From Department of Clinical Science, Intervention and Technology,
Division of Renal Medicine

Karolinska Institutet, Stockholm, Sweden

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Erik Nilsson

Karolinska Institutet

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By
Erik Nilsson

Principal Supervisor:
Prof. Peter Stenvinkel
Karolinska Institutet
Department of Clinical Science, Intervention and Technology (CLINTEC)
Division of Renal Medicine

Co-supervisors:
Dr. Juan-Jesus Carrero-Roig
Karolinska Institutet
Department of Medical Epidemiology and Biostatistics (MEB)

Dr. Olof Hellberg
Örebro University
School of Medical Sciences
Department of Internal Medicine

Dr. Olof Heimburger
Karolinska Institutet
Department of Clinical Science, Intervention and Technology (CLINTEC)
Division of Renal Medicine

Opponent:
Prof. Vladimir Tesar
Charles University
First Faculty of Medicine
Department of Nephrology

Examination Board:
Dr. Sergiu-Bogdan Catrina (coordinator)
Karolinska Institutet
Department of Molecular Medicine and Surgery

Dr. Gregor Guron
University of Gothenburg
Department of Molecular and Clinical Medicine

Dr. Sverker Ek
Karolinska Institutet
Department of Clinical Science, Intervention and Technology (CLINTEC)
Division of Obstetrics and Gynecology

I must finish what I've started, even if, inevitably, what I finish turns out not to be what I began.

Salman Rushdie, Midnight's Children.

ABSTRACT

Several hormonal systems are disrupted in chronic kidney disease (CKD) and disturbances of the growth hormone axis could contribute to increased morbidity and mortality through effects on cardiovascular health, energy metabolism and inflammation. The overall aim of this thesis was to increase knowledge about hormonal alterations and their consequences in CKD, focusing on cardiovascular disease (CVD), mortality and the growth hormone axis in end-stage renal disease. We tested cross-sectional associations between different growth hormones and known risk factors for CVD. We also analyzed longitudinal associations between blood hormone levels and outcomes. In addition we studied potassium disturbances in a large healthcare-based cohort.

Paper I was a cohort study of insulin-like growth factor 1 (IGF-1) levels and mortality in patients starting hemodialysis. We found that patients with IGF-1 levels in the lowest tertile were more often female, had lower creatinine, lower serum albumin and higher degree of inflammation. Low IGF-1 levels were associated with increased mortality and this association remained when adjusted for age, sex and comorbid conditions [diabetes mellitus (DM), CVD, heart failure]. Our results show that low IGF-1 levels at dialysis initiation are associated with increased mortality.

Paper II was a cohort study of incident dialysis patients investigating pregnancy-associated plasma protein-A (PAPP-A) in relation to mortality and CVD. We also tested whether body composition, DM or inflammation would act as effect modifiers on this association. Higher PAPP-A levels showed a moderate association with mortality when adjusted for cardiovascular risk factors and body composition but when also including high-sensitivity C-reactive protein (hs-CRP) the association was weakened. In survival analysis, interactions with PAPP-A were found for hs-CRP, DM and fat tissue index. This indicates that higher PAPP-A levels in patients starting dialysis are associated with increased mortality, and that this association is modulated by inflammation, DM and body composition.

In **paper III** we describe incidence and determinants of hyperkalemia and hypokalemia in a large healthcare based cohort including adult individuals from Stockholm accessing healthcare in 2009. Estimated glomerular filtration rate (eGFR) was included as a measure of kidney function. During three years follow-up, 13.6% had at least one episode of hypokalemia. Hyperkalemia of any degree of severity was detected in 7%. Frequency of potas-

sium testing was naturally associated with dyskalaemia risk. In adjusted analysis, lower hyperkalemia risk was seen in women and in loop/thiazide diuretics users while hyperkalemia risk was higher in older age, lower eGFR, diabetes, heart failure and use of renin-angiotensin-aldosterone system inhibitors. Women, those of younger age, with higher eGFR or use of diuretics had higher risk of hypokalemia.

In **paper IV**, we investigated PAPP-A levels and mortality in prevalent HD patients and sought specifically to test our previous exploratory findings that inflammation and DM modulated the effect of PAPP-A on mortality. Higher PAPP-A was associated with increased mortality both in univariable analysis and when adjusted for confounders and cardiovascular risk factors. An interaction between PAPP-A and DM was found, implying greater prognostic utility of PAPP-A in patients with DM.

In **paper V**, we hypothesized that combining a function measurement of muscle strength (handgrip strength, HGS) with a biochemical nutritional marker (plasma IGF-1 levels) would have a stronger association to mortality in CKD than either marker alone. Patients in the low IGF-1 and low HGS category had increased mortality rate compared to the other categories and this association was robust when adjusted for Framingham's CVD risk score, CVD, malnutrition, smoking, hs-CRP, albumin and lean body mass index. The predictive utility of IGF-1 was somewhat enhanced but still weak in the low HGS group. Our results indicate that low HGS predicts higher mortality risk in CKD and that adding IGF-1 levels may marginally improve risk prediction in the low HGS group.

LIST OF SCIENTIFIC PAPERS

The thesis is based on the following publications, which will be referred to in the text by their Roman numerals:

I **Nilsson E**, Carrero JJ, Heimbürger O, Hellberg O, Lindholm B, Stenvinkel P. A cohort study of insulin-like growth factor 1 and mortality in haemodialysis patients. Clin Kidney J 2016; 9: 148–52

II **Nilsson E**, Cao Y, Lindholm B, Ohyama A, Carrero JJ, Qureshi, Stenvinkel P. Pregnancy-associated plasma protein-a predicts survival in end-stage renal disease-confounding and modifying effects of cardiovascular disease, body composition and inflammation. Nephrol Dial Transplant 2017; 32: 1776

III **Nilsson E**, Gasparini A, Ärnlöv J, Xu H, Henriksson KM, Coresh J, Grams ME, Carrero JJ. Incidence and determinants of hyperkalemia and hypokalemia in a large healthcare system. Int J Cardiol 2017; 245: 277–84

IV **Nilsson E**, Rudholm T, Stenvinkel P, Ärnlöv J. Pregnancy-associated plasma protein A and mortality in haemodialysis. Eur J Clin Invest 2018; e12959

V Chen Z, **Nilsson E**, Lindholm B, Heimbürger O, Barany P, Stenvinkel P, Chen J, Qureshi AR. Low plasma insulin-like growth factor-1 associates with increased mortality in chronic kidney disease patients with reduced muscle strength. MANUSCRIPT.

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LIST OF ABBREVIATIONS

Abbreviation	Denotes
BMI	Body mass index
CAD	Coronary artery disease
CeVD	Cerebrovascular disease
CKD	Chronic kidney disease
CKD-MBD	Chronic kidney disease-mineral and bone disorder
CVD	Cardiovascular disease
CVE	Cardiovascular event
DM	Diabetes mellitus
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
FBM	Fat body mass
FBMI	Fat body mass index
FTI	Fat tissue index
GH	Growth hormone
HD	Hemodialysis
HDL	High density lipoprotein
HGS	Handgrip strength
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
LBM	Lean body mass
LBMI	Lean body mass index
LTI	Lean tissue index
PAPP-A	Pregnancy associated plasma protein A
PD	Peritoneal dialysis
PEW	Protein-energy wasting
PTH	Parathyroid hormone
PVD	Peripheral vascular disease
SGA	Subjective global assessment
TNF	Tumor necrosis factor

Chapter 1

Background

1.1 Introduction

The focus of this chapter is the growth hormone (GH)/insulin-like growth factor 1 (IGF-1) axis in chronic kidney disease (CKD), specifically components of this system that we have investigated in the thesis papers. A brief vignette exemplifying hormonal alterations in CKD is given, along with reflections on biomarkers in general and the challenge of utilizing them in research concerning end-stage renal disease (ESRD). A section on potassium dysequilibrium is also included.

CKD is defined as "abnormalities of kidney structure or function, present for >3 months, with implications for health" [1]. Causes of CKD include for example diabetes mellitus (DM), hypertension and inflammatory renal diseases. If renal function deteriorates and reaches ESRD, continued renal replacement therapy is needed [2]. Dialysis, either hemodialysis (HD) or peritoneal dialysis (PD), is usually selected as the initial renal replacement therapy and renal transplantation performed for some patients at a later time [3]. CKD can cause alterations in bone, brain, heart, vasculature and other organs [4], as well as dysregulation of acid-base homeostasis and electrolytes such as potassium [5,6]. CKD and especially ESRD is therefore associated with increased mortality and morbidity [5,7]. Importantly, CKD is associated with a dramatic increase in cardiovascular disease (CVD). To illustrate this, **Figure 1.1** shows the incidence of cardiovascular events in different degrees of kidney failure, based on results from Go et al [8]. Note that patients on dialysis or renal transplant were

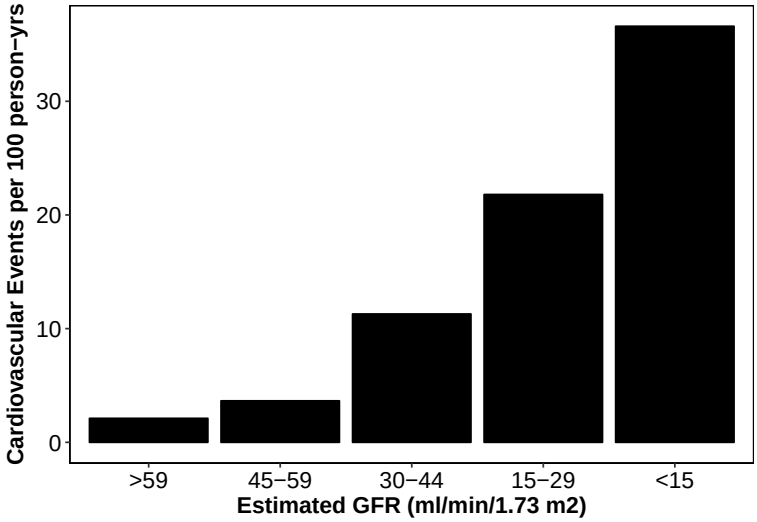

Figure 1.1: *Age-Standardized Rates of Cardiovascular Events in renal failure*

excluded.

1.2 Causes of hormone dysequilibrium in CKD

Several hormonal systems are dysregulated in CKD. Well known examples include a relative deficiency of erythropoetin in renal anemia [9] and elevated parathyroid hormone levels due to retention of phosphate, hypocalcemia and decreased 1,25-dihydroxyvitamin D production [10]. The mechanisms by which endocrine alterations appear in CKD are diverse. For example, stimulation or inhibition of hormone synthesis can be seen for parathyroid hormone (PTH) [10] and erythropoetin [9], respectively. Post-translational modifications also affect PTH signalling, leading to altered effect on the hormone receptor [11]. Accumulation of hormones or hormone fragments due to reduced renal clearance is yet another way in which CKD alters hormone homeostasis, exemplified by retention of PTH fragments [11] and decresed clearance of prolactin (PRL), the latter leading to secondary dysregulation of other sex hormones [12]. PRL also exemplifies the disturbance of normal pulsatile secretion that is present for many pituitary hormones, in that elevated PRL inhibits the rhythmicity

Table 1.1: *Example mechanisms of hormonal dysregulation in CKD*

Effect on hormone	Mechanisms
Synthesis	Inhibition
	Stimulation
	Post-translational modifications
Release	Stimulation
	Inhibition
	Rythmicity
Transport	Altered levels of hormone binding proteins
	Altered function of hormone binding proteins
Degradation and elimination	Proteolysis
	Reduced glomerular filtration
Action on receptor	Receptor number
	Resistance
	Local sequestration/release
Intracellular action	Alteration of intracellular pathways

of gonadotropin-releasing hormone, with secondary effects on sex hormone secretion. Alterations of hormone transport can also occur in renal failure. IGF-1, which mediates growth hormone effects, binds to a number of different IGF-1 binding proteins (IGFBPs). Retention of these binding proteins affects hormone availability, -degradation and -action [13]. Finally, the effect of a hormone on its target tissue is influenced by receptor density and intracellular pathways, mechanisms which are also disturbed in IGF-1 signalling in CKD [14]. Example mechanisms of hormonal dysregulation in CKD are listed in **Table 1.1**.

