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PEPTIDE AND NON-PEPTIDE BIOMARKERS OF MORTALITY RISK IN PATIENTS WITH ADVANCED CHRONIC KIDNEY DISEASE

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Stockholm 2016

Published by Karolinska Institutet.
Printed by Eprint AB 2016
Front page: The Kidney. Designed by Jingze Du.

ISBN 978-91-7676-518-0

Peptide and Non-Peptide Biomarkers of Mortality Risk in Patients with Advanced Chronic Kidney Disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Abstract

Over the last decade, multiple studies have attempted to identify better risk prediction tools specific to the chronic kidney disease (CKD) population. In many cases, these studies have evaluated the use of various peptide biomarkers as prediction tools. This is common practice in clinical medicine, but in CKD patients these studies are complicated by the fact that most small-to-medium sized compounds are thought to be at least partly metabolized in the kidneys. In fact, a majority of polypeptides smaller than 80 kDa circulate in elevated concentrations in these patients and appear to rise contemporaneously with a drop in glomerular filtration rate (GFR). Thus, elevated levels of many biomarkers may reflect not only increased production as in other patient populations, but also a prolonged half-life due to impaired clearance in the proximal tubules.

In **Study 1**, we analyzed the mortality risk associated with an elevated level of plasma pentosidine in 746 patients with CKD stages 1 - 5 including patients undergoing hemodialysis and peritoneal dialysis treatments. Our main finding is that pentosidine is markedly elevated in CKD and associates with low GFR, oxidative stress and inflammation, but it remains a statistical predictor of mortality in CKD patients also after correction for these confounders.

In **Study 2**, we investigated biomarkers in 543 CKD stage 5 patients initiating dialysis treatment in terms of their ability to classify patients with clinically overt cardiovascular disease (CVD) at baseline and predict all-cause mortality during follow-up for median 28 months. Following adjustments for confounders such as renal function, none of investigated biomarkers were better than simple demographics in predicting adverse outcomes.

In **Study 3**, we again assessed the usefulness of biomarkers, now in 224 prevalent hemodialysis patients. We assessed their ability to classify presence of clinically overt CVD at baseline, as well as predict CVD-related and all-cause mortality during a median 41 months follow-up. We found that while circulating pro-brain natriuretic peptides and insulin-like growth factor 1 predicted baseline CVD, interleukin 6 predicted CVD-related mortality during follow-up, while all-cause mortality was predicted by levels of 8-hydroxy-2'-deoxyguanosine. Thus, no biomarker was useful for all three outcomes, and none of the 13 was statistically

better than demographic data in determining baseline CVD or mortality risks in these patients.

In **Study 4**, renal biopsies from 15 patients with glomerulonephritis and three biopsies from healthy living donors were analyzed for expression of the proximal tubular endocytotic proteins megalin and cubilin. Using both immuno-electron microscopy and standard immunohistochemistry, we show that megalin and cubilin are not just two components of a receptor-endocytosis complex, but rather appear to play distinct roles during albuminuria, as evidenced by their differing expression levels. To gain further understanding, we generated a proteinuric zebrafish model and showed that megalin and cubilin indeed are influenced by tubular albumin levels.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers. They are referred to in the text by their Roman numerals:

I. Machowska A, **Sun J**, Qureshi AR, Isoyama N, Leurs P, Anderstam B, Heimbürger O, Bárány P, Stenvinkel P and Lindholm B. Plasma pentosidine and its association with mortality in patients with chronic kidney disease. PLOS ONE. 2016 Oct 4; 11(10)

II. **Sun J**, Axelsson J, Machowska A, Heimbürger O, Bárány P, Lindholm B, Lindström K, Stenvinkel P, Qureshi AR. Biomarkers of Cardiovascular Disease and Mortality Risk in Patients with Advanced CKD. Clin J Am Soc Nephrol. 2016 Jul 7;11(7):1163-72

III. **Sun J**, Danielson K, Snaedal S, Lindström K, Axelsson J, Lindholm B, Bárány P, Heimbürger O, Stenvinkel P and Qureshi AR. Impact of biomarkers in cardiovascular disease and mortality risk in prevalent hemodialysis patients. Manuscript

IV. **Sun J**, Hultenby K, Axelsson J, Nordström J, He B, Wernerson A, Lindström K. Proximal tubular expression patterns of megalin and cubilin in proteinuric nephropathies. (Submitted manuscript under revision)

CONTENTS

List of Abbreviations

ACEi/A2RB	Angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers
AGEs	Advanced glycated end-products
ANOVA	Analysis of variance
BMI	Body mass index
BP	Blood pressure
CAPD	Continuous ambulatory peritoneal dialysis
CI	Confidence intervals
CKD	Chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CV	Coefficient of variation
CVD	Cardiovascular disease
ELISA	Enzyme-linked immunosorbent assay
ESA	Erythropoiesis-stimulating agents
ESRD	End stage renal disease
GFR	Glomerular filtration rate
HD	Hemodialysis
HGS	Handgrip strength
HOMA-IR	Homeostasis model assessment of insulin resistance index
hsCRP	High-sensitivity C-reactive protein
iEM	Immuno-electron microscope
IgAN	Immunoglobulin A nephropathy
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin 6
LD-Rtx	Living donor kidney transplantation
MCNS	Minimal change nephrotic syndrome
MIA	Malnutrition, inflammation and atherosclerosis
MIMICK	Mapping of inflammation markers in chronic kidney disease
OPG	Osteoprotegerin
PD	Peritoneal dialysis

PEW	Protein energy wasting
PLT	Platelet count
proBNP	Pro brain-type natriuretic peptide
PTH	Parathyroid hormone
PTX3	Pentraxin 3
ROC	Receiver operating characteristics
RR	Relative risk ratio
RRT	Renal replacement therapy
SD	Standard deviation
SGA	Subjective global nutritional assessment
sICAM-1	Soluble intercellular adhesion molecule 1
sVCAM-1	Soluble vascular cell adhesion molecule 1
TMD	Thin membrane disease
TNF-α	Tumor necrosis factor α
TnT	Troponin T
USRDS	United States Renal Data System
WBC	White blood cell
8-OH-dG	8-hydroxy-2’-deoxyguanosine

Chapter 1 Introduction

1.1 The kidney

1.1.1 Kidney anatomy and function

The kidneys are retroperitoneal structures located under the dorsal ribs, at the thoracic 12 to lumbar 3 vertebral level, and approximately with the size of a human fist. The left kidney is typically somewhat higher than the right on account of the liver. Structurally, each kidney is composed of an outer cortex and an inner medulla, each containing parts of the functional unit of the filtration apparatus, called a nephron and consisting of a glomerulus and paired tubular system. A healthy human is thought to possess approximately 1 million glomeruli in each kidney.

The kidneys can be described as one of the metabolic centers of the body. It filters water soluble compounds from the blood thus removing both endogenous and exogenous toxins, excess salts and waste products, while also balancing total body water and pH. This occurs through a two-step process, passive filtration over the glomerular filtration barrier followed by reabsorption and secretion along the tubuli. The filtration product, the primary urine, is approximately free from proteins (e.g. albumin) and blood cells as they are too large to pass the glomerular filter, but otherwise similar in composition to the systemic circulation, and measuring about 180 liters/day. Along the tubules, most of this liquid is reabsorbed along with the recycling of many compounds, meaning that the final daily urine output usually is about 1 - 2 liters/day. The kidney needs a continuous blood supply to enter the glomerulus in order to maintain the filtration pressure, and it is also an important organ in regulating blood pressure.

1.1.2 Glomerular filter

The glomerulus of the kidney is a highly developed microvascular bed that acts as a filter. It is composed of three different cell types, the endothelial cells closest to the vascular space, the epithelial cells (podocyte) that is integrated to the glomerular basement membrane (GBM) and the mesangial cells holding the capillaries together [1] (Figure 1). The barrier allows small molecules, such as water, sugars, electrolytes and small proteins, to pass

through while retaining very large macromolecules [2]. It is build up by the endothelial cells lining the capillary space and normally has small gaps, fenestration, allowing passage of fluid into the GBM. The GBM is built up by collagen fibers and negatively charged macro molecules. Podocytes line the outside of the glomerular capillaries and thus face the primary urine collected in the space called Bowman's capsule. The podocytes wrap around the adjacent capillaries with foot processes, that in turn interdigitate to form filtration slits. The slit pore size was originally reported to be 35 Å with some variation in size [3]. These structures are often involved in nephrotic diseases [4, 5]. For example, in minimal change nephrotic syndrome (MCNS, Figure 1c), the slit diaphragms loose surface area due to foot process effacement [6].

