol-3 kinase
MAPK	Mitogen-activated protein kinase
ADAM	A disintegrin and metalloprotease
MMP	Matrix metallo proteinase
PSA	Prostate specific antigen
RXR	Retinoid X receptor
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide-gel electrophoresis
IL-6	Interleukin-6
PAPP-A	Pregnancy-associated plasma protein A
$K_D$	Dissociation constant
kDa	kilo Dalton
RIA	Radioimmunoassay
ELISA	Enzyme-linked immunosorbent assay
DELFIA	Delayed enhanced lanthanide fluorescent immunoassay
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription

# INTRODUCTION

## Historical background

The Insulin-like growth factors (IGF-I and -II) are abundant peptide hormones that act in an endocrine, paracrine and autocrine way to affect growth and metabolism. They are found in early vertebrates and are conserved among mammals. This indicates that they have important biological functions. The IGFs were discovered as growth promoting factors almost fifty years ago (Salmon and Daughaday 1990). Salmon and Daughaday found that serum from healthy rats stimulated sulphate ( $^{35}\text{S}$ ) incorporation into cartilage cells *in vitro*, while serum from hypophysectomized rats did not. Furthermore, they found that Growth hormone (GH) administration to hypophysectomized rats increased growth, while it had no effects when given directly to cells. In 1963 Froesch et al (Froesch, Buergi et al. 1963) discovered an insulin-like activity in human serum that was not suppressible by antibodies against insulin. Ten years later a serum fraction containing proliferative as well as insulin-like activity was partially purified indicating that one factor might be responsible for both these effects (Pierson and Temin 1972). This led to the Somatomedin hypothesis (Daughaday, Hall et al. 1972). It stated that the growth promoting effects of GH was mediated by a circulating factor with insulin-like activity as well as growth promoting effects. Some years later the IGF-I and IGF-II were isolated and their amino acid sequence characterized (Rinderknecht and Humbel 1976; Rinderknecht and Humbel 1978; Rinderknecht and Humbel 1978). IGF-I and IGF-II were peptides of 70 and 67 amino acids (approximately 7.5 kDa) that shared 62 % amino acid identity. In addition, the mature IGF-I/ -II shared approximately 40 % amino acid identity with pro-insulin. During the protein purifications IGFs were found in fractions of higher molecular weight (Froesch, Buergi et al. 1963; Daughaday and Kipnis 1966; Jakob, Hauri et al. 1968). This led to the hypothesis that IGFs are bound to plasma proteins. These were later discovered as the IGF binding proteins (IGFBPs). The IGFBPs circulate in nM concentration in serum and limit the bioactivity of IGFs. Even though it's been almost 30 years since the IGFs were characterized it is still not fully understood how their bioactivity is regulated.

## The IGFBP family

The IGF binding proteins (IGFBPs) are a family of six proteins (IGFBP-1 to -6) that share similar structure and function. Most importantly, they all have a strong affinity for IGF-I and IGF-II. Furthermore, they share the same protein structure with conserved N- and C-terminal domains and a variable middle region. The N- and C-terminal domains contain several well-conserved cysteine residues that form intra-domain disulfide bonds. Several other proteins have lately been found to share some of the structural features of IGFBPs, e.g. conserved N-terminal cysteines. Some of them display weak IGF binding *in vitro*. There is now a consensus to call them IGFBP related proteins (IGFBPrp). However, the relative importance of these with regard to the IGF system is unknown. Other structural motifs in the IGFBPs include an Arg-Gly-Asp (RGD) sequence in the C-terminal of IGFBP-1 and -2. These mediate integrin binding and thereby cell association. Several of the IGFBPs also contain heparin-binding motifs in the C-terminal region that mediate binding to proteoglycans in extracellular matrix or on cell surfaces (IGFBP-3, IGFBP-4 and IGFBP-5). The functional significance of this is illustrated by the fact that cell surface bound IGFBP-3 has a lower affinity for IGF compared to soluble IGFBP-3 (McCusker, Busby et al. 1991). IGFBP-3 interaction with endothelial cells may therefore affect IGF bioavailability in serum (Booth, Boes et al. 1995). This interaction may localize IGF to the vascular wall and release IGF close to cell surface receptors.

Extensive work has been performed in order to elucidate the IGF-binding site in the IGFBPs. A model of IGF interaction was presented in 1998 by Kalus et al (Kalus, Zweckstetter et al. 1998). They determined the secondary structure of  $^{40-92}\text{IGFBP-5}$  by nuclear magnetic resonance spectroscopy and discovered that several amino acids in this region were important for IGF association. These amino acid residues are well conserved in all IGFBPs suggesting that this might be the main IGF binding site. This was later confirmed in IGFBP-3 (Imai, Moralez et al. 2000). In addition, C-terminal residues are important for stabilizing the



IGF-IGFBP complex and achieving high affinity binding (Firth, Ganeshprasad et al. 1998; Qin, Strong et al. 1998). The IGFs, IGFBPs and the main receptors mediating IGF effects are illustrated in Figure 1. Additional information about the IGFBPs can be found in the following reviews (Jones and Clemmons 1995; Hwa, Oh et al. 1999; Firth and Baxter 2002).

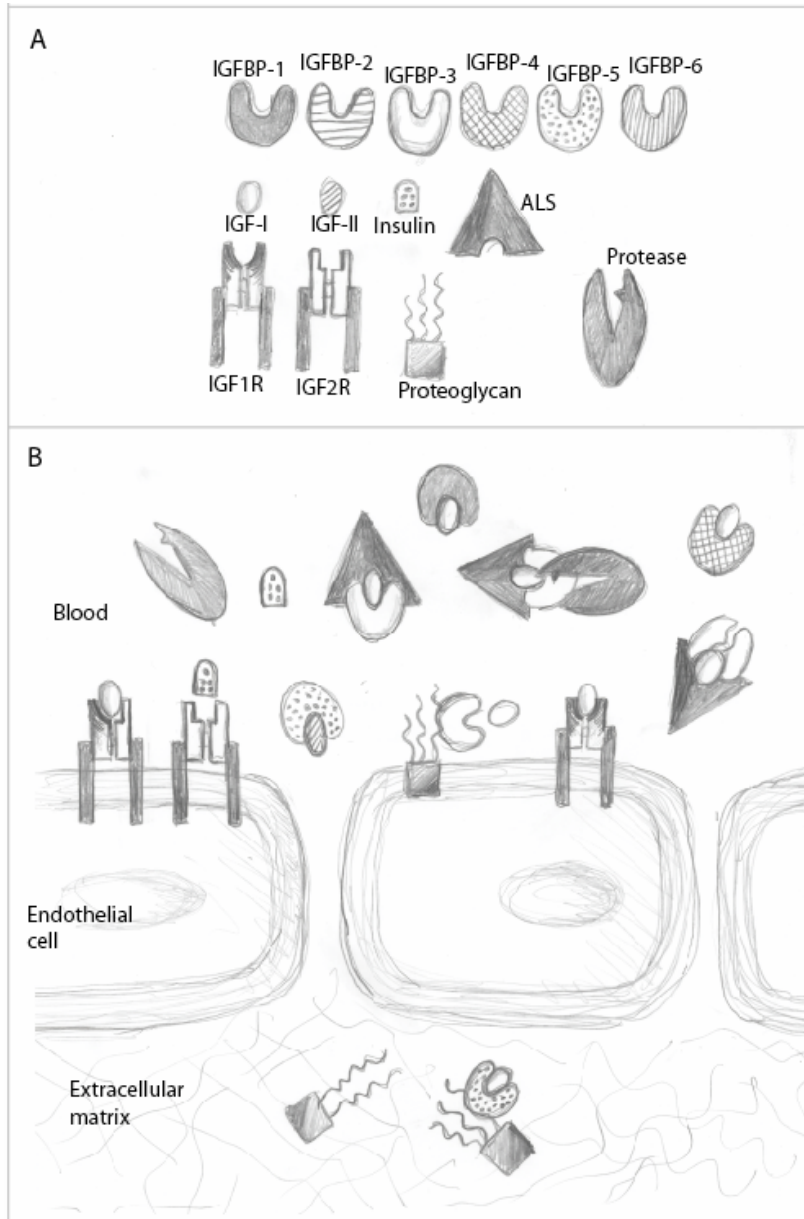
### **Posttranslational modifications of IGFBPs**