1.3 The growth hormone/insulin-like growth factor 1 axis

GH is secreted from the anterior pituitary, regulated by neuroendocrine mechanisms including stimulatory GH-releasing hormone (GHRH) and inhibitory somatostatin (SS), both originating from the hypothalamus [15]. Historically, it was postulated that the effect of GH on skeletal muscle was mediated by some other substance [16], later isolated and termed "Somatomedin" [17], further characterized as "nonsuppressible insulin-like activity" [18] and named insulin-like growth factor (IGF) [19]. It has later been found that GH also has direct effects apart from those mediated through IGF-1 and that IGF-1 has both auto- and paracrine functions [20]. Further, there are two types of IGFs, IGF-1 and

IGF-2, that bind with different affinity to the IGF-1 receptor, although GH action is mediated primarily by IGF-1 [20]. GH induces production of its effector hormone in multiple tissues, but the liver is the main source of circulating IGF-1 [21,22].

Several IGFBPs have been identified [23]. These prevent degradation and elimination of IGF-1, providing a plasma pool of the bound hormone, and interact in a complex manner to regulate local IGF-1 availability [24]. Paracrine IGFBPs sequester IGF-1, which can be made available to the target tissue through proteolysis of the IGF-1-binding protein complex [25]. Intriguingly, it has been found that IGFBPs also have diverse IGF-1 independent functions [26–28]. For example, IGFBP1 promotes cell migration [29] and IGFBP-3 has IGF-1 independent effects on cell growth [28]. Locally, IGF-1 action is also regulated by the membrane bound matrix metalloproteinase pregnancy-associated plasma protein-A (PAPP-A), which cleaves IGFBP-4 and thereby releases IGF-1 from its binding protein, making it available to the IGF-1 receptor at the target cell surface [25]. IGF-1 acts on most cell types and has both mitogenic and anti-apoptotic effect [28], typically inducing hypertrophy and hyperplasia [30]. Apart from regulating growth during adolescence, GH and IGF-1 have multiple other actions in adults, including effects on energy metabolism [31], bone metabolism [32] and on the cardiovascular system [33]. Further, reduced IGF-1 signalling has been associated with longevity in a number of organisms [34].

A concept of reduced levels of "free" IGF-1 has been suggested [14], where elevated IGFBP-levels would lead to lower concentrations of unbound IGF-1 and thereby decreasing IGF-1 signalling. This can be challenged, however, since IGFBPs can also enhance IGF-1 action [13,35]. In addition, there is a substantial local IGF-1 production and -regulation [36]. Thus, disturbances in IGFBP levels are not easily interpreted in terms of reduced or increased IGF-1 signalling in the target tissues.

1.3.1 Pregnancy-associated plasma protein-A

PAPP-A is a membrane bound matrix-metalloproteinase which cleaves IGFBPs 3-, 4- and 5 and thereby releases IGF-1 from its binding protein, making it available to the IGF-1 receptor at the target cell surface [37]. A second form of PAPP-A, named PAPP-A2, has also been identified. It has proteolytic activity on IGFBPs 3- and 5 but IGFBP4 is specifically cleaved by PAPP-A [38]. Since IGFBPs have higher affinity than the IGF-1 receptor

for IGF-1, proteolysis of the IGFBPs increases IGF-1 activity on the receptor. Enzymes like PAPP-A that exhibit proteolytic activity against IGFBPs are therefore important regulators of IGF-1 action [39]. The role of PAPP-A in regulating growth is supported by animal experiments. For example overexpression of PAPP-A has anabolic effect on muscle in mice [40] while PAPP-A knock-out mice exhibit reduced IGF1 activity, primarily in the kidney [41]. Modulating PAPP-A expression also affects bone growth [42] and muscle function [43].

PAPP-A is expressed in most tissues, with the highest abundance in kidney, bone and placenta [44]. During pregnancy PAPP-A is synthesized in placental tissue and complex-bound to the proform of eosinophil major basic protein. Elevation of non-complexed plasma PAPP-A in other conditions may represent up-regulation of tissue expression, induced by injury or inflammation, and increased escape of otherwise membrane-bound PAPP-A into the circulation [25]. A number of regulators of PAPP-A synthesis and -activity have been identified. PAPP-A is affected in inflammatory injury responses and tissue remodeling [44], where PAPP-A expression is stimulated by tumor necrosis factor (TNF) and interleukin-1b (IL-1b) and inhibited by interferon gamma and stanniocalcin-2. It should be noted that PAPP-A regulation by cytokines may be cell-specific. For example, in vascular smooth muscle cells, IL-1b is more potent than TNF in stimulating PAPP-A expression. However, the opposite has been demonstrated in human coronary artery endothelial cells, in which IL-1b has a weaker stimulatory effect than TNF [45]. There is some evidence of interaction between PAPP-A and other hormonal systems. Induction of PAPP in acute phase response may be dependent on sex hormones with progesterone in females and oestradiol in males being permissive of PAPP increase while testosterone inhibits PAPP-elevation in response to injury [46]. Further, PTH inhibits PAPP-A activity in vitro and this suppression is alleviated by estradiol [47]. However, early studies on PAPP-A are limited by polyspecific antibodies obtained from immunization with purified protein from plasma, which may recognize both PAPP-A and pro-myelin basic protein, and should therefore be interpreted with caution [25].

To summarize, PAPP-A regulates local IGF-1 action and may be up-regulated in pathological conditions such as tissue inflammation.

1.3.2 GH/IGF-1 axis and cardiovascular disease

The presence of GH-receptors in heart and vasculature indicates that the GH-IGF1-axis has effects on these systems. In experimental models, GH and IGF-1 induces cardiac myocyte hypertrophy and enhance cardiac contractility [48,49]. Conditions with GH excess or -deficiency are associated with CVD and CVD-related mortality [50,51] and in the general population, both high and low IGF-1 levels associate to increased cardiovascular mortality [52]. On the basis of its mitogenic effect on smooth muscle cells, it has been proposed that IGF-1 promotes development of atherosclerotic lesions [53] and the finding of IGF-1 and IGFBP expression in human atherosclerotic plaques suggested that these play a role in coronary artery disease [54]. In apparent conflict with this hypothesis, *low* IGF-1 levels are also associated with increased risk of ischemic heart disease [55]. Further, in a study on Apo E knock-out mice, treatment with an IGF-1 analog reduced carotid stenosis as well as signs of plaque instability such as cap to core ratio and rate of intraplaque hemorrhage [56]. Others have found that the beneficial effect from IGF-1 may be mediated through anti-inflammatory effects on the vasculature, with reductions in both interleukin-6 (IL-6) and TNF expression [57]. Thus, it is not clear if IGF-1 activity promotes or inhibits atherosclerosis and there may be differential effects on development of arterial stenosis and plaque instability, respectively.

In rodent models, lower PAPP-A activity has been associated with longer lifespan, less vascular cell proliferation after injury, reduced plaque area and less luminal occlusion as well as inhibited atherosclerotic plaque progression in atherosclerosis [41,58]. In a study by Harrington et al atherosclerosis-prone mice with deletion of the PAPP-A gene were generated [59]. While the mice retaining PAPP-A expression had progression of atherosclerotic lesion area, with increases in PAPP-A, IGF-1 and IGFBP-4 mRNA in these lesions, the PAPP-A knock-out mice had less progression of atherosclerosis and lower IGF-1 activity in aortic tissue. Increased PAPP-A activity has in rodents been linked to vascular smooth muscle cell proliferation and atherosclerosis [60]. An increased plasma PAPP-A concentration is associated with extent of coronary artery disease in humans, as well as with mortality in chronic stable angina pectoris and acute coronary syndromes [61]. An interaction between PAPP-A and the anti-inflammatory cytokine interleukin-10 (IL-10) levels has been observed, indicating that elevated PAPP-A is associated with worse outcomes only when IL-

10 levels are low. Increased PAPP-A levels have also been linked to carotid- and peripheral artery disease [61,62]. Sangiorgi et al analyzed PAPP-A in atherosclerotic lesions removed at endarterectomy [63]. They found that both serum levels and expression of PAPP-A was higher in vulnerable plaques compared to stable ones. Others have found that PAPP-A levels are inversely associated with plaque thickness, but positively correlated with echogenicity and plaque inflammation [61].

The GH-IGF-1 axis may also be linked to cardiovascular disease via effects on energy metabolism and the metabolic syndrome [31,64]. GH affects protein metabolism, increases lipolysis and glucose output from the liver [31] and among other features, persons with GH deficiency exhibit central adiposity and decreased muscle mass [65]. In a randomized controlled trial of GH treatment in postmenopausal women with abdominal obesity, Franco et al showed that GH reduced abdominal visceral fat and improved insulin sensitivity as well as reducing low-density lipoprotein (LDL) concentrations [66]. In line with these associations between GH and metabolic profile, lower serum IGF-1 levels are associated with an increased prevalence of insulin resistance and risk factors for metabolic syndrome in nondiabetic persons [67] and IGF-1 treatment increases insulin sensitivity in type 2 DM [68].

1.3.3 Links to body composition, malnutrition and inflammation

In line with the influence of GH-IGF on energy metabolism described above, these hormones also affect body composition [69]. GH has protein anabolic effects and preserves muscle mass in the fasting state [70]. Persons with GH deficiency exhibit central adiposity and decreased muscle mass [65]. In a study on adult onset GH deficiency, Bengtsson et al investigated the effects of recombinant GH on body composition [71] and found that GH treatment reduced body fat and that visceral fat was reduced more than subcutaneous fat. Also, GH treatment increased muscle volume, increased serum phosphate and reduced PTH and thyroxine levels. In turn, body composition and nutritional factors can also influence the GH-IGF-1 axis. GH production rate and frequency of pulsatility is reduced in obesity [72] and undernutrition causes GH resistance with lower IGF-1 levels and elevated GH [73,74]. Consequently, IGF-1 levels correlate with nutritional markers such as triceps skinfold thickness [75]. PAPP-A is also related to body composition. It is more abundantly expressed in visceral- than in

subcutaneous fat and in PAPP-A knock-out mice mesenteric fat depots are reduced [76]. Thus, there is evidence of a bidirectional link between fat tissue and PAPP-A. In ESRD, there is a negative association between PAPP-A and body mass index (BMI) and a link to protein-energy wasting (PEW) has been suggested [77].

The concept of PEW describes loss of protein mass and energy stores [78] and is common in hemodialysis [79]. Notably, surrogate markers for PEW are associated to CVD and increased mortality. Hypoalbuminemia is viewed as a marker of PEW, but its utility as a biomarker is limited since it is affected by inflammation and urinary losses [80–82]. Compared to albumin, IGF-1 correlates more strongly to biochemical- and anthropometric markers of PEW and malnutrition [83]. In ESRD, IGF-1 activity is thought to be reduced due to increased levels of IGF-1 binding proteins (IGFBP's) as well as altered receptor- and post-receptor signaling [84]. These GH/IGF-1-axis disturbances may contribute to PEW in ESRD.

The growth hormone axis interconnects with inflammatory pathways. A number of different inflammatory mediators, such as interleukin-1, TNF, and IL-6, can modulate the effects of IGF-1 on target tissue [85] and conversely, IGF-1 infusion has been found to reduce vascular expression of IL-6 and TNF, as well as reducing markers of oxidative stress [57]. Further, GH replacement in GH deficiency reduces inflammatory markers c-reactive protein (CRP) and IL-6 [86]. Chronic inflammation is thought to cause GH-axis dysregulation through multiple mechanisms, including changes in IGFBP levels and disrupted intracellular signalling pathways [87]. There is also evidence of an interaction between IGF-1 levels and inflammation on nutritional parameters [88].