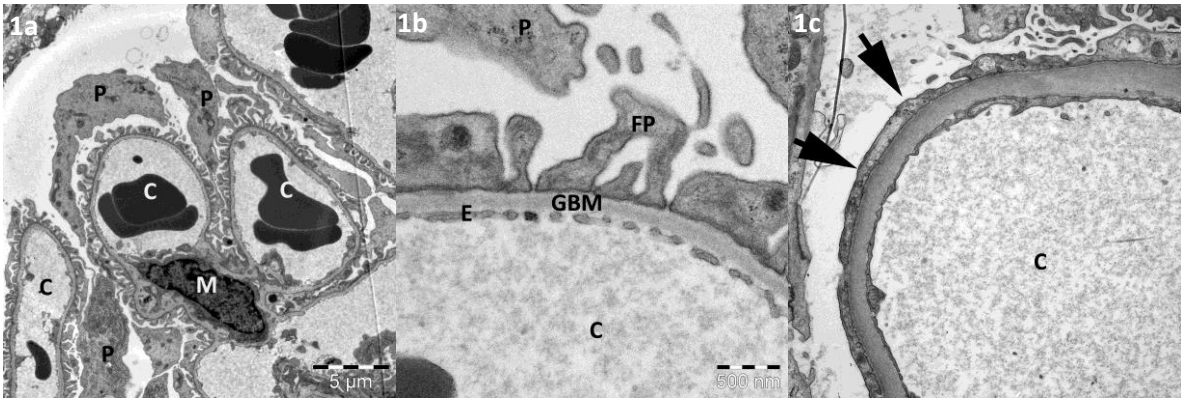

Figure 1. Electron microscopy images of the glomerular filtration barrier with normal structure (1a and 1b) and effacement (1c). C=capillary, E=endothelium, FP=foot process, GBM=glomerular basement membrane, M=mesangial.

1.1.3 Proximal tubules

The proximal tubule begins abruptly at the urinary pole of the glomerulus and comprises an initial convoluted portion, the pars convoluta, which is a direct continuation of the parietal epithelium of Bowman's capsule, followed by a straight portion, the pars recta, which is located in the medullar border. Finally, the distal convoluted tubules and collecting duct pass through the medulla to reach the renal calyx. The proximal convoluted tubule plays a major role in the reabsorption of small peptides, Na^+, HCO_3^-, Cl^-, K^+, Ca^{2+}, PO_4^{3-}, water, and organic solutes such as vitamins, glucose, and amino acids. Indeed, approximately 70% of the ultrafiltration from renal glomeruli is removed already in the proximal tubules [7].

1.1.4 Renal peptide metabolism

For peptides, recycling in the proximal tubules is thought to be mainly mediated by the two receptors megalin (also known as low density lipoprotein-related protein 2, Lrp2) and cubilin [8, 9]. They mediate endocytic uptake into clathrin-coated pits, after which the proteins are transferred through endosomes to lysosomes for degradation. The receptors are thought to return to the apical cell membrane rather than undergoing lysosomal proteolysis [10]. This reabsorptive process is extremely efficient as evidenced by the virtual protein-free urine in humans. Indeed the most abundant protein in human urine, Tamm-Horsfall protein, is secreted further along the tubules [11]. The process of peptide recycling serves not only to remove bioactive and osmogenic proteins from the tubule, but also conserves a variety of essential substances inside (e.g. amino acids) and attached to (e.g. vitamins) the plasma proteins [12].

The role of megalin and cubilin in this process was recently reviewed in detail by Christensen [13]. Briefly, it is thought that megalin serves as a receptor for numerous ligands including low-molecular-weight proteins [12], polypeptide hormones [14], albumin [15], vitamin-binding proteins [16] and polybasic drugs such as aminoglycosides [17]. Cubilin is thought to co-localize with megalin in order to anchor to the membrane, but to have several ligands that appear to partly overlap those reported for megalin, including albumin and various vitamin-binding proteins [12]. A ligand of particular interest is albumin, the most abundant serum protein and an important part of proteinuric urine. Albumin has been reported to bind to both receptors [15, 18], or essentially to cubilin [19].

1.1.5 Proteinuria

Urinary protein excretion up to 150 mg per day may be normal; however, the limits used to define presence of albuminuria are usually set at values below 30 mg. A decreasing GFR may be associated with an increase in the albumin-to-creatinine ratio and overt albuminuria in the general population [20]. When the urinary protein concentration exceeds 3.5 g protein per 24 hours, a patient is diagnosed as having nephrotic proteinuria [21]. Physiologically, two mechanisms must operate in order to get proteinuria including dysfunction of the selective permeability of the glomerular filtration barrier [22, 23] and saturation of proximal tubular reabsorption capacity [24]. The hypothetical negative influence of increased tubular

endocytosis in glomerular diseases is not fully clear yet [25-28]. However, it is clear that proteinuria is either a marker or mediator of harm to the kidney [29] which in the long term appears to lead to renal fibrosis, kidney failure and to have implications for systemic vascular function.

1.1.6 Glomerular filtration rate

The GFR is defined as the volume of fluid filtered from the kidney glomerular capillaries into the Bowman's capsule per unit of time. An exact measurement of GFR is very difficult to achieve; instead, minimally invasive and relatively reliable methods are used to estimate GFR in the clinical setting.

In the clinic, even faster and less impractical methods are used based on demographic parameters along with a blood sample to determine creatinine that provide estimated GFR (eGFR) through various equations. Recent publications have suggested using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula [30].

1.2 Chronic kidney disease

1.2.1 Classification

The strong correlation of phenotype with GFR has led to a system classifying CKD into 5 stages, regardless of etiology, shown in Table 1 [31].

According to the Kidney Disease Quality Outcome Initiative (K/DOQI), CKD is defined as abnormalities of kidney structure or function, present for 3 months or more, with implications for health [32]. GFR < 60 ml/min/1.73m^2 is referred as decreased GFR and GFR < 15 ml/min/1.73m^2 is referred as end-stage kidney failure. Once CKD becomes manifest, we currently do not have therapy to reverse it but only to retard progression. Also, the CKD phenotype is solely dependent on residual renal function, and thus is very little altered by the primary cause of kidney damage. CKD is associated with adverse outcomes, especially premature CVD and death [33]. Even in the early stages, CKD confers a risk of fatal and non-fatal cardiovascular events far exceeding that which occurs in the general population of the same age [34].

Table 1. Stages of chronic kidney disease.

Stages	GFR (ml/min/1.73m^2)	Description
Stage 1	> 90	Structural damage with normal or high GFR
Stage 2	60 - 89	Structural damage with mildly decreased GFR
Stage 3a	45 - 59	Mildly to moderately decreased GFR
Stage 3b	30 - 44	Moderately to severely decreased GFR
Stage 4	15 - 29	Severely decreased GFR
Stage 5	< 15	Kidney failure and requiring renal replacement therapy

1.2.2 Epidemiology of CKD

According to the 2015 annual report by the United States Renal Data Systems (USRDS), the overall prevalence of CKD in the U.S. increased from 12% in 1988 - 1994 to 14% 2007 - 2012, and 117 162 patients were living with ESRD in 2013. These had adjusted annual mortality rates of 14%, 17% and 4% for ESRD, dialysis and transplant patients, respectively [35]. While a recent large-scale in Europe that attempted to carefully characterize CKD prevalence identified substantial variation in CKD prevalence between countries, these differences appear to be caused by factors other than the prevalence of diabetes, hypertension and obesity [36].

In the Stockholm region it is estimated that 6% of adult individuals have CKD insert 2006 - 2011 [37]. In Sweden, there were 11 373 known CKD patients in 2014, most of whom (94%) had CKD stage 3 - 5 [38].

1.2.3 Effects of declining renal function

As may be expected, the many roles of the kidney in human physiology mean that a decline in function impacts normal body function in multiple ways. First, metabolic acidosis is normally counteracted by renal excretion of hydrogen ions. With a loss of renal function acidosis invariable appears [39]. Furthermore, renal generation of bicarbonate is also impaired when the damage progresses, worsening the metabolic acidosis [39].

