BIOMARKERS IN IGA NEPHROPATHY

Hilde Peters
Biomarkers in IgA nephropathy

Proefschrift

Ter verkrijging van de graad van doctor aan de Radboud Universiteit Nijmegen op gezag van rector magnificus prof. dr. Th.L.M. Engelen, volgens besluit van het collega van decanen in het openbaar te verdedigen op dinsdag 19 mei 2015 om 13.30 uur precies

door

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geboren op 21 maart 1977
te Doornenburg
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Introduction
Chapter 1

**IgA nephropathy**

Immunoglobulin A nephropathy (IgAN) is the most common noninfectious glomerular disease worldwide. Based on biopsy registries IgAN constitutes 30-40% of all glomerulopathies in Europe. In the Netherlands, IgAN is diagnosed in 12% of patients undergoing a kidney biopsy. The reported incidence of biopsy proven IgAN is 19 patients per 1*10^6 adults. Since many patients with IgAN present with isolated, asymptomatic hematuria, geographical differences in reported incidence and prevalence rates are largely explained by differences in biopsy policies, and to a lesser extent by genetic variations. In North America 8% of patients with a glomerular disease are diagnosed with IgAN since it is less common in African Americans than in Caucasians. In Europe prevalence rates are 13-20%, and in Asia prevalence is reported to be as high as 30-50%. The impact of IgAN is best illustrated by its contribution to ESRD rates. IgAN is the cause of ESRD in 3-6% of patients treated with renal replacement therapy. Since a large number of patients with IgAN are young and without significant co-morbidity, they make good candidates for a kidney transplant, for example in 2005 22% of patients receiving a kidney transplant in Australia or New Zealand suffered from IgAN. In North America ESRD was due to IgAN in only 1-4% of kidney transplant patients. In the Netherlands, IgAN accounted for 3% of patients on dialysis, and for 7% of kidney transplant patients (data obtained from Renine database).

**Clinical presentation and outcome**

Clinical presentation of IgAN varies widely. Most patients present with isolated hematuria, but some present with rapidly progressive glomerulonephritis. The high prevalence of IgAN combined with its early age of onset, makes this glomerulopathy a significant renal disease. Studies conducted before implementation of routine use of ACE inhibitors report that 25-50% of patients with recurrent macroscopic hematuria or persistent microscopic hematuria progress to end-stage renal disease (ESRD) in 10-30 years. The risk of development of ESRD in patients with IgAN is highly variable and associated with the clinical presentation (table 1). Patients presenting with microscopic hematuria without proteinuria have an excellent prognosis with <5% developing ESRD, whereas on the other end of the spectrum, 40-75% of patients with crescentic IgAN progress to ESRD.

**Diagnosis and pathogenesis**

A kidney biopsy and immunofluorescence studies are needed to diagnose IgAN. Histologically, IgAN is defined by dominant staining of IgA in the glomeruli and should include staining of the mesangium. The pathogenesis of IgAN has been considerably clarified by recent studies. Four processes are needed to come together to induce renal injury resulting in IgAN (figure 1). First, the normal glycosylation pattern of IgA1 is altered...
and characterised by a deficiency of galactose in the hinge region of the IgA1 heavy chains. Second, IgG (or IgA) autoantibodies directed against galactose deficient IgA1 must be synthesized. Binding of galactose deficient IgA1 with IgG autoantibodies results in the formation of immune complexes, which accumulate in the mesangium. Finally, mesangial cells proliferate and produce matrix resulting in fibrosis.

**Prediction of outcome**

The individual outcome of patients with IgAN remains difficult to predict. Previous longitudinal studies have associated proteinuria, hypertension, and impaired kidney function at the time of diagnosis with progression of IgAN. The degree of proteinuria is one of the strongest predictors of outcome. Whereas in other types of glomerulonephritis subnephrotic proteinuria at presentation is not associated with progression of disease, such lower levels of proteinuria are of prognostic significance in IgAN. Proteinuria at presentation <1g/d is associated with a risk of ESRD at 10 years ~10%, whereas this is up to 30-40% in patients with proteinuria 1-3g/d, and even 60% in those with proteinuria >3g/d (table 1) . Although proteinuria at presentation is an important factor, proteinuria over time even more closely correlates with disease outcome . If proteinuria over time is maintained <1g/d, the 10 year risk of ESRD is <5% . Therefore, a reasonable therapeutic goal is achieving a consistent level of proteinuria <1g/d. Hypertension at presentation is beneficial and blood pressure averaged over time is a better predictor of optimal supportive therapy. These patients are therefore at increased risk of progressive chronic kidney disease. Several randomized controlled trials suggest that a 6-month course of steroid therapy can reduce proteinuria and the risk of ESRD. This treatment is most suited for patients with well-preserved eGFR. Treatment guidelines are less well defined for patients who had failed steroid therapy or patients with moderate kidney failure. Treatment of IgAN with corticosteroids in combination with other immunosuppressive agents remains controversial due to the limited number of trials, conducted in an era before the standard use of optimized supportive care. A small study in patients with progressive IgAN showed that treatment with prednisolone and cyclophosphamide followed by azathioprine resulted in a reduction in proteinuria and improvement in renal survival. Since side effects occur more frequently with double immunosuppression, the risks of treatment need to be weighed against the risk of ESRD. Identifying those patients who are most likely to progress to ESRD is therefore essential.

**Therapy**

Despite recent advances in clarifying the pathogenesis of IgAN, there is currently no treatment at our disposal to specifically halt the production of aberrant IgA or prevent the mesangial deposition of the IgA containing immune complexes. An optimized supportive regimen constitutes the cornerstone of therapeutic approach in patients with IgAN (figure 2). Main goals are control of blood pressure and reduction of proteinuria preferably by renin-angiotensin blockade. As mentioned above, prognosis is excellent if proteinuria is significantly and persistently reduced. However, in a significant number of patients lowering of proteinuria to the target of <1.0g/d will prove impossible despite

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**Figure 2. Suggested therapeutic approaches in patients with IgA nephropathy based on KDIGO Clinical Practice Guideline for Glomerulonephritis 2012**

- Normal eGFR, proteinuria <1g/d, no hypertension
- eGFR reduced and/or proteinuria >1g/d and/or hypertension
- Supportive therapy, most importantly:
  - Control of blood pressure (<125/75 mmHg)
  - ACEi or ARB therapy
  - Protein restriction
  - Sodium restriction
- Exclusion of acute kidney injury due to prolonged macroscopic hematuria or other causes of progression of renal insufficiency than IgAN
- eGFR >50 ml/min progressive loss of eGFR and/or proteinuria >1g/d after optimal supportive therapy during at least 3-6 months
- Additon of corticosteroids during 6 months
- Continuation of supportive therapy, no immunosuppressive treatment
- Rapid decline of renal function
- Immunosuppressive therapy consisting of prednisone monotherapy for nephrotic syndrome/ combined therapy with prednisone and cyclophosphamide for crescentic glomerulonephritis

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**Biopsy-proven IgA nephropathy**

- Normal eGFR, proteinuria <1g/d, no hypertension
- eGFR reduced and/or proteinuria >1g/d and/or hypertension
- Supportive therapy, most importantly:
  - Control of blood pressure (<125/75 mmHg)
  - ACEi or ARB therapy
  - Protein restriction
  - Sodium restriction
- Exclusion of acute kidney injury due to prolonged macroscopic hematuria or other causes of progression of renal insufficiency than IgAN
- eGFR >50 ml/min progressive loss of eGFR and/or proteinuria >1g/d after optimal supportive therapy during at least 3-6 months
- Additon of corticosteroids during 6 months
- Continuation of supportive therapy, no immunosuppressive treatment
- Rapid decline of renal function
- Immunosuppressive therapy consisting of prednisone monotherapy for nephrotic syndrome/ combined therapy with prednisone and cyclophosphamide for crescentic glomerulonephritis
Chapter 1

Role of biomarkers
As described above, clinicians treating patients with IgA nephropathy face a difficult task. On one hand, the majority of patients with hematuria and proteinuria <1g/d have a good prognosis, yet some develop a progressive decline of kidney function. On the other hand, patients with proteinuria >1g/d clearly have an increased risk of developing ESRD, but kidney function will remain stable over decades in a substantial number of patients. Thus, specific and sensitive biomarkers that allow prediction of outcome and treatment response are desperately needed.

In patients with membranous nephropathy, measurement of low molecular weight proteins, which are markers of tubular damage, predict progression of renal failure, thus allowing identification of patients who are likely to benefit from immunosuppressive treatment. Kidney injury molecule-1 (KIM-1) is a sensitive marker of early tubular injury. KIM-1 expression is absent in a healthy kidney, but induced in both acute and chronic kidney damage. In human kidney disease, elevated urinary KIM-1 levels seem to be a sensitive marker of tubulointerstitial damage.

In 2005, neutrophil gelatinase-associated lipocalin (NGAL) was first described as a biomarker for acute kidney injury after cardiac surgery. This 25-kD protein is released in blood and urine from injured tubular cells due to various stimuli and as such it is an early and sensitive marker of renal damage. Urinary NGAL also predicts progression of renal disease in human proteinuric kidney disease.

Hepcidin
Hepcidin, a key hormone of iron homeostasis, has gained interest as a potential biomarker of iron status in chronic kidney disease (CKD) and dialysis patients over the last decade. Hepcidin was originally described as a peptide with antibacterial and antifungal properties, yet focus shifted as hepcidin was discovered to regulate iron metabolism. The potential role of urinary hepcidin as a biomarker for kidney disease or kidney injury was supported by studies that showed that urinary levels of hepcidin predicted renal lupus nephritis flares and acute kidney injury after coronary surgery.

Synthesis, structure, and kinetics
Hepcidin is mainly produced by hepatocytes as a 25 amino-acid peptide (MW of 2.8 kDa) and is secreted in the circulation. Subsequent amino-terminal processing of the 25 isoform creates two smaller hepcidin fragments of 22 and 20 amino-acids. These smaller isoforms have no known biological function. Under normal circumstances, substantial amounts can be detected in urine, but not in blood, suggesting that these isoforms are merely formed by urinary degradation of hepcidin-25. Recent studies demonstrate hepcidin expression by other cells, such as kidney tubules, heart, fat cells, and monocytes.

Although hepcidin synthesis by these cells may exert local effects, it does not influence systemic circulating concentrations.

Evidence that circulating hepcidin is bound to α2-macroglobulin with high affinity is derived from a single study, suggesting that approximately 90% of hepcidin was protein bound. Clearance of hepcidin is assumed to take place via the kidneys. Unbound hepcidin is likely to be freely filtered by the glomerulus due to its low molecular weight. In humans fractional excretion of hepcidin is as low as 0.5%, possibly due to tubular reabsorption or impairment of free filtration (e.g. by protein binding).

Function and regulation
Hepcidin-25 has been shown to participate in iron regulation by binding to the sole cellular iron exporter ferroportin, which is present in the membranes of macrophages, hepatocytes and enterocytes. Hepcidin-25 induces the internalization and degradation of ferroportin, resulting in a decreased dietary iron uptake by the gut and sequestration of iron by reticulo-endothelial macrophages. As a consequence, an increase in hepcidin production leads to a decrease in plasma iron concentrations (figure 3). In addition, hepcidin peptides are able to bind divalent metals, suggesting a non-hormonal role for hepcidin in iron metabolism.

Figure 3. Hepcidin and ferroportin interaction.

Hepcidin acts by binding to ferroportin, expressed by duodenal enterocytes, macrophages and hepatocytes. Binding of hepcidin results in internalization and subsequent degradation of ferroportin. Binding of hepcidin to ferroportin expressed on enterocytes leads to a decreased iron absorption from the gut. Binding of hepcidin to hepatocytes and macrophages inhibits iron release from these cells. Iron deficiency and erythropoiesis suppress the production of hepcidin.

tension and inflammatory cytokines. An increased demand for iron - as is the case in conditions such as iron deficiency, hypoxia, anemia, and erythropoiesis - inhibits hepcidin synthesis, with subsequent release of stored iron and an increase in dietary iron uptake, finally resulting in increased plasma iron concentrations. In contrast, iron administration and inflammation both enhance hepcidin production, thereby limiting iron uptake, increasing macrophage iron sequestration and lowering plasma iron levels.

Assays
In the past, research on hepcidin was hampered by the lack of a reliable assay, but the development of new assays allowed study of the role of hepcidin in iron metabolism in humans. The first assay to detect hepcidin-25 was a semi quantitative Surface-Enhanced Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry assay (SELDI-TOF MS). Mass spectrometry assays are labour-intensive and require expensive equipment, yet they distinguish hepcidin-25, -22 and -20. Enzyme-linked immune sorbent assays (ELISA) are easier to use, but measure total hepcidin concentration with different contributions of the isoforms, depending on the specificity of the antibody. Notably, it is not clear whether these current assays measure free, bound or total hepcidin.

Hepcidin in renal disease, as an iron biomarker, and in acute kidney injury
Anemia and disturbances in iron homeostasis are common in patients with CKD. Most patients can be effectively treated with erythropoietin stimulating agents (ESA), yet approximately 10% is hypo- or non-responsive to ESA. Iron deficiency contributes to ESA resistance and the majority of hemodialysis patients receive intravenous iron, aiming to maintain adequate ferritin levels. Of note, high doses of iron or ESA are potentially dangerous. Hepcidin may contribute to ESA resistance through trapping of iron in the reticuloendothelial system. As such, hepcidin gained a lot of interest as a tool to guide treatment with ESA and iron in anemic CKD patients, instigating explorative clinical studies. Serum hepcidin levels were found to be elevated in a small series of patients with renal impairment. In 2005 Kulaksiz et al. suggested a role for the kidney in the production of hepcidin by showing that hepcidin is predominantly present in the distal rat tubule. Interestingly, in humans, lower urine hepcidin 24 hours after ischemic injury was found to be associated with an increased risk of renal failure after several days. Thus, locally available hepcidin, due to local synthesis, activation, degradation or delivery via glomerular filtration and tubular reabsorption, may promote renal recovery.

Connective tissue growth factor

Tubulointerstitial fibrosis is a common final pathway for many kidney diseases, including glomerulopathies, and ultimately results in loss of renal function. Transforming growth factor-β (TGF-β) is generally regarded as the key fibrogenic cytokine, and connective tissue growth factor (CTGF), a 36 to 38 kDa peptide, is an important downstream mediator of TGF-β. CTGF seems a candidate biomarker for monitoring ongoing fibrosis; it is readily quantifiable in body fluids by using an ELISA.

In renal biopsies of patients with diabetes mellitus type 2, tubular expression of CTGF correlated with serum creatinine, proteinuria, and interstitial fibrosis. Elevated urinary levels of CTGF are reported in patients with diabetic nephropathy, chronic allograft nephropathy, IgA nephropathy, focal segmental glomerulosclerosis, and idiopathic membranous nephropathy and correlate with proteinuria and glomerular filtration rate in several studies. Although urinary CTGF may reflect local production, it cannot be excluded that increased urinary CTGF levels are the consequence of altered glomerular or tubular handling.

Aim and outline of the thesis
Aim of this thesis is to investigate the role of known biomarkers as predictors in IgA nephropathy (part 1). Furthermore we evaluate the renal handling of new, potential biomarkers (part 2), since this information is critical to its appropriate use in animals and/ or humans.

Part 1. Biomarkers in IgAN
Serum creatinine and proteinuria remain the most important clinically used parameters for predicting risk of progression to end-stage renal disease in patients with IgA nephropathy. Unfortunately, these established biomarkers have insufficient sensitivity and specificity. Tubulointerstitial injury plays a key role in the progression of IgA nephropathy and low molecular weight proteins are thought to reflect the degree of interstitial damage. In patients with idiopathic membranous nephropathy, measurement of low molecular weight proteins, established markers of tubular injury, allows identification of patients who are at high risk for progression of renal disease with a reasonable sensitivity and specificity. In chapter 2 we evaluate the prognostic value of urinary excretion of low molecular weight proteins, for predicting progression of kidney disease in patients with IgA nephropathy. In chapter 3 we evaluate the prognostic value of two recently discovered alternative markers of active tubular damage, KIM-1 and neutrophil gelatinase-associated lipocalin (NGAL), for predicting end-stage renal disease in patients with IgA nephropathy. In chapter 4 we describe a pilot study that evaluated the relations of intratubular GMP-17 positive T-lymphocytes with urinary markers of tubular damage and its prognostic value.
for predicting ESRD. In chapter 5 we evaluate the effect of immunosuppressive therapy in patients with IgAN on renal function and we sought to identify predictors of response to therapy.

**Part 2. Renal handling of the novel biomarkers hepcidin and CTGF**

An ideal renal biomarker allows simple, accurate and reproducible quantification, is a sensitive indicator of renal injury, is inexpensive, and provides relevant clinical information above and beyond other already available tests. Biomarkers can be measured in fluids, such as serum, urine, saliva, sweat, but also in kidney biopsy material. Serum or urine biomarkers are preferred because they are readily obtainable. Urine is more likely than serum to contain biomarkers reflecting processes in the kidney, yet urine markers may degrade in the bladder or in a test tube, and degradation may depend on urinary pH.