1.3.4 GH/IGF-1 axis dysregulation in CKD

In CKD, the GH-IGF-1 is dysregulated in multiple ways [14]. Although animal experiments have shown that stimulation of GH release via GHRH from the hypothalamus may be reduced in renal failure [89], the daily secretion rate of GH is elevated in uremic patients [90] and GH is eliminated to a large extent through the kidneys [91]. Veldhius et al found that GH half life was prolonged in uremic patients compared to controls, with a half life of 17 $\pm$1.4 minutes in controls versus 21 $\pm$1.3 minutes in uremia [90], and that GH pulsatility was at a higher frequency in uremia. Others have confirmed that metabolic clearance of GH is determined by glomerular filtration rate (GFR) and plasma concentration of the

hormone [92]. GH is ultrafiltrated in the kidney and to a large extent absorbed by endocytocis into the tubular cells where it undergoes proteolysis. IGF-1 on the other hand, is not filtered to any significant extent by the kidneys [93]. The pattern of hormone levels in CKD suggest a state of GH resistance, with reduced levels of IGF-1 and normal or elevated GH [94,95]. Tönshoff et al studied GH and IGF-1 levels in children with pre-terminal or end-stage renal disease and found that while GH levels were increased in children with CKD, IGF-1 levels were not elevated compared to controls, indicating that IGF-1 producing tissues did not respond to GH stimuli [96]. Reduced IGF-1 expression has been demonstrated in some tissues in uremic rats [97] and uremic serum has reduced IGF-1 activity [98]. Several perturbations may contribute to GH resistance in CKD, for example altered IGFBP levels [99], reduced GH receptor density [97], and impaired post-receptor signalling [100].

Chronic inflammation is common in ESRD and contributes to GH-axis dysregulation as noted above. In addition, down-regulation of GH- and IGF-1 receptors in inflammation and uremia has been hypothesized [87,97]. However, there is some evidence to the contrary and in a study on 21 ESRD patients with 14 controls, Greenstein et al found no difference in GH receptor expression between the groups [101], although GHBP levels were reduced in uremia and correlated to the inflammatory marker CRP.

To summarize, GH axis disturbances could contribute to increased morbidity and mortality in ESRD through effects on cardiovascular health, energy metabolism and inflammation, or serve as markers associated with these conditions.

1.4 GH-IGF-1 axis hormones as biomarkers in ESRD

As described above, the GH axis dysregulated in CKD and linked to CVD, PEW and inflammation, conditions that are prevalent in ESRD and contribute to increased mortality in this population [102]. Components of the GH-IGF-1 axis that are possible to measure in blood could therefore be suitable candidate biomarkers for PEW and CVD as well as for predicting mortality in ESRD patients. Although GH levels in blood are difficult to assess due to pulsatility and diurnal variation, IGF-1, IGFBPs and PAPP-A are not subject to these limitations.

Table 1.2: *Characteristics of cohort studies on IGF-1 and mortality in dialysis*

Author	Year	Population	N
Himmelfarb	1994	Prevalent HD	52
Fernandez-Reyes	2002	Prevalent HD	64
Qureshi	2002	Prevalent HD	128
Hung	2005	Prevalent HD	158
Kalousova	2012	Prevalent HD	261
Beberashvili	2013	Prevalent HD	96
Jia	2014	Incident HD/PD	365
Nilsson	2016	Incident HD	265

GH has not been evaluated as a risk marker for mortality in ESRD. It has however been used in treatment studies with mortality as end-point. Kopple et al conducted a RCT of GH treatment in HD patients [103]. The study was terminated early due to slow recruitment and no subjects completed the planned 24 month treatment period. However, 712 patients were randomized and 695 received at least one dose of recombinant human GH (hGH). There was no effect of treatment on all-cause mortality, cardiovascular events or the combination of these two outcomes. Interestingly, a reduction of hs-CRP was seen with treatment, as well as effects on body composition, with reduction in body weight and total body fat, although there was no change in lean body mass.

The association of IGF-1 to outcomes in ESRD has been investigated in a few relatively small studies. Characteristics of these studies is presented in **Table 1.2**.

A summary of results from the same studies is presented in **Table 1.3**. Note that in the studies by Hung et al [104] and Nilsson et al [105], the risk associated with the low IGF-1 is shown, while others presented the (reduced) risk associated with high IGF-1.

1.4.1 Himmelfarb 1994

In 1994, Himmelfarb et al investigated nutritional parameters including IGF-1 in 52 prevalent HD patients (mean age 65 years, 48% male, 44% with diabetes) and found that low dialysis adequacy (Kt/V) and high cortisol but not IGF-1 levels were predictive of mortality [106]. A multivariable Cox proportional hazards model was used, including the parameters kt/V, serum albumin, predial-

Table 1.3: *Summary of results from cohort studies on IGF-1 and mortality in dialysis*

Author	Crude model	Adjusted model
Himmelfarb 1994	-	OR not presented (P 0.2)
Fernandez-Reyes 2002	-	OR 0.98-0.99
Qureshi 2002	Log rank P <0.01	-
Hung 2005	NS (t-test survivors)	OR 0.47-5.43†
Kalousova 2012	HR 0.532-0.814	-
Beberashvili 2013	HR 0.73-3.07	HR 0.53-2.81
Jia 2014	HR 0.31-0.57	HR 0.32-0.98 per SD
Nilsson 2016	OR 1.7–3.4†	OR 1.1-2.4†

† Odds ratios for lowest IGF-1 tertile.

ysis cortisol, IGF-1, triceps skin-fold thickness (TSFT), mid-arm circumference (MAC), the latter two treated as continuous variables. The authors used stepwise backward elimination to identify important predictors of outcome (hospitalization, death). However, it is not clear from the paper which variables were eliminated by this procedure and all variables listed above were included in the final model. Follow-up was 12 months and outcomes were hospitalization (n = 35) and death (n = 14), respectively. Notably, serum albumin was not associated to any of the outcomes in this study. IGF-1 levels were found to be higher in HD patients compared with age- and sex matched controls (178 $\pm$ 9 ng/mL vs 142 $\pm$ 58 ng/mL, t-test p $\leq$ 0.05). Also, postdialysis IGF-1 levels were elevated compared to predialysis values (217.8 $\pm$ 13.5, p $\leq$ 0.05). IGF-1 was not correlated with nutritional parameters in this study (mentioned in the discussion section - data not shown).

A major limitation, emphasized by the authors, is the small sample size. In survival analysis, a low number of events per variable in multivariable models increases the risk of type I and type II error [107]. A rule of thumb is a minimum of 10-20 events per variable [108], although it has been argued that this rule can be relaxed in certain circumstances, for example in sensitivity analysis when demonstrating adequacy of control for confounding [109]. In the study by Himmelfarb et al, there were 14 deaths and six covariables (again, it is not clear from the article if all six were entered into the final cox model or if some were eliminated through backwards exclusion), yielding 2.3 events per variable (5.8 for hospitalizations). In addition, potentially important confounders such as sex, age and concomitant diabetes were not considered. Thus, the risk of error as well as residual confounding must be considered high in this analysis.

1.4.2 Fernandez-reyes 2002

In a marginally larger study (N = 64), Fernandez reyes et al [110] studied IGF-1 as a predictor of mortality in prevalent HD patients. Nutritional and inflammatory parameters such as albumin, CRP, BMI, TSF, MAC and mid-arm muscle circumference (MAMC) were measured.

IGF- levels were at 194 ± 110 ng/mL (range 23-551). IGF-1 levels were lower in those with higher CRP values (t-test p-value = 0.02) but the association was not statistically significant in a multivariable adjusted logistic regression model. Using Pearson correlation coefficient IGF-1 was associated with age, albumin and cholesterol. Cox proportional hazards models were used for analyzing mortality rate, with two variables per model according to the number of events (18 deaths, yielding 6 events per covariable). With CRP as the only covariable, lower IGF-1 (p = 0.011) as well as higher CRP predicted higher mortality.

Again, a major limitation of this study is the low number of participants and consequently relatively few events during follow-up. The authors appropriately limited the number of covariables in the survival models, although from this follows instead a high risk of residual confounding. Furthermore, the large variety of statistical methods used for testing associations between variables make results less clear and exposes the analysis to the multiple testing problem. For example, baseline associations to CRP were tested using students t-test (for differences in IGF-1 levels between CRP categories), logistic regression (for adjusted analysis) and Pearson correlation.

1.4.3 Qureshi 2002

In a study investigating the association of nutritional status and inflammation and other comorbidities to mortality, Qureshi et al [111] measured IGF-1 levels in 128 prevalent HD patients. Nutritional status indices included subjective global nutritional assessment (SGNA) and anthropometric markers and serum albumin. Patients followed for 36 months with 57 deaths. IGF-1 below median (170 ng/mL) was associated with higher mortality (logrank p <0.01). In multivariable cox, parameters that exhibited non-proportional hazards were excluded and IGF-1 was not in the final model.

This study has a somewhat larger sample size that the ones above,

but a major limitation to inference based on the results is the absence of multivariable adjusted models for IGF-1. Baseline associations between IGF-1 and other variables were not reported.

1.4.4 Hung 2005

Hung et al [104] measured IGF-1 and other nutritional markers [albumin, subjective global assessment (SGA)] as well as markers of inflammation (IL-6, IL-1b, TNF, serum amyloid A and CRP) in 158 prevalent HD patients. Patients with obvious signs of acute infection were excluded, follow-up was 36 months and 31 patients died during this time. Covariables included dialysis adequacy (kt/V), age, sex, dialysis vintage, coronary artery disease (CAD), SGA and DM.

Variables were dichotomized into high and low-risk fractions respectively, which for IGF-1 was the lowest tertile (<32.6 ng/mL). IGF-1 in the lowest tertile was associated with an odds ratio for mortality of 1.6 (95% CI 0.47-5.43, p = 0.45) when adjusted for age, sex, DM, kt/V and dialysis vintage. In the fully adjusted models SGA and CRP remained predictive of mortality and adding both these markers to the model improved model likelihood ratio compared to including either. Of the variables listed above, IGF-1 was positively associated with albumin and diabetes and negatively with TNF, serum amyloid A and dialysis vintage.

As in some of the previously mentioned studies, the number of events were not sufficient for the number of covariables included in survival analysis (5.2 events per covariable in the minimally adjusted models), again making both type I and type II errors more likely. Testing of model assumptions are not stated in the article.

1.4.5 Kalousova 2012

With the chief aim of studying PAPP-A and mortality in HD, Kalousova et al also measured IGF-1 levels in 261 prevalent HD patients [112]. During 5 year follow-up 146 patients died. Univariable Cox proportional hazards models were used for overall mortality and death due to infection or cardiovascular causes, respectively. IGF-1 was associated with all types of mortality in univariable analysis ($p < 0.001$ for overall mortality) but, follow-

ing a backwards exclusion method, IGF-1 was not included in adjusted models.

1.4.6 Beberashvili 2013

Beberashvili [113] studied the interaction between IGF-1 levels and inflammation (CRP, TNF) in 96 prevalent HD patients followed for up to four years, during which 48 patients died. Survival analysis utilized Cox proportional hazards models with adjustment for age, gender, DM status, dialysis vintage and history of past cardiovascular disease. IGF-1 was converted into an age and sex adjusted standard deviation score (SDS). An SDS score below median was not associated with increased mortality in crude (OR 1.5, 95% CI 0.73-3.07) or adjusted model, but for the multiplicative interaction term of IGF-1 SDS below median and IL-6 above median there was a statistically significant association to survival in both crude (OR 4.27, 95% CI 2.10–8.68, $p < 0.001$) and adjusted (OR 3.32, 95%CI 1.58–6.97, $p = 0.002$) models. Similar results were seen for cardiovascular mortality.

In this study, the sample size was small and the number of events insufficient (six events per variable when including the three non-reference categories of the interaction term) for the multivariable analysis performed. It should also be noted that adjustment variables included age and sex, and since IGF-1 SDS was already derived from the same variables, there may be over-adjustment for these parameters.

1.4.7 Jia 2014

Jia et al measured IGF-1 in 365 CKD patients, including 115 in HD, 92 PD and 158 CKD stage 5 not on dialysis [114]. Follow up was 5 years follow-up and 28% (n ca 131) patients died during this time. Survival was analyzed Cox proportional hazard models for multivariable adjustment and and renal transplantation included as a competing risk. When adjusted for calendar year of inclusion, age, sex, DM, CVD, IL-6, and poor nutritional status (SGA >1), higher IGF-1 at baseline was associated with lower all-cause mortality when including all 365 CKD patients (HR, 0.57; 95% CI, 0.32 to 0.98) and when including only HD patients, but not when only PD patients were included.

In this study, baseline IGF-1 levels were negatively associated to age, DM, CVD history, PEW, IL-6, and osteoprotegerin and

positively- to serum phosphate, serum calcium, body fat mass, bone mineral density, and fibroblast growth factor-23.