High potassium level represents another major pathophysiological consequence of declining GFR. Potassium is to 90% excreted by the kidney [40], explaining why even a modest decline in renal function leads to hyperkalemia. Depending on the severity, hyperkalemia may result in neurological and heart rhythm problems.

Likewise, imbalances of calcium and phosphate linked to reduced excretion by the kidney as well as reduced production of active vitamin D results in a number of clinical complications [41]. Perhaps most noticeably, renal patient suffer osteoporosis and enhanced vascular calcification [42].

Uremic toxicity occurs in the later stages of CKD, and result from the failure of the kidneys to remove potentially toxic compounds from the blood stream, causing their accumulation in the body [43]. The exact toxicity of individual compounds as well as their effects on the body remain poorly characterized [44].

Hypertension is present in more than 80% of patients with CKD [45], constituting both a cause and a consequence of the progression of CKD and cardiovascular damage [46]. Furthermore, hypertension is often associated with proteinuria, which is the common phenotype of kidney disease. Proteinuria is considered both a marker of progression in renal disease [29] and a driver of cardiovascular events [47].

In a normal adult, approximately 3.5 - 4.5 g protein/kg body weight are synthesized and degraded each day with the majority being intracellular proteins [48]. In CKD, this amount may be reduced as evidenced both by signs of increased muscle catabolism but also by low circulating concentrations of high-abundance proteins such as albumin.

Muscle wasting itself is another major complication of CKD and is reported to be progressive [49]. The mechanism are incompletely understood, but involve impaired insulin/IGF-1 intracellular signaling, which stimulates protein degradation through altered ubiquitination [50].

1.2.4 Renal replacement therapy (RRT)

Patients with ESRD require RRT with either kidney transplantation or dialysis, in many cases both over a lifetime. The two main methods of conducting dialysis, hemodialysis (HD) and peritoneal dialysis (PD), have approximately similar outcomes but their application varies

between countries for both medical and logistic reasons. In Sweden where approximately 79% of dialysis patients are on HD and 21% on PD, the annual mortality rate in the dialysis population was 20% and for transplant patients is 2% [38].

Dialysis today entails the removal only of water and solutes. This leaves larger molecules such as proteins in place, with unknown consequences. Also the loss of residual renal function with time, a process that occurs also in patients on dialysis, has been found to contribute to poor clinical outcomes [51, 52]. Dialysis itself is associated with hypermetabolism and inflammation [53], while central dialysis catheters are a major cause of inflammation and infections leading to premature deaths [54]. Thus, it is not surprising that the survival of dialysis patients is relatively poor.

Transplantation of a kidney from a living or deceased donor constitutes the second form of RRT, and is associated with improved survival and quality of life as compared to other options. However, the renal allograft is constantly at risk of rejection and immunosuppressive therapy must therefore be maintained.

1.3 Mortality risk factors and their biomarkers

A risk factor is a variable that has association with a certain disease or disease process in an individual or a population. It is used for identifying subjects at increased risk of an adverse outcome. When it comes to CVD, the Framingham study has led the way in linking factors such as old age, smoking, hypertension, dyslipidemia, diabetes to CVD and increased mortality. However, in CKD patients the impact of these common risk factors is muted, despite a very high prevalence of left ventricular hypertrophy and hearth failure. Instead a number of novel risk factors have been proposed. These include protein energy wasting (PEW), circulating inflammatory cytokines, endothelial dysfunction, fluid overload and vascular calcification [55, 56]. However, while all of the above are associated with an adverse outcome, there is to date no single test or marker that may be used to predict which patients will do well and which will suffer CVD.

In this context, a number of circulating biomarkers have been proposed as risk markers in renal disease patients [57]. Most are only validated in small cohort studies and the impact of

residual renal function on them is not well established. Furthermore, except for the markers of GFR, quantitative indicators of pathologic processes that vary continuously with the progression of disease, including glomerular membrane leakage, sclerotic glomeruli and the amount of interstitial fibrosis are lacking [58].

Below, a number of common complications of CKD associated with adverse outcomes (in particular premature death) are discussed along with biomarkers that have been proposed.

1.3.1 Protein-energy wasting (PEW)

PEW is a relatively new term for describing the progressive depletion of protein and/or energy stores that occurs in patients with CKD [59], and is associated with adverse clinical outcomes [60]. According to a proposal by the International Society of Renal Nutrition and Metabolism (ISRNM), the optimal diagnosing of PEW is based on the presence of biochemical criteria, together with a reduced body mass and a reduced muscle mass [61].

The reported prevalence of PEW in maintenance HD and PD patients ranges from 18% to 75% [62, 63]. While PEW is a prognostic factor associated with mortality [64], to date no accurate biomarker exists to reflect PEW while interventions available to treat it are often ineffective. Studies suggest that PEW is accompanied by increased inflammation [65], resistance of skeletal muscle to insulin [66] and muscle atrophy [67], as well as reduced serum albumin concentrations [68, 69]. As mentioned, few interventions are successful and novel treatment strategies are needed [64].

Several biomarkers have been proposed to reflect PEW, but none is accepted in clinical practice. The markers range from the traditionally used serum albumin [69], cholesterol [70], creatinine [71] and IGF-1 [72], to newer markers such as IGF binding protein (BP)-1 [73] and myostatin [74].

1.3.2 Cardiovascular disease

CVD is the major cause of death in patients with CKD, and even a mildly reduced renal function results in a dramatically increased risk of premature CVD and death [75, 76]. The cardiovascular risk in CKD cannot solely be explained by traditional risk factors such as hypertension, dyslipidemia, smoking, DM or obesity [77]. The uremic phenotype itself entails a wide variety of metabolic disturbances known to increase cardiovascular risk such as

oxidative stress and inflammation [78], endothelial dysfunction [79] and vascular calcification [80]. Arteriosclerosis due to both intimal disease and medial calcification is a common finding in CKD and affects both small and large arteries [81].

Another pathway implicated in uremic CVD is the B-type natriuretic peptides (BNP) pathway. BNP belongs to a system of natriuretic peptide that has a major role in regulating blood pressure and volume through direct effects on the kidney and systemic vasculature [82, 83]. BNP is synthesized as an amino acid precursor protein by the heart, and then undergoes intracellular modification to a more stable N-terminal cleavage product (NT-proBNP). Prevalence of elevated BNP or NT-pro-BNP levels is high in patients with CKD [84] and has been linked to CVD and death [85].

Troponin T (TnT) is another biomarker implicated in CKD-CVD. It is one of the components of the contractile apparatus of striated muscle, and is mainly present in the heart [86]. TnT is detected in high concentrations in serum from patients with ESRD [87] in whom it predicts CVD [88].

1.3.3 Vascular ossification

Cardiovascular ossification of the intima and especially the media is common in CKD patients [89], while the proteins fetuin-A and osteoprotegerin (OPG; both mediating calcium homeostasis) are related to arterial stiffness and atherosclerosis in this group [90]. In medial calcification a differentiation from smooth muscle cells to osteoblast-like cells takes place [91] giving a bonelike matrix production and mineralization that happens in the presence of high calcium and phosphorus [92]. Vascular calcification and inflammation are interrelated in CKD, perhaps in part mediated via phosphate retention and/or bone disease [93]. Fetuin-A is often decreased in the inflamed uremic milieu and has a positive relation to survival in HD and PD patients [94], while OPG associates with progression of coronary artery calcification and mortality and is increased in HD patients with CVD [95].

1.3.4 Proteinuria

Proteinuria plays an important role in the pathogenesis of CKD progression [96, 97]. Epidemiological studies have demonstrated a graded relationship between increased proteinuria and mortality as well as kidney outcomes in diverse study populations [98]. This

association appears to be independent of low GFR and the role of proteinuria as a risk factor for CVD is well established [99].

The exact mechanisms that link proteinuria to kidney disease progression remain unclear [100]. Clearly albumin leakage into the final urine is pathological, and may reflect a combination of filter damage and reduced compensatory capacity of the proximal tubule endocytotic apparatus [101]. The damage is likely then propagated by the toxic effects of proteins (including albumin overload) on the proximal tubule epithelium, causing interstitial inflammation and fibrosis [102].