Posttranslational modifications are a common way of changing functional aspects of proteins. In the IGFBPs most modifications are located in the variable middle region demonstrating that the IGFBPs are differently modified by phosphorylation, glycosylation and proteolysis. IGFBP-1, IGFBP-3 and IGFBP-5 can be phosphorylated on serine residues. Phosphorylation of IGFBP-1 increase IGF affinity (Jones, D'Ercole et al. 1991). This is important since the affinity of IGFs for IGFBPs is only slightly higher than for the type 1 IGF receptor and even a small difference in binding affinity may affect IGF bioavailability. However phosphorylation of IGFBP-3 (Ser<sup>111</sup>, Ser<sup>113</sup>) has no effect on IGF affinity (Hoeck and Mukku 1994). Furthermore, IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6 can be glycosylated. N-linked glycosylation (Asp residues) is found in IGFBP-3 and in IGFBP-4, while O-linked glycosylation (Ser and Thr residues) has been found in IGFBP-5 and IGFBP-6. IGFBP-3 contains three glycosylation sites of which one is variably used (Asn<sup>89</sup>, Asn<sup>109</sup> and Asn<sup>172</sup>). The IGFBP-3 protein is therefore normally found glycosylated in serum and is detected as a doublet of 40-42 kDa in western immunoblots. Glycosylation does not affect IGF binding but it might affect cell association (Firth and Baxter 1999). In addition IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6 can be modified by proteases that catalyze the cleavage of peptide bonds. Proteolytic cleavage of IGFBP-2 (Ho and Baxter 1997), IGFBP-3 (Lassarre and Binoux 1994), IGFBP-4 (Cohick, Gockerman et al. 1993; Giudice, Conover et al. 2002), IGFBP-5 (Andress, Loop et al. 1993) and IGFBP-6 (Marinero, Neumann et al. 2000) produce IGFBP fragments that display reduced IGF affinity *in vitro*. In most cases reduced IGF affinity will increase IGF bioavailability and thereby IGF biological effects. An additional example of the functional importance of proteolysis comes from IGF-I. The proteolytic cleavage of three amino acids (Gly-Pro-Glu) at the N-terminal of IGF-I results in des(1-3)IGF-I. Des(1-3)IGF-I has reduced IGFBP affinity and therefore increased IGF biological effects (Carlsson-Skwirut, Lake et al. 1989). This naturally occurring form of IGF-I was isolated from brain demonstrating the functional importance of proteolysis as a natural way of changing hormone activity (Sara, Carlsson-Skwirut et al. 1986).

### **The ternary complex and IGFBP-3**

The binding affinity between IGFs and the IGFBPs ( $K_D$  of approximately  $10^{-10}$  M) is strong and it exceeds that between IGFs and the type 1 IGF receptor ( $K_D$  of approximately  $10^{-9}$  M). The high affinity binding explains why IGFs are almost exclusively bound to IGFBPs in the circulation and in extracellular fluids. More than 90% of circulating IGF is bound in a ternary complex of 150 kDa containing IGF-I/-II, IGFBP-3/-5 and the acid labile subunit (ALS). However, since IGFBP-3 is much more abundant than IGFBP-5 most IGFs are bound to IGFBP-3. The molar concentration of IGFBP-3 equals the concentration of IGF-I plus IGF-II. The remaining IGF circulate in binary complexes of approximately 50 kDa with other IGFBPs. The quantity of free IGF-I is estimated to less than 1% (Frystyk, Skjaerbaek et al. 1994).

IGFBP-3 is expressed in several tissues. However, kupffer cells in the liver produce most of the circulating IGFBP-3 and this production is stimulated by GH. In addition, insulin and IGF-I may affect the expression level of IGFBP-3 (Villafuerte, Zhang et al. 1996). Cytokines like IL-6 can also change the expression of IGFBP-3 in the liver (Lelbach, Scharf et al. 2001). Besides regulating IGFBP-3, GH also increases the expression of IGF-I and ALS. This demonstrates that GH largely determines the quantities of ternary complexes.



**Figure 1.** A) The different components of the IGF-system as well as important binding partners. B) The endothelial lining and the different IGF complexes are shown. Most IGF is bound in ternary complexes with IGFBP-3 and ALS. A small quantity of IGF is present in binary complexes or free. Circulating proteases cleave IGFBP-3 and increase IGF bioavailability by decreasing IGF binding affinity. IGFBP-3 can also interact with proteoglycans at the cell surface or in extracellular matrix. Binding to proteoglycans decrease IGF affinity and increase bioavailability. The ternary complex can not be transported from the circulation. However, binary complexes are transported over the endothelial wall but the mechanism is not clear. IGF binding to the type 1 IGF receptor (IGF1R) initiates signal transduction. In addition, IGF binding to the insulin receptor (IR) or IGF1R/IR hybrid receptors may stimulate IGF effects.

The role of the ternary complex is to limit IGF efflux from the vascular space. The ternary complex can not pass the endothelial wall. However, another important function is to increase IGF half-life. The half-life of the ternary complex is 12-15 h while it is approximately 10 min for unbound IGF (Guler, Zapf et al. 1989). In this way the ternary complex serve as a stable reservoir of IGFs in serum. The quantities of ternary complexes largely determine the serum level and bioactivity of IGFs. It is therefore important to understand the mechanisms that regulate IGFBP-3 and ALS. However, the serum level of IGFBP-3 is related to changes in IGF and ALS, since ternary complex formation also reduces IGFBP-3 clearance.

### **IGFBP-3 proteolysis**

Human adult serum contains large quantities of IGFBP-3 and some of it is always detected as proteolytic fragments. In pregnancy serum where IGFBP-3 proteolysis was first discovered IGFBP-3 is completely degraded to fragments of 30 kDa (Giudice, Farrell et al. 1990; Hossenlopp, Segovia et al. 1990). This 30-kDa fragment retains IGF binding affinity and the ternary complexes are intact (Baxter, Suikkari et al. 1993). However, the affinity for IGF is decreased and this results in increased IGF bioavailability in serum (Blat, Villaudy et al. 1994; Lassarre and Binoux 1994). Biosensor analysis of synthesized IGFBP-3 fragments also demonstrate lower IGF affinity (Vorwerk, Hohmann et al. 2002). Increased IGF-I bioavailability may also result in increased tissue availability since IGF-I present in a “proteolyzed ternary complex” is more easily transported over endothelial cells (Payet, Firth et al. 2004). The mechanism of IGF transport over the endothelium has still not been demonstrated.

Attempts have been made to isolate and characterize the IGFBP proteases in pregnancy serum. Bang et al (Bang and Fielder 1997) isolated two IGFBP-3 protease activities in pregnancy serum of which one corresponded to plasminogen (70-90 kDa), as determined by N-terminal sequence. It was activated by tPA and uPA and inhibited by plasmin inhibitors. Kubler et al characterized a 50 kDa IGFBP-3 gelatinase that displayed disintegrin immunoreactivity in human pregnancy serum (Kubler, Cowell et al. 1998). A candidate IGFBP-3 protease in pregnancy serum is ADAM 12-S. This is a disintegrin metalloproteinase of 68 kDa that is present in human pregnancy serum but not in human normal serum and it degrades IGFBP-3 *in vitro* (Loechel, Fox et al. 2000). However, the finding that ADAM 12-S transgenic mice does not display increased IGFBP-3 proteolytic activity questions the significance of ADAM 12-S (Kawaguchi, Xu et al. 2002).