In a subset of patients, a follow-up IGF-1 was measured after 1 year. IGF-1 increased after initiation of dialysis in both patients starting on PD and patients starting on HD. Mortality rate was increased in patients with persistently low or decreasing IGF-1 concentrations compared with those who had persistently high or increasing IGF-1 concentration.

This was the largest study to date reporting data on IGF-1 and mortality in ESRD (N = 365), although CKD stage 5 patients not on dialysis was also included (n on HD or PD = 207). Survival analysis utilizing the full cohort (N = 365) had a sufficient number of events (16 events/covariable) for the adjusted models but the number of deaths in subgroups were not reported. A limitation of this study is the pooling of data from different cohorts, introducing a risk of bias due to differences between studies. However, this may to some extent be ameliorated due to mean IGF-1 levels being similar in patients on PD (187.2 ± 75.3 mkg/L), HD (191.3 ± 93.9 mkg/L) and the total number of participants (191.9 ± 90.5 mkg/L). Further, the authors also report risk estimates in the different subgroups.

1.4.8 Nilsson 2016

This study is part of the thesis [105]. Briefly, 265 HD patients were followed for a minimum of three years and 134 deaths occurred during follow-up [105]. IGF-1 was categorized into low or non-low based on tertiles and predicted mortality in both crude and adjusted models. Adjustment for confounders was incremental in a series of Cox proportional hazards models with the full model including age, sex, diabetes mellitus, cardiovascular disease, heart failure, high-sensitivity CRP (hs-CRP), serum creatinine and serum albumin. The number of events per variable in the statistical models ranged from 13 to 67, which was deemed sufficient. Testing of model assumptions was not reported. A major limitation of this study was the lack of body composition- or nutritional parameters.

1.4.9 Other cohorts

Carrero at al [115] reported associations between IGF-1 and thyroid hormone levels in ESRD. They found positive correlations to triiodothyronine (T3) and free T3 and a negative correlation to

Table 1.4: *Characteristics cohort studies on PAPP-A and mortality in dialysis*

Author	Year	Population	N
Kalousova	2004	Prevalent HD	40
Etter	2010	Prevalent HD	170
Kalousova	2012	Prevalent HD	261
Kalousova	2014	Prevalent HD with DM	1255
Nilsson	2017	Incident HD and PD	286

thyroxin-binding globulin, but did not report on the association between IGF-1 and mortality.

1.4.10 Summary of studies on IGF-1 levels and outcomes in ESRD

The two previous studies on IGF-1 and outcomes in ESRD that were adequately powered [112,114] showed an association between lower IGF-1 and higher mortality in ESRD. However, Kalousova et al [112] did not report multivariable adjusted results for IGF-1 on mortality and in the study by Jia et al [114] pooling of different cohorts, whose participants had different stages of kidney disease, is an important limitation. Our study [105] addresses some of these limitation through including only incident HD patients, providing sufficient power for the analyses made and presenting results adjusted for potential confounders. Nevertheless, we did not have access to data on body composition and concerns about residual confounding therefore remain.

1.5 PAPP-A as a biomarker in ESRD

The association of PAPP-A to outcomes in ESRD has been investigated in a few relatively small studies and one larger study on prevalent HD patients, where PAPP-A levels are increased and associated with all-cause mortality [77,112,116,117]. Characteristics of these studies is presented in **Table 1.4** and a summary of results from the same studies is shown in **Table 1.5**.

Table 1.5: *Summary of results from cohort studies on PAPP-A and mortality in dialysis*

Author	Crude model	Adjusted model
Kalousova 2004	Higher PAPP-A non-survivors	-
Etter 2010	-	OR 1.009–1.088†
Kalousova 2012	HR 1.002–1.345	HR 1.060–1.444
Kalousova 2014	HR 1.04-1.19	HR 1.06-1.23
Nilsson 2017	-	HR 0.99–1.41

Hazard ratios are per standard deviation increase, except for those marked †, which are hazard ratios per unit increase.

1.5.1 Kalousova 2004

In 2004 Kalousova et al reported preliminary results [116] from a study on prevalent HD patients published in 2012 [112]. The authors found differences in PAPP-A levels between survivors (n=18) and non-survivors (n=22), with median PAPP-A at 26.8 (IQR 21.6-36.8) vs 20 (IQR 14.9-26.6) mU/l, and Mann-Whitney U test p-value = 0.034. The low number of participants and the lack of censoring analysis are obvious limitations of this preliminary analysis, although lack of censoring may be less problematic since all participants were followed for the duration of the 20 months follow-up period.

1.5.2 Etter 2010

Etter et al [117] studied PAPP-A levels in relation to outcomes in 170 prevalent HD patients from the the *monitor!* trial (N = 174). PAPP-A was lower in survivors (Mdn 20, IQR 15–25) compared to non-survivors (Mdn 28, IQR 19–35, Mann– Whitney U p-value = 0.008) and in multivariable adjusted analysis using logistic regression (using 159 complete cases with 21 deaths). Higher PAPP-A was associated with increased risk of death (OR 1.048, CI 1.009–1.088, p = 0.015). Adjustment (with the standard simultaneous entry method) was for age, sex, number of comorbidities, dialysis vintage, IL-6, CRP, PTH, calcium-phosphate product, and total serum cholesterol. In addition Kt/V was added to the model in a separate sensitivity analysis (n = 110 with 17 deaths, OR not reported, CI 1.014–1.111, p = 0.01). This yields 2.1 events per variable indicating that lack of power may be a problem in this study, which is acknowledged by the authors. Odds ratio (OR) is not reported for the univariable association between PAPP-A and mortality. Cardiovascular mortality and morbidity were stated as

secondary outcomes by the authors but not reported in results, perhaps due to the limited number of events.

1.5.3 Kalousova 2012

Kalousova et al [112] studied the association between PAPP-A levels and outcomes in 261 long-term HD patients followed up for 5 years with censoring at renal transplantation (n = 52). In a multivariable Cox proportional hazards analysis including cardiac troponin-I, albumin, creatinine, retinol, age, DM and CVD and with stratification for dialyzer membrane type (low- or high-flux), PAPP-A levels predicted all-cause mortality (n = 138) and death due to infection (n = 36) but not cardiovascular death (n = 70). The preliminary results of this study, with short-term follow-up, were published separately in 2004 [116].

Selection of covariables for the adjusted models was done in three steps: First, the authors selected biochemical parameters that were associated with outcome in univariable analysis. These were then entered into a multivariable analysis eliminating parameters using backward exclusion. The remaining independent biochemical predictors were then entered together with demographic and clinical variables in yet another stepwise backwards exclusion procedure. The use of a stepwise procedure for variable selection is flawed due to risk overfitting and inflated p-values, and puts the results of the adjusted models in study into question [118]. Additionally the number of events per variable was 3.3-13 (depending on outcome) in the first step (with only biochemical variables), 2.3-8.6 in the second step and 4.5-17 in the final multivariable model. Thus, the study was under-powered for analysis of separate outcomes for cardiovascular death and especially for death due to infection, leading to increased risk of type I and type II errors. Notably, lack of power may have influenced the stepwise selection procedure. However, PAPP-A was associated to mortality in univariable analysis (HR 1.161, CI 1.002–1.345) and with the log-rank test PAPP-A levels in the fourth quartile (>30.8 mIU/l) was associated with worse survival compared to values in the first to third quartiles.

1.5.4 Kalousova 2014

Kalousova et al investigated the association between PAPP-A and cardiovascular events in diabetic hemodialysis patients [77]. The

data was from a previous randomized controlled trial investigating atorvastatin in 1255 patients with type 2 diabetes mellitus study (the 4D study) including subjects on hemodialysis for less than 2 years and with the primary endpoint defined as a composite of cardiac death, non-fatal myocardial infarction (MI) or stroke.

The analysis of PAPP-A in relation to outcomes aimed to investigate the association to different outcomes and consequently the combined cardiovascular events (CVE) category from the original 4D study, sudden death, MI, stroke, all-cause mortality and deaths due to infection were analyzed separately. In some of the prediction models presented, PAPP-A was entered as a continuous variable and hazard ratios computed per standard deviation. In others, PAPP-A quartiles were used.

The authors found that higher PAPP-A levels were associated with increased risk of all-cause mortality as well as with the combined cardiovascular events endpoint. Elevated PAPP-A levels were also associated with stroke, sudden cardiac death and death due to infection, but not with myocardial infarction.

A total of 1098 patients, who had a PAPP-A measurements available, were included in the analysis and 534 of these persons died during follow-up (141 from sudden cardiac death and 114 due to infection). Regarding non-fatal outcomes, 398 patients had CVE, 169 had MI and 85 suffered a stroke. This yields 5.7-36 events per variable in the fully adjusted models, depending on outcome (or 4.7-30 events per variable if PAPP-A quartiles are used). Consequently, the study may be somewhat underpowered for separate analysis of stroke, but sufficient for the other outcomes.

In multivariable analysis, adjustment was for age, sex, smoking status, body mass index, atorvastatin treatment, systolic blood pressure, dialysis vintage, coronary artery disease, and levels of hemoglobin, hemoglobin A1c, albumin, phosphate, creatinine and total cholesterol. It may be noted that adjusting for both CAD and the treatment thereof (atorvastatin treatment), as well as risk factors for CAD such as cholesterol levels may lead to violation of the assumption of no multicollinearity required for the Cox proportional hazards model, especially since PAPP-A levels were associated with both CAD and cholesterol levels. Testing of model assumptions was not reported.

1.5.5 Nilsson 2017

This study is part of the thesis [119]. Briefly, PAPP-A levels, inflammation biomarkers and body composition indices were measured in 286 incident dialysis patients. During follow-up 60 months follow-up, 86 patients died and in an adjusted model including cardiovascular risk factors and body composition, higher PAPP-A was associated with mortality. This association was in part confounded by inflammation. Multivariable models included 9 to 11 covariables, resulting in 7.8 to 9.6 events per variable. It should be noted that this is short of the minimum 10 events per variable recommended [108].

1.5.6 Summary of studies on PAPP-A levels and outcomes in ESRD

To summarize, PAPP-A has been investigated in relation to outcomes in prevalent HD patients in a few studies but not in incident dialysis patients (prior to our work included in this thesis). Due to high short-term mortality after dialysis initiation [120], studies on prevalent patients are not representative for the incident dialysis population.

The association of PAPP-A to all-cause mortality is consistent in the studies reviewed above and analyses have been adequately powered in two of these. However, some authors have also aimed to study the association of PAPP-A to other outcomes such as cardiovascular death, cardiovascular events and death due to infection. Most of the studies have been under-powered for such analysis and used methods for covariable selection that are not recommended [112,117]. A single study was adequately powered to analyze other outcomes than all-cause mortality in multivariable models [77]. Here, the authors found that higher PAPP-A levels predicted a combined cardiovascular events endpoint, sudden cardiac death and death due to infection, but not myocardial infarction. There was also an association to stroke but due to an inadequate number of such events the evidence on this point must be considered weak.

Based on the above, there is a lack of research on PAPP-A as a predictor in incident dialysis patients and a scarcity of adequately powered studies in prevalent dialysis patients. The association of PAPP-A to all-cause mortality in prevalent dialysis patients would benefit from being replicated using multivariable models

with a thoughtful approach to covariable selection. The association between PAPP-A levels and death due to infection is intriguing but has only been reported in one study with adequate power and should therefore also be replicated if possible. A conservative approach to selection of covariables may enable analysis of separate outcomes in smaller cohorts. Some of these issues were addressed in one of the studies included in this thesis, which used incident dialysis patients and where covariable selection was based on previous knowledge [119].

1.6 Potassium dysequilibrium in CKD

Under normal conditions, the kidneys maintain potassium homeostasis, which is important for physiological functions such as acid-base balance, cardiac electrical conduction, smooth muscle tone and neuronal signaling [121,122]. The main regulatory mechanism for potassium excretion depends on the mineralocorticoid hormone aldosterone, which stimulates excretion of potassium in the renal tubules and collecting ducts [6]. CKD increases the risk of hyperkalemia and high potassium levels in CKD are associated with increased mortality [123]. Medications interfering with the renin-angiotensin-aldosterone system (RAAS) are also associated with increased risk of hyperkalemia [124,125].