1.3.5 Oxidative stress

Oxidative stress - loosely defined as an excess of oxidative compounds and insufficient antioxidant defense mechanisms [103] appears to increase as CKD progresses and may correlate inversely with renal function [104, 105]. In CKD, more stable forms of oxidative damage such as advanced glycation end products (AGEs) accumulate [106]. Retention of AGEs and other pro-oxidants is likely to contribute to a pro-inflammatory milieu as renal function declines [107]. The specific interaction between AGEs and their receptors further specifically contributes to generation of intracellular oxidative stress [108]. Likewise, pentosidine (mainly in the form of a combined AGE-protein complex [109]) is remarkably increased in dialysis patients and predicts CVD and disorders of bone mineralization [110]. Another biomarker 8-hydroxy-2'-deoxyguanosine (8-OH-dG) is one of the most abundant oxidative products of nucleic acids [111]. The circulating level of 8-OH-dG is elevated in patients with CVD and predict all-cause mortality in dialysis patients [112].

1.3.6 Inflammation

The phenotype of CKD is often accompanied by systemic inflammation, which may promote progression [113], premature ageing [76] and CVD [114]. In the general population, persistent inflammation is related to poor clinical outcomes, mainly cardiovascular disease [114], and the same risk is found in CKD patients, especially after the start of dialysis [115].

The causes of inflammation in CKD are not known. One hypothesis is that retention of circulating cytokines [116], AGEs [107], and/or other pro-oxidants [104] contribute to a pro-inflammatory milieu as renal function declines. The chronic low-grade inflammation

engendered may play a central role for the uremic phenotype, as evidenced by the high prevalence of micro inflammation [117]. Various clinical events are also common causes of inflammation in CKD [118].

Reviewing the literature, Kalantar-Zadeh has reported more than 40 inflammatory markers which have been studied in patients with CKD [119]. In this thesis, pentraxins, several pro-inflammatory cytokines and adhesion molecules will be discussed. Amongst them, CRP is an acute phase reactant produced in the liver that already in the 1990s was shown to be a powerful predictor of CVD related death and all-cause mortality in HD [120] and PD patients [121]. Today, CRP is widely used in as a marker of inflammation in the clinic due to its low cost, high reliability, and extensive availability for detection [122]. Pentraxin 3 (PTX3), which belongs to the pentraxin family of proteins that also includes CRP, is an acute phase reactant involved in pathogen recognition, complement activation and regulation [123]. PTX3 is elevated in dialysis patients compared to healthy controls and reflects endothelial dysfunction [124]. It is associated with cardiovascular disease and mortality risk [125].

IL-6 is a cytokine produced by various cells including T-cells, mast cells, macrophages, and endothelial cells [126]. It is also well-known to be a strong predictor of mortality in incident and prevalent HD patients [127]. Another pro-inflammatory cytokine TNF-α has a pivotal role in regulating both pro- and anti-inflammatory mediators [128]. Elevation of TNF-α also predicts clinical outcomes in dialysis patients [116], even after controlling for age and serum albumin level [57].

Finally, adhesion molecules including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are found on endothelial cells [129]. Several are known to be elevated in patients on dialysis [130]. As the formation of atherosclerosis is partly caused by the adhesion of leukocytes to the vascular endothelium following various pro-inflammatory signals [131] they also represent a possible biomarker for CVD in the setting of uremia.

Chapter 2 Aims

The overall aim of this thesis is to investigate the impact of impaired renal function (possible related to proximal tubular endocytosis) on the usefulness of selected biomarkers of outcomes in patients with CKD.

Specifically, we had the following objectives:

Study 1: To investigate the statistical performance of a single measurement of the plasma concentration of pentosidine - in relation to plasma albumin to which pentosidine is bound - as a predictor of mortality risk in CKD patients.

Study 2: To evaluate the usefulness of several common peptide and nucleotide biomarkers as predictors of clinical outcomes in ESRD patients.

Study 3: To evaluate a similar panel of biomarkers in prevalent HD patients.

Study 4: To investigate expression patterns of megalin and cubilin in proximal tubules from patients with different glomerulopathies and varying grades of albuminuria.

Chapter 3 Materials and Methods

3.1 Ethical considerations

All studies in this thesis adhere to the Declaration of Helsinki for human studies. The Ethics Committee of the Karolinska Institute at the Karolinska University Hospital Huddinge, Stockholm, Sweden, approved the study protocol of the Prima control, Prima, MIA, MIMICK 1, MIMICK 2 and LD-Rtx cohort studies, as well as the use of healthy individuals and renal biopsies. Informed consent was obtained from each patient and healthy individuals. The study of zebrafish embryos was performed by Zebrafish Core Facility, Karolinska Institute.

3.2 Study protocols

3.2.1 Study 1

The study is based on post hoc analysis of baseline data and subsequent longitudinal follow-up of 746 participants from the six cohorts - the Prima control, Prima, MIA, MIMICK 1, MIMICK 2 and LD-Rtx - with varying degrees of renal failure as follows: 37 individuals in CKD stage 1 - 2, 54 in CKD stage 3 - 4, 386 in CKD stage 5 but were investigated prior to starting on dialysis (CKD 5 - ND), 195 were prevalent HD patients and 74 were prevalent PD patients. Plasma pentosidine, biomarkers of inflammation, oxidative stress and nutritional status were investigated, and mortality data collected.

3.2.1.1 CKD stage 1 - 2

The Prima control cohort represents a population based sample with signs of mild CKD (micro- or macro- albuminuria or reduced GFR) and served as control group for the Prima study cohort. The Prima control cohort comprised 37 individuals from the Stockholm region, randomly selected by Statistics Sweden (a government agency) and accepting to participate. Recruitment was carried out by mail between February 2003 and April 2004. More detailed description of the study has previously been published [132]. The median age was 68 years, median eGFR was 79 ml/min/1.73m^2 and the prevalence of CVD was 8%, while 6% of

participants were classified as malnourished (SGA >1). Over a median 60 months of follow-up, 2 individuals died.

3.2.1.2 CKD stage 3 - 4

The Prima cohort consists of 54 CKD stage 3 - 4 patients recruited from December 2001 until March 2004. The description of the study has previously been published [132]. In brief, patients being seen at the outpatient clinic of the Karolinska University Hospital were asked to enroll after informed consent. The median age was 60 years, 74% were males, median eGFR CKD-EPI was 27 ml/min/1.73m^2, 21% were malnourished, 44% had diabetes and 26% had CVD. The most common causes of CKD were diabetic nephropathy (25%) and glomerulonephritis (23%). Over a median 60 months of follow-up, 24% of patients died.

3.2.1.3 CKD 5 - ND

MIA (Malnutrition, Inflammation and Atherosclerosis in CKD) is a cohort coordinated by the Division of Renal Medicine, Department of Clinical Science, Intervention and Technology, Karolinska Institutet that recruits incident patients starting renal replacement therapy. This ongoing cohort study is described in detail elsewhere [65].

There are 386 incident dialysis patients were recruited (from June 1994 through October 2012). The median age was 55 years, 60% were male, median eGFR CKD-EPI was 6 ml/min/1.73m^2, 30% were malnourished, 31% had CVD and 28% had DM. The causes of CKD were chronic glomerulonephritis (23%) and diabetic nephropathy (25%). The majority of patients were on antihypertensive medications; 59% of patients were prescribed a beta (β) - blocker, 54% angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers (ACEi/A2RB), 45% calcium channel blocker and 21% received statin treatment. The majority of patients were on antihypertensive medications and other commonly used drugs in CKD, such as phosphate and potassium binders, diuretics, iron substitution and vitamin B, C, and D supplementation. During 32 months follow up, 27% of patients died.

3.2.1.4 Prevalent HD

A total of 195 prevalent HD patients were included in the MIMICK 1 (Mapping of Inflammation Markers in Chronic Kidney Disease 1) cohort, which was recruited from October 2003 until September 2004. A description of the study was presented previously

[133]. The median age was 64 years, 57% were males, 23% had diabetes, 61% had CVD, 45% were malnourished (SGA >1) while most of the patients in this cohort were functionally anuric with no or minimal residual renal function. The most common causes of CKD were glomerulonephritis (20%), diabetic nephropathy (18%) and hypertension/renal vascular disease (13%). Most patients were on anti-hypertensive medications such as β-blockers (48%), calcium channel blockers (27%), ACEi/A2RB (34%) while some were taking statins (32%). Most also received medications common to CKD stage 5 with dialysis, such as phosphate and potassium binders, diuretics, and vitamin B, C, and D supplementation. During a median follow-up of 41 months 48% died.