In the context of renal disease, detailed understanding of the renal handling of a biomarker is critical to its appropriate use in animals and/or humans. In chapter 6, we assess the intra-individual variability of serum hepcidin in haemodialysis patients and aim to identify significant determinants of the variability of hepcidin. In chapter 7, we evaluate the influence of eGFR on serum levels of hepcidin-25 and its isoforms in patients with CKD. In chapter 8, we evaluate whether tubular reabsorption processes influence urinary excretion of hepcidin. In chapter 9, we examine whether urinary CTGF is filtered by the glomeruli and reabsorbed by the tubules and whether tubular dysfunction results in increased urinary CTGF levels. In chapter 10, we summarize and discuss our principle findings.
Chapter 1


Urinary excretion of low molecular weight proteins as prognostic markers in IgA nephropathy

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Chapter 2

ABSTRACT

Background
Immunoglobulin A nephropathy (IgAN) is characterised by high variability in clinical course and outcome. Accurate prediction of prognosis is needed to optimise treatment. Urinary α1-microglobulin and β2-microglobulin are markers of tubulointerstitial injury and predict the risk of end-stage renal disease (ESRD) in idiopathic membranous nephropathy. We questioned the relevance of these markers in IgAN.

Methods
We included patients with biopsy proven IgAN, who were evaluated for proteinuria in our centre between 1995 and 2007. Data were analysed using univariate and multivariate Cox regression for the outcome variables ESRD and progression (rise in serum creatinine of >50% or start of immunosuppressive therapy).

Results
Seventy patients (71% men) were selected. Median age was 39 years, median serum creatinine 140 μmol/l, and median proteinuria 2.4 g/day. Median urinary α1-microglobulin excretion was 23.5 μg/min (range 3.5-275.3) and median urinary β2-microglobulin excretion was 0.4 μg/min (range 0.1-62.1). Both α1m and β2m correlated significantly with serum creatinine (r = 0.65, p<0.01 and r = 0.62, p<0.01) and total proteinuria (r = 0.35, p<0.01 and r = 0.28, p<0.05). During follow-up (median 75 months) 25 patients (36%) developed ESRD, and 46 patients (66%) showed progression. 19 patients (27%) were treated with immunosuppressive agents. In univariate analysis urinary α1- and β2-microglobulin predicted ESRD and progression. In multivariate analysis only serum creatinine and urinary protein were independent predictors of both outcomes.

Conclusion
Urinary excretion of low molecular weight proteins did not offer an advantage over total proteinuria and serum creatinine in predicting prognosis in patients with IgAN.

Introduction
IgA nephropathy (IgAN) is the most common glomerulonephritis worldwide. The natural history is quite variable and published data must be interpreted with caution since many patients with mild disease may never come to clinical attention or undergo a renal biopsy. End-stage renal disease (ESRD) develops in 20-30% of patients with IgAN within 20 years after diagnosis.

Although there is a lack of randomized controlled trials, data suggest that a minority of patients may benefit from immunosuppressive treatment. Ideally, such treatment should be restricted to patients who will progress to ESRD. Several variables have been identified as predictors of prognosis i.e. elevated serum creatinine concentration, arterial hypertension, arterial hypertension and histological characteristics. Few studies have showed that clinical features evaluated after one year of follow-up predicted prognosis more accurately than at the time of presentation. Recently Reich et al. reported that persistent proteinuria is the strongest predictor of poor renal outcome in IgAN and that sustained reduction of proteinuria to <1 g/24 h is associated with a good prognosis. Unfortunately, most markers are not very accurate, with low sensitivity and specificity.

In idiopathic membranous nephropathy, high-risk patients can be identified in an early stage by measuring low molecular weight proteins such as α1-microglobulin (α1m) and β2-microglobulin (β2m) with a sensitivity of 83% and a specificity of 97%. We aimed to determine whether the excretion of low molecular weight (LMW) proteins adds to predicting prognosis in patients with IgAN.

Subjects and methods
Population
Since 1995, we have performed standardized protein measurements in patients with proteinuria due to glomerular diseases. Patients are referred to our medical center from hospitals located mainly in the south-eastern part of the Netherlands. For the present study we analyzed the data of adult patients with biopsy proven IgA nephropathy who were evaluated for proteinuria in our center between 1995 and 2007, and followed thereafter. Patients with other causes of IgA-positive glomerular staining (systemic lupus erythematosus, Henoch-Schönlein purpura or liver disease) or a follow-up of less than 12 months, were excluded from analysis.

Baseline measurement
Gender, ethnicity, age, body weight and height were recorded at the time of measurement. Details of the measurements have been described. Two 24-h urine samples were...
obtained for measurement of creatinine and total protein. The excretion of the low and high molecular weight proteins was measured under standardized conditions. A urinary pH >6.0 is necessary to allow reliable measurements of urinary β₂m. Therefore, patients used 4000 mg of oral sodium bicarbonate the evening before the measurement. On arrival at the ward an additional 2000-4000 mg sodium bicarbonate was used and up to 500 ml of tap water was given to enforce diuresis. The patients remained supine during 2 h except for voiding. Blood pressure measurements were taken using an automated device (DINAMAP, Criticon, Tampa FL) with six consecutive readings registered every 5 min after 10 min rest; these readings were used to calculate the mean arterial pressure (MAP). Measurement of urinary pH, β₂m, α₁m, immunoglobulin G (IgG), transferrin, albumin, total protein and creatinine was performed. Beta₂-microglobulin excretion was measured only in urine with a urinary pH >6.0. Laboratory parameters were measured in blood samples collected in the middle of the urine collection period.

The use of angiotensin-converting enzyme inhibitors (ACEIs) and/or angiotensin II type 1 receptor antagonists (ARBs), calcium channel blockers, other antihypertensive agents, diuretics, and non-steroidal anti-inflammatory drugs (NSAIDs), as well as HMG-CoA-reductase inhibitors, was recorded. Current or previous use of corticosteroids, other immunosuppressive agents or fish oil was registered.

Serum creatinine, cholesterol, urinary total protein, and creatinine were measured with standard automated techniques. Urinary proteins were measured as described before.

Follow-up

After baseline measurements, patients were commended to the care of their local physicians. Immunosuppressive therapy was advised to patients with progressive renal disease. We collected data on serum creatinine, albumin, cholesterol and urea, total urinary protein and creatinine levels, blood pressure, body weight and exposure to medication during follow-up from medical records.

Calculations and definitions

Body mass index (BMI) was calculated from body weight and height at baseline. MAP during follow-up was calculated as the diastolic pressure plus one third of the pulse pressure. The glomerular filtration rate (GFR) at baseline and follow-up was estimated (eGFR) using the abbreviated MDRD equation. Start of follow-up was defined as the time of standardized measurement of proteinuria, regardless of the first assessment suggestive of renal disease. We defined the following two renal outcomes: ESRD and progression of renal disease. ESRD was defined as initiation of dialysis, renal transplantation or an eGFR <15 ml/min per 1.73m². Progression of renal disease was defined as an elevation in serum creatinine of 50% or more since the baseline measurement, the start of immunosuppressive therapy or the development of ESRD.

Statistical Analysis

Missing values for urinary protein concentration in the 24 hour urine samples were imputated by using the urinary protein-creatinine ratio which was obtained from the 2 hour sample, and by using serum albumin. Missing values for low-molecular weight proteins were not imputed.

All baseline variables were compared for patient groups by χ²-test if dichotomous, one way ANOVA if continuous and after log transformation if skewed. Each continuous baseline variable was divided into tertiles and plotted in a Kaplan-Meier curve for visual inspection.

Possible collinearity for univariate significant predictors was checked. Predictors that had a Spearman’s rho smaller than 0.800 were entered into a multivariate Cox model. A backward stepwise selection algorithm, criteria for exclusion being a likelihood ratio test with p-value greater than 0.05 and smaller than 0.10 for inclusion, was used. Possible interactions, based on plausible mechanism, were entered and tested too. The most parsimonious model with the best fit, using generalized R², was considered most appropriate. Internal validation of the selected model was done with a bootstrapping procedure using 1000 samples. The predictive value of this model was investigated by the area under the receiver operating characteristics (ROC) curve.

Results

Baseline characteristics and outcome variables

Initial demographic, clinical and laboratory data of 70 patients are listed in Table 1. In the majority of patients (57%) proteinuria was >2.0 g/d and the estimated GFR <60 ml/min/1.73m². Eighty percent of the population were taking ACEIs or ARBs at the time of evaluation for proteinuria. Median duration of follow-up was 74 months. The time period between onset of renal disease or biopsy and subsequent referral to our center varied. In 60% of patients the time between biopsy and referral was less than 6 months.

During follow-up all patients were treated with ACEIs and/or ARBs. Immunosuppressive therapy was initiated in 19 patients, the majority of them (74%) were treated with cyclophosphamide combined with prednisone. Twenty-five patients (36%) developed ESRD, with the shortest survival time being two months. Five and eight year renal survival rates from baseline were 78% and 66% (figure 1). In 46 (66%) patients progression of renal disease occurred based on an increase in serum creatinine of >50% (n=35), or initiation of immunosuppressive therapy (n=11). Thus, of 19 patients who received immunosuppressive therapy during follow-up, 8 patients were treated because of a rise in serum creatinine of more than 50%. In 11 patients treatment was started earlier. These patients were characterised by higher serum creatinine values at baseline and more severe proteinuria (table 1).
Chapter 2

Low molecular weight proteins

Alpha-1-microglobulin levels were not available for two patients, while β2m levels could not be measured in nine patients due to a urinary pH <6.0. The urinary excretion of both α1m and β2m was increased in patients with IgAN, with median levels of 23.5 µg/min (reference value <10 µg/min) and 0.4 µg/min (reference value <0.2 µg/min).

There was a high correlation between α1m and β2m (r =0.86, p <0.01). Both α1m and β2m correlated significantly with serum creatinine (r =0.65, p <0.01 and r =0.62, p <0.01), IgG excretion (r =0.59, p <0.01 and r =0.58, p <0.01), and total proteinuria (r =0.35, p <0.01 and r =0.28, p <0.05).

Predictors of outcome

End-Stage Renal Disease: Urinary α1-microglobulin, β2-microglobulin and IgG excretion, serum creatinine and urea levels, total urinary protein, eGFR and the use of diuretics before baseline were all significantly associated with ESRD. When evaluating tertiles of α1m, renal survival in the highest tertile was markedly lower compared with that in the lowest and middle tertiles (94% versus 63% at 5 years, 85% versus 44% after 8 years, p=0.001) (figure 2). Only one patient within the lowest tertile of urinary β2m developed ESRD. Therefore, renal survival in the lowest tertile of β2m was higher than in the middle and highest tertile (figure 3). After multivariate Cox regression analysis only baseline serum creatinine and total proteinuria proved significant predictors of ESRD when correcting for therapy (table 2). Thus, neither α1m nor β2m were independent predictors of ESRD.

Of note, patients whom were treated with immunosuppressive agents during follow-up were less likely to develop ESRD.

### Table 1. Clinical and demographic characteristics at baseline

<table>
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<th>Progression</th>
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<td>Rise in serum creatinine</td>
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<td></td>
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<tr>
<td>A</td>
<td>% male</td>
<td>71.4%</td>
<td>74.3%</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>39 [17-70]</td>
<td>36.0 [17-70]</td>
<td>37 [25-59]</td>
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<td>BMI (kg/m²)</td>
<td>26.2 [19.4 - 41.0]</td>
<td>26.1 [20.4 - 31.1]</td>
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<td>MAP (mm Hg)</td>
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<td>104 [83 - 133.7]</td>
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<td>Urinary protein (g/d)</td>
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<td>2.9 [0.5 - 12.6]</td>
<td>3.6 [0.4 - 24.3]</td>
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<td>Protein-creatinine ratio (g/10mmol)</td>
<td>2.9 [0.5 - 18.2]</td>
<td>3.5 [1.0 - 16.1]</td>
<td>4.5 [0.6 - 18.2]</td>
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<td>Serum creatinine (µmol/l)</td>
<td>139.5 [70 - 366]</td>
<td>149 [76 - 366]</td>
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<td>Serum urea (µmol/l)</td>
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<td>Serum albumin (g/l)</td>
<td>38.0 [21 - 46]</td>
<td>38.0 [21 - 46]</td>
<td>37.0 [29 - 45]</td>
</tr>
<tr>
<td>Serum cholesterol (mmol/l)</td>
<td>5.7 [3.5 - 8.9]</td>
<td>5.9 [3.6 - 8.7]</td>
<td>5.1 [3.6 - 8.9]</td>
</tr>
<tr>
<td>MDRD (ml/min/1.73 m²)</td>
<td>48.2 [16.5 - 95.5]</td>
<td>44.9 [16.5 - 86.7]</td>
<td>37.1 [22.3 - 82.0]</td>
</tr>
<tr>
<td>α₁-microglobulin excretion (µg/min)</td>
<td>23.5 [3.5 - 275.3]</td>
<td>30.6 [3.5 - 275.3]</td>
<td>48.0 [7.8 - 192.0]</td>
</tr>
<tr>
<td>β₂-microglobulin excretion (µg/min)</td>
<td>0.4 [0.1 - 62.1]</td>
<td>1.1 [0.1 - 62.1]</td>
<td>1.1 [0.1 - 36.0]</td>
</tr>
<tr>
<td>IgG excretion (mg/d)</td>
<td>111.4 [11.3 - 1327.2]</td>
<td>144.8 [16.2 - 1327.2]</td>
<td>111.4 [11.3 - 900.1]</td>
</tr>
<tr>
<td>ESRD (%)</td>
<td>35.70%</td>
<td>65.70%</td>
<td>18.20%</td>
</tr>
<tr>
<td>Time until ESRD (months)</td>
<td>74.6 [2.2 - 145.6]</td>
<td>72.7 [2.2 - 145.6]</td>
<td>97.5 [6.1 - 135.5]</td>
</tr>
<tr>
<td>Time until progression or last follow-up (months)</td>
<td>39.4 [0.2 - 125.9]</td>
<td>28.1 [0.5 - 114.5]</td>
<td>2.1 [0.2 - 75.6]</td>
</tr>
<tr>
<td>Interval between biopsy and referral (months)</td>
<td>2.0 [0 - 209.7]</td>
<td>5.0 [0 - 187.5]</td>
<td>0.7 [0 - 98.9]</td>
</tr>
<tr>
<td>Interval between onset and referral (months)</td>
<td>29.3 [219.7 - 2.0]</td>
<td>38.6 [195.9 - 4.1]</td>
<td>36.0 [179.7 - 2.3]</td>
</tr>
<tr>
<td>Use of ACEi/ARBs before baseline</td>
<td>80.00%</td>
<td>80.00%</td>
<td>81.80%</td>
</tr>
<tr>
<td>Use of diuretics before baseline</td>
<td>30.00%</td>
<td>34.30%</td>
<td>27.30%</td>
</tr>
<tr>
<td>Use of other anti-hypertensive medication</td>
<td>34.30%</td>
<td>37.10%</td>
<td>45.50%</td>
</tr>
<tr>
<td>Use of immunosuppressive treatment before baseline</td>
<td>4.30%</td>
<td>5.70%</td>
<td>9.10%</td>
</tr>
<tr>
<td>Use of ACEi/ARBs during follow-up</td>
<td>100.00%</td>
<td>100.00%</td>
<td>100.00%</td>
</tr>
<tr>
<td>Use of diuretics during follow-up</td>
<td>68.60%</td>
<td>71.40%</td>
<td>90.90%</td>
</tr>
<tr>
<td>Use of other anti-hypertensive medication during follow-up</td>
<td>54.30%</td>
<td>62.90%</td>
<td>63.60%</td>
</tr>
<tr>
<td>Use of immunosuppressive drugs during follow-up</td>
<td>27.14%</td>
<td>22.86%</td>
<td>100.00%</td>
</tr>
</tbody>
</table>

Data are expressed as median [range]. *In a few patients biopsy was performed after evaluation for proteinuria. P-values are from χ2-test or ANOVA comparing the three groups: rise in serum creatinine, therapy and non-progressors. BMI: body mass index, MAP: mean arterial pressure, MDRD: modification of diet in renal disease equation, ESRD: end-stage renal disease, ACEI: angiotensin-converting enzyme inhibitors, ARB: angiotensin II type I receptor antagonist.

Renal survival was defined as onset of ESRD.

Figure 1. Renal survival curve in patients with IgAN.

Figure 2. Renal survival curve in patients with IgAN.
Progression of renal disease: Urinary α₁-microglobulin, β₂-microglobulin and IgG excretion, serum creatinine and urea levels, total urinary protein, eGFR and age, were significant predictors of progression in univariate analysis. Multivariate Cox regression showed that only serum creatinine and total urinary protein were independent significant predictors of progression (table 2).