Several IGFBP-3 proteases have been reported. Plasmin, as well as the plasminogen activators tPA and uPA have been shown to degrade IGFBP-3 *in vitro* (Lalou, Silve et al. 1994; Booth, Boes et al. 1996; Bang and Fielder 1997; Lalou, Sawamura et al. 1997). Furthermore, *in vitro* studies have shown that thrombin (Zheng, Clarke et al. 1998), MMP -1, -2, -3, -7, -19 (Fowlkes, Enghild et al. 1994; Sadowski, Dietrich et al. 2003; Miyamoto, Yano et al. 2004), cathepsin D and G (Claussen, Kubler et al. 1997; Gibson and Cohen 1999), elastase (Gibson and Cohen 1999), ADAM 12-S (Loechel, Fox et al. 2000), ADAM 28 (Mochizuki, Shimoda et al. 2004), human kallikrein-2 (Koistinen, Paju et al. 2002), trypsin (Koistinen, Paju et al. 2002) and Prostate-specific antigen (PSA) (Fielder, Rosenfeld et al. 1994; Koistinen, Paju et al. 2002) cleave IGFBP-3. Different IGFBP-3 proteases have different recognition sites and they generate different fragments (Booth 1996). Protease inhibitors indicate that different proteases are activated in different states of elevated serum IGFBP-3 proteolysis. For example Bang et al found that IGFBP-3 proteolysis was inhibited by TIMP-1 in human post-operative serum but not in pregnancy serum (Bang, Nygren et al. 1998).

Another approach to characterize the IGFBP-3 protease is to isolate IGFBP-3 *in vivo* generated fragments. By determining the specific cleavage site the protease may be unraveled. Booth et al isolated IGFBP-3 fragments generated by pregnancy serum (Booth, Boes et al. 1996). They demonstrate that the fragmentation pattern was different from that caused by plasmin and thrombin. In addition, IGFBP-3 fragments generated *in vivo* have been isolated in hemofiltrate from patients undergoing dialysis (Kubler, Draeger et al. 2002; Schebek-Furstenberg, Standker et al. 2004). These studies have reported N-terminal

sequences and the molecular mass of the different IGFBP-3 fragments. However, C-terminal amino acid sequencing is necessary to confirm the full structure of these fragments. The cleavage sites in IGFBP-3 have therefore not been convincingly demonstrated. So far the proteases responsible for elevated IGFBP-3 proteolysis in the post-operative state, critical illness and type 2 diabetes remain unknown.

### **IGF independent effects of IGFBP-3**

*In vitro* generated IGFBP-3 fragments have been used in order to study potential effects of IGFBP-3 proteolysis. Lalou et al found that a plasmin-generated IGFBP-3 fragment of 22/ 25 kDa had reduced affinity for IGFs, while a 16 kDa fragment had no IGF affinity (Lalou, Lassarre et al. 1996). Interestingly they found that the 16 kDa fragment inhibited IGF-I induced DNA-synthesis despite the lack of IGF affinity. The use of IGF1R negative cells further demonstrated that this was an IGF-independent effect (Zadeh and Binoux 1997). Booth et al further showed IGF-independent effects of IGFBP-3 fragments. They isolated two plasmin-derived IGFBP-3 fragments (20 kDa, 8 kDa) that stimulated glucose uptake in the absence of IGFs (Booth, Boes et al. 1999). This effect was not observed with intact IGFBP-3. However, IGF independent effects have also been reported for intact IGFBP-3. IGFBP-3 was shown to inhibit growth (Valentinis, Bhala et al. 1995) and induce apoptosis in IGF1R negative cells (Rajah, Valentinis et al. 1997). When IGFBP-3 was found in the nucleus of lung cancer cells it was proposed that some of the IGF independent effects of IGFBP-3 may be mediated by interaction with nuclear proteins (Jaques, Noll et al. 1997). The nuclear transport of IGFBP-3 is facilitated by a nuclear localization sequence in IGFBP-3 and the transport to the nucleus is probably mediated by importin- $\beta$  (Schedlich, Le Page et al. 2000). Yeast two-hybrid system has further demonstrated that IGFBP-3 binds nuclear proteins such as RXR $\alpha$  (Liu, Lee et al. 2000). A recent study suggests that IGFBP-3/ RXR association lead to the translocation of Nurr77 and a subsequent induction of apoptosis (Lee, Ma et al. 2005).

### **IGF receptors**

The IGFs exert their effects by binding to cell surface receptors. IGFs can bind to three different receptors i.e. the type 1 IGF receptor (IGF1R), the type 2 IGF receptor (IGF2R) and the insulin receptor (IR). The IGF1R is the main receptor mediating the IGF effects. It is expressed in most tissues except in the classical insulin responsive organs such as fat and liver. The IGF1R is a tyrosine kinase receptor. It is composed by  $\alpha$ - and  $\beta$ -subunits ( $\alpha_2\beta_2$ ). The  $\alpha$ -subunit contains the extracellular ligand binding part and the  $\beta$ -subunit contains the membrane-spanning domain as well as the intracellular catalytic tyrosine kinase domain. The IGF1R has a high affinity for IGF-I and IGF-II. In addition it has a low affinity for insulin (Massague and Czech 1982; Steele-Perkins, Turner et al. 1988). The IGF2R does not share the same structure as the IGF1R. It is a single chain polypeptide with an extracellular repetitive unit and it is also known as the mannose 6-Phosphate receptor. This receptor binds IGF-II with high affinity and IGF-I with much lower affinity. It has no affinity for insulin (Massague and Czech 1982). As suggested by the name it also binds mannose-6-Phosphate containing proteins. This receptor has mainly been associated with internalization and lysosomal degradation of ligands. The importance of IGF-II as a ligand for the IGF1R was shown in IGF2R knock-out mice. These mice have increased serum IGF-II due to the lack of clearance. The excess IGF-II results in an increased birth weight (135%) due to activation of IGF1R (Ludwig, Eggenschwiler et al. 1996). The IR is like the IGF-IR a tyrosine kinase receptor with the same subunit structure ( $\alpha_2\beta_2$ ). There are two isoforms of the insulin receptor (IR<sub>A</sub> and IR<sub>B</sub>) generated by alternative splicing. Interestingly, apart from the high affinity for insulin the IR<sub>A</sub> receptor has high affinity for IGF-II (Frasca, Pandini et al. 1999). However, the IR<sub>A</sub> isoform is mainly expressed in fetal tissues and in tumors. The IR<sub>B</sub> receptor has a high affinity for insulin and a very low affinity for IGF-I and IGF-II. Due to structural similarities one half of an IGF-IR ( $\alpha\beta$ ) can associate with one half of an IR ( $\alpha\beta$ ) to form a complete signaling hybrid receptor ( $\alpha_2\beta_2$ ). If both IR and IGF-IR are expressed in the same cell they are assembled at random. Hybrid receptors containing IR<sub>A</sub> bind IGF-I, IGF-II and insulin while hybrid receptors containing IR<sub>B</sub> only bind IGF-I with high affinity (Pandini, Frasca et al. 2002). It has not been studied whether changes in the ternary complexes introduced by IGFBP-3 proteolysis changes the interactions with the IGF1R, the IGF2R and the IR<sub>A</sub>-IGF1R/ IR<sub>B</sub>-IGF1R. Ligand binding to the IGF1R or

the IR induces a conformational change that will lead to activation of the tyrosine kinase domain and auto phosphorylation of the receptor. The catalytic tyrosine kinase domain of the IGF-IR and IR share approximately 80% amino acid identity. Activation of the receptor recruits IRS and start two downstream intracellular signaling cascades i.e. the PI3-kinase cascade as well as the MAPK cascade. The intracellular signaling that is activated by the IGF-IR and IR is very similar and it is not clear how the metabolic and mitogenic signal diverge. For a full review of the IGF/ insulin signaling differences consult reviews (Dupont and LeRoith 2001; Siddle, Urso et al. 2001).