3.2.1.5 Prevalent PD

A total of 74 prevalent PD patients were recruited from March 2008 to March 2011 to take part in the MIMICK2 (Mapping of Inflammation Markers in Chronic Kidney Disease 2). A description of the study has previously been published [112]. In summary, the median age was 61 years, 64% were males and median eGFR CKD-EPI was 6 ml/min/1.73m^2. Further 20 % had diabetes, 22 % had CVD, and 42% were malnourished (SGA score >1). CAPD was used by 75% of patients and the remaining 25% patients were treated by automated peritoneal dialysis (APD). The most common causes of CKD were glomerulonephritis (13%), diabetic nephropathy (12%) and hypertension/renal vascular disease cause (12%). Most patients were on anti-hypertensive medication β-blockers (68%), calcium channel blockers (31%), ACEi/A2RB (69%). Most patients received medication such as phosphate and potassium binders, diuretics, and vitamin B, C, and D supplementation. After a median follow up 25 months, 38% of patients had died.

3.2.1.6 LD-Rtx

In **Study 1**, 59 CKD patients are from LD-Rtx (undergoing living donor kidney transplantation) cohort, which was recruited at the Department of Transplantation Surgery of the Karolinska University Hospital between May 2009 and October 2013. More details about this ongoing cohort have been published previously [134]. There are 24 patients allocated to CKD stage 5 - ND, 17 to prevalent HD and 18 to prevalent PD.

3.2.2 Study 2

In **Study 2**, 543 incident dialysis patients from the MIA cohort (from June 1994 through November 2014) were included. The median age was 56 years, 63% were male, median eGFR CKD-EPI was 9.5 ml/min/1.73m^2, 33% were malnourished, 37% had CVD and 31% had DM. The causes of CKD were chronic glomerulonephritis in 126 patients (23%), diabetic nephropathy in 159 patients (29%; of which 125 were treated with insulin), hypertension/renal vascular disease in 113 patients (21%) and other or unknown etiologies in 145 patients (27%). The majority of patients were on antihypertensive medications; 63% of patients were prescribed β-blocker, 60% ACEi/A2RB, 49% calcium channel blocker and 27% received statin treatment. During median follow up 28 months follow up, 28% of patients died.

The final selection of biomarkers to be included was based on the availability of previous measurements and their clinical utility (commercial kits available for analysis and reported to predict either CVD or mortality in the CKD 5 population).

3.2.3 Study 3

The **Study 3** was comprised 224 prevalent hemodialysis patients from MIMICK 1 (from August 2003 until September 2004). The median age was 66 years, 55% were males, 26% had diabetes, 63% had CVD, 47% were malnourished (SGA >1) while most of the patients in this cohort were functionally anuric with no or minimal residual renal function. The most common causes of CKD were glomerulonephritis (17%), diabetic nephropathy (18%) and hypertension/renal vascular disease (17%). Most patients were on anti-hypertensive medications such as β-blockers (50%), calcium channel blockers (25%), ACEi/A2RB (34%) while 32% were taking statins. During a median follow-up of 41 months 53% died.

The **Study 3** uses similar criteria as in **Study 2** to select biomarkers that were studied as predictors of CVD, CVD-related mortality and all-cause mortality.

3.2.4 Study 4

3.2.4.1 Human biopsies

Briefly, using patient records we identified 15 patients (median age 29 years, 10 males) diagnosed with specific kidney diseases by diagnostic renal biopsy at the Karolinska University Hospital between 2000 and 2013. There were nine biopsies from patients with IgA nephropathy (IgAN) presenting different degrees of albuminuria, three biopsies from patients with minimal changes nephritic syndrome (MCNS) and three biopsies from patients with thin membrane disease (TMD). Biopsies from three healthy living kidney donors were used as controls. Biopsies were performed under ultrasound guidance and using standard equipment. Control kidney biopsies were taken immediately after removal of the donor kidney and before flushing with the preservative solution.

For light microscopy, the specimens were fixed in 4% phosphate buffered formalin, then dehydrated and embedded in paraffin according to standard procedures. Sections of 1.5 µm were cut on a microtome and stained with hematoxylin and eosin, periodic-acid Schiff, Ladewig's trichrome, and periodic acid silver. Materials for diagnostic transmission electron microscopy were fixed in a mix of 2.5% glutaraldehyde and 0.5% paraformaldehyde and embedded in an epoxy resin. The blocks were cut into approximately 60 nm thick sections and evaluated in a transmission electron microscope (TEM). A small piece of each kidney biopsy was also collected for immuno-electron microscope (iEM). The tissue was fixed in a solution containing 0.1M phosphate-buffered 1% paraformaldehyde and 0.5% glutaraldehyde and processed for low temperature embedding into K11M [135].

The histopathology of the included patients' biopsies was re-examined by an experienced renal pathologist to confirm the previously reported diagnoses. Tubular atrophy and interstitial fibrosis were semi-quantified according to Banff criteria [136]. Thus, tubular atrophy was graded between 0 - 3; 0 = none, 1 = affecting 0 - 25%, 2 = affecting 26 - 50%, and 3 = affecting >50% of the cortical area. Interstitial fibrosis was graded 0 - 3; 0 = affecting 0 - 5%, 1 = affecting 6 - 25%, 2 = affecting 26 - 50%, and 3 = affecting >50% of the cortical area.

3.2.4.2 Zebrafish biopsy

A proteinuric zebrafish model was generated by morpholino-mediated knock-down of nphs2 as described earlier [137]. The morpholino antisense oligo (MO) targeting nphs2 (5'-TAGACT TACCTTCTCCAGGTCCCTC) and a standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA) were obtained from GENE TOOLS, LLC (Philomath, OR, USA). Two doses (50 µM and 100 µM) of nphs2 MO were injected into one- or two-cell embryos, respectively. As a control, 100 µM of control MO was similarly injected. We used embryos from a wild-type AB zebrafish line, which is maintained and raised in the Zebrafish Core Facility, Karolinska Institutet, as previously described [138].

Whole zebrafish embryos were observed using a Leica MZ12 dissecting stereomicroscope with an attached digital camera. Pericardial edema of the morphants was analyzed and photographed at 4 days post fertilization (dpf). For TEM and iEM, 4-dpf morphants were fixed with 3% PFA and kept at 4°C until the next procedure for dehydration and embedding. Materials for diagnostic transmission electron microscopy were fixed in a mix of 2.5% glutaraldehyde and 0.5% paraformaldehyde and embedded in an epoxy resin. The blocks were cut into approximately 60 nm thick sections and evaluated in TEM.

We classified the biopsied patients according to their diagnosis and albuminuria: Group 1 comprised IgAN with microalbuminuria (IgANL, albuminuria >30 mg/24h and <300 mg/24h, n=3) [139, 140] along with TMD (no albuminuria); Group 2 comprised IgAN with macro albuminuria (IgANM, albuminuria >300 mg/24h but <3500 mg/24h, n=3) [140]; and Group 3 with IgAN with nephrotic degree of albuminuria (IgANH, albuminuria >3500 mg/24h, n=3) along with MCNS (albuminuria >3500 mg/24h, n=3). The expression of megalin and cubilin in proximal tubular sections was investigated by iEM and IHC, while a similar study was performed in proteinuric zebrafish as supplementary confirmation or comparison.

3.3 Methods

3.3.1 Anthropometric and metabolic evaluation

Height and body weight were obtained at baseline and BMI was recorded. SGA was used to evaluate overall PEW as previously described [49]. PEW was defined as SGA score >1. HGS was measured using a Harpenden Handgrip Dynamometer (Yamar, Jackson, MI, USA) in the dominant hand. The individual values for HGS were expressed as % of healthy subjects, adjusting for the gender.

Arterial systolic and diastolic blood pressures (BP) were measured three times in the morning after a 15 min resting period, and the mean pressure used. In non-diabetic patients, insulin resistance was calculated by the homeostasis model assessment for insulin resistance [141]. Insulin resistance was not assessed in diabetic subjects.

3.3.2 Biochemical Analysis

Blood samples were collected at baseline evaluation. The plasma was separated within 30 minutes, and samples were kept frozen at –70° C if not analyzed immediately.