Rates of hyper- and hypokalemia differ between studies [124–126] and reports on dyskalaemia incidence and risk factors in the broad healthcare setting are few and based on North American data [123,125,127]. In **paper III**, we investigated hyper- and hypokalemia in a Swedish healthcare system including associations to renal function and medications affecting the RAAS.

1.6.1 Studies on hyperkalemia incidence

Previous studies have reported on hyperkalemia incidence in the North American healthcare setting [123,125,127]. Chang et al investigated hyperkalemia in patients undergoing blood pressure testing and found three year incidence proportions of 10.8% for K >5.0 and 2.3% for K>5.5 [127]. Similarly, Einhorn et al investigated hyperkalemia in healthcare users with one or more inpatient hospital visits and found that as many as 13.7% had hyperkalemia $\geq$ 5.5 mmol/L within one year [123]. In **paper III** we found that during three years follow-up, approximately 7.0% had mild- and 2.5% had moderate/severe hyperkalemia and

that one in three of these cases had recurrence of hyperkalemia. Chang et al and Einhorn et al also investigated hyperkalemia recurrence and found comparable numbers, although it should be noted that these studies were on different populations and with different definitions of recurrence than ours [123,127].

After the publication of our **paper III**, Thomsen et al described the occurrence, risk factors and clinical outcomes of elevated potassium levels in a Danish population-based cohort study [128]. A total of 157,766 patients with CKD diagnosis were included. The one-year cumulative incidence of hyperkalemia was 4.8% for K >5.0 and 2.1% for K >5.5. The corresponding three-year incidence was 22% and 10%, respectively. Risk factors for hyperkalemia included DM, heart failure, ACEi, potassium supplements and spironolactone. The authors found worse outcomes for CKD patients with hyperkalemia.

Chapter 2

Research aims

The overall aim of this thesis was to increase knowledge about hormonal alterations in CKD and their consequences, focusing on CVD, mortality and growth hormones. We therefore tested cross-sectional associations between different hormones and known risk factors for CVD, with the aim of elucidating pathophysiological processes contributing to high mortality in CKD. We also analyzed longitudinal associations between blood hormone levels and outcomes, to assess these hormones as markers of increased mortality risk.

2.1 Aims of each sub-study

In **paper I** [105] we investigated the association between IGF-1 and mortality as well as its association to inflammation and albumin. Serum hs-CRP and serum albumin were considered potential confounders and the association between serum IGF-1 levels and mortality, above established cardiovascular risk factors, was assessed.

In **paper II** [119] we investigated another component of the GH/IGF-1 system, PAPP-A, in relation to mortality and CVD in HD patients. Due to the lack of previous research on incident HD patients, an additional aim of the study was to present data on PAPP-A and mortality for incident as opposed to prevalent dialysis patients. Based on theoretical and previously observed links between PAPP-A, body composition DM and inflammation, a further aim was to test if the hypothesized association between

PAPP-A and mortality would be modulated by these factors. We also reported results on associations between PAPP-A and CVD risk factors.

In **paper IV** [129], we sought to add to existing research on the association between PAPP-A and mortality in prevalent HD patients and specifically to test our previous exploratory finding that inflammation and DM and modified the effect of PAPP-A on mortality. In addition, we attempted to replicate a previously observed association between PAPP-A and inflammatory markers.

In **paper V** we tested whether combining a function measurement of muscle strength (handgrip strength, HGS) with a biochemical nutritional marker (plasma IGF-1 levels) would have a stronger association to mortality in CKD than either marker alone.

In **paper III** [130], we originally planned to estimate hyperkalemia risk after initiation of mineralocorticoid receptor antagonist (MRA) treatment in a large healthcare cohort and specifically to study the influence of renal failure on that risk. Instead, we described hyperkalemia and hypokalemia risk in general (ie not only in MRA treatment) and identified risk factors for these events, including reduced renal function and MRA treatment.

Chapter 3

Subjects and methods

3.1 Subjects and study designs

3.1.1 Örebro risk marker cohort

This cohort was based on biobank samples collected routinely from patients starting HD at a single dialysis clinic (Örebro University hospital) during the years 1991–2009. Those with prior history of dialysis treatment or renal transplantation were excluded and the study population was 265 persons. In 2013, data on survival time and comorbid conditions were retrieved from diagnoses in the Swedish National Patient Register. Demographic data, time to renal transplantation, cases of regained renal function and cause of renal failure were collected from the Swedish Renal Registry. Follow-up was 3 years and survival time was censored at renal transplantation or regained renal function. This cohort was utilized in **paper I**. Additional results from this cohort are presented in chapters 3 and 4 and covered by the original ethics approval from the Regional Ethical Review Board in Uppsala (Dnr 2009/082).

3.1.2 Malnutrition, inflammation, and atherosclerosis (MIA) cohort

This ongoing cohort was started in 1994 and includes CKD stage 5 (GFR <15 mL/min) patients planned for initiation of dialysis treatment at the Karolinska University Hospital at Huddinge,

Sweden [80]. Exclusion criteria were: Age <18 years or >70 years, clinical signs of acute infection, active vasculitis or liver disease. Patients were sampled in proximity to the start of renal replacement therapy (either HD or PD) and followed until death or renal transplantation. Body composition was measured using dual-energy X-ray absorptiometry (DEXA). This cohort was utilized in **paper II**.

3.1.3 Mapping of inflammatory markers in chronic kidney disease (MIMICK-1 and MIMICK-2) cohorts

The MIMICK-1 cohort was utilized in **paper IV** and includes prevalent HD patients with a minimum of 3 months of HD treatment from the Karolinska University Hospital at Huddinge, Stockholm, Sophiahemmet, Danderyds Hospital and Uppsala Academic Hospital. Inclusion was from October 2003 through March 2004. Patients undergoing regular HD treatment at any of the units (n=254) were invited to participate. Six patients declined, and one patient with human immunodeficiency virus infection was excluded. The original aim of this study was to assess variability of inflammatory parameters over time and after 12 weeks follow-up, eleven patients were excluded from the cohort due to insufficient baseline clinical data; seven were excluded due to lack of hs-CRP measurements and one patient who had died was also excluded from further analysis. The remaining 228 patients were followed for 5 years and causes of death retrieved from death certificates. Ninety-two of these had frozen plasma samples available for analysis and were included in **paper IV**.

The MIMICK-2 cohort was designed similarly to MIMICK-1, but included prevalent PD patients at Karolinska University Hospital and Danderyds Hospital, Stockholm, Sweden [131]. All patients had been treated with continuous ambulatory peritoneal dialysis or automated peritoneal dialysis for a minimum of 3 months before inclusion. Recruitment was from March 2008 to April 2011. Of 82 patients originally included in MIMICK-2, blood samples for IGF-1 analysis were available for 70 patients, constituting part of the material for **paper V**.

3.1.4 Stockholm CREAtinine Measurements (SCREAM) cohort

The SCREAM cohort data includes residents in the region of Stockholm who had serum creatinine measured during 2006–11. The study reported in **paper III** includes adult individuals (>17 years of age) accessing healthcare in 2009 with at least one ambulatory measurement of serum creatinine in the preceding year and with at least one follow-up potassium test during three years follow-up, amounting to a total of 364 955 persons. Renal function was estimated using the CKD-EPI creatinine-based equation and comorbid conditions defined from previous diagnoses in the healthcare system, using ICD codes. Concomitant medications were retrieved from complete information of drugs dispensed at Swedish pharmacies [132]. Outcomes were defined as follows: Hypokalemia was defined as potassium <3.5 mmol/L; hyperkalemia as potassium >5 mmol/L and further classified as moderate/severe if >5.5 mmol/L.

3.1.5 Other cohorts

In **paper V**, data from multiple CKD cohorts was combined and included 75 CKD 3-4 patients (PRIMA cohort), 361 incident dialysis patients (MIA-cohort, described above), 70 prevalent PD patients (MIMICK-2 cohort, described above) and 179 prevalent HD patients (MIMICK-1 cohort, described above) with a total of 685 CKD patients. IGF-1 levels and HGS values were dichotomized at cut-offs determined by receiver operating characteristic (ROC) curve analysis. These categories were then combined and tested for prediction of death (n = 208) during the 5 year follow up time.

In the PRIMA cohort, patients with CKD were recruited from the renal outpatient clinic of Karolinska University Hospital. Exclusion criteria were: Clinical signs of acute infection, active vasculitis, or liver disease. This cohort has been described previously, for the initial 50 patients included [133].

3.2 Laboratory methods

3.2.1 Immunometric assays of growth hormone axis components

In the Örebro risk marker cohort as well as the MIA and MIMICK cohorts, IGF-1, IGF-1 binding protein-3 (IGFBP-3) were analyzed using immunometric assays on an Immulite 1000 Analyzer (Siemens Healthcare Diagnostics, Los Angeles, CA, USA). In the MIA and MIMICK cohorts, PAPP-A was measured using ELISA (R&D Systems, Minneapolis, USA).

3.2.2 Other methods

Handgrip strength was assessed with a Harpenden Handgrip Dynamometer (Yamar, Jackson, MI, USA). Measurements were made in the dominant hand for patients without an arteriovenous (AV)-fistula and otherwise in non-AV-fistula arm. Values were normalized using measurements from healthy subjects. In prevalent HD patients (MIMICK-1 cohort), the measurements were made post dialysis.

In the MIA cohort, GFR was determined using the mean of renal urea and creatinine clearances during a 24-hour urine collection, which is considered a reasonably accurate estimate of GFR in patients starting on dialysis [134,135].

Dual-energy x-ray absorptiometry was used in the MIA cohort (**paper II and V**). Measurements performed using a DPX-L device (Lunar Corp, Madison, WI).

3.2.3 The drawing of blood

In the MIA and MIMICK2 cohorts, blood samples were collected after an overnight fast while in the MIMICK1 cohort samples were drawn before the dialysis session after the longest interdialytic period. Plasma samples were stored at –70°C.

In the Örebro risk marker cohort, samples were retrieved up to 11 days prior to dialysis initiation and stored frozen at -20°C if not analyzed immediately. Storage time may slightly affect levels of IGF-1 in stored samples [136]. Consequently, to test the effects of storage time on serum IGF-1 values, a variable for the period of inclusion was computed and mean IGF-1 values in patients

Figure 3.1: *Storage time and IGF-1 levels*

recruited during the different time periods compared (**Figure 3.1**).

3.3 Register data

In the Örebro risk marker cohort (**paper I**), register data was used. Demographic data, time to renal transplantation, cases of regained renal function and cause of renal failure were collected from the Swedish Renal Registry, which is maintained by the Swedish Society of Nephrology. International Classification of Diseases (ICD) codes for comorbid conditions were retrieved from inpatient diagnoses in the Swedish National Patient Register, which has been validated for a number of diagnoses and has complete coverage of inpatient care since 1982 [137,138]. Time until death and cause of death were retrieved from the Swedish Cause of Death Register. In the SCREAM cohort [139] (**paper III**), two additional registers were utilized: Regional register data from Stockholm county council was used for retrieval of ICD codes and the Swedish Pharmaceuticals Registry was used for data on medication dispensation [132].

While registers provide cheap access to data, there are a number of challenges associated with use of register data. Naturally, the researcher has no control over the data entry procedure and it is therefore difficult to assess the quality of data. Since diagnoses are sometimes put in error or before conditions are fully confirmed, there is also a risk of misclassification that may introduce bias in

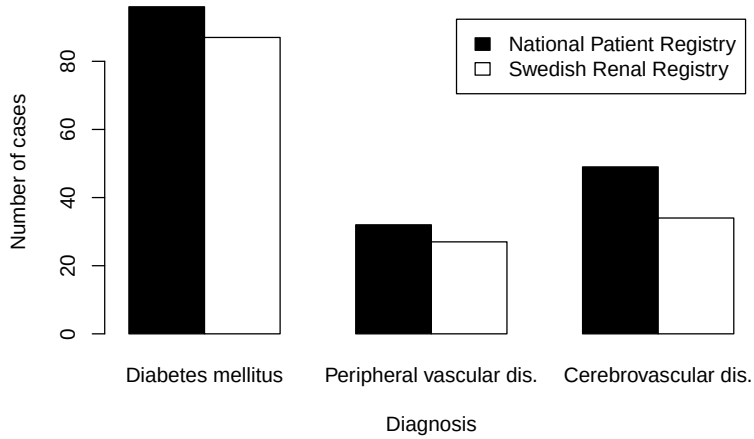

Figure 3.2: *Comparison of classifications of diagnoses based on different registries in 265 hemodialysis patients*

study results. An advantage to the Swedish national registers is the complete coverage of all deaths and dispensed medications and of all diagnoses entered in the local healthcare administrative systems. They are therefore not dependent on a single investigator catching and noting diagnoses and are therefore likely to have a high sensitivity for known medical conditions. This is illustrated in **Fig 3.2**, which shows a comparison of classifications using the national patient register and the Swedish renal registry, respectively, based on data used for the Örebro risk marker cohort (**paper I**). It can be noted that the Swedish renal registry has a lower number of positive classifications in all disease categories. For **paper I** we used the national patient register data for classification of comorbid conditions.