### **IGF biological effects**

Even though most of the circulating IGFs are derived from the liver the IGFs are abundantly expressed in most tissues (Daughaday and Rotwein 1989). At the cellular level IGFs stimulate growth by increasing proliferation, differentiation as well as by inhibiting apoptosis. In addition IGFs stimulate metabolic effects such as glucose and amino acid uptake. The effects on glucose uptake has been demonstrated *in vivo* in humans (Guler, Zapf et al. 1987; Zierath, Bang et al. 1992). Knock-out studies of the IGF-I, IGF-II and IGF1R further demonstrate that the IGFs are important for pre- and postnatal growth *in vivo* (DeChiara, Efstratiadis et al. 1990; Liu, Baker et al. 1993). The importance of IGFs for longitudinal growth is further illustrated in patients with GH receptor deficiency (Laron syndrome). Due to the negative feedback loop between pituitary GH and hepatic IGF-I these patients have increased levels of GH and decreased levels of IGF-I. These growth-retarded patients increase their growth rate when they are treated with IGF-I but not with GH (Wilton 1992). The main effects of IGF-I in humans are also illustrated in a patient with a IGF-I gene deletion (Camacho-Hubner, Woods et al. 1999). This boy has no endogenous IGF-I production and subsequently undetectable serum IGF-I levels. The lack of IGF-I increases his GH levels. The IGFBP-3 and ALS levels are also elevated demonstrating that GH regulates IGFBP-3 and ALS even in the absence of IGF-I. Even though he has high IGF-II levels and high GH levels he had pre- and postnatal growth retardation. Furthermore, the high levels of GH and insulin resulted in insulin resistance. IGF-I treatment has increased his longitudinal growth and reduced insulin, IGF-II, ALS, IGFBP-3 and GH levels in serum.

### **IGFBP-3 biological effects**

The importance of ternary complex formation with ALS was demonstrated by Firth et al (Firth, McDougall et al. 2002). They showed that an IGFBP-3 mutant with reduced ALS binding properties does not decrease the hypoglycemic effect of IGF-I as efficiently as normal IGFBP-3. This demonstrates that the formation of the ternary complex is an important mechanism to reduce the acute metabolic effects of IGF-I. This also explains that IGF-I given intravenously is not as potent as insulin in increasing glucose uptake (Guler, Zapf et al. 1987) despite the fact that IGF-I and insulin are equipotent in stimulating glucose uptake into human muscle strips *ex vivo* (Dohm, Elton et al. 1990). Consequently, circulating levels of IGF are 100 to 1000-fold higher than insulin without causing hypoglycemia. The importance of the ternary complex as a reservoir of IGFs was further demonstrated when co-administration of IGF-I/ IGFBP-3 to type 1 diabetic patients resulted in less side-effects compared to IGF-I alone (Cheetham, Holly et al. 1995; Clemmons, Moses et al. 2000). However, the IGF-I/ IGFBP-3 treatment resulted in reduced blood glucose and insulin requirements. On the other hand IGFBP-3 transgenic mice have fasting hyperglycemia, impaired glucose tolerance and insulin resistance suggesting reduced IGF bioavailability (Silha, Gui et al. 2002). These mice also have a modest reduction in birth weight and postnatal growth despite slightly elevated IGF levels (Modric, Silha et al. 2001). These studies demonstrate that IGFBP-3 and the ternary complex are important for glucose metabolism and for pre- and postnatal growth. Whether IGFBP-3 independent effects contribute to these findings remains unknown. Knock-out mice lacking ALS and hepatic IGF-I have low levels of circulating IGF-I. These mice are growth retarded suggesting that the ternary complexes are important for maintaining growth (Yakar, Rosen et al. 2002). However, mice only lacking hepatic IGF-I was in the same size as their littermates suggesting that local production of IGF-I is equally important (Yakar, Liu et al. 1999).

### **Clinical states of elevated IGFBP-3 proteolysis**

Increased serum IGFBP-3 proteolysis has not only been found in pregnancy but also in the post-operative state (Davenport, Isley et al. 1992), diabetes type-I and -II (Bang, Brismar et al. 1994; Bereket, Lang et al. 1995), severe illness (Davies, Wass et al. 1991), arthritis (De Benedetti, Meazza et al. 2001) and in cancer (Frost, Macaulay et al. 1993).

Surgery is a commonly used treatment in a broad variety of diseases. Post-operative recovery represents a big part of hospital care. Several factors affect the length of hospital stay e.g. insulin resistance. Post-operative insulin resistance is a way to ensure energy supply to the brain after trauma. The etiology is unknown but it has been speculated that cytokines released after a trauma might induce insulin resistance. The serum levels of IGFs and the IGFBPs are altered after surgery and in critically ill patients. Serum IGF-I and IGFBP-3 is decreased (Cotterill, Mendel et al. 1996; Mesotten, Wouters et al. 2004). However, low levels of IGFBP-3 are explained by increased IGFBP-3 proteolysis. Increased IGFBP-3 proteolysis after surgery has been correlated to the increase in C-peptide (Bang, Nygren et al. 1998). This suggests a link between IGFBP-3 proteolysis and post-operative insulin resistance. It has also been shown that an insulin infusion after surgery resulted in a further increase in IGFBP-3 proteolytic degradation (Nygren, Carlsson-Skwirut et al. 2001). It was further shown that the relative increase in proteolysis was correlated to the relative increase in free dissociable IGF-I. However, insulin induced changes in IGFBP-1 was identical before and after surgery. This suggests that insulin may regulate IGFBP-3 proteolysis during insulin resistance and that this might affect IGF-I bioavailability. Insulin resistance may increase IGFBP-3 proteolysis to increase IGF bioavailability and compensate for reduced glucose uptake. The post-operative state, critical illness and arthritis have elevated IGFBP-3 proteolysis as well as Interleukin-6 (IL-6) (de Benedetti, Massa et al. 1991; Thorell, Nygren et al. 1996; de Groof, Joosten et al. 2002). The factors that activate proteases resulting in increased serum IGFBP-3 proteolytic activity in these states are still not known and the proteases responsible for IGFBP-3 proteolysis *in vivo* have not yet been identified. Benedetti et al. suggested that elevated IGFBP-3 proteolysis in arthritis was mediated by IL-6 (De Benedetti, Meazza et al. 2001). They found that transgenic mice overexpressing IL-6 have elevated proteolytic degradation of IGFBP-3. It was suggested that elevated IGFBP-3 proteolysis increase IGF clearance and that this might explain reduced growth in children with arthritis. In addition, *in vitro* studies have shown that IL-6 increases the activity of an IGFBP-3 protease i.e. matrix metalloproteinase-2 (MMP-2) (Janowska-Wieczorek, Marquez et al. 1999; Kossakowska, Edwards et al. 1999).

# AIM & HYPOTHESIS

The overall aim of this project was to identify proteases and inductors of proteases that result in IGFBP-3 cleavage *in vivo*, and to study their impact on IGF-I bioavailability. Specifically we hypothesized that:

**Paper I:** Limited vascular damage during blood sampling activates thrombogenic and/ or fibrinolytic enzymes sufficiently to increase IGFBP-3 proteolysis and increase free dissociable IGF-I in humans. This effect is dependent on the level of IGFBP-3 proteolysis (normal versus elevated e.g. post-operatively).

**Paper II:** Infusion of IL-6 increases IGFBP-3 proteolysis and increases free dissociable IGF-I by activating specific proteases in human serum. IL-6 effects circulating IGFBP-1 by changing the serum levels of insulin and/ or cortisol.

**Project report III:** Isolation and structural characterization of IGFBP-3 proteolytic fragments from post-operative patients will demonstrate specific cleavage sites in IGFBP-3 and disclose the protease. IGF binding kinetics of the post-operative IGFBP-3 fragment compared with full-length IGFBP-3 will help to identify domains in IGFBP-3 important for IGF-IGFBP interactions.

# METHODS

## Subjects

In the present thesis the IGF-system was studied in post-operative patients (I, III) as well as in healthy subjects (II). The post-operative state was chosen since it is well characterized regarding changes in the IGF-system and it represents a catabolic condition with insulin resistance. The post-operative patients in this thesis represent a population with a mean age of 62 years. The age of the subjects are important since the serum IGF and IGFBP-3 peak in puberty and decline with age. In contrast, the healthy subjects investigated in paper II were much younger and were expected to have higher IGF levels. The advantage of studying younger individuals in paper II is that changes in IGF-I and intact IGFBP-3 by IL-6 should be easily detectable. Although age related changes in IGFBP-3 proteolysis has not been reported it may be assumed that elderly people are more catabolic and thus may be expected to have relative increased IGFBP-3 proteolytic activity. It is therefore advantageous to study younger individuals for the detection of increased IGFBP-3 proteolysis. A younger patient group is not possible in paper I and III since large abdominal surgery is not common in a younger population. Many of the post-operative patients were operated because of malignancies. This might be important since serum IGFBP-3 proteolysis has been reported to be elevated in patients with for example breast cancer (Frost, Macaulay et al. 1993) and because some tumors over express IGF-II.