Concentrations of hemoglobin and serum albumin (intra-assay CV: 3 - 4%) (bromcresol purple), creatinine, ferritin (intra-assay CV: 6%), hemoglobin A1c (HbA1c%), hsCRP (intra-assay CV: 5%; inter-assay CV: 6%) (nephelometry), orosomucoid (intra-assay CV: 5 - 7%), PLT (intra-assay CV: 5 - 7%), TnT (intra-assay CV: 8%), urea, WBC (intra-assay CV: 6%) and plasma concentrations of cystatin C and glucose were determined by routine methods at the Department of Laboratory Medicine, Karolinska University Hospital. Commercial ELISA kits were used to determine sVCAM-1 (intra-assay CV: 3.5%; inter-assay CV: 6%) and sICAM-1 (intra-assay CV: 4%; inter-assay CV: 5%) (R&D Systems Europe, Ltd, United Kingdom). Plasma concentrations of insulin, IGF-1 (intra-assay CV: 4.3%; inter-assay CV: 6.9%), IL-6 (intra-assay CV: 4%; inter-assay CV: 5%) and TNF-α (inter-assay CV: 5%) were measured on an Immulite TM Automatic Analyzer (Siemens Healthcare, Diagnostics Products Ltd. United Kingdom), using assays manufactured for this analyzer and according to the manufacturer's instructions. Plasma pentosidine was analyzed by reverse-phase high performance liquid chromatography (HPLC) as described previously [107]. Circulating pentosidine is mainly present in protein bound form with albumin [142]; therefore, the total plasma pentosidine concentration

measured in nmol/L was corrected for albumin and expressed as nmol of plasma pentosidine per gram of albumin.

3.3.3 Antibodies

Following light microcopy to determine the integrity and structure of the proximal tubules in each biopsy, we studied human samples using iEM employing sheep anti-megalin and rabbit anti-cubilin antibodies (gift from Professor Renata Kozyraki, Institute de la Vision, INSERM, Paris, France) as well as rabbit anti-albumin antibodies (Sigma, cat. No. A3293-2ML). To detect the signal of reabsorbed protein in the larval zebrafish proximal tubules, a sheep anti full-length rat albumin antibody (Bethyl Laboratories, Inc; cat. no. A110-134A) was used. For immunohistochemistry staining, we used antibodies to megalin (Atlas, cat. no. HPA005980) and cubilin (Biorbyt, cat. no. orb4997). The same antibodies were also used for zebrafish iEM analysis as a Blast analysis showed that zebrafish homology of the immunogenic peptides for megalin and cubilin was 80% and 83% similar to human, respectively. Normal sheep serum (Dako, cat. no. X 0503) and normal rabbit IgG (Dako, cat. no. X0903) were used as negative controls. To detect bound antibodies, a secondary gold-conjugated protein A (10 nm) or gold-conjugated donkey anti-sheep (10 nm) antibody (British Biocell International, cat. no. 7687 and 15249) were used.

3.3.4 Immunohistochemistry (IHC)

IHC was performed on paraffin sections from all biopsies using the standard IHC protocol in the Department of Pathology and staining was analyzed by using a Bond-III microscope (Leica Biosystem, Germany). Antibodies to megalin (1:500) and cubilin (1:200) were used.

3.3.5 iEM and semi-quantification of protein expression

All sections were analyzed in a Tecnai 10 microscope (FEI, Eindhoven, The Netherlands) at 100 kV and digital images were captured with a Veleta camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

To determine the number of images needed for an appropriate sampling, we used a cumulative mean plot as previously described [143]. In the present study, ten proximal tubules were chosen randomly and in each tubule three different areas were selected. In each area one image at a primary magnification of 39 000 × covering brush border and one

image covering the adjacent apical cytoplasmic vesicles were taken. Thus, thirty images were analyzed in each compartment, respectively. The area of the corresponding compartments was calculated by point counting using a 1 x 1 cm square lattice, and expressed as μm^2 [143]. The number of gold particles was counted in the images and the concentration of each protein was calculated by dividing the total number of gold particles by the area, and expressed as gold particles/μm^2 (Au/μm^2). The mean concentration was then calculated in each compartment.

The method described [143] was used to calculate the images of zebrafish samples. Since the zebrafish nephron only has two tubules, four consecutive sections were analyzed in each zebrafish tubuli, and in each section five areas including brush border and cytoplasm were randomly selected. Thus, twenty images were analyzed in each compartment, respectively.

3.3.6 Statistical analysis

Data are expressed as median (10th to 90th percentile) or percentage or as appropriate. Statistical significance was set at the level of $p<0.05$. All statistical analyses were performed using statistical software GraphPad Prism version 7.0 (GraphPad Software Inc, CA, USA) and SAS version 9.4 (SAS Campus Drive, Cary, NC, USA).

Comparisons between two groups were assessed with the nonparametric Wilcoxon test, while for three or more groups Kruskal-Wallis ANOVA test was used, followed by Dunn's test. Fischer exact test or Chi-square test was used for nominal variables. Non-parametric Spearman rank correlation analysis was used for continuous and ordinal variables.

To ensure that the best possible cut-off for each biomarker was used for each biomarker, separate receiver operating characteristics (ROC) curves were plotted and areas under the curves (AUC) calculated prior to each dichotomous analyses and using the same outcome variable. ROC curves were generated by plotting the relationship of the true positivity (sensitivity) and the false positivity (1-specificity) at various cutoff points, and AUC was defined as the area under the resulting curve. An AUC of 1.0 was defined as perfect prediction with no false positives, while an AUC of 0.5 indicated no diagnostic value.

In **Study 1**, to study the associations between 1 standard deviation (1-SD) increments of pentosidine/albumin we used linear multivariable regression analysis. To ascertain the

adjusted RR for death associated with each 1-SD higher pentosidine/albumin, multivariable GENMOD regression analysis was performed. A multiple imputation of missing values was performed using the function PROC MI, with all variables in the covariate section used to produce the values for imputation. ROC curves were analyzed allowing calculation of areas under the curves (AUCs) and cut-off values for pentosidine/albumin in relation to all-cause and CVD-related deaths.

In **Study 2**, adjudication of outcomes included determination of optimal cut-off values for each biomarker to be used for dichotomous analysis, followed by multivariable GENMOD regression analysis and multinomial logistic regression analysis. The predictive strength, expressed as pseudo r, of biomarkers to classify presence of clinically overt cardiovascular disease and all-cause mortality risk during follow-up of up to 60 months.

In **Study 3**, to study the associations between the presence of clinically overt CVD and other 20 parameters, a multivariable GENMOD regression analysis was performed. A multiple imputation of missing values was performed using the function PROC MIANALYZE, with all variables in the covariate section used to produce the values for imputation. The results for each imputation were generated using PROC GENMOD, and then combined using PROC MIANALYZE. As sensitivity analyses showed minimal effects of imputation, reported results are based on an imputed complete-case analysis (n=224).

In **Study 4**, comparisons between six groups among patient biopsies and three groups among zebrafish biopsies were performed using the general linear models (GLM) test.

Chapter 4 Results and discussion

4.1 Overall summary

In the present thesis, we investigated the hypothetical role of proximal tubular protein metabolism on the predictive value of common and novel circulating biomarkers of CVD risk and mortality in patients with different degrees of reduced renal function and albuminuria. We found that demographic factors were more sensitive than most protein biomarkers when predicting risks in the CKD population. Furthermore, studies suggest that proximal tubular protein endocytosis is active also in disease states of micro albuminuria, suggesting that it may be also important to assess urinary biomarkers. At present, we do not consider any of the investigated biomarkers to give additional information on mortality risk above and beyond that obtained from careful clinical assessments and demographic data, presumably because none of them directly reflect the pathophysiological pathways that mediate CVD in CKD.

4.2 Most protein biomarkers do not perform better than demographics

In **Study 1 - 3**, we investigated the ability of multiple biomarkers to predict clinical outcomes in various cohorts of CKD patients. We found the predictive power, even optimized by a ROC-maximized cut-off, to be relatively low. In fact, most protein biomarkers were no longer significant predictors following adjustment for age, gender, diabetic status and nutrition status. This was true both in early and late stage CKD, and in classifying baseline CVD, CVD-related mortality and all-cause mortality.