Table 2. Hazard ratios (HR) and confidence intervals (CI) of significant baseline predictors of ESRD or progression of renal disease after univariate and multivariate analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>ESRD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₁-microglobulin (μg/min)</td>
<td>1.015 (1.008 - 1.023)</td>
<td></td>
</tr>
<tr>
<td>β₂-microglobulin (μg/min)</td>
<td>1.06 (1.022 - 1.099)</td>
<td></td>
</tr>
<tr>
<td>IgG excretion (mg/d)</td>
<td>1.002 (1.001 - 1.004)</td>
<td></td>
</tr>
<tr>
<td>Use of diuretics before referral</td>
<td>1.002 (1.001 - 1.004)</td>
<td></td>
</tr>
<tr>
<td>MDRD (ml/min/1.73m²)</td>
<td>1.002 (1.001 - 1.004)</td>
<td></td>
</tr>
<tr>
<td>Protein-creatinine ratio (g/10mmol)</td>
<td>1.002 (1.001 - 1.004)</td>
<td></td>
</tr>
<tr>
<td>Serum creatinine (µmol/l)</td>
<td>1.009 (1.005 - 1.013)</td>
<td>1.009 (1.005 - 1.013)</td>
</tr>
<tr>
<td>Total urinary protein (g/d)</td>
<td>1.160 (1.061 - 1.268)</td>
<td>1.241 (1.029 - 1.781)</td>
</tr>
<tr>
<td>Immunosuppressive therapy</td>
<td>0.843 (0.336 - 2.115)</td>
<td>0.179 (0.010 - 0.921)</td>
</tr>
<tr>
<td>Generalized R²=0.508</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₁-microglobulin (μg/min)</td>
<td>1.013 (1.008 - 1.018)</td>
<td></td>
</tr>
<tr>
<td>β₂-microglobulin (μg/min)</td>
<td>1.033 (1.006 - 1.061)</td>
<td></td>
</tr>
<tr>
<td>IgG excretion (mg/d)</td>
<td>1.002 (1.001 - 1.003)</td>
<td></td>
</tr>
<tr>
<td>MDRD (ml/min/1.73m²)</td>
<td>1.002 (1.001 - 1.003)</td>
<td></td>
</tr>
<tr>
<td>Protein-creatinine ratio (g/10mmol)</td>
<td>1.002 (1.001 - 1.003)</td>
<td></td>
</tr>
<tr>
<td>Serum creatinine (µmol/l)</td>
<td>1.009 (1.005 - 1.013)</td>
<td>1.009 (1.005 - 1.013)</td>
</tr>
<tr>
<td>Total urinary protein (g/d)</td>
<td>1.216 (1.116 - 1.325)</td>
<td>1.213 (1.112 - 1.362)</td>
</tr>
<tr>
<td>Immunosuppressive therapy</td>
<td>0.843 (0.336 - 2.115)</td>
<td>0.179 (0.010 - 0.921)</td>
</tr>
<tr>
<td>Generalized R²=0.368</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Discussion

Our data clearly indicate that the urinary excretion of low molecular weight proteins does not predict prognosis in IgAN more accurately than total proteinuria. To our knowledge we are the first to report on the prognostic value of urinary excretion of α₁-microglobulin and β₂-microglobulin in IgAN. Others have found a highly significant relation between tubulointerstitial damage and the presence of unspecified, urinary LMW proteins, in a small cohort of patients with IgAN. Woo et al. reported a higher incidence of chronic renal failure in 60 patients with IgAN who presented with LMW proteinuria and were followed for 6 years. However, patients with LMW proteinuria had more severe proteinuria and higher serum creatinine levels.

In our patients with IgAN urinary excretion of α₁m and β₂m exceeded normal values. Renal survival was significantly lower in patients with values of urinary α₁m and β₂m in higher tertiles. We observed a difference when comparing renal survival curves for tertiles of urinary α₁m and β₂m excretion. This seemingly discrepancy can be explained by the fact that β₂m could not be measured in 9 patients due to a low urinary pH. These patients were characterised by higher serum creatinine levels and more severe proteinuria. Thus, missing values are not at random but reflect an impairment of renal function and bicarbonate excretion. This illustrates the limitations of β₂m as a prognostic marker. Both urinary α₁m and β₂m predicted ESRD and progression of renal disease in univariate analysis. However, in multivariate analysis they did not prove to be independent predictors of either outcome. These findings are in contrast with previous reports on the good performance of LMW proteins as prognostic markers in patients with idiopathic membranous nephropathy. Urinary α₁m and β₂m are considered to reflect tubulointerstitial injury. In general, the presence and extent of tubulointerstitial injury determines renal outcome. From this perspective, the difference in the predictive value of LMW proteins between IgAN and idiopathic membranous nephropathy is remarkable. Of note, the predictive value of LMW proteins (such as α₁m) in idiopathic membranous nephropathy was validated in patients with no or moderate renal impairment, defined as a serum creatinine <135 μmol/l. However, even within the subgroup of patients with IgAN and a serum creatinine level of <135 μmol/l, α₁m does not allow identification of high-risk patients. This difference is illustrated in the panels of figure 4. From the figure it is evident that levels of α₁m are higher in patients with idiopathic membranous nephropathy. These patients presented more frequently with nephrotic range proteinuria. Thus, the prognostic value of these LMW proteins may be confined to glomerulopathies characterized by nephrotic range proteinuria.

We found baseline serum creatinine and proteinuria to predict ESRD and progression of renal disease. The relation between serum creatinine and ESRD is to be expected, since a patient with a higher serum creatinine concentration will develop ESRD at an earlier time-point, even if the rate of renal function deterioration is similar. To overcome this problem we defined a 50% or more increase of serum creatinine concentration as progression. We chose a 50% rise to be sure that patients who had a minor increase were not marked as progressors. Since multivariate analysis regarding the outcome ESRD implied that the natural progression of IgAN is influenced by immunosuppressive therapy, this was considered an end-point. The use of initiation of immunosuppressive therapy as an end-point can be debated. In our study, 19 out of 70 patients received immunosuppressive therapy during follow-up. In 8 patients, treatment was started after serum creatinine had increased by 50% or more. The remaining 11 patients received immunosuppressive treatment before reaching this 50% rise in serum creatinine. These patients were characterized by high serum creatinine and more severe proteinuria at baseline. Further delay of treatment was considered inappropriate by their physicians. As such, these patients reflect current treatment practice in our region. At the time of start of therapy, mean serum creatinine was 221μmol/l, clearly pointing to the severity of IgAN. Even with progression as outcome, serum creatinine level and total urinary protein excretion remained significant, independent predictors.

The observation that serum creatinine concentration is a significant, independent predictor of progression of renal disease implies that an accelerated rather than a linear decline in renal function occurs in the course of the disease. In order to correct for the...
possible confounding effect of using initiation of immunosuppressive therapy as an end-point, we reanalyzed the data using an increase of serum creatinine of >50% as only end-point. Serum creatinine concentration remained a significant predictor, which possibly reflects that patients with an increased serum creatinine are more likely to progress or progress at a faster rate than those with no renal impairment. Our findings support observations reported by others and is in line with the hypothesis that loss of nephrons gives rise to hyperfiltration of a reduced number of nephrons leading to further destruction of nephrons and an accelerated deterioration of renal function\textsuperscript{29, 30}.

Although data are scarce, immunosuppressive medication may be of benefit for patients failing a supportive approach and at high risk for progressive loss of renal function. In order to avoid unnecessary immunosuppressive therapy, a model with a high specificity is required to guide clinical decisions regarding treatment. When constructing an receiver operating characteristics curve (ROC), our model predicting ESRD using serum creatinine concentration and urinary protein concentration has an area under the curve (AUC) of 0.88 (95% CI 0.78-0.95). When predicting progression of renal disease using the same variables, the AUC was 0.80 (95% CI 0.69-0.91). Although these values indicate a reasonably good performance of our models, closer examination of data learns that a specificity of 90% is accompanied by a low sensitivity (50-60%). Our models are therefore unsuitable to guide clinical decisions. The identification of more accurate prognostic markers remains essential.

Admittedly, this study has several limitations. First, it describes a relatively small number of patients. Second, when compared to other reported populations, eGFR is lower and proteinuria is more severe in our cohort despite a comparable blood pressure. A large percentage of patients shows fast progression and many patients developed ESRD. This may be due to a selection bias since patients with stable serum creatinine and moderate proteinuria are less likely to be biopsied and/or referred to our medical center. Since many patients were biopsied in another hospital and material was unavailable, we were unable to correlate urinary α1m and β2m with histopathological characteristics. On the other hand, in contrary to earlier populations examined, this cohort is comprised of patients who were all treated with ACEIs and/or ARBs, an important element of therapy nowadays. Furthermore, we ruled out an effect of therapy while analysing data. Finally, the lack of a validation group, as in every other study evaluating the prognostic predictors for progression of IgAN, was corrected for by bootstrapping.

**Conclusion**

Urinary excretion of the low molecular weight proteins α1- microglobulin and β2- microglobulin does not add to predicting prognosis of IgAN. Serum creatinine concentration and urinary protein excretion are the most potent predictors of progression of IgA nephropathy.
Chapter 2

Acknowledgments

H.P.E. Peters is supported by a grant from the Dutch Kidney Foundation.
We thank G. Feith and M. den Hartog, Hospital Gelderse Vallei Wageningen; J. Beutler, D. Hollander, J. Jansen, M. Koolen, Jeroen Bosch Hospital ’s-Hertogenbosch; M. Ten Dam, I. Go, J. van de Leur, Canisius Wilhelmina Hospital Nijmegen; A. van den Wall Bake, St. Joseph Hospital Veldhoven; R. van Leusen, L. Reichert, Hospital Rijnstate Arnhem; W. van Kuijk, V. Verstappen, VieCurie Medical Center Venlo; R. Smeets, St Anna Hospital Geldrop; A. Lückers, Maas Hospital Boxmeer; H. Krepel, Hospital Lievensberg Bergen op Zoom, for their participation in this study.

References

High urinary excretion of Kidney Injury Molecule-1 is an independent predictor of end-stage renal disease in patients with IgA nephropathy

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# both authors contributed equally
Chapter 3

KIM-1 is a predictor of ESRD in IgAN

ABSTRACT

Background
The variable course of immunoglobulin A nephropathy (IgAN) warrants accurate tools for the prediction of progression. Urinary kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) are markers for the detection of early tubular damage caused by various renal conditions. We evaluated the prognostic value of these markers in patients with IgAN.

Methods
We included patients (n=65, 72% male, age 43±13 yrs) with biopsy proven IgAN who were evaluated for proteinuria. Urinary KIM-1 and NGAL were measured by enzyme-linked immunosorbent assay. We analysed data using Cox regression for the outcome end-stage renal disease (ESRD).

Results
Median serum creatinine was 142 µmol/L and proteinuria 2.2 g/day. During follow-up (median 75 months), 23 patients (35%) developed ESRD. In patients with IgAN median urinary KIM-1 excretion was 1.7 ng/min and NGAL excretion was 47 ng/min, both significantly higher than in healthy controls. KIM-1 and NGAL were correlated with proteinuria (r=0.40 and 0.34 respectively, p<0.01) and each other (r=0.53, p<0.01) but not with estimated glomerular filtration rate (eGFR). Interestingly, KIM-1 was not significantly correlated with the excretion of α1-microglobulin and β2-microglobulin, known markers of tubular injury. Univariate analysis showed that baseline serum creatinine, urinary excretion of total protein, α1-microglobulin, β2-microglobulin, IgG, KIM-1 and NGAL were significantly associated with ESRD. By multivariate analysis, serum creatinine and urinary KIM-1, but not proteinuria, were independent predictors of ESRD.

Conclusion
KIM-1 and NGAL excretion are increased in patients with IgAN and correlate with proteinuria but not with estimated glomerular filtration rate (eGFR). Baseline serum creatinine and urinary KIM-1, but not proteinuria, are independent predictors of ESRD.

Introduction
The presentation and course of IgA nephropathy (IgAN) is extremely variable. Long-term studies report that up to 30% of patients with IgAN progress to end-stage renal disease (ESRD) by 20 years1-3. Obviously, published data must be interpreted with caution since many patients with mild disease may never come to clinical attention or undergo a renal biopsy. The optimal treatment of IgAN remains debatable due to a lack of randomized controlled trials. Recent studies suggest that a subset of patients benefits from immunosuppressive agents4-6. Ideally, such therapy should be confined to patients who will eventually progress to ESRD. However, accurate prediction of ESRD remains a challenge.

Elevated serum creatinine concentration7-10, severe proteinuria11-14, arterial hypertension7;12;14 and histological characteristics5;8;11;15 have been identified as baseline predictors of prognosis. A few trials have indicated that clinical features evaluated after one year of follow-up predicted prognosis more accurately than characteristics at the time of presentation5;11-15. Recently Reich et al. reported that persistent proteinuria is the most potent predictor of poor renal outcome in IgAN and that a sustained reduction of proteinuria < 1g/day is associated with a good prognosis17. Unfortunately, these prognostic markers all have low sensitivity and specificity. More accurate prognostic tools are thus needed to predict the course of IgAN and guide decisions regarding treatment.

Tubulointerstitial injury plays an important role in the progression of IgAN16, as in many other renal diseases17. Low molecular weight proteins are thought to reflect the amount of interstitial damage. In idiopathic membranous nephropathy, high-risk patients can in fact be identified in an early stage by measuring low molecular weight proteins such as α1-microglobulin (α1m) and β2-microglobulin (β2m) with a sensitivity of 83% and a specificity of 97%-100%. Remarkably, the excretion of these low molecular weight proteins does not predict renal outcome in patients suffering from IgAN18. Therefore, other biomarkers for tubular damage are needed to predict outcome in IgAN. Kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) are recently discovered biomarkers for renal injury, foremost studied in the setting of acute kidney injury23-24. KIM-1 is selectively expressed by injured proximal tubular cells, providing a strong impetus for using KIM-1 as a biomarker of early tubular damage25. Moreover, elevated urinary KIM-1 levels are strongly related to tubular KIM-1 expression in experimental and in human renal disease25-28. KIM-1 has prognostic value not only in acute kidney injury, but also in renal transplant recipients, predicting graft loss independent of proteinuria29. Also, urinary NGAL has recently emerged as a marker for the detection of early tubular damage, predicting progressive renal function decline in human proteinuric disease30-32. We therefore investigated whether urinary excretion of KIM-1 and NGAL predict prognosis in patients with IgAN.
**Materials and Methods**

**Population**

In our center, patients with proteinuria due to glomerular diseases are evaluated using a standard protocol since 1995\(^1\). For the present study we analysed the data of adult patients with biopsy-proven IgAN who were evaluated for proteinuria in our center between 1995 and 2007, and followed thereafter. Patients with other causes of IgA-positive glomerular staining (systemic lupus erythematosus, Henoch-Schönlein purpura or liver disease) or a follow-up of less than 12 months, were excluded from the analysis. Patients were recruited from hospitals located mainly in the south-eastern part of the Netherlands.

Controls were matched for age and gender and were considered healthy if they had no history of cardiovascular and/or renal disease, used no medication, had a normal blood pressure (systolic blood pressure <140 and diastolic blood pressure <90 mmHg) and an estimated glomerular filtration rate (eGFR) >60 ml/min/1.73m\(^2\). This study was performed in adherence to the declaration of Helsinki and approved by the Hospital Ethical Committee. All subjects gave written informed consent.

**Baseline measurement**

All measurements were carried out in the morning, after an overnight fast. Details of the measurements have been described elsewhere\(^2\). Gender, ethnicity, age, body weight and height were recorded at the time of measurement. Two 24-h urine samples were obtained for measurement of creatinine and total protein. A urinary pH >6.0 is needed to prevent degradation of urinary β\(_2\)m. Therefore, patients used 4000 mg of oral sodium bicarbonate the evening before the measurement. Upon arrival an additional 2000-4000 mg sodium bicarbonate was given and patients drank 375-500 ml of tap water to enforce diuresis. The patients remained supine during 2 h except for voiding. Blood pressure measurements were taken using an automated device (Dinamap, Criticon, Tampa FL) with six consecutive readings registered every 5 min after 10 min rest; these readings were used to calculate the mean arterial pressure (MAP). In controls, measurement of urinary protein, KIM-1, NGAL, immunoglobulin G (IgG), albumin and β\(_2\)m was performed in a 24 h urine sample.

Measurement of urinary pH, β\(_2\)m, q, IgG, transferrin, albumin, total protein and creatinine was performed. Urine was immediately stored at -80°C until measurement of KIM-1 and NGAL. Urinary KIM-1 and NGAL were measured by enzyme-linked immunosorbent assy (ELISA) (intra-assay coefficient of variation 7.4%, 6.8%). Antibodies were obtained from R&D systems, Minneapolis, USA. Urine samples were diluted two times for KIM-1 and 10 or 100 times for NGAL. Detection limit for KIM-1 was 0.042 ng/ml, for NGAL 0.63 ng/ml. Laboratory parameters were measured in blood samples collected in the middle of the urine collection period. Serum creatinine, cholesterol, urinary total protein, and creatinine were measured with standard automated techniques. Urinary proteins were measured as described before\(^3\). Only in urine with a urinary pH >6.0 β\(_2\)m excretion was measured.

The use of angiotensin-converting enzyme inhibitors (ACEIs) and/or angiotensin II type 1 receptor antagonists (ARBs), calcium channel blockers, other antihypertensive agents, diuretics, and non-steroidal anti-inflammatory drugs (NSAIDs), as well as HMG-CoA-reductase inhibitors, was recorded. Current or previous use of immunosuppressive agents or fish oil was registered.

**Follow-up**

After standardized protein measurements, patients were entrusted to the care of their local physicians. Immunosuppressive therapy was advised to patients with progression of renal disease. During follow-up we collected data from medical records on serum creatinine, albumin, cholesterol, urea, total urinary protein and creatinine levels, blood pressure, body weight and use of medication.

**Histological classification**

Patients were included for evaluation of renal biopsy material if the interval between the time of renal biopsy and baseline measurement was ≤ 6 months. Light microscopic assessment of renal biopsies was performed in accordance with the Oxford Classification of IgA nephropathy\(^4\) by a single experienced renal pathologist.

**Calculations and definitions**

Body mass index (BMI) was calculated as the ratio between baseline weight and height squared. MAP was calculated as the diastolic pressure plus one-third of the pulse pressure. The glomerular filtration rate (GFR) at baseline and follow-up was estimated using the abbreviated modification of diet in renal disease (MDRD) equation\(^5\). Start of follow-up was defined as the time of standardized measurement of proteinuria, regardless of the first assessment suggestive of renal disease. We defined the outcome ESRD as initiation of dialysis, renal transplantation or an eGFR <15 ml/min per 1.73m\(^2\).