## IGFBP-3 determinations

### *IGFBP-3 western blot (I, II and III)*

Western immunoblot is a common method used to detect proteins in various tissues. In regard of the IGFBPs it is the simplest and best way of determining whether different molecular forms of IGFBP-3 exist in serum. Serum is separated on SDS-PAGE and then transferred to nitrocellulose membranes. A specific antibody detects IGFBP-3. The primary antibody ( $\alpha$ -hIGFBP-3) used in this study recognizes intact IGFBP-3 as well as several IGFBP-3 fragments. The polyclonal  $\alpha$ -hIGFBP-3 antiserum cross-reacted with human serum albumin and displayed a band of 60 kDa on the gels. By comparing the intensity of IGFBP-3 fragments related to intact IGFBP-3 we get an estimate of endogenous IGFBP-3 fragmentation in serum (IGFBP-3 *in vivo* fragmentation). However, the antibody may have different affinities for intact and fragmented IGFBP-3. Western immunoblot is therefore a semi-quantitative method that should be used to detect relative differences. In order to compare the result of several different gels (I, II) IGFBP-3 bands need to be quantified. Gels are scanned and the intensity of bands determined using different programs. In the present thesis we have used Micro Image (I) and Image J (II). This may explain that we found different fragmentation levels paper I and II. To be able to compare results of several different gels data should be normalized to an internal standard. In paper I we investigated the relative difference in IGFBP-3 proteolysis in response to cannulation and stasis. The relative change was therefore determined by comparing against the individual mean. In paper II IGFBP-3 proteolysis was compared to human normal serum (reference sample).

### *IGFBP-3 protease assay (I, II)*

By *in vitro* degradation of recombinant labeled IGFBP-3 the protease activity in serum samples can be estimated (IGFBP-3 *in vitro* proteolytic activity). Recombinant IGFBP-3 labeled with  $^{125}\text{I}$  is incubated for 5 h (37°C) with the sample of interest. The sample mixture is then separated on SDS-PAGE and the radioactivity is detected on film. In this way different proteolytic fragments can be detected. This is also a semi-quantitative method that should be used to detect relative differences. Importantly, protease activity should not be analyzed in EDTA plasma, since EDTA inhibits several proteases. For both western immunoblot and the protease assay it is important that samples are kept cold at all times. Increasing temperature will affect the proteases present (activate/ inactivate) and affect the circulating forms of IGFBP-3. As of today there are no absolute quantitative ways of determining IGFBP-3 proteolysis. Serum IGFBP-3 western blot (IGFBP-3 *in vivo* proteolysis) and IGFBP-3 protease assay (IGFBP-3 *in vitro*



proteolysis) complement each other since western immunoblot shows how much of the circulating pool of IGFBP-3 that has been proteolyzed *in vivo* and the protease assay show the *in vitro* protease activity of recombinant IGFBP-3.

#### *Western ligand blot*

Western ligand blot was developed by Hossenloop et al to detect IGFbps in serum (Hossenlopp, Seurin et al. 1986). This method has been extensively used in the IGF research field. Serum is separated on SDS-PAGE and proteins are then transferred to nitrocellulose membranes. IGF-I labeled with  $^{125}\text{I}$  is then hybridized to the membrane. The conserved secondary structure of the IGFbps allows binding to  $^{125}\text{I}$ -IGF-I. Radioactivity is then detected on films. However, it was early recognized that ligand blot of pregnancy serum displayed no intact or fragmented IGFBP-3. When a 30 kDa IGFBP-3 band was identified in western immunoblot it was discovered that the 30 kDa fragment is not detected in ligand blot. Suikkari et al (Suikkari and Baxter 1991) demonstrated that the 30 kDa fragment binds IGF-I but not  $^{125}\text{I}$ -IGF-I probably due to a conformational change. Western ligand blot is a good way of characterizing the different IGFbps in a sample, but it should not be used for studies of IGFBP proteolysis.

#### *IGFBP-3 immunoassays*

There are commercially available assays for determinations of IGFBP-3. However, the antibodies used in these assays have not been fully characterized regarding their binding to different IGFBP-3 fragments. A change in IGFBP-3 proteolysis may therefore give false results. Lasserre et al have reported a specific assay that detect IGFBP-3 proteolysis due to different characteristics of the antibodies (Lassarre, Lalou et al. 1994).

### **IGF-I determinations**

#### *Total IGF-I; RIA (III) and DELFIA (II)*

Total IGF-I in serum was analyzed with two different methods, a RIA and an IGF-I DELFIA. For both these methods an acid ethanol extraction was first performed in order to remove the IGFbps. This was done as described by Bang P et al (Bang, Eriksson et al. 1991). The RIA is a classic competitive immunoassay that use des(1-3)IGF-I labeled with  $^{125}\text{I}$  as a tracer. Des(1-3)IGF-I and IGF-I in the sample compete for binding the primary antibody. The use of des(1-3)IGF-I as a tracer has the advantage that it does not interact with IGFbps. The drawback with the RIA is that radioactivity is used. A more sensitive assay to determine IGF-I was recently established in our laboratory modified from (Frystyk, Dinesen et al. 1995). The IGF-I DELFIA offers a greater sensitivity and can be performed without radioactivity in microtiter plates. This is a sandwich assay where a pre-bound monoclonal IGF-I antibody captures IGF-I in the sample of interest. A second monoclonal IGF-I antibody labeled with europium then detects IGF-I. With the addition of enhancement solution the europium molecules are released and form stable fluorescent chelates that can be measured. This allow for “time-resolved” fluorescence to be measured.

#### *Free IGF-I ELISA (II)*

Free dissociable IGF-I was analyzed with commercial ELISA. Serum samples are directly analyzed without acid-ethanol extraction. This is a sandwich assay with two monoclonal IGF-I antibodies. The second one is labeled with horseradish peroxidase. When substrate is added the enzymatic reaction produce a colored product. A disadvantage with this method is that the binding of free IGF-I to the pre-bound antibody may shift the binding equilibrium and release more IGF-I from binary and ternary complexes. These assays may therefore turn out not to be valid (Bang, Ahlsen et al. 2001). However, in order to study relative changes over time the method is acceptable. An alternative assay has been developed by Frystyk et al (Frystyk, Ivarsen et al. 2001). They use ultra filtration in the first step to separate free IGF-I from IGF-I in binary and ternary complexes. This is followed by a DELFIA.

### **Protein purifications (III)**

#### *Acid size exclusion chromatography*

This is a standard method used for separating IGFs from IGFbps previously described (Giudice, Farrell et al. 1990; Bang, Eriksson et al. 1991). Even though Sephadex G-50 is commonly used for separating IGFs from IGFbps we found that the 30 kDa IGFbp-3 fragment and IGF-I partly overlapped during elution. A disadvantage in using this as a first step when purifying serum proteins is that only a small quantity can be loaded on the column every time. A second disadvantage is that acidic fractions often have to be lyophilized and neutralized before the next step.

#### *Ultra filtration*

Ultra filtration represents another method to separate IGFs from IGFbps. Filters with a 30 kDa as well as a 10 kDa cut-off were evaluated. We found that the 30 kDa filters gave a better recovery even though most IGF was retained in the filter. Filters have been used to separate free IGF-I from bigger complexes before assessment of free IGF-I (Frystyk, Ivarsen et al. 2001).

#### *IGF-I affinity chromatography*

IGF-I affinity chromatography is a good way to isolate IGFbp-3 fragments that retain IGF affinity. It selectively captures IGF binding proteins. However, it is important to remove IGFs before this step otherwise IGF in solution will compete with the column. However, small quantities of IGF may facilitate the isolation of IGFbp-3 fragments since IGFs preferable bind intact IGFbp-3.