3.4 Statistical methods used in the different studies

In **paper I**, we assessed the distribution of baseline variables by visual inspection of density- and Q–Q plots (**Fig 3.3**). The Normal or Non-Normal distribution of data then guided the selection of descriptive point- and variance estimates, ie mean and SD for Normal data and median and IQR for Non-Normal data. The method used for hypothesis testing or comparison of baseline parameters in two groups was also selected based on these

assessments, whereby we used Student's t-test for Normal distributed continuous variables and Wilcoxon–Mann–Whitney test for Non-Normal data.

Although the method of visual inspection or other testing of normality is often used for statistical method selection, this approach is flawed [140]. As an example, see **Fig 3.3**, which displays data on IGF-1 levels used in paper I. The non-transformed IGF-1 values (panels **A** and **C**) are approximately normally distributed, but do not display a perfect fit to normality. It is apparent that the decision if one should accept the non-transformed data or not has some measure of subjectivity to it. Note that square-root transformation (panels **B** and **D**) in this case improves fit to normality. However, although such transformation would perhaps optimize performance of certain statistical tests, it would also impair interpretation of results.

Furthermore, the testing of normality (irrespective of the method used - visual or by significance testing) introduces an extra level of testing which by itself contributes to the uncertainty of the final results. In other words, the tests used for assessing normality are subject to the risks of type I and type II error. It can be argued that any increase in statistical power gained through using a parametric method on the basis of such assessment is at risk of being lost due to the extra "layer" of testing. Alternative approaches include: Firstly, deciding on the Normal- or Non-Normal distribution of variables based on previous literature. This removes the formal testing from the current analysis, but if existing evidence is conflicting on this point some measure of uncertainty will be introduced anyway and influence the validity of the study results. Such an approach may be suitable if there is ample pre-existing data on the distribution of a certain parameter. The log-scale of CRP may serve as an example. The second approach is to a-priori decide to use non-parametric methods. This eliminates the step of normality testing altogether, but may result in loss of statistical power due to inherent properties of non-parametric statistics. This approach may be suitable if sample size is relatively large and the variable in question cannot be assumed to have Normal distribution based on previous studies. It has the further benefit of simplicity - the same statistics can be used for all variables of the same type. As an example, in **paper II** we used quantile regression models for describing associations between baseline variables. When it comes to descriptive statistics it is probably useful to show both mean (SD) and median (IQR) [141].

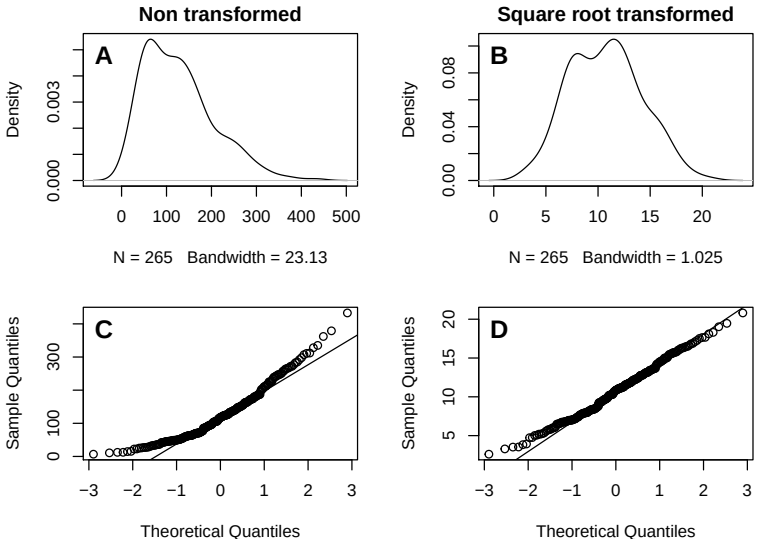

Figure 3.3: *Density plots and Q-Q plots for testing distribution of IGF-1 levels*

The focus on p-values has been discussed and challenged in scientific press [142] and it should be noted that for some statistical methods such as quantile regression, the confidence interval of estimates as well as the p-values are computed using bootstrapping methods and therefore subject to some variation between different runs of the same data. Although we did in some of our papers try to put less emphasis on a specific cut-off for statistical significance, reviewers did not approve.

For survival analysis we used the popular Cox proportional hazards model in **paper I**, **II** and **IV**. To be valid, these models must be tested for a number of assumptions, the foremost of which is proportional hazards. Different methods exist for such assessment, and in line with the discussion on normality above, it can be argued that such testing is also subject to uncertainty. In fact, statements on testing of model assumptions are often omitted from scientific publications. As exemplified in one of the papers on IGF-1 and mortality cited above, Himmelfarb et al tested the proportional hazards assumption using a product term between time and the explanatory variables, although it was not stated which other assumptions were tested [106]. In our **paper II**, non-proportional hazards mandated the use of a time-varying coefficient for CVD, providing further information that CVD was predictive of mortality in day 0-400 after dialysis initiation, but not

thereafter. Again, "non-parametric" methods for survival analysis are available and in **paper IV**, we utilized quantile regression as a complementary analysis. This has the added benefit of giving more easily interpretable statistics (ie the proportion of individuals surviving past a certain time point).

In **paper I**, we used dichotomization of IGF-1 levels to categorize participants into "low" and "non-low" groups. This practice may be intuitively appealing, for example to facilitate interpretation, being able to characterize a group with relative IGF-1 deficiency and to remove the effects of outliers. However, it is generally not recommended due to loss of statistical power, risk of spurious findings, reduced comparability to other studies and risk of missing non-linear relationships [143]. In **paper II**, we tested associations between PAPP-A and cardiovascular risk factors in a cross-sectional analysis using a quantile regression method, which may be a preferable approach to reducing the effect of outliers without the inherent disadvantages of dichotomization.

Selection of explanatory variables is another important aspect of statistical model and method selection. Automated methods for exclusion and inclusion of explanatory variables exist (such as step-wise forward or backward exclusion), but are fraught with problems [144,145]. For example, only a select set of variables are entered into the model in the first place and unmeasured variables that could have influenced the model selection procedure may be absent. Sometimes, explanatory variables for multivariable analysis are selected based on their association with the outcome in univariable analysis, a method we used in **paper I**. However, this is generally not recommended [146]. A priori selection of explanatory variables may be a more reasonable approach. Then at least it is possible to argue for and against including certain variables based on what is already known. This, of course, does not eliminate the problem of unmeasured confounding. Since the number of explanatory variables is limited by statistical power (sample size and number of events) it is often advantageous to a-priori remove factors that are not regarded as confounders. Certain theories may help in eliminating some confounders from the model selection in a structured manner and using Directed Acyclic Graphs is such a method [147].

Over-adjustment may also also present a problem. For example the commonly used eGFR estimate of renal function includes age in its calculation, and if one also uses Age as such as a explanatory variable, it will be entered two times in the model. Another example is albumin, which is lower in inflamed states,

and sometimes used in conjunction with CRP, thus introducing a dual adjustment for inflammation. One approach, if uncertainties on what variables to include in analysis, is to present sensitivity analyses utilizing a somewhat different set of explanatory variables, to assess the consistency of findings.

Chapter 4

Results and discussion

4.1 IGF-1

In the first published work (**paper I**) [105], we found that patients with low IGF-1 levels had worse outcomes in hemodialysis. In multivariable analysis on mortality, including adjustment for cardiovascular risk factors (age, sex, DM, heart failure and history of CVD) and biochemical markers (hs-CRP, s-creatinine, s-albumin), we found that low IGF-1 remained associated with higher mortality risk when adjusted for these factors.

In **paper V**, we hypothesized that combining a function measurement of muscle strength (HGS) with a biochemical nutritional marker (plasma IGF-1 levels) would have a stronger association to mortality in CKD than either marker alone. We found that patients in the combined low IGF-1 and low HGS category had increased mortality rate compared to the other categories and that this association was robust when adjusted for CVD risk factors (Framingham's CVD risk score, previous CVD, smoking) inflammation (hs-CRP, plasma albumin) and markers of PEW [SGA, lean body mass index (LBMI)]. The predictive utility of IGF-1 was somewhat enhanced but still weak in the low HGS group (area under the curve changed from 0.6 to 0.66). A major limitation to this study was the use of pooled data from different cohorts with diverse characteristics that may impact both predictors (HGS, IGF-1) and outcome. Indeed, median HGS was markedly lower in prevalent dialysis patients compared to the other groups. To somewhat ameliorate these methodological concerns, a term for dialysis status (on dialysis vs not on dialysis) was included as a

Table 4.1: *Multivariable regression model on IGF-1 levels in 265 hemodialysis patients*

Variable	Estimate	CI [5%, 95%]
Intercept	230	[190, 270]
hs-CRP, mg/L	-0.15	[-0.25, -0.051]
Female	-30	[-45, -14]
Age, years	-1.1	[-1.7, -0.57]
Diabetes mellitus	-15	[-31, 0.29]

Linear regression with adjustment for all variables shown.
Abbreviations: hs-CRP, high-sensitivity C-reactive protein.

covariable in the regression analysis on mortality.

Our results showing an association between IGF-1 levels and mortality are in line with two adequately powered previous studies on IGF-1 and outcomes in ESRD [112,114]. However, Kalousova et al [112] did not report multivariable adjusted results for IGF-1 on mortality and in the study by Jia et al [114] pooling of different cohorts, whose participants had different stages of kidney disease, is an important limitation. **Paper I** addresses some of these limitations through including only incident HD patients, providing sufficient power for the analyses made and presenting results adjusted for potential confounders. Nevertheless, in **paper I** we did not have access to data on body composition and concerns about residual confounding therefore remain. Thus, we provide novel results in the form of survival models adjusted for important confounders and with validity for incident HD patients. Further, in **paper V** we show that low HGS is associated with mortality in a mixed CKD-ESRD population and that adding IGF-1 levels may perhaps improve prediction in the group with low HGS.

4.1.1 Factors associated with IGF-1 levels

In **paper I**, low IGF-1 was associated with female sex, lower IGFBP-3 levels, inflammation (higher hs-CRP, lower albumin) and lower creatinine (which may be regarded as a marker of PEW in this population) but not with CVD, age or DM. Multivariable analysis on IGF-1 levels was not reported in the paper but is shown here in **Table 4.1**.

In **paper V**, IGF-1 was positively correlated with muscle function (HGS), muscle mass (LBMI) and BMI while it was negatively asso-

Table 4.2: *Associations between IGF-1 levels and selected variables in dialysis*

Parameter	Nilsson 2016	Chen	Jia 2014	Hung 2005	Fernandez 2002
Age	0	Neg	Neg	0	Neg
Female	Neg	0	0	0	NA
DM	0	Neg	Neg	Pos	NA
CVD	0	Neg	Neg	0	NA
CRP	Neg	Neg	NA	0	Neg
IL-6	NA	Neg	Neg	0	NA
Creatinine	Pos	Pos	NA	NA	0
Albumin	Pos	Pos	NA	Pos	Pos
PEW	NA	Neg	Neg	Neg	NA
HGS	NA	Pos	NA	NA	NA

PEW was defined as SGA >1. Abbreviations: Pos, positive; Neg, negative; zero (0) denotes no association.

ciated with SGA >1, supporting its role as a marker of nutritional status and protein-energy wasting. Consequently, IGF-1 was also positively associated with other markers influenced by nutrition and PEW such as albumin, creatinine and calcium-phosphate product. IGF-1 was negatively correlated with cardiovascular risk factors (age, DM, smoking, history of CVD, mean blood pressure and Framingham's CVD risk score) as well as inflammatory markers (IL-6, hs-CRP, TNF). In multivariable analysis, low IGF-1 was independently associated with lower HGS, lower LBMI, lower albumin and higher Framingham's CVD risk score.