**Statistical Analysis**

Missing values for total urinary protein concentration in the 24-h urine samples were imputed by using the urinary protein-creatinine ratio, which was derived from the 2-h sample, and by using serum albumin. Parameters between groups were compared using the Mann–Whitney or Kruskal–Wallis test for non-parametric continuous data, independent t-test for parametric data and the chi-square test for categorical data. Spearman’s bivariate correlation test was used to examine correlation between
nonparametric data. Possible collinearity for univariate significant predictors was checked. Predictors that had a Spearman’s rho < 0.80 were entered into a multivariate Cox model. A backward stepwise selection algorithm, criteria for exclusion being a likelihood ratio test with p-value greater than 0.05 and smaller than 0.10 for inclusion, was used. The predictive value of this model was investigated by the area under the receiver operating characteristics (ROC) curve. Statistical analysis was performed using SPSS for Windows software, version 16.0 (SPSS Inc., Chicago, IL).

**Results**

We studied 65 patients with IgAN and 65 matched healthy controls. Baseline characteristics are presented in Table 1. IgAN patients had a higher BMI, higher blood pressure (despite frequent use of antihypertensive medication), and serum creatinine. In more than 70% of the patients, the baseline measurement was performed within 1 yr after renal biopsy. In the majority of patients (57%) proteinuria was >2.0 g/d and the eGFR <60 ml/min/1.73m². Eighty-two percent of the population was using ACEIs or ARBs at the time of evaluation for proteinuria. Two patients had previously been treated with steroids. Median duration of follow-up was 75 (range 3-146) months. During follow-up all patients were treated with ACEIs and/or ARBs. Immunosuppressive therapy was initiated in 19 patients who had either progressive deterioration of renal function or persistent proteinuria. The majority of them (74%) received cyclophosphamide combined with prednisone. Twenty-three patients (35%) reached the predefined end point of ESRD. Six of these patients had received immunosuppressive therapy. Overall renal survival was 78% at 5 years, and 70% at 8 years. The renal survival curve is depicted in Figure 1.

**Figure 1. Renal survival curve in patients with IgAN. Renal survival was defined as onset of ESRD.**

### Table 1. Clinical and demographic characteristics at baseline

<table>
<thead>
<tr>
<th>Variable</th>
<th>IgAN patients n=65</th>
<th>Healthy controls n=65</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>gender (% male)</td>
<td>72</td>
<td>69</td>
<td>0.70</td>
</tr>
<tr>
<td>age (yrs)</td>
<td>42±13</td>
<td>39±12</td>
<td>0.97</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27±4</td>
<td>25±3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>101±13</td>
<td>91±8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>serum albumin (g/l)</td>
<td>38 (21-46)</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
<td>serum cholesterol (mmol/l)</td>
<td>5.8±1.3</td>
<td>4.8±0.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>serum creatinine (µmol/l)</td>
<td>142 (70-362)</td>
<td>78 (52-96)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73 m²)</td>
<td>50±21</td>
<td>92±12</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>proteinuria (g/d)</td>
<td>2.2 (0.4-24.4)</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
<td>KIM-1 excretion (ng/min)</td>
<td>1.7 (0.8-3.1)</td>
<td>0.6 (0.4-0.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>NGAL excretion (ng/min)</td>
<td>47 (21.7-104.0)</td>
<td>15.6 (10.4-19.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>α₁-microglobulin excretion (µg/min)</td>
<td>24.3 (12.1-48.3)</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
<td>β₂-microglobulin excretion (µg/min)</td>
<td>0.4 (0.1-2.4)</td>
<td>0.06 (0.03-0.08)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IgG excretion (mg/d)</td>
<td>105 (50-201)</td>
<td>0 (0-5.21)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>albumin excretion (µg/d)</td>
<td>2389 (1044-3360)</td>
<td>77 (60-113)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>interval between biopsy and referral* (months)</td>
<td>2.0 (53-209)</td>
<td>- -</td>
<td></td>
</tr>
<tr>
<td>use of ACEI/ARBs at baseline (%)</td>
<td>82</td>
<td>0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>use of diuretics at baseline (%)</td>
<td>31</td>
<td>0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>use of other anti-hypertensive medication (%)</td>
<td>35</td>
<td>0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>use of immunosuppressive treatment before baseline (%)</td>
<td>3</td>
<td>0</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Data are expressed as means±SD, median (range) or median (interquartile range). Abbreviations are: BMI, body mass index; MAP, mean arterial pressure; eGFR, estimated glomerular filtration rate; IgG, immunoglobulin G; KIM-1, Kidney Injury Molecule 1; NGAL, Neutrophil Gelatinase-Associated Lipocalin, n.a.: not available.

*In few patients biopsy was performed after evaluation for proteinuria.

**KIM-1 and NGAL excretion**

In patients with IgAN median urinary KIM-1 excretion was 1.7 (interquartile range (IQR) 0.8-3.1) ng/min and urinary NGAL excretion was 47 (IQR 21.7-104.0) ng/min, both significantly higher than values in healthy controls (KIM-1 0.6 (IQR 0.3-0.9) ng/min, NGAL 16.2 (IQR11.3-21.5) ng/min) (Figure 2). KIM-1 and NGAL were significantly correlated with proteinuria (r=0.40 and 0.35 respectively, p<0.01, Figure 3A) and each other (r=0.53, p<0.01) but not with serum creatinine or eGFR (Table 2). Interestingly, KIM-1 was not significantly correlated with urinary β₂m (Figure 3B) and α₁m. There was only a weak correlation between NGAL and urinary β₂m and α₁m.
Predictors of ESRD

In univariate Cox regression analysis, urinary KIM-1, NGAL, α₁-microglobulin, β₂-microglobulin and IgG excretion, total urinary protein, serum creatinine, and eGFR were all significantly associated with ESRD (Table 3). By multivariate Cox regression analysis baseline serum creatinine, KIM-1 excretion, and immunosuppressive therapy, but not proteinuria, proved significant predictors of ESRD. Thus, KIM-1 excretion is an independent predictor of ESRD. When constructing a ROC curve, our model predicting ESRD using serum creatinine concentration and KIM-1 excretion had an area under the curve (AUC) of 0.86 (95% confidence interval 0.77-0.95).

Correlations with histology

Renal biopsy material, obtained within 6 months before or after baseline measurement, was available for 36 of 65 patients. In this subgroup of patients (69% male), mean age was 42±14 years, mean MAP 101±13 mmHg, median serum creatinine 145 µmol/L (range 70-276), median eGFR 45 (range 22-95) ml/min/1.73m² and median proteinuria 3.2 (range 0.5-24.2) g/d at baseline. During a median follow-up of 76 months, 9 patients (25%) received immunosuppressive therapy and 13 patients (36%) developed ESRD. The median number of glomeruli per biopsy was 12. Data are given in Table 4. The tubulointerstitial score correlated with eGFR and with the urinary excretion of the low molecular weight proteins α₁m and β₂m. A higher tubulointerstitial score was also associated with a higher risk of ESRD (67% in patients with T2 versus 21% in patients with T0+1, p=0.01). In contrast, the tubulointerstitial score did not correlate with KIM-1 or NGAL excretion.

Discussion

Our data indicate that serum creatinine and urinary excretion of KIM-1, but not proteinuria, are independent predictors of renal outcome in patients with IgAN. To our knowledge, we are the first to report long-term follow-up data on the prognostic value of urinary KIM-1 and NGAL excretion in primary renal disease.

KIM-1 is a recently discovered type I transmembrane protein that is not detected in normal kidneys but is up regulated in renal proximal tubules after both acute and chronic injury due to various renal diseases. Cleavage of KIM-1 by metalloproteinases leads to shedding of its soluble 90-kDa ectodomain in the urine. Urinary KIM-1 levels are strongly correlated with tubular KIM-1 expression in experimental and human renal disease. KIM-1 is thought to be involved in the development of tubular cell injury and interstitial fibrosis. It remains unclear if KIM-1 is actively regulating inflammation or a response to tubular damage, reflecting tubular repair mechanisms. Experimental data suggest that KIM-1, located on epithelial cells, mediates phagocytosis of apoptotic and necrotic cells by binding to phosphatidylserine and oxidized lipid epitopes on the apoptotic cell.
KIM-1 is a predictor of ESRD in IgAN

Chapter 3

Overall, experimental and human data strongly suggest that KIM-1 reflects tubulointerstitial injury and repair. Since urinary KIM-1 is strongly correlated with renal tubular expression, it seems a promising biomarker.

NGAL is a soluble 25-kDa acute phase protein and was originally purified from neutrophils. It is expressed at low levels in several human tissues including the kidney and its expression is induced by epithelial injury. Urine and plasma NGAL levels are reported to be independent early predictors of acute kidney injury in several studies, mostly performed in patients undergoing cardiac surgery. Ding et al. measured urinary NGAL in 70 non-hypertensive IgAN patients with normal renal function and proteinuria >1.0 g/day while not on ACEIs/ARBs. They found significantly increased urinary NGAL levels in patients with Lee grade III IgAN. Recently, urinary NGAL excretion was shown to be a strong predictor of progressive renal function decline during follow-up in human proteinuric renal disease.

In the present study urinary KIM-1 and NGAL levels were elevated in patients with IgAN compared to healthy controls. Furthermore, KIM-1 and NGAL excretion were significantly associated with the development of ESRD, as were known prognostic markers such as serum creatinine and proteinuria. Yet, by multivariate analysis KIM-1 along with serum creatinine, and not proteinuria nor NGAL, proved to be independent predictors of ESRD. Others have reported that proteinuria at diagnosis is not a predictor of renal outcome. Moreover, a recent retrospective study performed in over 500 patients with IgAN, showed that proteinuria during follow-up was a strong predictor of renal outcome.

Patients who reached proteinuria <1 g/day had an excellent prognosis, regardless of

### Table 2. Correlation of urinary KIM-1 with other demographic and biochemical variables.

<table>
<thead>
<tr>
<th>Variable</th>
<th>ρ</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>0.10</td>
<td>0.41</td>
</tr>
<tr>
<td>Age</td>
<td>-0.11</td>
<td>0.39</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.11</td>
<td>0.39</td>
</tr>
<tr>
<td>MAP</td>
<td>-0.11</td>
<td>0.41</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>-0.52</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Serum cholesterol</td>
<td>0.23</td>
<td>0.07</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>-0.24</td>
<td>0.06</td>
</tr>
<tr>
<td>eGFR</td>
<td>0.24</td>
<td>0.06</td>
</tr>
<tr>
<td>Urinary protein</td>
<td>0.40</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Urinary NGAL</td>
<td>0.53</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Urinary α₁-microglobulin</td>
<td>0.15</td>
<td>0.25</td>
</tr>
<tr>
<td>Urinary β₂-microglobulin</td>
<td>0.12</td>
<td>0.36</td>
</tr>
<tr>
<td>Urinary IgG</td>
<td>0.41</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Urinary albumin</td>
<td>0.48</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

### Table 3. Hazard ratios (HR) and confidence intervals (CI) of baseline predictors of ESRD after univariate and multivariate regression analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>1.005 (0.95-1.06)</td>
<td>1.018 (1.011-1.025)</td>
</tr>
<tr>
<td>Serum albumin (g/l)</td>
<td>0.930 (0.89-1.00)</td>
<td>1.029 (1.011-1.047)</td>
</tr>
<tr>
<td>Serum cholesterol (mmol/l)</td>
<td>0.66 (0.44-0.99)</td>
<td>1.011 (1.002-1.021)</td>
</tr>
<tr>
<td>Serum creatinine (µmol/l)</td>
<td>0.79 (0.59-1.08)</td>
<td>0.999 (0.994-1.004)</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73m²)</td>
<td>0.96 (0.85-1.09)</td>
<td>0.96 (0.85-1.09)</td>
</tr>
<tr>
<td>Proteinuria (g/day)</td>
<td>1.143 (1.033-1.265)</td>
<td>1.217 (1.087-1.302)</td>
</tr>
<tr>
<td>Urinary KIM-1 (ng/min)</td>
<td>1.132 (1.035-1.238)</td>
<td>1.217 (1.087-1.302)</td>
</tr>
<tr>
<td>Urinary NGAL (ng/min)</td>
<td>1.007 (1.002-1.012)</td>
<td>1.007 (1.002-1.012)</td>
</tr>
<tr>
<td>Urinary α₁-microglobulin (µg/min)</td>
<td>1.015 (1.007-1.024)</td>
<td>1.015 (1.007-1.024)</td>
</tr>
<tr>
<td>Urinary β₂-microglobulin (µg/min)</td>
<td>1.183 (1.031-1.357)</td>
<td>1.183 (1.031-1.357)</td>
</tr>
<tr>
<td>Urinary IgG (mg/day)</td>
<td>1.003 (0.993-1.014)</td>
<td>1.003 (0.993-1.014)</td>
</tr>
<tr>
<td>Urinary albumin (g/day)</td>
<td>1.196 (1.043-1.372)</td>
<td>1.196 (1.043-1.372)</td>
</tr>
<tr>
<td>Immunosuppressive therapy</td>
<td>0.865 (0.341-2.198)</td>
<td>0.216 (0.068-0.686)</td>
</tr>
</tbody>
</table>

*In ten patients data on α₁m excretion were not available
**In nine patients β₂m excretion could not reliably be measured due to a urinary pH<6.0
the level of baseline proteinuria. We have previously analysed the data of this patient cohort and reported urinary protein as an independent predictor of ESRD, along with serum creatinine\(^2\). The current analysis clearly indicates that KIM-1 excretion is a better predictor than proteinuria, suggesting that KIM-1 provides additional information on tubular processes involved in progressive renal failure. Urinary excretion of α1m and β2m - established markers of tubulointerstitial injury - is increased in IgAN, but does not predict outcome in these patients. KIM-1 was not correlated with α1m and β2m and did prove to be an independent predictor of ESRD. This finding underscores the potential of KIM-1. One could hypothesize that KIM-1 may reflect the process of active tubular damage that precedes the development of fibrosis, whereas low molecular weight proteins reflect chronic tubulointerstitial injury in patients with IgAN. Analysis of the correlation between pathological lesions and baseline characteristics confirms this hypothesis. As expected, the tubulointerstitial score correlated with eGFR and with the urinary excretion of the low molecular weight proteins. In contrast, the tubulointerstitial score did not correlate with KIM-1 excretion, although KIM-1 levels were numerically higher in patients with T2 score. When constructing an ROC curve, our model predicting ESRD that uses urinary KIM-1 excretion and serum creatinine has an area under the curve of 0.86. This indicates a reasonable accuracy. However, a specificity of 90% is accompanied by a sensitivity of only 60%. Thus, the predictive value of the model is still limited and should not be used to guide decisions regarding immunosuppressive therapy in individual patients. Moreover, in this cohort of patients with moderate to severe renal impairment at baseline indicating the presence of chronic tubulointerstitial injury, serum creatinine was, not unexpectedly, the most powerful predictor of ESRD. Our data suggest that urinary KIM-1 excretion may be of particular value in patients with normal or mildly impaired renal function, since high KIM-1 excretion (i.e. above the median value) predicted progression of ESRD in a subgroup of patients with a serum creatinine ≤135 μmol/l (Figure 4). In 15 of these patients, a renal biopsy was performed within 6 months of baseline. The vast majority (n=12) was scored as having no or absent tubulointerstitial fibrosis (T0), indicating that the patients depicted in Figure 4 are indeed expected to have no or minimal tubulointerstitial damage. This needs further study.

Admittedly, this study has several limitations. First, it describes a small number of patients and no follow-up data on the course of KIM-1 and NGAL were obtained. Second, when compared to other reported populations, renal impairment and proteinuria are more severe in our cohort despite a similar blood pressure. Fast progression of renal disease was observed in a large percentage of subjects and eventually many patients developed ESRD. Since patients with stable serum creatinine and moderate proteinuria are less likely to be biopsied and/or referred to our hospital, this may be due to a selection bias. On the other hand, contrary to previously reported populations, this cohort is comprised of patients who were all treated with ACEIs and/or ARBs, an important element

<table>
<thead>
<tr>
<th>MEST-score</th>
<th>Number of patients</th>
<th>KIM-1 (ng/min)</th>
<th>NGAL (lg/min)</th>
<th>α1m (µg/min)</th>
<th>β2m (µg/min)</th>
<th>eGFR (ml/min/1.73m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>22</td>
<td>1.60(0.3-16.6)</td>
<td>65.56(72)</td>
<td>0.58</td>
<td>0.60(1.27)</td>
<td>0.99</td>
</tr>
<tr>
<td>M1</td>
<td>14</td>
<td>1.86(0.4-11.7)</td>
<td>33.64(22)</td>
<td>0.57</td>
<td>0.60(1.30)</td>
<td>0.54</td>
</tr>
<tr>
<td>E0</td>
<td>18</td>
<td>1.60(0.3-16.6)</td>
<td>48.56(22)</td>
<td>0.48</td>
<td>1.00(1.30)</td>
<td>0.08</td>
</tr>
<tr>
<td>E1</td>
<td>18</td>
<td>1.60(0.3-16.6)</td>
<td>48.03(62)</td>
<td>0.47</td>
<td>0.56(1.30)</td>
<td>0.08</td>
</tr>
<tr>
<td>S0</td>
<td>7</td>
<td>2.10(0.5-11.7)</td>
<td>47.64(22)</td>
<td>0.47</td>
<td>1.00(1.30)</td>
<td>0.08</td>
</tr>
<tr>
<td>S1</td>
<td>15</td>
<td>1.70(0.3-16.6)</td>
<td>47.64(22)</td>
<td>0.47</td>
<td>1.00(1.30)</td>
<td>0.08</td>
</tr>
<tr>
<td>T0</td>
<td>9</td>
<td>1.50(0.3-3.2)</td>
<td>68.62(22)</td>
<td>0.30</td>
<td>0.60(1.27)</td>
<td>0.54</td>
</tr>
<tr>
<td>T1</td>
<td>12</td>
<td>2.50(0.3-11.7)</td>
<td>61.64(22)</td>
<td>0.45</td>
<td>0.60(1.27)</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Data are expressed as median (range) or mean±SD.
of current therapy. Lastly, we cannot exclude a confounding effect of immunosuppressive therapy, since 19 patients received immunosuppressive agents and multivariate analysis implied that these patients were less likely to develop ESRD. Yet, KIM-1 excretion was significantly higher in patients who received immunosuppressive therapy compared to those who received supportive treatment (median 3.1 (IQR 1.3–5.5) versus 1.5 (IQR 0.7–2.2) ng/ml, p=0.02). Thus, KIM-1 excretion remained a predictor of ESRD, despite a possible beneficial effect of immunosuppressive therapy in a subgroup of patients exhibiting increased urinary KIM-1 excretion. Therefore, if any confounding effect has occurred, the use of immunosuppressive therapy may have attenuated the predictive value of KIM-1 excretion in this cohort. The results of our study should be confirmed in a larger population, preferably consisting of patients with varying degrees of renal impairment.