#### *Reversed phase chromatography*

This method separates proteins based on their hydrophobicity and it is a good way of separating different IGFbps and IGFbp fragments. Two different columns, with different hydrophobic properties were used in this study, a C1/ C8 column and a C2/ C18 column. The IGFbps were eluted with an increasing concentration of acetonitrile.

# RESULTS & DISCUSSION

## Paper I

In the present study we found that there was no difference in IGFBP-3 *in vitro* and *in vivo* fragmentation in serum samples collected directly after venous cannulation compared to samples collected from a previously inserted catheter. This indicates that cannulation does not affect IGFBP-3 proteolysis. Furthermore, there was no difference in IGFBP-3 proteolysis in samples collected with or without a standardized venous stasis. We also examined whether the post-operative state of elevated IGFBP-3 proteolysis was further affected by venous cannulation and stasis. However, we found no such effect. We conclude that venous stasis and cannulation does not affect IGFBP-3 proteolysis. However, four subjects out of six displayed elevated IGFBP-3 *in vitro* fragmentation directly after the first cannulation before surgery but the mean level was not significantly elevated. This is in accordance with our initial observation of elevated IGFBP-3 proteolytic activity in some diabetic adolescents when a new venous catheter was inserted (unpublished observation). This might represent normal variation in small population. However, it might reflect a true increase in IGFBP-3 protease activity since the variation was very low at all the other time points. Due to this variability we found no increase in IGFBP-3 *in vitro* fragmentation after surgery at this time point. The fact that the variable response was not observed after surgery may reflect that a small difference in IGFBP-3 proteolytic activity is difficult to detect in an elevated state of IGFBP-3 proteolysis. In accordance with previous studies we found an increase in IGFBP-3 proteolytic activity after surgery in the other time points studied (Davenport, Isley et al. 1992). Due to the variability at the first sampling we suggest that serum samples should be collected approximately 15 min after cannulation when comparing pre and post-operative IGFBP-3 proteolysis.

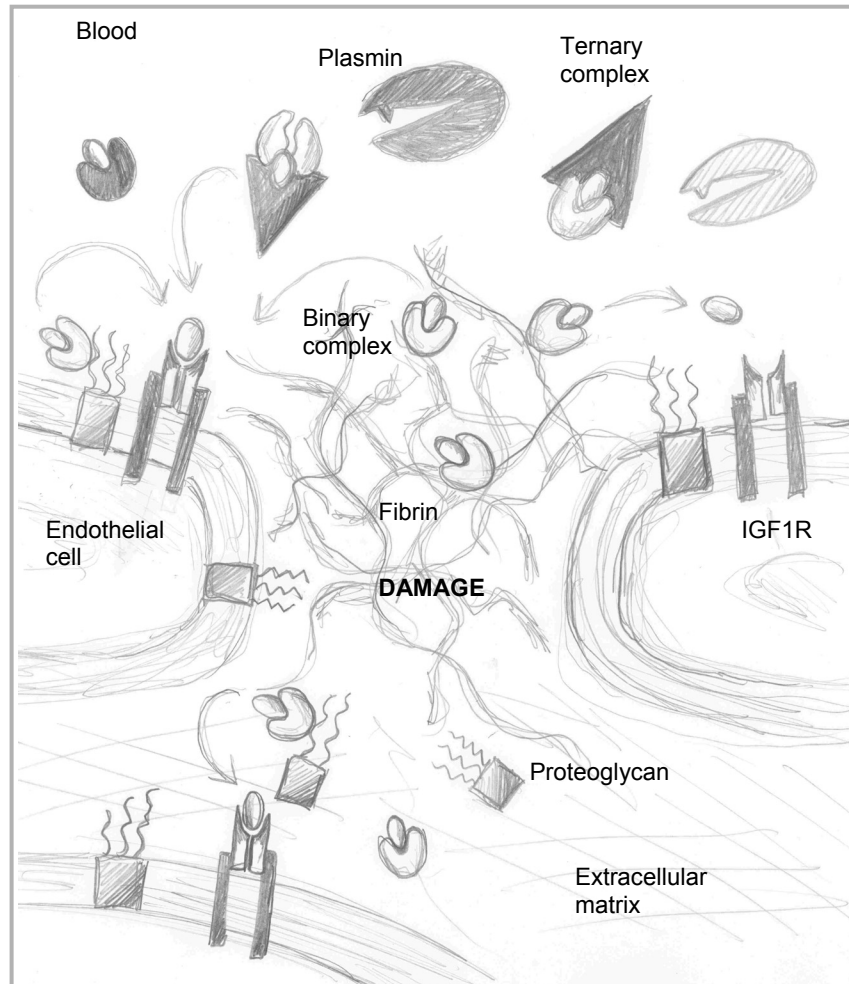
Vascular endothelial damage (mechanical injury or atherosclerosis) activates thrombogenic enzymes and subsequently fibrinolytic proteases. In addition, venous stasis may activate these enzymes by altering mechanical factors such as blood flow and shear stress (Turitto and Hall 1998; Sokabe, Yamamoto et al. 2004). Several of these proteases have been shown to cleave IGFBP-3 *in vitro* e.g. plasmin and thrombin (Lalou, Silve et al. 1994; Booth, Boes et al. 1996; Bang and Fielder 1997). Studies of plasmin digested IGFBP-3 fragments have demonstrated that increasing concentrations of plasmin increase the number of small fragments and reduce the quantity of intact IGFBP-3. This indicates that there is a progressive degradation to smaller fragments. Plasminogen binds IGFBP-3 and the IGF-IGFBP-3 binary complex (Campbell, Durham et al. 1998) and plasmin dissociates binary complexes (Campbell, Novak et al. 1992). A recent study characterized a 92 kDa IGFBP-3 protease in human plasma (Cohn fraction IV) that was inhibited by aprotinin and  $\alpha$ 2-antiplasmin (Oesterreicher, Blum et al. 2005). This further suggests that plasmin might be the IGFBP-3 protease responsible for IGFBP-3 proteolysis in serum from healthy subjects. Activation of fibrinolytic and thrombogenic enzymes may therefore result in elevated IGFBP-3 proteolysis. However, a small vascular damage such as that produced by cannulation may not be a sufficient activator of these enzymes. This may explain our results. Still, the aim of this study was to determine whether blood sampling might increase IGFBP-3 proteolysis. It was important from a methodological point of view; to know whether venous stasis and cannulation activated thrombogenic, and fibrinolytic proteases to such an extent that IGFBP-3 proteolysis occurred *in vivo*. This could have questioned earlier studies of IGFBP-3 proteolysis. However, we show that this is not the case.

Based on these results we cannot exclude that thrombogenic and fibrinolytic enzymes cleave IGFBP-3 *in vivo*. A large vascular insult e.g. venous thrombosis or myocardial infarction may increase serum proteolytic degradation of IGFBP-3 by activating these enzymes to a greater extent. To our knowledge elevated serum IGFBP-3 proteolysis has not been demonstrated in cardiovascular disease. However, both coagulation and fibrinolysis is increased in type 2 diabetes suggesting that these enzymes may affect IGFBPs (Aso, Matsumoto et al. 2002). In accordance, type 2 diabetic patients have elevated levels of

serum IGFBP-3 proteolysis (Bang, Brismar et al. 1994). Whether the elevated IGFBP-3 proteolysis depends on fibrinolytic enzymes or other proteases remain unknown.