In **Study 1**, 1-SD higher pentosidine/albumin independently predicted all-cause mortality (RR=1.04, 95% CI: 1.01 - 1.08, p=0.01) and CVD mortality (RR=1.03, 95% CI: 1.01 - 1.06, p=0.03) after adjusting for all confounders in CKD patients with different stages and therapies. However, the nutrition, CVD and DM status respectively were each even stronger predictors of all-cause mortality and CVD mortality. In **Study 2**, IL-6 is a classifier of clinically overt CVD (RR=1.10, 95% CI: 1.02 - 1.19, p=0.01) and predictor of all-cause mortality (RR=1.79, 95% CI: 1.20 - 2.67, p=0.01) after adjusting for all confounders in patients with

stage 5 CKD. However, age (RR=1.31, 95% CI: 1.22 - 1.41, p<0.001), smoking (RR=1.15, 95% CI: 1.07 - 1.23, p<0.001), diabetic status (RR=1.24, 95% CI: 1.14 - 1.34, p<0.01) and nutrition status (RR=1.10, 95% CI: 1.02 - 1.19, p<0.001) are even better predictors of presence of baseline CVD, while nutrition status (RR=2.28, 95% CI: 1.56 - 3.33, p<0.001) is the best predictor for all-cause mortality. In **Study 3**, after adjustment of all confounders, proBNP (RR=1.25, 95% CI: 1.10 - 1.42, p<0.001) and IGF-1 (RR=1.14, 95% CI: 1.01 - 1.29, p=0.03) are classifier for clinical CVD; IL-6 predicted CVD mortality (RR=2.80, 95% CI: 1.34 - 5.87, p=0.006) and 8-OH-dG predicted all-cause mortality (RR=1.56, 95% CI: 1.02 - 2.36, p=0.04). Nevertheless, age, smoking and DM are all better predictors of CVD mortality and all-cause mortality.

In interpreting our data, the possibility of confounding through urinary protein losses must also be considered. Indeed, **Study 4** demonstrates the complexity of proximal tubular endocytotic receptor expression, which suggests that the amount of albumin in the urine must reflect multiple pathologies that may or may not affect circulating biomarkers. Prevalent HD patients in **Study 3** unlikely have residual renal function, therefore the results obtained in **Study 3** were neither qualitatively nor quantitatively different from those obtained in the other cohorts, this may suggest that other factors than proximal tubular metabolism are more important for their predicative ability. However, this does not mean that they reflect the actual pathophysiological processes that drive mortality in the CKD population. Indeed, one can argue that the poor performance of all the markers in the studies suggests that we still have to elucidate real risk factors.

4.3 The prediction of IL-6

Of those markers that remained significant predictors after adjustments for basic demographic factors and eGFR, IL-6 stands out. In **Study 2**, an IL-6 concentration above the ROC-calculated cut-off (7.0 and 6.7 pg/ml respectively) was classify of baseline CVD (RR=1.10, 95% CI: 1.02 - 1.19, p=0.01) and predictive of all-cause 5 years mortality (RR=1.79, 95% CI: 1.20 - 2.67, p=0.01). Likewise, in **Study 3** an IL-6 concentration above the ROC-calculated cut-off (8.3 pg/ml) predicted 5 years CVD-related mortality (RR=2.80, 95% CI: 1.34 - 5.87, p=0.006).

These findings confirm several previous reports [127, 144]. One may speculate that links between IL-6 and the premature ageing phenotype in uremia could be one reason that explains why IL-6 has a notable strong performance as risk predictor in patients with CKD. Uremic toxins are thought to mediate premature ageing via driving vascular smooth muscle cell damage [145] and phenotypic changes [76] and these changes involve IL-6.

4.4 Pentosidine and 8-OH-dG performed better than peptide biomarkers

In **Study 1**, we performed an analysis of plasma pentosidine. Pentosidine is a combined AGE-protein complex [109] which is reported to related with inflammation and malnutrition [107], as well as with CVD and poor clinical outcomes [146, 147]. But the contribution of pentosidine to the development of cardiovascular events and mortality in CKD patients has been disputed [148] and traditional risk factors reported to be more important for cardiovascular outcomes in this population [149]. Our data does not address the issue of importance as a long-term risk factor, but does show that an elevated plasma pentosidine measured in 746 patients with different stages of CKD (some on PD and HD) does predict both all-cause and cardiovascular mortality over 5 years follow-up.

Plasma pentosidine was corrected for serum albumin, but despite this, the pentosidine levels were higher in CKD stage 5 patients (who are usually hypoalbuminemic) and correlated negatively with eGFR CKD-EPI. In GENMOD, a 1-SD higher pentosidine/albumin independently predicted all-cause mortality (RR=1.04; 95% CI: 1.01 - 1.08, p=0.01) and CVD mortality (RR=1.03; 95% CI: 1.01 - 1.06, p=0.03) also after adjusting for confounders. As a comparison, 1-SD higher age was associated with a predicted increase in all-cause mortality (RR=1.08, 95% CI: 1.04 - 1.11, p<0.001) and did not predict CVD mortality (RR=1.01, 95% CI: 0.98 - 1.05, p=0.22) after adjusting for confounders.

Thus, plasma pentosidine/albumin was a more useful marker of CVD mortality than age in this cohort, while both were associated with similar increases in risk for all-cause mortality. This does not mean that plasma pentosidine/albumin can replace demographic parameters. Indeed, while we adjusted for the eGFR based cohorts, our analysis in **Study 1** did not adjust for eGFR itself. As serum albumin tends to decrease and protein pentosidine increases as

GFR falls, we may have inadvertently measured something reflecting the risk of declining renal function along with pentosidine.

Another marker that performed well in **Study 1 and 3** was the oxidative stress marker 8-OH-dG, which is the most abundant oxidative product of nucleic acids [111]. In **Study 1**, 8-OH-dG was also an independent predictor of all-cause mortality (RR=1.04, 95% CI: 1.01 - 1.09, p=0.03) along with age, pentosidine, baseline CVD, DM and malnutrition. However, 8-OH-dG did not significantly predict CVD mortality (RR=1.03, 95% CI: 0.99 - 1.06, p=0.06) after adjustments. A similar result was obtained in **Study 3**: all-cause mortality in 224 prevalent HD patients was predicted by amongst others 8-OH-dG (above vs. below 1.35 ng/ml, RR=1.56, 95% CI: 1.02 - 2.36, p=0.04) also after adjustments. Again, 8-OH-dG did not significantly predict CVD-related mortality (RR=1.74, 95% CI: 0.91 - 3.31, p=0.09).

Comparing pentosidine to 8-OH-dG, both are thought to reflect increased oxidative stress [108, 111] and have been reported to be associated with mortality risk in CKD previously [110, 112]. Also, previous data suggests that enhanced production and accumulation of pentosidine among patients with impaired renal function is mainly related to conditions other than hyperglycaemia [108], possibly because protein glycation is diminished in uremia [142]. Thus, levels of AGEs in circulation and in tissues such as skin, in patients with more advanced CKD are influenced by factors such as oxidative stress, inflammation and decreased renal clearance rather than hyperglycemia [150]. Indeed, the similar performance of both 8-OH-dG and pentosidine, including an independent effect of pre-existing DM, suggests that it is the molecular size and likely the connection to oxidative damage that is most important.

Plasma pentosidine is usually found attached to protein amino acids and it is therefore unclear why it performed better in our studies than did other protein biomarkers (such as CRP). One hypothesis is that the combined measurement of free and protein bound pentosidine corrected for the confounding of declining GFR. But it is also possible that pentosidine more accurately reflects the pathophysiological pathways involved in uremic CKD than do for example CRP.

4.5 Proximal tubular endocytosis is unchanged in highly albuminuric states

In **Study 4**, we investigated mechanisms of importance for circulating peptides in the form of endocytic receptors. These receptors, megalin and cubilin, are thought to mediate most re-uptake of freely filtered protein [9]. Their function and regulation in albuminuric states is poorly investigated. Supporting a complex relationship between circulating protein, albuminuria and proximal tubular health we report that nephrotic albuminuric states are characterized by an expression of megalin that is significantly lower than in micro-albuminuric states and approaches that in healthy kidneys. While, the expression of cubilin increases in all patients compared to living donors. In **Study 4**, we did not measure activity of the receptors, but the ratio of membrane bound to cytoplasmic megalin and cubilin was relatively stable between the groups. We can thus speculate that pathologic or protective downregulation of megalin occurs in the nephrotic tubule, leading to reduced endocytosis and increased urinary losses. This has implications primarily for larger molecules with a limited distribution volume.

4.6 Consequences for current therapies

Each of the investigated risk biomarkers has been demonstrated to independently predict both clinical CVD and mortality in various populations across the world. To this list we now added pentosidine, which in **Study 1** independently predicted all-cause mortality in a large cohort of CKD patients. However, as illustrated in **Study 2**, where we investigated 12 biomarkers in 543 incident CKD patients starting dialysis, as well as in **Study 3** of 224 prevalent HD patients, the predictive value of even the best biomarkers may be statistically significant but are rarely of clinical relevance. Notably the relative risk ratios for baseline CVD is usually predicted almost as well using only demographic factors such as age and DM (**Study 2 and 3**). The same holds true for CVD-related and all-cause mortality, although these outcomes are currently hard to predict using any method.