Conclusion

Urinary KIM-1 and NGAL excretion are increased in patients with IgAN and correlate with proteinuria but not with eGFR, urinary β2m and α1m. Baseline serum creatinine and urinary KIM-1 are independent predictors of ESRD. Future studies should be undertaken to verify the predictive value of KIM-1 excretion in patients with IgAN.

Acknowledgements

We thank G. Feith and M. Den Hartog, Hospital Gelderse Vallei Wageningen; J. Beutler, D. Hollander, J. Jansen, M. Koolen, Jeroen Bosch Hospital ’s-Hertogenbosch; M. Ten Dam, I. Go, J. van de Leur, Canisius Wilhelmina Hospital Nijmegen; A. van den Wall Bake, St. Joseph Hospital Veldhoven; R. van Leusen, L. Reichert, Hospital Rijnstate Arnhem; W. van Kuijk, V. Verstappen, VieCurie Medical Center Venlo; R. Smeets, St. Anna Hospital Geldrop; A. Lückers, Maas Hospital Boxmeer; H. Krepel, Hospital Lievensberg Bergen op Zoom, for their participation in this study.
References


Interstitial GMP-17-positive T-lymphocytes and disease progression in IgA nephropathy

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²Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands.
ABSTRACT

Background

The clinical course in patients with IgA nephropathy (IgAN) is highly variable and accurate predictors of prognosis are lacking. Recent studies suggest that urinary excretion of low-molecular weight (LMW) proteins, kidney injury molecule-1 (KIM-1) and neutrophil gelatinase associated lipocalin (NGAL) is increased in patients with progressive IgAN. We previously showed that the presence of GMP-17 positive T-lymphocytes in intact kidney tubules predicted outcome in patients with IgAN and a normal or near normal estimated glomerular filtration rate (eGFR).

The objective of this study was to validate the prognostic value of GMP-17 positive T-lymphocytes for predicting progression of kidney disease in IgAN and to correlate their presence with urinary markers of early tubular damage. We also evaluated whether GMP-17 positive lymphocytes were present in kidney tubules of patients with idiopathic membranous nephropathy (IMN).

Methods

Adults with IgAN or IMN who had undergone standardised measurements of proteinuria in our center within 6 months of kidney biopsy were included in this study. Kidney biopsy specimens were evaluated for the presence of GMP-17 positive T-lymphocytes in intact kidney tubules. Intra-epithelial GMP-17 positive cells were scored in a semi-quantitative manner and categorized as 0-1, 2-3 or ≥4 positive cells within the kidney tubules in the whole section.

Results

Kidney biopsy material of 24 patients with IgAN allowed reliable scoring of GMP-17 positive T-lymphocytes. Intratubular GMP-17 positive T-lymphocytes were present in nine patients. GMP-17 positive patients had more severely impaired kidney function (median eGFR 37 vs 57 ml/min/1.73m²), and a higher urinary excretion of LMW proteins and KIM-1 than GMP-17 negative patients. However, there was no statistically significant difference regarding progression of kidney disease between GMP-17 positive and GMP-17 negative patients – 4/9 (44%) versus 9/15 (60%). In three out of six patients with idiopathic membranous nephropathy GMP-17 positive T-lymphocytes were observed in the tubules.

Conclusion

We failed to confirm the prognostic value of intratubular GMP-17 positive T-lymphocytes in predicting progression of IgAN. GMP-17 positive T-lymphocytes can also be detected in kidney tubules of patients with idiopathic membranous nephropathy.

Introduction

IgA nephropathy (IgAN) is the most common glomerulonephritis worldwide. In the majority of patients the disease manifests as glomerular hematuria and runs a non-progressive course (so-called benign hematuria). However, 25-50% of patients will progress to ESRD after 10-30 years of follow up(1). Thus, the clinical course is highly variable, with some patients attaining a spontaneous remission, and others developing end-stage kidney disease (ESRD) within a few years after diagnosis. This variable course hinders the development of clinical trials and the use of standardised treatment protocols. Accurate prediction of progression is instrumental in developing individualized treatment protocols. Thus far, clinical variables are insufficient to predict prognosis.

A kidney biopsy is required for the detection of glomerular IgA immune deposits, the diagnostic hallmark of IgAN. A variety of grading systems has been developed for classifying histopathological changes in IgAN patients(5). An international consortium identified a set of distinct pathological variables as independent prognostic markers in patients with IgAN, known as the Oxford classification(6,7). The four predictive pathological features were; mesangial hypercellularity, the presence of endocapillary proliferation, segmental glomerulosclerosis/adhesion, and severity of tubular atrophy/interstitial fibrosis. Validation of these prognostic histopathological variables has been conducted in different patient populations(8-11). Results vary, but tubulointerstitial fibrosis appears the most reproducible prognostic marker. This is hardly surprising since tubulointerstitial injury has long been known to predict progression more accurately than glomerular damage in glomerulonephritis(11,12). Interstitial fibrosis is preceded by interstitial inflammation, a process involving lymphocytes and macrophages and the production of pro- and anti-inflammatory cytokines. In 2008, Van Es et al. showed that the presence of cytotoxic GMP-17-positive T-lymphocytes in the interstitium and within kidney tubules predicted progression towards kidney failure in patients with IgAN and normal or near-normal glomerular filtration rate(13).

Urinary excretion of low molecular weight (LMW) proteins - such as α1-microglobulin (α1m) and β2-microglobulin (β2m) - kidney injury molecule-1 (KIM-1), and neutrophil gelatinase-associated lipocalin (NGAL) are thought to reflect the severity of interstitial damage. In previous studies we found that low molecular weight proteins, urinary KIM-1 and NGAL levels were elevated in patients with IgAN compared to healthy controls(14,15). In multivariate analysis, urinary KIM-1 along with serum creatinine proved to be independent predictors of ESRD. Yet, our model had a limited predictive value, and lacked sufficient accuracy to predict prognosis in individual patients. Therefore, we explored whether identification and scoring of GMP-17 positive T-lymphocytes, would be of use to improve our model for predicting ESRD.
The purpose of this study was to validate the prognostic value of GMP-17 positive T-lymphocytes in an independent cohort of patients with IgAN and to correlate their presence with urinary markers of tubular dysfunction. Furthermore, we evaluated the presence of GMP-17 positive T-lymphocytes in kidney biopsies of patients with idiopathic membranous nephropathy.

Materials and methods

Population

Patients with proteinuria due to glomerular diseases are evaluated in our center using a standard protocol for almost two decades. For the present study, we analysed the data of adult patients with biopsy-proven IgAN who were evaluated for proteinuria in our centre between 1995 and 2007 and followed thereafter. Patients with other causes of IgA-positive glomerular staining (systemic lupus erythematosus, Henoch-Schönlein purpura or liver disease) or a follow-up of <12 months were excluded from the analysis. We excluded patients in whom the interval between the kidney biopsy and the evaluation of proteinuria was >6 months. The study was carried out in accordance with applicable rules in the Netherlands concerning the review of research ethics committees and informed consent.

Baseline measurement

All measurements were carried out in the morning, after an overnight fast. Details of the measurements have been described elsewhere(16). Gender, ethnicity, age, body weight and height were recorded at the time of measurement. Two 24-h urine samples were obtained for measurement of creatinine and total protein. A urinary pH >6.0 is needed to prevent degradation of urinary β2 m. Therefore, patients used 4 g of oral sodium bicarbonate the evening before the measurement. Upon arrival, an additional 2–4 g sodium bicarbonate was given and patients drank 375–500 ml of tap water to enforce diuresis. The patients remained supine during 2 h except for voiding. Blood pressure measurements were taken using an automated device (Dinamap; Criticon, Tampa, FL) with six consecutive readings registered every 5 min after 10-min rest; these readings were used to calculate the mean arterial pressure (MAP).

In the freshly collected urine samples measurement of urinary pH, β2 m, α1m, immunoglobulin G (IgG), transferrin, albumin, total protein and creatinine was performed. Only in urine with a urinary pH >6.0, β2 m excretion was measured. Urine was immediately stored at -80°C until the measurement of KIM-1 and NGAL. Urinary KIM-1 and NGAL were measured by enzyme-linked immunosorbent assay as described previously15. Laboratory parameters were measured in blood samples collected in the middle of the urine collection period. Serum creatinine, cholesterol, urinary total protein and creatinine were measured with standard automated techniques.

Follow-up

After standardised protein measurements, patients were entrusted to the care of their local physicians. Immunosuppressive therapy was advised to patients with progression of kidney disease. During follow-up, we collected data from medical records on serum creatinine, albumin, cholesterol, urea, total urinary protein and creatinine levels, blood pressure, body weight and the use of medication.

Histological studies

Biopsy specimens were stained with hematoxylin-eosin, periodic acid Schiff and silver methenamine. A single kidney pathologist (IB), who was blinded to information on outcome, scored GMP-17-positive T-lymphocytes.

Sections measuring 4 mm were deparaffinized in 96% ethanol, blocked for endogenous peroxidases in a mixture of 1 ml of 30% H2O2 in 250 ml of methanol for 20 min, and subsequently hydrated in decreasing ethanol concentrations. Sections were boiled in 1 mM ethylene diamine tetraacetic acid pH 9 for 12 min. Sections were then incubated with antibodies directed against GMP-17 (2550 IgG clone 2G9; Immunotech, Marseille, France). Binding of these primary antibodies was visualized using horseradish peroxidase-labeled anti-mouse antibodies (Envision; Dako), and a mixture of 3.3 aminobenzidine (0.05%) and NiCl2 (0.07%). Sections were rinsed between each step with phosphate-buffered saline. Sirius red was used as counterstain to accentuate the tubular basement membrane. Ileal biopsies from a patient with Crohn’s disease were used as a positive control for GMP-17 staining.

Intra-epithelial GMP-17-positive cells were scored as follows: 0, no cells or one cell within the kidney tubules in the whole section; 1, two to three positive cells within tubules; and 2, four or more positive cells within the tubules. In case of different scores in different tissue sections, the highest score was noted as the final score.

Calculations and definitions

Body mass index (BMI) was calculated as the ratio between baseline weight and height squared. MAP was calculated as diastolic pressure plus one-third of the pulse pressure. The glomerular filtration rate at baseline and follow-up was estimated using the abbreviated Modification of Diet in Renal Disease equation . Start of follow-up was defined as the time of standardised measurement of proteinuria, regardless of the first assessment.
suggestive of kidney disease. We defined the outcome ESRD as initiation of dialysis, kidney transplantation or an eGFR <15 ml/min/1.73m². Progression of kidney disease was defined as an increase in serum creatinine > 50%.

**Statistical analysis**
Parameters between groups were compared using the Mann–Whitney or Kruskal–Wallis test for nonparametric continuous data, independent t-test for parametric data and the chi-square test for categorical data. Spearman’s bivariate correlation test was used to examine correlation between nonparametric data. Statistical analysis was performed using SPSS for Windows software, version 16.0 (SPSS Inc., Chicago, IL).

**Results**

**IgA nephropathy**
In the period 1995-2007 70 patients with IgAN were evaluated in our center(14, 15). In 36 patients the interval between kidney biopsy and protein measurements was < 6 months. Of 24 patients kidney biopsy material was still available to prepare fresh histology slides, which were suitable for GMP-17 staining and allowed reliable scoring. Baseline characteristics are depicted in Table 1. Of these 24 patients 13 patients had a rise in serum creatinine of >50%, and eight developed ESRD. Median follow-up was 102 (7-146) months. During follow-up seven patients were treated with immunosuppressive drugs because of progression of IgAN.

In nine out of 24 (38%) patients GMP-17 positive T-lymphocytes were present in the kidney tubules (Figure 1). All of these nine patients were scored as 1, meaning two to three GMP-17 positive cells within the kidney tubules. We therefore categorised patients as either GMP-17 positive or GMP-17 negative. Baseline characteristics of both groups are depicted in Table 2. When comparing both groups, GMP-17 positive patients had more severely impaired kidney function, and although severity of proteinuria did not differ, GMP-17 positive patients did show a significantly higher urinary excretion of α₁m (Figure 2) and KIM-1.

**Table 1. Baseline characteristics of IgAN patients**

<table>
<thead>
<tr>
<th></th>
<th>N (% male)</th>
<th>24 (71%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>38±13.5</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27±4</td>
<td></td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>99±13</td>
<td></td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>141 (70-276)</td>
<td></td>
</tr>
<tr>
<td>MDRD (ml/min/1.73m²)</td>
<td>50 (22-95)</td>
<td></td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>7.6 (4-36)</td>
<td></td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>38.5 (21-46)</td>
<td></td>
</tr>
<tr>
<td>Proteinuria (g/day)</td>
<td>2.1 (0.47-24.4)</td>
<td></td>
</tr>
<tr>
<td>α₁-microglobulin excretion (μg/min)</td>
<td>16.6 [8.4-48.6]</td>
<td></td>
</tr>
<tr>
<td>β₂-microglobulin excretion (ng/min)</td>
<td>260 [111-1238]</td>
<td></td>
</tr>
<tr>
<td>KIM-1 excretion (ng/min)</td>
<td>1.6 [0.7-3.2]</td>
<td></td>
</tr>
<tr>
<td>NGAL excretion (ng/min)</td>
<td>57 [22-110]</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD, median (range), median [interquartile range].
Kidney outcome between GMP-17 positive and GMP-17 negative patients did not differ: three out of nine (30%) GMP-17 positive patients developed ESRD, whereas 5 out of 15 (30%) GMP-17 negative patients did (Table 3). Progression of disease was seen in four out of nine (44%) GMP-17 positive patients and nine out of 15 (60%) GMP-17 negative patients, which was not a statistically significant difference. Survival analysis with Kaplan Meier curves showed no difference between the two groups. As mentioned previously, seven patients out of 24 received immunosuppressive treatment during follow-up. Four of these seven patients were GMP-17 positive, three patients received treatment before progression of kidney disease occurred, three patients were treated and did not show progression, whereas one patient was treated after attaining an eGFR compatible with ESRD. To correct for possible bias, we also analysed data for the combined outcome of ESRD or therapy, and progression of renal disease or therapy, whichever occurred first. Again, outcome did not differ between GMP-17 positive and negative patients (Table 3).

We separately analysed patients with maintained kidney function. Only nine out of 24 patients had an eGFR>60ml/min/1.73m². None of them developed ESRD, three patients showed progression of kidney disease. Two out of nine patients were GMP-17 positive, one of these two patients showed progression.

### Idiopathic membranous nephropathy

Kidney biopsies of six patients with idiopathic membranous nephropathy were evaluated for the presence of GMP-17 positive T-lymphocytes in the tubules. Median serum creatinine was 105 (range 61-128) µmol/L, median eGFR was 61 (range 28-75) ml/min/1.73m², median proteinuria was 4.75 (range 3.0-11.4) g/d, median urinary excretion of β₂ m was 436 (range 250-22200) ng/min and median urinary excretion of α₁m was 53 (range 23-108) µg/min. In three out of six patients GMP-17 positive T-cells were detected in intact kidney tubules. When comparing GMP-17 positive and GMP-17 negative patients, GMP-17 positive patients had a slightly better kidney function (median eGFR 71 vs 54 ml/min/1.73m², and less proteinuria (median proteinuria 4.1 vs 5.4 g/d), yet excretion of LMW proteins was higher (β₂ m 578 vs 293 ng/min and α₁m 61 vs 44 µg/min, Figure 3).

![Figure 3. Boxplot of α₁m excretion in GMP-17 positive and negative patients with IgAN](image-url)
Chapter 4  

Discussion

Our data failed to confirm the prognostic significance of the presence of GMP-17 positive T-lymphocytes in intact kidney tubules for predicting progression of IgAN. The predictive role of GMP-17 positive cells was previously evaluated in a study of 50 IgAN patients. This study showed that GMP-17 positive intra-epithelial T-cells predicted progression in a subgroup of patients with an eGFR ≥60 ml/min (n=31) with higher positive and negative predictive values than conventional markers of progression such as proteinuria or interstitial fibrosis. The current study was conducted to validate these findings and to correlate the presence of GMP-17 positive T-cells with other urinary markers of disease progression such as LMW proteins and KIM-1 and NGAL. Unfortunately, the interval between kidney biopsy and proteinuria measurements was >6 months in a large number of patients. There also was a lack of suitable biopsy material due to varying methods of fixation of material in different centres. Ultimately, only 24 out of our cohort of 70 IgAN patients could be included. Kidney outcome between nine GMP-17 positive patients and 15 GMP-17 negative patients did not differ. Notably, 30% of GMP-17 negative patients developed ESRD, and even 60% showed progression of kidney disease. Admittedly, the majority of these patients had an eGFR <60 ml/min/1.73 m² at presentation. Of the nine GMP-17 positive patients, and even 60% showed progression of kidney disease. Again, also two out of the seven GMP-17 negative patients showed progression. Despite the small number of patients and time lapse between kidney biopsy and measurement of urine markers, the results of this pilot study certainly raise doubts concerning the previously reported predictive value of GMP-17 positive intra-epithelial T-lymphocytes.