Serum levels of IGFBP-3 are high (100 nM) and a substantial effect on the circulating pool of IGFBP-3 may require extensive activation of proteases. It is therefore possible that these proteases only affect the IGFBPs at the site of injury. IGFBP-3 proteolytic activity has been demonstrated in rat wound fluid (Robertson, Pickering et al. 1996). The level of IGFBP-3 was significantly lower than in plasma but there was relatively more binary complexes compared to ternary complexes. This suggests an increased IGF bioavailability. With western ligand blotting they also discovered a 24 kDa IGFBP that was abundant in wound fluid. This probably reflects IGFBP-4. In addition, a role of IGFBP-4 proteolysis in wound healing was recently suggested by Chen et al (Chen, Leiferman et al. 2003). They found increased expression of the IGFBP-4 protease PAPP-A in healing skin wounds. IGFBP-3 binding to fibrin/ fibrinogen has further demonstrated that IGF is localized to sites of endothelial damage (Campbell, Durham et al. 1999). The binding of IGFBP-3 to fibrin is mediated by the heparin binding domain of IGFBP-3. This suggests that fibrin binding also reduce IGF affinity. Increased expression of proteoglycans/ fibrin at the site of injury may therefore be another mechanism to increase IGF bioavailability during wound healing. Susceptibility to proteolysis may be affected by heparin binding since the binding of IGFBP-3 to endothelial cells prevents proteolytic cleavage by plasmin and thrombin (Booth, Boes et al. 2002). This suggests that proteolysis only affect soluble IGFBPs. Possible mechanisms of increased IGF bioavailability during wound healing are illustrated in Figure 2.

Extensive activation of fibrinolytic enzymes during cardiovascular disease may affect the IGFBPs in damaged endothelium/ atherosclerotic plaque. In addition to the fibrinolytic/ thrombogenic enzymes, MMPs are increasingly activated in atherosclerotic plaque (Chen, Eriksson et al. 2005). MMPs cleave IGFBP-3 *in vitro* (Fowlkes, Enghild et al. 1994). However, whether MMPs affect IGFBP proteolysis within the atherosclerotic plaque remains unknown. Increased IGF bioavailability at the endothelial wall may increase cell proliferation and survival and affect disease progress. The role of IGFs in atherosclerosis is reviewed in (Bayes-Genis, Conover et al. 2000).



**Figure 2.** A schematic picture showing IGF and IGFBPs in damaged endothelium. Increased activation of thrombogenic and/ or fibrinolytic proteases may increase IGFBP-3 proteolysis and increase IGF bioavailability. However, IGFs may also be localized to the site of injury by binding to the fibrin network. In addition, IGFs bound to proteoglycans may increase IGF bioavailability.

## Paper II

Collaboration provided us with serum samples from healthy men that had received a 3-hour IL-6 infusion, and control subjects that received a saline infusion (Keller, Keller et al. 2003). We found that an IL-6 infusion (n=6) reaching plasma concentrations of 0.1 ng/ ml did not alter IGFBP-3 *in vitro* or *in vivo* proteolysis up to 5 hours after the end of infusion as compared to saline infusion (n=6). However we found that serum IGFBP-1 was significantly elevated 4-5 hours after the start of infusion in response to IL-6. To study the mechanism of IGFBP-1 induction insulin and cortisol was measured. We found that cortisol was increased during infusion (after 1-2 hours) in response to IL-6. An IL-6 infusion has previously been shown to increase the levels of cortisol (Petersen, Carey et al. 2005). In addition cortisol is a well-known regulator of IGFBP-1 transcription and it increases serum IGFBP-1 in humans (Conover, Divertie et al. 1993). Cortisol may therefore be responsible for the elevated IGFBP-1 that we found. However, in the limited amount of data we did not find any correlation between the change in cortisol and IGFBP-1. This may depend on the small size of the study population. Insulin decreases the hepatic expression of IGFBP-1 (Powell, Suwanichkul et al. 1991) and serum IGFBP-1 is largely regulated by insulin. However, the change in IGFBP-1 was not dependent on insulin since these remained unchanged. We found no difference in insulin levels between the IL-6 and saline group. Despite the increase in IGFBP-1 we did not find any change in total or free dissociable IGF-I.

Several clinical states are characterized by elevated IL-6 and IGFBP-3 proteolysis e.g. post-operatively, critical illness, arthritis and type 2 diabetes. In addition, Benedetti et al demonstrated that IL-6 transgenic mice had increased IGFBP-3 proteolysis (De Benedetti, Meazza et al. 2001). IL-6 can also increase the activity of MMP-2 (Janowska-Wieczorek, Marquez et al. 1999; Kossakowska, Edwards et al. 1999). This suggests that IL-6 may increase IGFBP-3 proteolysis by activating IGFBP-3 proteases such as MMPs. The fact that we could not demonstrate elevated IGFBP-3 proteolysis may depend on the IL-6 concentrations or the time of exposure. Patients with elevated serum IL-6 are often exposed to the cytokine for a longer time compared to in our study. The IL-6 concentrations in the clinical states mentioned above are elevated but there is huge difference in concentrations. As an example obese insulin resistant subjects have plasma IL-6 of 2 pg/ ml (Kern, Ranganathan et al. 2001) while critically ill children (sepsis) have 50-1200 ng/ ml (de Groof, Joosten et al. 2002). In contrast, healthy subjects have undetectable levels. The IL-6 concentrations in this study were 0.1 ng/ ml which is relatively low. However, infusing higher levels of IL-6 in humans may not be ethically acceptable. In addition, an *in vitro* study found no effect of IL-6 concentrations of 100 ng/ ml (20 hours) on IGFBP-3 proteolysis suggesting that the concentrations may not be of importance (Lelbach, Scharf et al. 2001). This supports our results of unchanged IGFBP-3 proteolysis. However, based on these results we cannot exclude that prolonged exposure to high concentrations of IL-6 may affect IGFBP-3 and IGF-I.

It has been speculated that elevated IL-6 may be responsible for the changes in expression of IGF-I, IGFBP-1 and IGFBP-3 in critical illness. Lelbach et al observed a dose-dependent inhibition of IGF-I mRNA by IL-6 in rat hepatocytes (Lelbach, Scharf et al. 2001). In addition they found that IL-6 stimulated IGFBP-3 expression. Thissen et al found that IL-6 stimulated IGF-I mRNA expression in rat hepatocytes *in vitro* (Thissen and Verniers 1997). However, IL-6 did not further increase GH stimulated IGF-I expression. *In vivo* studies also show different results. A study by Benedetti et al show that IL-6 transgenic mice have normal IGF expression but reduced IGF-I levels in plasma possibly due to increased IGFBP-3 proteolysis (De Benedetti, Meazza et al. 2001). On the other hand Lieskovska et al show that IL-6 transgenic mice have reduced expression of IGF-I and GH receptors in the liver (Lieskovska, Guo et al. 2002). In addition they have demonstrated that the expression of SOCS3 is increased in these mice but that this did not affect GH induced STAT5 activation. However, SOCS3 may interact with the IGF1R and IR and reduce their signaling (Dey, Furlanetto et al. 2000; Zong, Chan et al. 2000; Ueki, Kondo et al. 2004). This suggests that GH dependent effects of IL-6 on IGF-I and IGFBP-3 may be mediated by reduced GH sensitivity. However, as judged by the *in vitro* data mentioned above IL-6 may also affect IGF-I and IGFBP-3 independently of GH. We did not observe any effect of IL-6 infusion on the serum

level of IGF-I or IGFBP-3. This may depend on the IL-6 concentration since these were much higher in the studies mentioned above.

IL-6 has been shown to induce the expression of IGFBP-1 *in vitro* (Samstein, Hoimes et al. 1996; Lang, Nystrom et al. 1999; Lelbach, Scharf et al. 2001). This is in agreement with our results of increased IGFBP-1 *in vivo*. We speculate that IL-6 directly increased IGFBP-1 since we found no correlation between the increase in cortisol and the increase in IGFBP-1. A study of critically ill patients further indicated that IL-6 is a stronger regulator of IGFBP-1 compared to insulin (Mesotten, Delhanty et al. 2002). They found that insulin treatment failed to reduce elevated IGFBP-1. Our data suggests that IGFBP-1 is more sensitive to elevated IL-6 compared to IGFBP-3 and IGF-I. The effects of IL-6 on the IGF-system are summarized in Table 1.