Table 4.2 shows associations between IGF-1 levels and selected variables in cohort studies reviewed in section 1.4. The studies by Himmelfarb et al [106], Beberashvili et al [113] and Qureshi et al [111] are not included, since they did not report on associations to IGF-levels. Multivariable analysis was reported only in the studies by Chen et al (**paper V**) and Fernandez-reyes et al [110].

Low IGF-1 was associated with inflammation (higher hs-CRP) in both of the studies included in this thesis. Data on the association between IGF-1 and inflammation has been reported by others. Jia et al [114] found in unadjusted analysis that IL-6 levels were weakly and negatively associated with IGF-1 levels; similarly, Fernandez-reyes et al [110] reported a negative correlation to CRP while Hung et al [104] found no association to IL-6. Taken together, these results indicate lower IGF-1 levels in inflammatory states. **Figure 4.1** shows the quantile regression coefficients for hs-CRP on IGF-1 levels in the data set used for **paper I** and provides some additional information on the association between

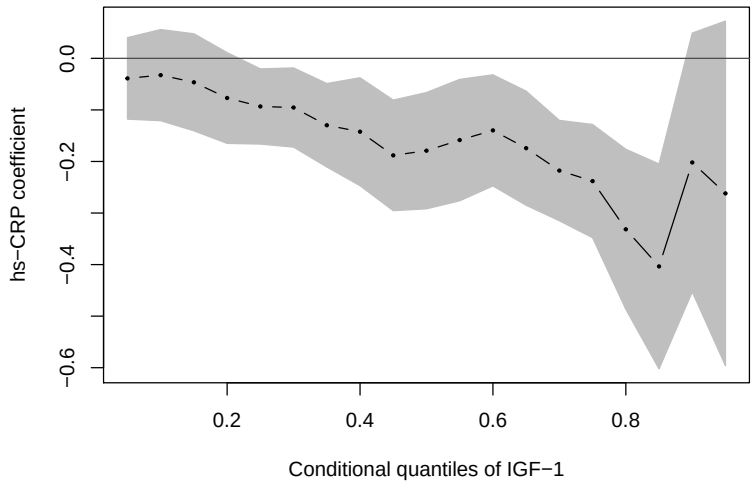

Figure 4.1: *Association between hs-CRP and quantiles of IGF-1 in 265 hemodialysis patients*

IGF-1 and inflammation. It can be noted that the regression coefficient becomes increasingly negative with increasing quantiles of IGF-1, leading to the interpretation that it is perhaps mainly IGF-1 levels in the higher end of the spectrum that are reduced in inflamed dialysis patients.

Although CVD was somewhat more prevalent in the "low IGF-1" group in **paper I**, the differences were not statistically significant. In **paper V** IGF-1 was negatively correlated to CVD and independently associated with CVD risk factors. Others have found no association [104] or a negative association [114]. Thus, based on somewhat weak evidence, IGF-1 levels appear to be reduced in dialysis patients with CVD, possibly due to its association with CVD risk factors.

Higher age was associated with lower IGF-1 levels in **paper V**, and in the data presented in **Table 4.1**, although the difference between "low" and "non-low" IGF-1 groups were not statistically significant when tested using Student's t-test in **paper I**. Others found a negative association [110,114] or no association [104]. Thus, higher age appears to be associated with lower IGF-1 levels in dialysis patients.

In our first study [105], female sex was associated with lower IGF-1 levels. However, in **paper V** and in the two additional studies reporting on sex differences [104,114] there was no such association.

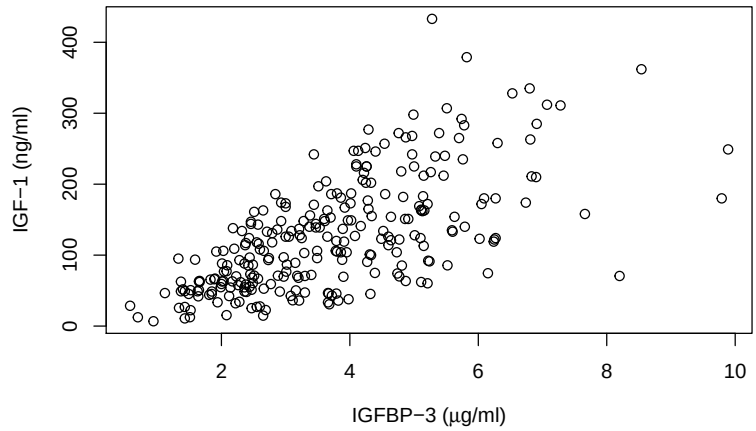

Figure 4.2: *IGF-1 and IGFBP-3 levels in 265 hemodialysis patients*

Although DM was more common in the low IGF-1 group in **paper I**, the difference between groups was not statistically significant. However, in **paper V** and in the study by Jia et al [114] there was a negative association between DM and IGF-1 levels. Conversely, in the study by Hung et al, the association was positive in [104]. Thus, results on the association between DM and IGF-1 in dialysis patients are inconsistent.

Markers of PEW (lower creatinine, lower LBMI and SGA >1) were associated with lower IGF-1 in unadjusted analysis in both our studies, although statistical significance was not reached for SGA >1 in adjusted analysis. It may be argued, however, that the multivariable model in **paper V** may have over-adjusted for PEW due to including multiple PEW-related markers (LBMI, SGA, HGS, and arguably albumin). Others have consistently found negative associations between IGF-1 and PEW [104,114].

It should be noted that IGF-1 levels correlate strongly to levels of IGFBP-3. **Figure 4.2** shows the correlation between IGF-1 and IGFBP-3 levels at baseline in the Örebro risk marker cohort. The strong correlation between these two markers signals that they do not contain differential prognostic information and furthermore, if included in the same regression model may cause problems with collinearity. It also indicates that the IGF-1 to IGFBP-3 ratio, which is sometimes used to measure "free IGF-1" is not likely to discriminate disease states or prognosis better than the total IGF-1 values in patients starting HD.

To summarize, our findings that IGF-1 levels are lower in inflammatory states and PEW are in line with previous dialysis cohort studies. The negative association to age reported in **paper V** was consistent with previous findings, as was the positive association to albumin. DM and sex were not consistently associated with IGF-1 levels. Thus, inflammation, age, PEW and perhaps DM should be considered potential confounders when analyzing effects of IGF-1 levels on mortality in dialysis.

4.2 PAPP-A

In **paper II** we studied the association between PAPP-A levels and mortality in patients starting dialysis [119]. We also described potential confounding and modifying effects of DM, body composition and inflammation on this association. In cross-sectional analysis, PAPP-A levels were associated with lean tissue index (LTI) and high-sensitivity C-reactive protein (hs-CRP) but not with fat tissue index (FTI) or history of CVD. In survival analysis, higher PAPP-A levels were associated with higher mortality, although there was a confounding effect of inflammation on this association. Interestingly, when the analysis was stratified by diabetes status, higher PAPP-A was associated with mortality in diabetic patients but not in those without diabetes. This finding was robust when adjusted for inflammation and other confounders and was further explored in another cohort in **paper IV** (see below). There are no previous studies on the association between PAPP-A and mortality in incident dialysis patients and, importantly, due to the high early mortality after initiation of dialysis [120], studies on prevalent patients are not representative for the incident dialysis population. Thus we provide novel evidence that PAPP-A is associated with mortality when sampled at the initiation of dialysis and results are therefore applicable to assessment of prognosis in patients planned for dialysis treatment.

In **paper IV**, we studied PAPP-A levels and mortality in prevalent HD patients. Based on findings in our previous work on incident dialysis patients, we hypothesized that concomitant DM and inflammation would modulate the association between PAPP-A and mortality. In univariable Cox proportional hazards analysis higher PAPP-A was associated with increased risk of mortality. In multivariable analysis, explanatory variables selected on the basis of prior knowledge were entered, including age and sex in the minimally adjusted model and then in addition DM, BMI and

hs-CRP in a model regarded as "fully" adjusted for confounders. Finally, to assess the association between PAPP-A and mortality beyond established risk markers, adjustment was also made for dialysis vintage and cardiovascular risk factors and we found that the association remained statistically significant in all models. There were 37 deaths during follow-up, 16 of which were deaths due to cardiovascular causes. This equates to 12.3, 6.6 or 3.4 events per variable, depending on model. The model adjusted for only age and sex is therefore sufficiently powered, while the other models should be regarded with caution. For interaction testing, a limited set of explanatory variables was used and we found statistically significant positive interactions between PAPP-A levels and DM. Subsequent analysis stratified on DM status indicated that PAPP-A predicted mortality only in diabetic HD patients. Death due to cardiovascular causes was analyzed as a secondary outcome and was sufficiently powered in the univariable model, in which PAPP-A levels failed to predict the outcome.

There is limited research on PAPP-A in prevalent HD patients. Most studies include a mix of diabetic and nondiabetic patients and show that PAPP-A levels are associated with all-cause mortality [112,116,117]. In the largest available study on PAPP-A as a marker of mortality in HD only patients with DM were included [77] and in **paper II** we observed that in incident HD patients, the association between PAPP-A and mortality appeared stronger in those with DM [119]. In **paper IV** we replicated these findings and provided novel evidence that there is effect modification from DM also in prevalent HD patients. The distinction between prevalent and incident patients is important due to the high early mortality after dialysis initiation [120], which contributes to differences in characteristics. Notably, the proportion of patients with DM is likely to be lower in prevalent compared to incident HD, due to DM being a risk factor for mortality. Also, there may be differences in types of CVD mortality between incident and prevalent HD patients [120]. Thus, results from incident cohorts are not automatically valid in cohorts including prevalent patients and vice versa.

In **paper IV**, we found no interaction between PAPP-A and CRP, which is in contrast with **paper II** [119] and some previously published research [61]. Potential explanations for these discrepancies in results include differences between prevalent and incident HD patients, a limited sample size and large variance in CRP levels, which may yield insufficient power to detect weak interactions.

It remains unclear if the association of PAPP-A to mortality in HD

is due to an association with cardiovascular mortality. Although increased PAPP-A activity may have a causative role in cardiovascular disease [148], it should be considered that rather than being causal in the atherosclerotic process, elevated plasma levels of PAPP-A may represent local up-regulation and escape of PAPP-A into the circulation due to tissue injury or inflammation [25]. Association between circulating levels of PAPP-A and outcomes therefore do not necessarily support a causative role of PAPP-A in CVD. In line with this, Kalousova et al [112] found that PAPP-A was associated with all-cause mortality and death due to infection but not with cardiovascular mortality and in a subsequent study found an association with sudden death, stroke and death due to infection, but not with risk of myocardial infarction [77]. Similarly, there was no association between PAPP-A levels and cardiovascular mortality in **paper IV** (the limited power for analyzing this specific outcome should be noted) and we found no association between history of CVD and PAPP-A levels in **paper II** or in the data used for **paper IV** (linear regression estimate of CVD on PAPP-A levels at baseline = 0.19, CI: -0.056-0.44, p-value = 0.2). The question therefore remains which pathophysiological process underlies the association between higher PAPP-A levels and worse outcomes in dialysis patients.

A number of important limitations apply to our results on PAPP-A in dialysis patients. There was a relatively small number of participants in both studies, resulting in low statistical power restricting the number of independent variables in the statistical models, which may lead to residual confounding. Limited statistical power also increases the propensity for type I and type II error. However, a high event rate somewhat balanced the relatively small number of participants and results in the fully adjusted models were consistent with models using a more conservative number of independent variables. An additional limitation was that our definition of DM included both type 1 and type 2 DM, which precludes differentiation between these two conditions. Further, the *post-hoc* analysis of existing cohort data and stored samples may contribute to publication bias if all findings are not published. To ameliorate this, hypotheses and statistical models were formulated prior to analysis. Acknowledging these limitations, our results from **paper II** and **IV** are nevertheless concordant and in line with previous evidence [77] of higher PAPP-A levels being associated with increased all-cause mortality in dialysis patients with DM.