Admittedly, there are important differences between this study and the previous one. First, the current study included a smaller number of patients (24 vs 50), had a different primary outcome (ESRD or a rise in serum creatinine of >50% vs progression defined as a decline of the slope of regression of 1000/serum creatinine of >0.2 units per year), with less patients reaching the actual outcome (8/24, 33% vs 27/50, 54%). Moreover, seven patients in our study were treated with immunosuppression, whereas none were treated in the previous study, and only 9/24 (38%) of our patients had an eGFR ≥60 ml/min/1.73 m² (vs 31/51 62%). Lastly, only two out of our nine patients (22%) with an eGFR ≥60 were GMP-17 positive versus 14 out of 31 patients (45%) in the previous study. This could be due to a difference in the number of tissue slices available for evaluation. Since our study was retrospective and tissue samples had to be obtained from different centres, it is possible that there was simply less renal tissue available for detecting GMP-17 positive cells. Although the above mentioned disparities could account for the lack of a predictive value of GMP-17 positive cells in this study, we feel that the role of GMP-17 positive cells for predicting progression and guiding therapy in individual patients, is limited if there is no data on the presence of GMP-17 positive intra-epithelial T-cells in patients with other types of glomerulonephritis, we evaluated the presence of these cells in six patients with idiopathic membranous nephropathy with normal or near normal kidney function. We found GMP-17 positive T-cells in the kidney tubules in three of these patients, so their occurrence in not specific for tubular injury in IgAN. Naturally, it would be of interest to study their prognostic value in idiopathic membranous nephropathy, or other glomerular diseases.

Altogether, the results of this small retrospective study put the prognostic value of GMP-17 positive T-lymphocytes in IgAN into question. We can state that the occurrence of these cells is not limited to patients with IgAN, since we also observed them in patients with membranous nephropathy.

Ideally, a larger study should be conducted in patients with IgAN with only mild impairment of kidney function.

Acknowledgements.

We thank L.A. Van Es for his participation in this study.
References


Immunosuppressive therapy in patients with IgA nephropathy

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ABSTRACT

Background
There is limited evidence to support cytotoxic therapy in patients with IgA nephropathy (IgAN) and renal insufficiency. We studied the effect of cytotoxic therapy in patients with IgAN and renal insufficiency, and evaluated possible predictors of response.

Methods
Retrospective analysis of patients with IgAN who received immunosuppressive therapy. Primary outcome measure was progression of renal disease, defined as an increase in serum creatinine levels of ≥50% or development of end-stage renal disease (ESRD).

Results
From 1996 to 2008, nineteen patients with biopsy-proven IgAN were treated with cytotoxic agents and prednisone because of renal insufficiency and/or severe proteinuria. Characteristics of patients at start of therapy: age 42±11 years, serum creatinine 208 (96-490) μmol/l, estimated glomerular filtration rate (eGFR) 33 (12-65) ml/min/1.73m², and protein-creatinine ratio 3.8 (0.6-18.2) g/10 mmol. Follow-up after initiation of therapy was 35 (7-133) months. Ten patients had progressive renal disease, whereas eGFR was stable in nine. Serum creatinine levels and proteinuria at the start of treatment were not significantly different between responders and non-responders. Proteinuria response at six months after start of therapy proved a good predictor: proteinuria decreased by ≥50% to levels below 1 g/day in 8/9 responders. In contrast, proteinuria decreased by more than 50% and reached levels <1 g/day in only 3/10 non-responders (p<0.01).

Conclusion
Prolonged immunosuppressive therapy with cytotoxic agents and prednisone may benefit a subgroup of patients with progressive IgAN. A reduction of proteinuria ≥50% to levels below 1 g/day within 6 months predicts a favorable long-term response.

Introduction
IgA nephropathy is the most common glomerular disease worldwide. Its natural course is highly variable and far from benign in many patients. Overall, approximately 25% of patients experience a lasting remission, whereas 30% of patients develop end-stage renal disease (ESRD) within 20 years. Risk factors associated with disease progression include heavy proteinuria, impaired baseline renal function, and renal morphological lesions such as the presence of segmental sclerosis and the degree of tubular atrophy/interstitial fibrosis.

Many patients with IgAN can be effectively treated with Angiotensin Converting Enzyme inhibitors (ACEI) or Angiotensin II Receptor Blockers (ARB) (KDIGO guideline 2012). There is evidence to support the use of corticosteroids in patients with persistent proteinuria and minimal renal impairment. It is debated if patients with IgAN and renal insufficiency benefit from more aggressive treatment consisting of corticosteroids combined with alkylating agents. The evidence is derived from small studies, mostly conducted in a period with less defined blood pressure targets and variable use of ACEI/ARBs as opposed to current guidelines. More specifically, evidence favoring the use of cyclophosphamide in patients with non-crescentic IgAN and severe renal insufficiency is derived from a single small controlled trial. During the last decade, we have used cytotoxic therapy including cyclophosphamide in patients with advanced or severe IgAN. In the present study, we aimed to determine the response to immunosuppressive therapy in patients with progressive IgA nephropathy. Specifically, we sought to identify possible predictors of response.

Methods
Patients
We retrospectively analyzed data of adults with biopsy-proven IgAN who were referred for therapeutic advice to the Radboud University Medical Centre and the Leiden University Medical Centre and were subsequently treated with cytotoxic agents because of progressive IgAN. Patients with evidence of systemic disease, such as systemic lupus erythematoses, chronic liver disease and Henoch-Schönlein purpura or a follow-up of less than 12 months were excluded. Relevant clinical and biochemical data were retrieved from the patients’ records. Baseline was defined as date of first presentation. During follow-up, quantitative analysis of proteinuria was assessed by the ratio of urinary protein to urinary creatinine in the majority of patients. This parameter is closely correlated with 24-hour urinary protein excretion.

This study has been carried out in the Netherlands in accordance with the applicable rules concerning the review of research ethics committees and informed consent.
Treatment protocol

Standard immunosuppressive therapy for IgAN consisted of prednisone 40 mg per day with monthly tapering for a maximum period of 24 months plus cyclophosphamide (1.5 mg/kg) during the first 3 months orally followed by azathioprine (1.5 mg/kg) for 18 months (derived from study treatment protocol by Ballardie et al.18). During prednisone therapy, concomitant treatment with H₂-blockers or proton pump inhibitors was used. Co-trimoxazole was prescribed to prevent Pneumocystis jiroveci infections during the administration of cyclophosphamide. Young women were treated with azathioprine and not with cyclophosphamide because the latter is associated with infertility. Severe leukopenia, infections or active malignancy were regarded as stringent reasons to discontinue immunosuppressive treatment.

Assessment of response

Primary outcome measure was progression of renal disease defined as an increase in serum creatinine levels of ≥ 50% or development of ESRD (serum creatinine ≥ 500 μmol/l, dialysis or renal transplantation). Proteinuria responses were evaluated in terms of a reduction in proteinuria of ≥ 50% or a persistent urine protein-creatinine ratio < 1g/10 mmol.

Calculations

The glomerular filtration rate was estimated (eGFR) by the simplified Modification of Diet in Renal Disease (MDRD) formula(21). Mean arterial pressure (MAP) was calculated as the diastolic pressure plus one third of the pulse pressure.

Statistical Analysis

Data values are expressed as mean± standard deviation or median (range) as appropriate. Parameters between groups were compared using the Mann-Whitney test for non-parametric continuous data, the independent t-test for parametric data, and the chi-square test for categorical data. A p-value of less than 0.05 was considered to be statistically significant. Statistical analysis was performed using SPSS for windows software, version 20 (IBM SPSS).

Results

Baseline characteristics

Between 1996 and 2008 19 patients received immunosuppressive therapy because of progressive IgA nephropathy.

Standard immunosuppressive treatment consisting of prednisone plus cyclophosphamide during the first 3 months orally followed by a cytotoxic agent was prescribed in 11 out of 19 patients. Five patients received cyclophosphamide without subsequent alternative cytotoxic therapy. This was due to side-effects (leucopenia, infection) or failure of improvement of already severely impaired renal function (n=1). Three patients were started on azathioprine plus prednisone instead of cyclophosphamide plus prednisone. Clinical characteristics at presentation are depicted in Table 1.

At presentation mean age was 39±11 years, median serum creatinine was 128 μmol/l, a median eGFR was 55 ml/min/1.73m², and median proteinuria was 4.5 g/10 mmol creatinine. All patients but one were treated with ACEi or ARBs prior to the initiation of immunosuppressive therapy. Three patients had previously been treated with prednisone monotherapy. In the majority of patients therapy was started because of an increase in serum creatinine of 15% or more in the previous 12 months (n=12), or because of a serum creatinine > 135 μmol/l and proteinuria > 1 gram per day (n=6). In one patient therapy was initiated because of severe impairment of renal function at time of presentation.

The median time between baseline and start of therapy was 19 months. At start of therapy, patients were 42±11 years and had a median serum creatinine of 208 μmol/l, a median eGFR of 33 ml/min/1.73m², and a median urine protein-creatinine ratio of 3.8 g/10 mmol (Table 1).

Table 1. Clinical characteristics of patients receiving cytotoxic therapy

<table>
<thead>
<tr>
<th></th>
<th>Presentation</th>
<th>Start of therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Male /female</td>
<td>14/5</td>
<td>14/5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>39±11</td>
<td>42±11</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>100±10</td>
<td>106±10</td>
</tr>
<tr>
<td>Serum creatinine (μmol/l)</td>
<td>128 (69-285)</td>
<td>208 (86-490)</td>
</tr>
<tr>
<td>Creatinine (mg/10 mmol creatinine)</td>
<td>4.5 (0.9-18.2)</td>
<td>3.8 (0.6-18.2)</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73m²)</td>
<td>55 (22-87)</td>
<td>33 (12-65)</td>
</tr>
<tr>
<td>Use of ACEis or ARBs (%)</td>
<td>89</td>
<td>95</td>
</tr>
<tr>
<td>Change in serum creatinine (%)</td>
<td>-</td>
<td>39(-19-209)</td>
</tr>
<tr>
<td>Change in UP/UCr (%)</td>
<td>-</td>
<td>-21 (-70-163)</td>
</tr>
<tr>
<td>Time from baseline to start of therapy (months)</td>
<td>-</td>
<td>19 (0.2-119)</td>
</tr>
<tr>
<td>Follow-up after start of therapy (months)</td>
<td>-</td>
<td>35 (7-133)</td>
</tr>
</tbody>
</table>

Data expressed as median (range) or mean±SD, MAP; mean arterial pressure, UP; urinary protein, UCr; urinary creatinine, eGFR; estimated glomerular filtration rate, ACEis; angiotensin converting enzyme inhibitors, ARBs; angiotensin II receptor blockers
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Immunosuppressive therapy in IgAN

Clinical outcome

Follow-up after initiation of therapy was 35 (7-133) months. In 10 patients renal disease was progressive despite the initiation of immunosuppressive therapy: 6 developed end-stage renal disease at a median follow up of 24 months (range 7-46) and 4 experienced doubling of serum creatinine after 17 (range 12-81) months. Renal function remained stable or improved in 9 patients.

Serum creatinine levels and proteinuria at the start of treatment were lower in responders (183 (120-381) versus 224 (96-490) μmol/l, p=0.23 and 2.2 (0.6-9.7) versus 4.1 (0.7-18.2) g/10 mmol creatinine, p=0.31 respectively), but these differences were not statistically significant (Table 2). There were no differences in other characteristics at baseline between responders and non-responders (Table 2). In 8 out of 9 responders proteinuria decreased by ≥ 50% within 6 months and attained levels below 1 g/day in 8 patients (figure 1A). In contrast, in non-responders proteinuria decreased by more than 50% and reached values < 1g/day in only 3 out of 10 patients (p<0.01, Figure 1B).

Table 3. Overview of treatment schedule and progressors/ non-progressors

<table>
<thead>
<tr>
<th>Treatment schedule</th>
<th>Progressors (n=10)</th>
<th>Non-progressors (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard course, i.e. prednisone plus cyclophosphamide for 3 months, followed by azathioprine, n=11</td>
<td>n=6 (54%)</td>
<td>n=5 (46%)</td>
</tr>
<tr>
<td>Shortened course, i.e. cyclophosphamide and prednisone ≤ 3 months without subsequent conversion to azathioprine, n=3*</td>
<td>n=2 (40%)</td>
<td>n=3 (60%)</td>
</tr>
<tr>
<td>Alternative, i.e. prednisone and azathioprine instead of cyclophosphamide, n=3*</td>
<td>n=2 (67%)</td>
<td>n=1 (33%)</td>
</tr>
</tbody>
</table>

*4 patients were treated for a total of 2.5-3 months with cyclophosphamide and prednisone, 1 patient was treated for a longer period thereafter with prednisone monotherapy, tduration of treatment at least 9 months

Data expressed as median (range) or mean ± SD. MAP; mean arterial pressure, UP; urinary protein, UCr; urinary creatinine, eGFR; estimated glomerular filtration rate. *the same patients

Treatment duration was 19 (range 3-81) months. Treatment duration was less than 12 months in two of the responders, and in 4 of the non-responders (table 3). Following the discontinuation of therapy, we observed no relapses of proteinuria.

Six patients had a serum creatinine level ≥250 μmol/l (the so-called point of no return) prior to start of therapy. In this small subgroup renal function remained stable after treatment in two patients during a follow-up of more than 8 years. The remaining four patients developed ESRD within 21-46 months after initiation of treatment.

Table 2. Clinical characteristics of progressors versus non-progressors at start of treatment with cytotoxic agents

<table>
<thead>
<tr>
<th>Variable</th>
<th>Progression of renal disease</th>
<th>Stable renal function</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (male/female)</td>
<td>7/3</td>
<td>7/2</td>
<td>0.70</td>
</tr>
<tr>
<td>Age (years)</td>
<td>44±14</td>
<td>40±7</td>
<td>0.42</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>104±10</td>
<td>104±10</td>
<td>0.96</td>
</tr>
<tr>
<td>UP/UCr (g/10 mmol creatinine)</td>
<td>4.1 (0.7-18.2)</td>
<td>2.2 (0.6-9.7)</td>
<td>0.31</td>
</tr>
<tr>
<td>Serum creatinine (μmol/l)</td>
<td>224 (96-490)</td>
<td>183 (120-381)</td>
<td>0.23</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73m²)</td>
<td>26 (12-65)</td>
<td>36 (17-46)</td>
<td>0.21</td>
</tr>
<tr>
<td>Duration of treatment (months)</td>
<td>16 (3-81)</td>
<td>22 (3-63)</td>
<td>0.50</td>
</tr>
<tr>
<td>Duration of follow-up after treatment</td>
<td>26 (6-81)</td>
<td>82 (28-133)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>50% reduction in proteinuria ≤ 6 months after start of therapy (n)</td>
<td>3*</td>
<td>8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Persisting proteinuria &lt; 1g/10mmol creatinine (n)</td>
<td>3*</td>
<td>8</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Data expressed as median (range) or mean ± SD. MAP; mean arterial pressure, UP; urinary protein, UCr; urinary creatinine, eGFR; estimated glomerular filtration rate. *the same patients

Figure 1A. Change in proteinuria after start of immunosuppressive therapy in patients with IgA nephropathy who attained a stable eGFR (so called responders).

Figure 1B. Change in proteinuria after start of immunosuppressive therapy in patients with IgA nephropathy who showed progression of renal disease.
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Immunosuppressive therapy in IgAN

**Chapter 5 Immunosuppressive therapy in IgAN**

**Adverse events**

Adverse events were reported in 11 patients (58%) and included anemia (n=3), leucopenia (n=4), thrombocytopenia (n=1), infection (n=4) or liver toxicity (n=1). In 2 patients this led to discontinuation of immunosuppressive therapy. No patients developed a malignancy during follow-up.

**Discussion**

This study suggests that immunosuppressive therapy may result in stabilization of renal function in approximately 50% of patients with IgA nephropathy at high risk for progression of renal disease. More importantly, a decrease in proteinuria of 50% or more within six months predicted a sustained response to therapy.

Over two decades, beneficial effects of corticosteroids have been reported by various studies in patients with no or mild renal impairment. However, in patients with advanced IgA nephropathy, administration of corticosteroids did not improve renal function. Few randomized controlled trials, enrolling a small number of patients, have been conducted investigating the efficacy of cytotoxic agents combined with prednisone. Only the study performed by Ballardie et al. showed that prednisone plus cytotoxic agents were effective in preserving renal function in patients with moderate, progressive disease. Thus, the optimal management of patients with advanced IgA nephropathy remains controversial.

Clearly, this small, retrospective and uncontrolled study cannot provide compelling evidence for benefit of corticosteroids and cyclophosphamide or azathioprine in high-risk IgAN patients. Yet, our data support the results reported by Ballardie; based on observational data on known major risk factors, at least 70-90% of this study population characterized by heavy, persistent proteinuria (median 3.1 g/d) and severe impairment of renal function (median serum creatinine 208 µmol/l) at the start of treatment with immunosuppressive drugs, would be expected to progress to ESRD. However, after immunosuppressive therapy progression of disease was halted and renal function remained stable in approximately 50% of patients.