As reviewed by Zoccali et al. [151] many biomarkers have been tested in the dialysis population, including ferritin [152], hsCRP [68, 153-156], IGF-1 [49, 72], IL-6 [68, 156, 157], sICAM-1, sVCAM-1 [158, 159], TnT [160], TNF-α [161], PLT [162] and WBC [163]. Kalantar-

Zadeh [119] counted more than 40 published peptide markers of inflammation that are elevated in CKD, and many of them correlated with outcomes as well. The present thesis suggests that CKD *per se* may cause as yet undefined far-reaching and systematic alterations that appear not to be well reflected by the circulating proteome, either before or after staring dialysis. Indeed, our mechanistic studies suggest that a high degree of albuminuria happens when the amount of endocytotic receptors available for uptake of proteins in the proximal tubules is reduced. Clearly, to identify better biomarkers, future studies should attempt to better elucidate and study the pathophysiological pathways specific to CKD patients.

Chapter 5 Future perspectives

The structured epidemiological assessment of mortality risk was greatly facilitated when in year 2002 a standardized definition and staging of CKD was first proposed [164]. Soon after, the degree to which the loss of renal function contributes to increased mortality risk became clear [165].

Since then much effort has been expended in identifying risk factors and biomarkers that could both allow more informed choices regarding therapies but also offer clues as to the pathophysiological mechanisms behind the increase in risk. After concluding that the traditional Framingham risk factors are relatively poor predictors of death in CKD [166] a lot of investigations have looked at the predictive power of less established risk markers, mainly circulating proteins.

The present thesis tries to link the performance of some of these many markers to proximal tubular renal function. However, we are unable to demonstrate a clear significance of this pathway. This suggests that another, so far elucidated, mechanism is behind the mortality increase while the observed biomarkers and demographic factors are merely minor modifiers.

Clearly, future studies must aim to go beyond what has been done previously and look with fresh eyes at what drives the mortality. In this regard, it is good to focus on systems that involve most cells in the body as most physiological processes seem affected in CKD.

Chapter 6 Acknowledgements

I would like to express my sincere gratitude and appreciation to all the participants for devoting samples which made this work possible. I wish to thank everyone who has supported me and contributed to this PhD thesis.

First I would like to thank my supervisor **Karin Lindström**. Your enthusiasm, positive energy and work ethic have been a big contribution to this work. In addition to the research, you guided me to build a resilient and positive attitude. I feel much grateful for all your help.

My co-supervisors:

Jonas Axelsson, thank you for inviting me to Sweden and introducing me to this project from the start. You shared your invaluable knowledge and creative mind, as well as talent on writing and presenting. I am grateful for your insightful comments which helped me in the research.

Annika Wernerson, thank you for your constructive, supportive and guidance during my studies. Your motto: 'Never ever give up' still inspires me today. It is great to experience warm and friendly atmosphere of the group.

Kjell Hultenby, thank you for your caring and patience, as well as your openness for discussions and your positive feedbacks. My time at the lab is remembered with fondness. I am forever grateful.

Tony Qureshi, thank you for all your suggestions and support in statistical analyses, as well as other thesis related topics. I appreciate you teaching me to have pragmatic views on regulations.

And my mentor **Jie Zhu**, I'm grateful for your constant warm presence whenever I need help and comfort.

Professor **Peter Stenvinkel**, thank you for providing a harmony and professional working environment for me and all students and researchers.

Professor **Bengt Lindholm**, I'm ever so grateful for your endless support during my study. I admire your energy, enthusiasm and immense knowledge.

Late Professor **Bengt Rippe**, thank you for offering me an excellent experience in your lab at Lund University. **Anna Rippe**, thank you for teaching me all skills and knowledge about animal experiment.

Peter Bárány, **Olof Heimbürger** and **Bing He**, I appreciate your comments and revision on the manuscripts. **Anna Witasp** and **Hannes Olauson**, thank you for your help with the collaboration.

Professor **Zhe Wang**, **Guowen Liu** and **Xiaobing Li**, my gratitude to your constant support during my stay in both China and Sweden.

Björn Anderstam, **Monica Ericsson** and **Ann-Christin Bragfors-Helin**, who work in the Renal Laboratory at Clinical Research Center, accept my endless gratitude for assisting and supervising me on biomarker assessment and coordination of sample handling. Many thanks to all the ladies working in the Pathology Laboratory, **Ingrid Lindell**, **Eva Blomén**, **Eva Idsund Jonsson** and **Anneli Ring** for assisting and supervising me in the lab. All of you are always so kind and generous with your time to help me with solving problems.

I am also in sincere appreciation to the administrators, **Maj Tofvesson**, late **Titti Lundmark** and **Evianne Larsson, Agneta Witlock, Ann-Charlott Nordström**, for your patients and kindness.

I would like thank everyone whom I was fortunate to work with at Baxter Novum. Many of you became my loving friends, and it has been my pleasure to spend all these years with you. I am ever so grateful to have your companies. To **Anna and Gabriela**, I will forever embrace much happiness that we shared. **Alessandro**, **Juliana**, **Rute**, **Filipa**, **Cindy**, **Desirée**, **Paul** and **Richard**, while we share our time at work, I also treasure the leisure time that we spent together. **Elvia**, **Xiang**, **Juan**, **Hong**, **Lu**, **Amaryllis**, **Christiaan**, **Gloria**, **Hideyuki**, **Pietro**, **Derk**, **Magdalena**, **Tae**, **Xiaoyan**, **Ting Jia**, **Ming**, **Thaine**, **Beatriz**, **Naohito**, **Kei** and **Edyta**, I am so happy to know you all and work with you at Baxtar Novum as well as all members who shared moments with us, **Jose Divno**, **Jacek**, **Martin**, **Louise**, **Karin**, **Sunna**, **Sonsoles**, **Tetsu**, **Ayumu**, **Sawako**, **Sung-Hee**, **Shirley**, **Marcelo, Jiangzi**, **Maria**, **Catalina** and **Sydney**.

All co-authors and colleagues not mentioned above.

My dear friends, **Xiaozhen**, **Ying**, **Simei**, **Tianwei**, **Jiaxue**, **Bin** and **Bojing**, we have spent most our free time together, I am so grateful to share so many wonderful moments with you. My plat mate, **Kirsten**, each time when I come back seeing you cooking through the kitchen window, I felt right at home. To all friends studying and working at NVS, **Gefei**, **Daniela**, **Ning Xu**, **Joanna**, **Carlos**, **Elena**, **Mingqin**, **Hongliang**, **Xinmei**, **Ruiqing**, **Jia Liu**, **Qiupin**, **Yang Ruan**, **Xiangyu**, **Xiuzhe**, **Huijie** and **Zhongshi**, and friends at BioNut, **Fei**, **Jiyu**, **Yumei, Miao** and **Ting Zhuang**, it was lovely to join you at all the parties and events. To my friends from China, **Meng**, **Xiaocui**, **Xiangsheng**, **Weidong**, **Xiaoxu**, **Chang**, **Yanhua**, **Peng**, **Xing**, **Xueli**, **Chao** and **Xia**, I'm grateful for our friendship and mutual understandings. To all my lovely friends who taught me playing cards, **Chen**, **Wei**, **Ning**, **Xiongzhuo**, **Xiao, Kun** and **Yunpo**, keep up your good spirit! My sincere gratitude to all friends I met during my Stockholm years, late **Zi**, **Wendy**, **Pui**, **Jing**, **Xiaohui**, **Jun**, **Yang Xuan**, **Jian**, **Jianping**, **Ning Wang**, **Xu**, **Yan**, **Lin**, **Yihe**, **Junzhe** and **Hantao**.

Finally thanks and boundless love to my grandma, who always thinks I am up to something big abroad. I miss you so much! Most of all I am so thankful to my parents, **Shuqin Zhang** and **Songshun Sun**, so much love for you! To my cousins, **Jingyi** and **Yu** as well as my nephew, **Jingze** (nice cover picture!), thank you for accompanying me and supporting me, love you! To all my family members, thank you for all support and understanding, I embrace you all.

Love and thanks to all who helped and supported me!

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