4.3 Hyperkalemia and hypokalemia

We originally planned to study hyperkalemia in mineralocorticoid users in the Stockholm CREAtinine Measurements (SCREAM) project but we decided instead to study the incidence and risk factors for potassium disturbances. In **paper III** we therefore aimed to describe the incidence, severity and recurrence of hyperkalemia and hypokalemia and to identify risk factors for dyskalaemia among demographic factors, comorbid conditions and concomitant medications.

We found that both hypokalemia and hyperkalemia were common. Hyperkalemia occurred in 7% of participants, comparable to studies from US healthcare [123,127]. The frequency of potassium testing was as expected a determinant of dyskalaemia risk, also described previously [127]. Other risk factors for hyperkalemia included diabetes mellitus, higher age, lower eGFR, previous myocardial infarction, heart failure or use of renin angiotensin-aldosterone system inhibitors (RAASi). Notably, female sex was associated with lower risk of hyperkalemia and higher risk of hypokalemia. The same pattern was seen for younger age, higher eGFR and use of loop/thiazide diuretics. The association of dyskalaemia related to different medications [6,123,127,149], and comorbid conditions [123,150–153], has been described previously.

Reduced kidney function was one of the strongest risk factors for hyperkalemia, in line with previous studies [151,152,154–156]. The use of creatinine based eGFR in our analysis is therefore especially important, due to lack of awareness for CKD and underutilization of ICD diagnoses in healthcare [157,158].

Limitations to this study include that data was lacking for some potential risk factors of hyperkalemia, such as blood pressure and body mass index, as well as its retrospective design. External validity is limited by the data being from a single large city region in Sweden, and findings may therefore not be applicable in other healthcare systems. There is also a risk of residual confounding due to unmeasured factors and confounding by indication, the latter precluding separating the risk of hyperkalemia associated with use of certain medications from risk due to the underlying condition for which the patient was treated.

Chapter 5

Summary and conclusions

In **paper III** on hyper- and hypokalemia, we evaluate the potassium testing in a large healthcare system, analyze the rates and likelihood of recurrence of both mild and moderate/severe hyperkalemia during a 3-year period. We show that both hyper- and hypokalemia is common in healthcare. Hyperkalemia was especially common in the presence of comorbidities, foremost in those with poor kidney function. Our results highlight the multitude of risk factors involved, including age, sex, diabetes, hypertension and cardiovascular disease. The use of RAASi remained strongly associated with occurrence of hyperkalemia after adjustment for comorbid conditions. Vigilant potassium monitoring may therefore be mandated in these conditions.

We add to existing evidence pointing to IGF-1 as a marker associated with worse outcomes in CKD by showing that this association is applicable to incident HD patients and are robust when adjusted for important confounders. We also show that low HGS is associated with mortality in a mixed CKD-ESRD population and that adding IGF-1 levels possibly improves in the group with low HGS. Further, we conclude that inflammation, age and PEW should be considered potential confounders when analyzing effects of IGF-1 levels on mortality in dialysis.

Previous research shows that PAPP-A levels are associated with outcomes in prevalent HD patients who have DM but it is less clear whether this is also true in patients about to start dialysis treatment and in nondiabetic HD patients, respectively. We

provide novel evidence that the association between PAPP-A and mortality in dialysis patients is modulated by concomitant DM and that there is effect modification from DM in both incident dialysis patients and prevalent HD patients. This implies that results concerning the association of PAPP-A to survival that are based on patients with DM may not be generalizeable to dialysis patients without DM. In addition, inflammation and body composition (FTI) were identified as potential effect modifiers for the association between PAPP-A and mortality.

Although disturbances in GH-IGF-1 signalling in CKD could hypothetically contribute to for example CVD and thereby contribute to higher mortality, interpretation of blood levels of these hormones is not straightforward. For example circulating levels of IGF-1 and PAPP may not reflect alterations in their activity on the target cell. Rather, increases may be compensatory to decreased IGF-1 signalling or secondary to confounders associated with CVD, such as inflammation, or in the case of PAPP-A tissue damage may lead to up-regulation and release into the circulation. Knock-out models could be more reliable but are limited to non-human subjects and knock-outs that are not tissue specific do not reflect changes in a specific organ such as the vasculature or heart. Also, it may not be appropriate to extrapolate from such models to ESRD where GH/IGF-axis is disturbed in other ways. It is also possible that the effect of GH/IGF-1 signalling is modulated by synergistic factors making it turn good or bad for vascular health depending on context. In fact, it appears likely that a hormone system with such pleiotropic effects would be contextually dependent, with myriad regulating factors on organ- and cellular level. Therefore, caution should be observed when interpreting our findings in relation to pathophysiology. This, however, does not preclude the possible usefulness of GH/IGF-1 axis hormones as biomarkers for identifying detrimental conditions such as PEW and in assessing prognosis in CKD.

5.1 Applicability of results

The utility of blood hormone levels in revealing underlying pathophysiological conditions of importance in ESRD is limited by the generally weak link between hormone pathophysiology and blood hormone levels. Systemically acting hormones (where hormone action is more tightly related to systemic levels), such as IGF-1, may be less limited by this than locally acting factors

(like PAPP-A), although local IGF-1 action is regulated by other components of the GH-IGF-1 axis, such as IGFBPs and PAPP-A. As markers of mortality risk, they must be compared to existing clinical biomarkers such as troponin and brain natriuretic peptide. However, blood biomarkers are easily retrieved, especially in HD patients who can be sampled during the HD session. If markers of clinical conditions, such as undernutrition and PEW, can be identified, they may serve as early warning signs and mandate interventions. In this context, GH-IGF-1 hormones could be of importance since associations between IGF-1 and multiple markers of nutrition and PEW have previously been shown. If IGF-1 is superior to for example albumin as a marker of these conditions, providing more evidence on the association between IGF-1 and mortality is relevant, since markers used for initiating interventions should ideally be associated with outcomes. It should be noted that the only large randomized controlled trial on IGF-1 replacement therapy in ESRD was not successfully executed, and that GH/IGF treatment as an option in PEW cannot be ruled out entirely [103]. However, the complex regulation of IGF-1 action through IGFBPs and other players indicate that treating relative IGF-1 deficiency may not be as simple as adding more of the hormone. Other possible interventions include nutritional counseling and increased physical activity.

The interpretation of PAPP-A levels is even less straightforward than for IGF-1. From a pathophysiological standpoint, PAPP-A is associated to atherosclerotic disease processes, but its function is *local* IGF-1 regulation. Indeed the enzyme is normally membrane-bound, and the association between blood levels and tissue PAPP-A activity is unclear. However, similarly to cardiac troponin PAPP-A may be useful as a biomarker for cardiovascular risk, hypothetically through release from injured vasculature tissue, unrelated to its pathophysiological action. If up-regulation of PAPP-A is first required it can be reasoned that it may have other attributes that cardiac troponin, which does not require prior up-regulation to be detected at injury.

5.2 Future perspectives

There are a number of components of the GH-IGF-1 axis that could be of interest as biomarkers in patients with chronic kidney disease. For example, the IGF-1 binding proteins as well as GH receptor levels are dysregulated in renal failure and could be tested

as markers for conditions such as PEW or as prognostic biomarkers [84,97,99]. However, as has been discussed, plasma biomarkers are generally limited by weak associations to pathophysiological conditions underlying increased morbidity and mortality in the CKD population, and few such markers presently have clinical utility. Novel techniques for detecting and studying modifiable risk factors in dialysis patients should therefore be explored.

5.2.1 Improving the utility of PAPP-A as a marker of cardiovascular disease

Although evidence points to a role of membrane bound PAPP-A in atherosclerotic disease, the link between serum concentrations of PAPP-A and its function in the pathophysiological pathways of atherosclerosis is tentative. Serum PAPP-A may reflect tissue damage causing release of PAPP-A into the circulation rather that up-regulation of the membrane-bound enzyme. Measurement of cell-membrane bound PAPP-A could therefore be a better proxy of its role in promoting cardiovascular disease, but is limited by the difficulty of obtaining tissue samples in human subjects. Interestingly, serum levels of PAPP-A are highly influenced by heparin administration. This may be due to heparin binding to the glucoseaminoglycan binding site, releasing membrane-bound PAPP-A into the circulation [77].

Based on the above, it can be proposed that elevation of PAPP-A after heparin administration could be a more accurate marker of cardiovascular dysfunction than are baseline serum levels, better reflecting the role of membrane bound PAPP-A in the atherosclerotic process. Hemodialysis treated persons have a high prevalence of cardiovascular disease and are routinely administered heparin at the start of each dialysis session. This provides a promising opportunity to study the association between heparin-induced elevation of PAPP-A and cardiovascular disease.

Atherosclerotic disease is rampant in ESRD, where existing tools for predicting cardiovascular disease such as electrocardiogram, troponin levels and exercise stress testing perform poorly. Better prognostic markers are therefore needed for identifying individuals that may benefit from intensified risk factor treatment or interventional procedures [159]. Heparin-induced increase in PAPP-A could prove to be a novel, powerful and easily measured biomarker for cardiovascular disease risk in hemodialysis patients.

Chapter 6

Populärvetenskaplig sammanfattning

Tillväxthormon (eng Growth Hormone, GH) reglerar omsättningen av fett, kolhydrater och proteiner i kroppen. Hormonet påverkar även hjärta och blodkärl. GH produceras i hjärnan och får levern och andra organ att frisätta "Insulin-like Growth Factor 1" (IGF-1), vilket är det hormon som främst förmedlar effekterna av GH i kroppen. IGF-1 regleras i sin tur av olika IGF-1-bindande proteiner i blodet och av en molekyl vid cellytan som kallas Pregnancy Associated Serum Protein-A (PAPP-A). Hos personer med njursvikt föreligger rubbningar i flera olika hormonsystem, bland annat tillväxthormonsystemet. Vi har studerat hur nivåer av IGF-1 och PAPP-A i blodet hos dialysbehandlade är kopplade till faktorer som ökar risken för hjärt- kärlsjukdom och till den kraftigt ökade dödlighet som ses vid dialysbehandling.

Våra resultat visar lägre nivåer av tillväxthormon (IGF-1) hos dialysbehandlade som är äldre, lider av undernäring eller har låg muskelmassa och hos dem som är inflammerade. Låga nivåer IGF-1 i blodet kan därför vara tecken på sådana tillstånd och vid analys av IGF-1 nivåer hos dialysbehandlade i samband med vetenskapliga studier behöver man ta hänsyn till dessa sk "förväxlingsfaktorer". Vi ser dock att även när man tar hänsyn till dessa faktorer är låga nivåer IGF-1 förknippade med sämre överlevnad i bloddialys. Eventuellt kan mätning av handgreppstyrka tillsammans med nivåer av IGF-1 vara av värde för att bättre bedöma prognosen för personer med njursvikt.

Liksom för IGF-1 påverkas nivåerna av PAPP-A i blodet av

inflammation och muskelmassa. I motsats till IGF-1 är dock dessa faktorer förknippade med höga nivåer av PAPP-A vilket i sin tur är kopplat till ökad dödlighet hos patienter som behandlas med dialys. Våra resultat visar att detta gäller för dem som skall starta dialys såväl som för dem som redan haft bloddialys en tid samt att kopplingen mellan PAPP-A nivåer och dödlighet föreligger främst hos dialysbehandlade som också har diabetes.

Vi har även studerat förekomsten av låga och höga nivåer av kalium hos personer som har kontakt med sjukvården och sett att avvikande kaliumnivåer är vanligt samt att det ofta är återkommande hos personer som någon gång haft ett avvikande kaliumvärde. Högt kalium visade sig vara särskilt vanligt hos dem med nedsatt njurfunktion, diabetes, hög ålder, hjärtsjukdom och vid användning av vissa läkemedel. Kvinnligt kön medförde lägre risk för högt kalium men istället ökad risk för lågt kalium, ett mönster som även sågs hos yngre, de med god njurfunktion och vid användning av vätskedrivande läkemedel. Dessa resultat visar på vikten av att vara medveten om riskerna för kaliumrubbingar hos personer som har kontakt med sjukvården och att följa upp viss läkemedelsbehandling med noggrann provtagning.

Chapter 7

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Chapter 8

Erratum

8.1 Paper I

In table 1, hs-CRP is noted in the "variables" column as presented using mean (SD), but the figures diplayed represent median (IQR).

In table 1, IGFBP-3 is noted in the "variables" column as presented using median (IQR), but the figures diplayed represent mean (SD).

Chapter 9

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