Thus, immunosuppressive combination therapy may benefit IgAN patients with poor prognosis. Unfortunately, side effects are frequently observed, and duration of treatment is long and 50% of patients are exposed to cytotoxic agents and prednisone to no avail. Therefore, immunosuppressive therapy seems to benefit only a subset of high-risk patients with IgAN. Currently it is impossible to identify this subgroup by means of clinical or pathological characteristics. Considering this, our finding that a 50% decrease in proteinuria within six months after start of therapy is indicative of a sustained response to immunosuppressive therapy is highly relevant. In patients lacking a substantial decrease in proteinuria after 6 months of therapy, discontinuation of treatment should strongly be considered in our opinion. Recently, it has been recognized that patients with IgA nephropathy have increased serum levels of galactose deficient IgA1 and that circulating auto-antibodies recognizing those galactose deficient IgA1 as an autoantigen or the levels of the autoantigen itself allow prediction of disease progression. Perhaps these and other promising biomarkers will allow identification of patients responsive to immunosuppressive therapy in the near future.

The results of this retrospective study differ to some extent from the data reported by Ballardie et al. A substantially larger number of patients suffered from therapy-related side effects in our study compared with the frequency reported by Ballardie and others (58% versus 7-12%). Although these side effects were mild and transient in the majority of cases, 2 patients withdrew from treatment. Second, several studies including Ballardie’s, reported that patients with a serum creatinine level ≥250 µmol/l inescapably develop ESRD despite therapy. After therapy, we observed a stabilization of renal function in 2 out of 6 patients beyond this so-called point of no return. Thus, even in patients with severe renal impairment, immunosuppressive therapy may be of benefit.

In conclusion, the optimal management of IgAN remains controversial and randomized controlled trials are much needed. Currently, a large randomized prospective controlled trial (STOP IgAN trial) is being conducted in order to determine whether immunosuppressive therapy is able to prevent progression of renal disease. The KDIGO Clinical Practice Guideline for Glomerulonephritis suggests corticosteroid therapy in patients with persistent proteinuria >1g/d despite 3-6 months of optimized supportive care (including ACEi/ARB) and eGFR >50ml/min. Combined immunosuppressive therapy is not advised unless there is crescentic IgAN with rapidly deteriorating renal function. In our opinion, immunosuppressive therapy can certainly be considered in IgAN patients with a decline in renal function and persistent proteinuria >1 g/d. Immunosuppressive therapy should only be continued beyond 6 months in those patients demonstrating a significant (>50%) decrease in proteinuria.

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References


Intra-individual variability of serum hepcidin-25 in haemodialysis patients using mass spectrometry and ELISA

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Nephrology Dialysis and transplantation 2012;27:3923-3929
ABSTRACT

Background
Measurement of serum hepcidin levels may provide a useful alternative to the current methods of determining iron status in chronic haemodialysis patients. However, the biological variability of this pivotal regulator of iron homeostasis is unclear, and the impact of inflammation, dialysis clearance, and iron therapy on hepcidin variability has not been established.

Methods
Two independent studies in chronic haemodialysis patients were conducted; serum hepcidin levels were measured at the start of dialysis sessions in 20 UK patients and in 43 Dutch patients by mass spectrometry (MS). Samples from UK patients were also analysed by a competitive enzyme-linked immunosorbent assay (cELISA). Coefficient of variance (CV) was calculated and potential factors affecting CV were also examined.

Results
The median CV (interquartile range) was 23% (17-28) for the UK MS, 26% (17-48) for the Dutch MS, and 23% (17-39) for the UK cELISA. The CV was similar in those patients receiving and those not receiving regular intravenous iron. The CV was not associated with the degree of inflammation. Hepcidin levels were higher following an inter-dialytic period of 3 versus 2 days (p=0.02).

Conclusions
These findings suggest considerable variability of serum hepcidin levels in haemodialysis patients. Inflammation and the use of iron did not impact on the degree of variability, and hepcidin levels were higher after an inter-dialytic period of 3 versus 2 days. These findings need to be taken into account in future studies assessing the utility of serum hepcidin as a guide to the use of iron or erythropoiesis stimulating agents.

Introduction
Hepcidin is a recently discovered small defensin-like peptide that regulates iron metabolism. Hepcidin degrades the cellular iron exporter ferroportin, which is expressed by enterocytes and macrophages, thereby decreasing intestinal iron uptake and causing iron sequestration in the reticulo-endothelial system. Hepcidin synthesis is upregulated in the presence of inflammation or iron overload and is reduced in the presence of anaemia, hypoxia and iron deficiency. Thus, inflammation decreases the availability of circulating iron, whereas hypoxia or anaemia increases iron release and absorption.

Chronic haemodialysis (HD) patients have been shown to have elevated hepcidin levels. This may partly explain why such patients have reduced erythropoiesis despite treatment with erythropoiesis stimulating agents (ESA) and a seemingly adequate iron status. Traditional markers of iron status are inaccurate for the detection of iron insufficiency in this population, since patients with normal or high ferritin levels and transferrin saturation (TSAT) still respond to intravenous (IV) iron. Moreover, these conventional parameters do not correlate well with bone marrow iron stores. Iron therapy might be a double-edged sword; although iron is necessary for erythropoiesis, excess iron promotes oxidative stress and affects immune effector function. Hepcidin has several potential advantages over conventional iron parameters such as ferritin in optimizing anaemia treatment in patients with renal insufficiency; (1) it directly reflects iron availability and demands for erythropoiesis, (2) it integrates the input from both inflammatory and erythropoietic pathways and (3) it reflects the status of iron homeostasis more accurately than single parameters such as TSAT and soluble transferrin. Hepcidin could thus become an important tool to predict ESA responsiveness and guide treatment with ESA and iron.

The potential role of serum hepcidin was questioned by the recent observations of Ford et al., who reported considerable intra-individual variability of serum hepcidin levels obtained at weekly intervals in a small number of HD patients. The authors noted a relationship with the inflammatory status. Importantly, hepcidin was measured using an ELISA assay, which is known to detect not only hepcidin-25 but also its isoforms hepcidin-20 and 22. These biologically inactive isoforms have been reported to accumulate in patients with end-stage renal disease (ESRD). Thus, the intra-individual variability reported by Ford et al. may partly be attributable to variability of these smaller isoforms.

In order to meaningfully interpret (changes in) serum hepcidin-25 levels, it is essential to obtain additional data on the variability of serum hepcidin-25, and not total hepcidin levels in HD patients. We have therefore extended these observations using a validated LC MS/MS assay, and also compared the results with a commercially available ELISA (Bachem, UK). Mass spectrometric techniques have been utilized for the quantitation of hepcidin isoforms. Such mass spectrometry assays are generally regarded as the “gold standard”, but are more technically demanding. The use of MS circumvents inherent problems in the
analysis since it allows the absolute levels of hepcidin-25 to be assessed.

Our aim was to determine the intra-individual variability of serum hepcidin in a cohort of haemodialysis patients by measuring the coefficient of variance (CV) for hepcidin using different assays. Furthermore, we aimed to identify significant determinants of intra-individual variability. Independent studies were designed and conducted in the UK and the Netherlands. Due to substantial differences in data collection, fully combining the analysis proved impossible. Therefore, the methods and results of the UK and Dutch study will be discussed separately.

Materials and methods, part I – UK study

Study design

All patients dialyzing on two successive days were invited to participate. Patients were excluded if they demonstrated any signs of acute or occult bleeding, had an acute bacterial infection within 4 weeks, a haematological dyscrasia other than anaemia, acute or chronic liver disease, or an active malignancy.

Nine consecutive pre-dialysis blood samples were scheduled over a period of 3 weeks during October 2010 at the Department of Renal Medicine, King’s College Hospital, London, UK. Two major factors known to affect serum hepcidin levels were prospectively assessed; the administration of IV iron and the degree of inflammation. High-sensitivity C-reactive protein levels (hsCRP) were measured at each of the 9 time-points. A single mid-study sample was taken from each patient for measurement of serum ferritin from each patient, at least one week after any administration of IV iron. The data were also examined according to the day of the week on which the samples were taken, to detect the influence of the inter-dialytic period. 10 patients were dialyzed on Mondays, Wednesdays and Fridays, and 10 were dialyzed on Tuesdays, Thursdays and Saturdays. The results obtained on a Monday or Tuesday were pooled (Mon/Tue), since these samples were taken after an inter-dialytic period of 3 days. Similarly, samples taken on a Wednesday or Thursday (Wed/Thurs) were pooled, as were samples taken on a Friday or Saturday (Fri/Sat).

All patients gave informed consent for participation in the study, which was conducted in accordance with the Declaration of Helsinki, and was approved by the London Research Ethics Committee 1 (LREC 09/H0718/034).

Sample analysis

All samples were drawn from the dialysis catheter or fistula immediately before the start of dialysis. The samples were centrifuged, and aliquots of sera were immediately stored in cryotubes at -80°C. Samples were then transferred to two separate laboratories (King’s College London, UK, and the University of Bialystok, Poland), for measurement of serum hepcidin levels (LC MS/MS and a commercially available ELISA, respectively). The samples were processed in a single run by two different operators. High-sensitivity Creactive protein (hsCRP) levels were determined using turbidimetry (P.Z. Cormay, Lublin, Poland).

Hepcidin levels were measured using a high-performance liquid chromatography tandem mass spectrometry technique, which has an intra-assay CV of 2.3% at hepcidin concentrations of 156 ng/ml and 4.0% at concentrations of 312 ng/ml.

The same samples were analysed by ELISA (Bialystok, Poland) using a commercially available kit (Bachem, UK). The reported normal range for this assay is 0.02-25 ng/ml, and both the calculated intra- and inter-assay variations are below 10%.

Statistical analysis

The CV (hepcidin) for every individual was calculated from all 9 hepcidin values acquired for each patient. The CV was defined as the standard deviation divided by the mean. The median and mean CV were calculated to determine and compare the variability in serum hepcidin for both assays.

The CV values of those receiving IV iron therapy were compared to those not receiving iron. High-sensitivity CRP values were correlated with the corresponding hepcidin values. Hepcidin values obtained after an inter-dialytic period of 3 days were compared to the values after an inter-dialytic period of 2 days.

Data were expressed as mean (±SD) or median (IQR – interquartile range) where appropriate. The Shapiro-Wilks test was used to test for normality of distribution. Comparisons between groups were performed using either a paired Student’s t-test or Wilcoxon rank sum for normally and non-normally distributed variables respectively. Bivariate correlation coefficients (r) were calculated using the Pearson’s product formula. A p-value of <0.05 was considered statistically significant.

Analyses were performed using Statistical Package for Social Science version 17.0 for Windows XP (SPSS Inc., Chicago Ill., USA).
Materials and methods, part II – Dutch study

Study design
The objective of the study was to assess intra-individual variability in serum hepcidin-25 concentration over 6 weeks in chronic HD patients and to identify significant determinants. Blood samples were collected once a week during 6 consecutive weeks, before the start of dialysis. Samples were collected from August to October 2010 at the Department of Nephrology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands. In order to obtain unbiased data we included all patients who agreed to participate. ESA and IV iron administration were continued according to current prescription during the study period.

In accordance with Dutch ethical regulations, all patients consented that blood samples would be used for medical research.

Sample analysis
All samples were drawn from the dialysis catheter or fistula immediately before the start of dialysis, and were processed and stored in polypropylene tubes at -80°C. Routine laboratory parameters and hepcidin levels were measured within 8 hours and 6 months of collection, respectively.

Serum hepcidin was measured by a combination of weak cation exchange chromatography and time-of flight mass spectrometry (TOF MS) as recently described. The intra-run coefficient of variance is 2.2% at 8.6 ng/ml, 3.7% at 22 ng/ml, 2.3% at 37.4 ng/ml. The inter-run coefficient of variance is 9.1% at 21.8 ng/ml and 3.9% at 36 ng/ml. Of note, all samples from an individual patient were measured in a single run. The median reference level of serum hepcidin-25 is 11.7 ng/ml, with a range of 1.4-38.8 ng/ml. All other laboratory parameters were measured using standard automated techniques.

Statistical analysis
Intra-individual variability in serum hepcidin and other variables was expressed as the coefficient of variance (CV, the standard deviation divided by the mean). Week-to-week CVs were calculated for each patient, as well as the mean, median, and range of CVs for serum hepcidin isoforms, haemoglobin, CRP, iron, TSAT and ferritin. Parameters between high and low variability (i.e. above and below median CV) groups were compared using the independent t-test for parametric data and the chi-square test for categorical data.

Correlation coefficients were used to assess associations between laboratory parameters. Correlations were calculated using Pearson’s correlation test. If data were skewed, log transformation was used. To determine independent baseline predictors of the intra-individual variability of serum hepcidin, multiple regression analysis was applied.

Predictors that showed high collinearity (rho > 0.8) were not simultaneously included in the analysis. Potential non-linear dose response relationships were checked using fractional polynomials. Robustness of the various models was checked with a jackknife resampling technique. The unicity of each regression solution was evaluated by interchanging highly correlated baseline predictors. A p-value <0.05 was considered significant for all analyses.

Results, part I – UK study
Patient demographics and baseline laboratory data are summarized in Table 1. 20 chronic HD patients (14 males) aged between 35 and 85 years were included. Each patient underwent dialysis three times weekly. Patients had been receiving haemodialysis for a median of 11.7 years, with a range of 2-37 years.

Table 1 – Demographics and baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>UK study</th>
<th>Dutch study</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (% male)</td>
<td>20 (70)</td>
<td>43 (56)</td>
</tr>
<tr>
<td>age (years)</td>
<td>63 ± 16</td>
<td>62 ± 15</td>
</tr>
<tr>
<td>Caucasian (%)</td>
<td>45</td>
<td>98</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26 ± 4.8</td>
<td>24 ± 3.8</td>
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<tr>
<td>antibiotic use previous month (%)</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>malignancy in history (%)</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>liver disease (%)</td>
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</tr>
<tr>
<td>ESA (%)</td>
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<td>98</td>
</tr>
<tr>
<td>ESA dose (x 1000 units)</td>
<td>6 (4-9)</td>
<td>12 (8 – 15)</td>
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<tr>
<td>iron supplementation (%)</td>
<td>60</td>
<td>95</td>
</tr>
<tr>
<td>iron dose (mg/week)</td>
<td>100 (100-150)</td>
<td>50 (25 – 50)</td>
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<tr>
<td>Laboratory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepcidin 25 (ng/ml)</td>
<td>168.2 (127.9-217.6)</td>
<td>46.7 (28.2 – 73.4)</td>
</tr>
<tr>
<td>Hepcidin 20 (ng/ml)</td>
<td>n/a</td>
<td>13.1 (8.9 – 19.2)</td>
</tr>
<tr>
<td>Ferritin (μg/l)</td>
<td>591.5 (321.9-861.1)**</td>
<td>376 (209 – 503)</td>
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<td>iron (μmol/l)</td>
<td>55.9 (39.1 – 72.6)</td>
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<td>transferrin saturation (%)</td>
<td>19.5 (16.7 – 30.2)</td>
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<tr>
<td>CRP (mg/l)</td>
<td>5.4 (3.8-18.9)</td>
<td>5 (5 – 24)</td>
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<tr>
<td>Hb (g/dl)</td>
<td>10.9 (9.8-11.8)</td>
<td>11.4 (10.5 – 11.9)</td>
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<tr>
<td>CV</td>
<td></td>
<td></td>
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<tr>
<td>Hepcidin-25 (%)</td>
<td>23 (17 – 28)</td>
<td>26 (17 – 48)</td>
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<tr>
<td>Hepcidin-20 (%)</td>
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<td>13 (9 – 21)</td>
</tr>
<tr>
<td>Ferritin (%)</td>
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<td>12 (11 – 26)</td>
</tr>
<tr>
<td>iron (%)</td>
<td>n/a</td>
<td>19 (14 – 30)</td>
</tr>
<tr>
<td>transferrin saturation (%)</td>
<td>20 (13 – 29)</td>
<td></td>
</tr>
<tr>
<td>CRP (%)</td>
<td>43.2 (26.9-58.1)</td>
<td>41 (8 – 63)</td>
</tr>
<tr>
<td>Hb (%)</td>
<td>n/a</td>
<td>4 (3 – 5)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sd and median (interquartile range). BMI; body mass index, ESA; erythropoietin stimulating agents, CRP; C reactive protein, Hb; haemoglobin, CV; coefficient of variance. To convert hepcidin-25 from ng/ml to nanomol/l divide by 2.789.
mean of 4.1 ±4.6 years, and all were stable for at least 3 months. All except one patient were
dialyzed in the afternoon. All except one patient had been on a stable dose of ESA for >4
weeks. Eight patients (40%) had received no IV iron for 4 weeks prior to commencement
of the study, and this was maintained for the 3-week study period.

*Intra-individual variability*

Both assays revealed a considerable degree of biological variability in this patient cohort
(Figure 1). The median CV, for the mass spectrometry assay was 23% (IQR 17-28), and for
the ELISA was 23% (IQR 17-39) (p=0.12). A CV, of less than 20% was seen in only 40% of
the patients for both assays, a CV, of 20-25% was found in 15% for both assays, a CV, of
25-30% was seen in 25% (MS) and 10% (ELISA), and a CV, of >30% was seen in 20% (MS)
and 35% (ELISA).

**Figure 1.** The coefficient of variance (CV1) of serum hepcidin-25 in 20 UK patients, suing MS and ELISA.

*Intermethod difference in hepcidin values*

The mass spectrometry assay used in this study previously reported serum hepcidin
levels of 4.6 ± 2.7 nmol/L (12.8 ± 7.5 ng/ml) in normal healthy individuals. In the cohort of
haemodialysis patients recruited to the present study, hepcidin levels were considerably
greater (mean 172 ± 70 ng/ml; median of 168 ng/ml (IQR128-218)). The data for the Bachem
ELISA were similar (mean 230±557;median 170 ng/ml (IQR 107-239 ng/ml), p=0.03).