The alterations in the IGF-system in critically ill patients cannot only be attributed to IL-6 since several other cytokines are simultaneously elevated. It has been shown that IL-6, TNF $\alpha$  and IL-1 $\beta$  have an additive effect of IGFBP-1 expression (Lang, Nystrom et al. 1999). Another study further show increased IGFBP-3 proteolysis in fibroblasts treated with TNF $\alpha$  and IL-1 $\beta$  (Liu, Tsushima et al. 1999). This indicates that other cytokines may be of equal importance. In addition, other hormones may affect IGFBP-3 proteolysis. Interestingly Nygren et al found an additional increase in IGFBP-3 proteolysis during insulin infusion after surgery (Nygren, Carlsson-Skwirut et al. 2001). It was further shown that the relative increase in proteolysis was correlated to the relative increase in free dissociable IGF-I. These data suggests that insulin is an inducer of IGFBP-3 proteolysis in the catabolic post-operative state of elevated cytokines. Elevated levels of insulin in type 2 diabetes may possibly explain elevated IGFBP-3 proteolysis. The elevated plasma IL-6 in these patients may induce insulin resistance since IL-6 impairs hepatic insulin signaling (Senn, Klover et al. 2002; Klover, Zimmers et al. 2003). This may be mediated by increased expression of SOCS proteins (Senn, Klover et al. 2003). IL-6 may induce insulin resistance and the resulting high insulin levels may signal to increase IGF bioavailability. This would explain the unchanged level of IGFBP-3 proteolysis and insulin that we found.

**Table 1.** IL-6 effects on the IGF-system

	<b>Effect</b>	<b>Reference</b>
<b><i>In vitro</i></b>		
IGFBP-3	Increased liver expression, unchanged proteolysis	Lelbach
IGFBP-1	Increased liver expression	Lelbach/ Samstein/ Lang
IGF-I	Increased/ reduced liver expression	Thissen/ Lelbach
Insulin	Reduced hepatic signaling	Senn
<b><i>In vivo</i></b>		
IGFBP-3	Increased serum proteolysis	Benedetti/ de Groof
IGF-I	Unchanged/ reduced expression, decreased in serum	Benedetti/ Lieskovska/ de Groof
IGFBP-1	Elevated in plasma	De Groof
Insulin	Insulin resistance	Klover
GH	Normal in serum, reduced quantities of GH receptors	Lieskovska

### Project report III

In the present study we have isolated the major 30 kDa IGFBP-3 fragment in human post-operative serum (post-op day 1-5) using the following methods; acid size exclusion chromatography/ ultra filtration, IGF-I affinity chromatography and reverse-phase chromatography. This purification scheme has previously been used to isolate IGFBP-3 fragments in pregnancy serum (Ahlsen, manuscript). N-terminal sequencing provided us with the amino acid sequence GASS. This 30 kDa fragment corresponded to the N-terminal end of IGFBP-3. This is in agreement with the major fragment generated by plasmin digestion (Booth, Boes et al. 1996; Lalou, Sawamura et al. 1997; Booth, Boes et al. 1999). Due to low recovery we were not able to obtain the C-terminal amino acid sequence. Based on these results we cannot provide any information about cleavage sites or the proteases responsible. However, based on the molecular weight of the fragment (30 kDa) we suggest that the cleavage site is in the end of the variable middle region. A recent study characterized a 92 kDa IGFBP-3 protease in human plasma (Cohn fraction IV) that was inhibited by aprotinin and  $\alpha$ 2-antiplasmin (Oesterreicher, Blum et al. 2005). This further suggests that plasmin might be the IGFBP-3 protease responsible for IGFBP-3 proteolysis in serum from healthy subjects. Plasmin has a broad specificity and catalyzes the cleavage of Lys-X and Arg-X. Potential cleavage sites in IGFBP-3 include Lys 160, Arg 95 and Arg 97. An IGFBP-3 fragment generated *in vivo* was recently characterized in plasma of a child with acute renal failure. This 17 kDa fragment started at Lys 160 (Schebek-Furstenberg, Standker et al. 2004). Furthermore Kubler et al (Kubler, Draeger et al. 2002) characterized two IGFBP-3 fragments generated *in vivo* in hemofiltrate. The structure of the 11 and 16 kDa fragments were predicted based on N-terminal sequencing and mass spectrometry. They concluded that they corresponded to non-glycosylated and glycosylated <sup>1-98</sup>IGFBP-3. If plasmin is responsible, the IGFBP-3 fragment isolated by Kubler et al may turn out to be 1-97, since the C-terminal was only predicted based on the molecular mass and since there is a predicted cleavage site at Arg 97. As of today IGFBP-3 fragments found in post-operative serum remain uncharacterized and we do not know which proteases that is responsible for elevated IGFBP-3 proteolysis after surgery. The study by Bang et al (Bang, Nygren et al. 1998) indicate that it might be a serine protease or MMP but this remain to be shown. However, surgical trauma and vascular injury increase the activity of fibrinolytic enzymes. This indicates that plasmin might be the mediator of elevated IGFBP-3 proteolysis after surgery.

The purification scheme used in the present study depended on the capture of the 30 kDa IGFBP-3 fragments by IGF-I affinity chromatography. This can be accomplished since the major IGFBP-3 fragment (30 kDa) retains high IGF-I affinity. For a successful purification with this approach it is crucial that we separate IGFs from the binding proteins before this step. The low recovery may be explained by the partial separation of IGFs from IGFBPs in size exclusion chromatography and in ultra filtration. However, the low recovery cannot only be attributed to this. It was also affected by the moderate recovery of the other chromatographic steps. The number of steps greatly affects overall recovery. The concentration of IGFBP-3 in the starting material may also affect recovery. The mean age of the study population was 65 years. This indicates lower basal levels of IGFBP-3, since serum levels of IGFBP-3 decline with age. Furthermore, since the degradation of IGFBP-3 after surgery is partial the concentration of IGFBP-3 fragments in relation to other serum proteins is very low. The fact that this approach was successful in isolating IGFBP-3 fragments in pregnancy serum may be because the basal level of IGFBP-3 is higher in young pregnant women and also that there is a 100% degradation of IGFBP-3. In order to improve recovery we attempted to try an alternative purification strategy i.e. anti-IGFBP-3 affinity chromatography. However, we failed to try this approach since the rabbits immunized with IGFBP-3 failed to generate antibodies against human IGFBP-3.

The different parameters tested in this study demonstrated that even though ultra-filtration did not fully separate IGFs from IGFBP-3 a higher recovery was accomplished compared to with size-exclusion chromatography. The reason that size exclusion chromatography did not work properly in separating IGFs from IGFBPs remain unknown. Sephadex G-50 is known to separate IGF and IGFBPs (Giudice, Farrell et



al. 1990; Bang, Eriksson et al. 1991). One reason may be the column size. The large amounts of proteins only allowed us to inject 0.25 ml every time. This resulted in 166 injections and 664 fractions that were pooled, lyophilized and resuspended. A larger column might increase recovery since fewer injections would be needed. Surprisingly, the ultra-filtration approach selectively separated the IGFBP-3 fragments. The reason for this may be that IGFs in solution (in our sample) have a higher affinity for intact IGFBP-3 and that this allowed IGFBP-3 fragments to bind the IGF-I column. Subsequently, this allowed us to selectively separate IGFBP-3 fragments.

# CONCLUSION

■ From paper I we conclude that the blood sampling procedure, i.e. venous cannulation and stasis does not increase serum IGFBP-3 proteolysis pre- or post-operatively. IGFBP-3 proteolytic activity was elevated after surgery. However, this increase could not be detected directly after the first sampling due to a variable response.

■ From paper II we conclude that short-term exposure to IL-6 does not increase IGFBP-3 proteolysis, or change the circulating levels of total and free IGF-I. On the contrary, IL-6 increased IGFBP-1 as compared to saline infusion. Interestingly, the change in IGFBP-1 was not due to changes in insulin. There was no correlation between elevated cortisol and IGFBP-1 indicating that IL-6 directly increased IGFBP-1.

■ From project report III we conclude that the 30 kDa IGFBP-3 fragment in post-operative serum correspond to the N-terminal end of IGFBP-3. Based on these results we cannot provide any information on protease cleavage sites.

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