**The effect of intravenous iron administration on CV1 and hepcidin levels**

The CV, was not affected by the administration of intravenous iron (Table 2). The median
CV, (MS) for hepcidin in the 8 patients who did not receive iron therapy was 26% (IQR 19-
28) compared to 23 % (IQR 2-32) in those who were receiving regular IV iron (p = 0.77). For
individual hepcidin profiles for these 8 patients see supplementary figure A. Similar results
were found using the ELISA assay: median CV, for the group not receiving iron was 23 %
(IQR 15-34) versus 23 % (IQR 17-57) for the IV iron group (p = 0.29).

Of the 12 patients receiving IV iron supplementation, 11 were having iron administered
on a weekly basis across haemodialysis. This provided an opportunity to compare
hepcidin levels across three time-points: immediately before administration of IV iron, and
immediately prior to the next two dialysis sessions. There was a small (non-significant)
increase in serum hepcidin in the sample taken before the next dialysis session, but
overall there was no clear impact of IV iron on serum hepcidin, possibly due to the fairly
modest dose administered (100 mg) and the high background levels of serum hepcidin.

One patient received IV iron during every dialysis session, but his hepcidin CV, values
were similar to those of the other patients: 25.6% (MS) and 20.0% (ELISA).

**Effect of inflammatory status on CV1 and hepcidin levels**

High sensitivity CRP levels were determined in all samples: median 6.1 mg/L (1.1, 18.9) with
a range of 0.1-140.8 mg/L. The correlation between hepcidin (MS) and hsCRP levels in this
analysis was weak (r= 0.15; p=0.04) (Figure 2). Mean serum hepcidin levels were significantly

![Figure 2. Correlation between hepcidin (MS assay) and high-sensitivity CRP (log transformed) in UK patients.](image-url)
increased in the highest hsCRP tertile when compared to the lowest hsCRP tertile (190.4 ± 91.1 versus 161.1 ± 50.7 ng/mL, respectively (p=0.04). We found no correlation between the CV, for hsCRP and the CV, for hepcidin (MS: r = 0.38, p = 0.10, ELISA: r = -0.18, p = 0.44).

**Effect of inter-dialytic interval on serum hepcidin**

Hepcidin levels were higher following a 3-day inter-dialytic interval compared to a 2-day inter-dialytic interval (ANOVA; p =0.02) (Figure 3). Both assays demonstrated the highest median hepcidin levels on the first day after the weekend (182.8 (138.8-235.0) and 184.1 (111.9-267.7)) ng/ml with MS and ELISA, respectively.

No relationship was found between serum hepcidin and dialysis quantity, haemoglobin, erythropoietin dosage, or serum ferritin levels (data not shown).

**Results, part II – Dutch study**

Patient demographics and baseline laboratory data are summarized in Table 1. We included 43 consecutive chronic HD patients (24 males), aged between 24 and 83 years. 36 patients received haemodialysis three times a week for ~4 hours, 22 patients were dialyzed in the morning, whereas 14 patients were dialyzed in the afternoon. Seven patients were treated with nocturnal dialysis four times a week for ~8h. Median time on dialysis was 2.3 years. The majority of patients were treated with IV iron sucrose (n=41) and epoetin beta (n=42). Iron sucrose was given once weekly, and blood samples were drawn at the start of the session during which IV iron was administered.

**Intra-individual variability**

One patient died during the study; for this patient only 5 samples were available for analysis. For the remaining 42 subjects all 6 samples were collected according to the study protocol. The median intra-individual coefficient of variation (CV) of hepcidin-25 levels over 6 weeks was 26% (IQR 17-48). CV, was higher in patients with low levels of serum hepcidin (Figure 4) and serum ferritin (Figure 5). Median CVs of ferritin and CRP were 12% (IQR 11-26) and 41% (IQR 8-63), respectively.

**Figure 4. Correlation of coefficient of variance (CV1) with baseline hepcidin-25 levels in Dutch patients.**

**Figure 5. Correlation between baseline ferritin and intra-individual coefficient of variance (CV1) of serum hepcidin-25 in Dutch patients.**
Determinants of intra-individual variability

Comparison of patients with intra-individual variability of hepcidin-25 above (high variability) and below (low variability) the median CV showed that patients with high intra-individual variability had significantly lower baseline hepcidin and ferritin levels and lower TSAT (Table 3). CRP levels were not significantly different between these 2 groups.

By multivariate regression analysis, we found baseline ferritin and CV of TSAT, but not CRP, to be independent predictors of intra-individual hepcidin-25 variability (R²=0.54). Because of collinearity, baseline ferritin can be substituted by baseline hepcidin and CV of TSAT by CV of iron. In the majority of patients there was no correlation between hepcidin-25 and ferritin, iron, TSAT, CRP, or haemoglobin (Figure 6). In the individual patient, change in hepcidin-25 cannot be predicted from change in ferritin or CRP (data not shown).

Limiting the analysis to patients with normal CRP levels did not decrease CV of serum hepcidin-25 levels: median CV, 22% (IQR 16-34). Likewise, repeating the analysis after exclusion of patients with hepatitis C (n=2), prostate carcinoma (n=1), blood loss or receiving blood transfusion in the month before or during the study (n=9), or patients who received an antibiotic in this period (n=8) did not alter the results (median CV of serum hepcidin-25 levels 27% (IQR 17-48)).

Hepcidin-25 variability was also not dependent on the time of dialysis, as illustrated by a median CV of serum hepcidin-25 levels of 34% (IQR 17-75) in patients on nocturnal dialysis compared to a median CV of 26% (IQR 17-31) in patients on diurnal dialysis (p=0.40).

Discussion

The results of these studies suggest that there is significant intra-individual variability of serum hepcidin in chronic haemodialysis patients, which is consistent with a previous report by Ford et al.² The study by Ford et al. had several limitations; the authors studied a small number (n=28) of predominantly African-American dialysis patients, and they...
did not report timing of the blood sample and the duration of the inter-dialytic interval. However, the most important limitation of this study by Ford et al. was the use of an ELISA assay to measure hepcidin. This is known to cross-react with hepcidin isoforms, and the contribution of this lack of specificity to their findings of hepcidin variability was impossible to ascertain. In the current studies however, we utilized specific MS assays for hepcidin-25, and confirmed significant hepcidin variability. We also attempted to examine factors that could potentially influence hepcidin levels (IV iron and the degree of inflammation).

The hepcidin results obtained using MS were generally lower than those obtained using the ELISA. This is likely to be due to the fact that the ELISA is detecting the smaller isomers of hepcidin as well as hepcidin-25. Nevertheless, as in previous studies, hepcidin levels in haemodialysis patients were consistently elevated compared to healthy individuals. Both assays showed a similar degree of hepcidin variability, and the median CV values were almost identical for MS versus ELISA.

Higher hepcidin levels (MS) were seen following a 3-day inter-dialytic interval versus a 2-day interval, possibly due to greater generation of hepcidin during the longer dialysis free period.

It has previously been demonstrated that inflammation (acute and chronic), IV iron, and erythropoietin administration influence hepcidin levels. In our parallel studies, we were unable to demonstrate a convincing association between CRP and hepcidin levels, in contrast to the findings of Ford et al. and the multivariate analyses by Zaritsky et al. Interestingly, when using a different MS assay and a different study design, very similar CV values (26%) were observed in the Dutch cohort to those seen in the UK study (24%).

We previously suggested that measurement of serum hepcidin might have a role in predicting CKD patients’ response to IV iron therapy. This was investigated by Tessitore et al. in 56 haemodialysis patients. Serum hepcidin did not predict the response to IV iron loading. In addition, it was demonstrated that mean serum hepcidin levels did not change following IV iron loading, confirming our findings and also those of Weiss et al. It is possible, therefore, that these negative results may be partly due to significant hepcidin variability.

It is presently unclear if the variability is related to specific, hitherto unexplained features that are characteristic of the dialysis procedure. Future studies must evaluate CV, in predialysis patients to answer this question. The large and unexplained variability suggests that it may be difficult to use serum hepcidin-25 levels to guide treatment of anaemia in the individual patient. However, we caution against premature conclusions. Measurements of serum hepcidin-25 in blood samples collected at shorter time intervals, and with and without administration of ESA and IV iron are needed to assess the role of serum hepcidin-25 in HD patients.

In conclusion, we have independently demonstrated that variability in serum levels of hepcidin-25 in haemodialysis patients exceeds 20%, even when using an MS assay. No major impact of IV iron or inflammatory status was observed, although a minor effect of length of intra-dialytic interval was seen. These data have implications for studies examining factors affecting hepcidin levels, and suggest that the utility of serum hepcidin as a marker of iron status in haemodialysis patients is limited.
References


Serum hepcidin-25 levels in patients with chronic kidney disease are independent of glomerular filtration rate

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* Both authors contributed equally.
**ABSTRACT**

**Background**

Hepcidin is a key regulator of iron homeostasis and levels are elevated in patients with chronic kidney disease (CKD). Hepcidin may explain the often observed imbalance in iron metabolism in patients with CKD. We evaluated the influence of estimated glomerular filtration rate (eGFR) on serum levels of hepcidin-25 and its isoforms in patients with renal dysfunction.

**Methods**

Serum levels of the biologically active hepcidin-25 and its isoforms were determined in CKD and dialysis patients by a mass spectrometry-based assay.

**Results**

In 83 patients with CKD not requiring dialysis, serum hepcidin-25 levels were not significantly increased (5.1 nM versus 4.2 nM in controls, \( p = 0.30 \)) and were positively correlated with ferritin (\( r = 0.74, p < 0.01 \)). Multiple regression analysis showed ferritin to be the only significant predictor of hepcidin-25 levels. Serum hepcidin-25 levels were not dependent on eGFR. In contrast, hepcidin-20 and total hepcidin levels showed an independent significant inverse correlation with eGFR. In 48 haemodialysis patients, median hepcidin-25 levels were significantly higher than in CKD patients (9.4 nM versus 5.1 nM, \( p < 0.001 \)) and again strongly correlated with ferritin (\( r = 0.79, p < 0.001 \)).

**Conclusions**

eGFR is not a major determinant of serum hepcidin-25 levels. In contrast, the hepcidin isoforms hepcidin-20 and hepcidin-22 accumulate in patients with renal impairment.

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**Introduction**

Hepcidin is a recently discovered low molecular weight (LMW) protein that plays an important role in iron homeostasis\(^1\). Hepcidin may explain the often observed imbalance in iron metabolism and the resistance to erythropoiesis-stimulating agents (ESA) in patients with impaired renal function. As such, hepcidin could become an important tool to predict ESA responsiveness, and to guide treatment with ESA and intravenous iron. In addition, hepcidin has the potential to become a target of treatment\(^2, 3\).

Hepcidin is primarily produced by hepatocytes as an 84-amino-acid (84 aa) pro-prohepcidin. Subsequent posttranslational processing results in the biological active 25 aa form (hepcidin-25) that is secreted in plasma and excreted in urine. Additional aminoterminal degradation results in two smaller isoforms (hepcidin-20 and 22)\(^4, 5\). The biological significance of hepcidin-20 and 22 is unknown, although in vitro at supraphysiologic concentrations, hepcidin-20 shows broad spectrum antimicrobial activity\(^4\). Hepcidin inhibits the release of iron from macrophages and the absorption of dietary iron from the intestine. It does so by causing the internalization and degradation of the cellular iron exporter ferroportin, which is highly expressed in macrophages and duodenal enterocytes\(^6\). The net effect of hepcidin is to increase intracellular iron stores, decrease dietary iron absorption, and decrease circulating iron concentrations.

Various physiologic and pathologic processes regulate the synthesis of hepcidin. An augmented demand for circulating iron due to iron deficiency, hypoxia\(^7\), anaemia\(^7\), conditions characterized by ineffective erythropoiesis, or the use of ESA\(^8\), leads to a decrease in hepcidin synthesis. On the other hand, hepcidin synthesis is increased by infection or inflammation\(^7\). This is believed to lead to the anemia of chronic disease that is characterized by a decrease in circulating iron available for erythropoiesis, despite apparently normal iron stores. These latter features are similar to those observed in patients with impaired renal function.

Hepcidin measurements in serum and urine have long proven to be difficult. Recently, both reliable mass spectrometry (MS) based assays and competitive immunoassays have become available\(^9, 10\). MS assays have the advantage of distinguishing between the three hepcidin isoforms, hepcidin-25, 22 and 20. Immunoassays, however, will measure total hepcidin levels, with (depending on the specificity of the antibody) different contributions from each of the isoforms.

Our recent findings suggest that hepcidin-25, like other small peptides such as \( \beta_2 \)-microglobulin, is freely filtered and subsequently reabsorbed in the proximal tubules\(^11\). Accordingly, both serum hepcidin-25 and total hepcidin levels have been found to be elevated in small series of patients with renal dysfunction\(^12, 13\). Furthermore, Ashby et al., exploiting a radio-immunoassay found total hepcidin levels to be elevated and positively correlated with ferritin and inversely correlated with estimated GFR (eGFR)\(^14\). Yet, it is
Hepcidin-25 levels are independent of eGFR in CKD

Chapter 7

Hepcidin-25 levels are independent of eGFR in CKD

were treated with peritoneal dialysis, using a pH neutral, lactate-buffered, low glucose
We measured hepcidin-25 levels in blood and dialysate samples of three patients who
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After the haemodialysis session, several filters were flushed with a physiologic salt solution
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erthropoietin (EPO) during dialysis. In order to assess the presence of hepcidin in the
session in a subgroup of 15 patients. These patients did not receive intravenous iron or
dialysate samples would be used for medical research. We measured the following
subjects and methods
Blood samples of 24 healthy controls were collected randomly throughout the day. We
obtained blood samples of 83 consecutive patients with CKD who visited the outpatient
clinic of the Radboud University Nijmegen Medical Centre to ensure coverage of the
full GFR range. Relevant clinical and biochemical data were retrieved from the patients’
records.
Additional blood samples were collected in 48 consecutive patients on haemodialysis,
using a biocompatible Fresenius polysulfone® dialysis membrane. In all patients, blood
samples were collected at the start of haemodialysis. To determine whether the artificial
kidney removed hepcidin, blood samples were drawn from both the arterial (blood
flowing to the dialyzer) and the venous (blood flowing to the patient) segment of the
dialysis circuit 5-15 minutes after start and 5-10 minutes before termination of the dialysis
session in a subgroup of 15 patients. These patients did not receive intravenous iron or
erthropoietin (EPO) during dialysis. In order to assess the presence of hepcidin in the
dialysate, dialysate samples were collected within 30 minutes after the start of dialysis.
After the haemodialysis session, several filters were flushed with a physiologic salt solution
and acetonitrile in order to determine the presence of hepcidin on the membrane.
We measured hepcidin-25 levels in blood and dialysate samples of three patients who
were treated with peritoneal dialysis, using a pH neutral, lactate-buffered, low glucose
degradation products solution (Stay-Safe® Balance system, Fresenius Medical Care,
Germany). The dialysate samples were taken after a 6-8 hour dwell time.
In accordance with the Dutch ethical regulation, all patients consented that blood
and dialysate samples would be used for medical research. We measured the following
parameters in the blood samples: serum creatinine, hemoglobin, serum iron, iron binding
capacity, ferritin, C-reactive protein (CRP), and hepcidin isoforms.
Serum hepcidin measurements by use of Matrix-Assisted Laser Desorption/Ionization
Time-Of-Flight mass spectrometry (MALDI-TOF-MS) were performed between July
2008 and July 2009 by an update from our previously described method11, 14. For the
quantification of hepcidin in serum an internal standard (synthetic hepcidin-24, Peptide
International Inc., Louisville, KY, USA) was added to 50 µL serum before total hepcidin
was isolated from the sample with Macro-Prep® CM Support beads (Bio-Rad laboratories,
Hercules, CA, USA) as described11. Next, hepcidin was applied to a MSP 96 polished steel
MALDI target plate followed by the addition of energy absorbing matrix, 5 mg α-Cyano-
4-hydroxy-cinnamic acid in 1 mL 50% acetonitrile and 0.1% Trifluoro-acetic acid, all in
nitrogen atmosphere. Mass–to-charge (m/z) spectra were generated using MALDI-TOF-
MS (Microflex LT, Bruker Daltonics GmbH, Bremen, Germany) in positive, linear ion mode
and 500 laser shots. Initial Laser Power; 60%, Laser Attenuator; Offset 25% and Range
20%. Pulsed ion extraction was set to 150 ns. Concentrations of serum hepcidin were
expressed as nanomoles per litre. Total hepcidin was calculated as the sum of hepcidin-25,
hepcidin-22 and hepcidin-20. The Lower Limit Of Detection (LLOD) of this method for
serum was 0.5 nM with an intra-run coefficient of variation (CV) of 3.7% at 79 nM, 2.3% at
13.4 nM and 2.2 % at 3.1 nM. The inter-run CV is 9.1% at 7.8 nM and 3.9% at 12.9 nM. The
median reference level of serum hepcidin-25 is 4.2 nM, range 0.5- 13.9 nM. The LLOD of
this method for dialysate was 0.05 nM.
Calculations
The glomerular filtration rate was estimated (eGFR) by the simplified Modification of Diet
in Renal Disease (MDRD) formula13, 14.
Statistical analysis
Linear regression analysis was conducted to evaluate the relation between serum
hepcidin and other relevant parameters. Correlations were calculated using Spearman’s
or Pearson’s correlation test. If data were skewed log transformation was used. Possible
collinearity for significant univariate predictors was checked. To determine independent
predictors of serum hepcidin, multiple regression analysis was applied. Predictors that
showed high collinearity (Spearman’s rho> 0.8) were not simultaneously included in the
analysis. Wilcoxon signed rank test was performed to assess differences between hepcidin
levels in haemodialysis patients. Parameters between groups were compared using the
Mann-Whitney or Kruskal-Wallis test for non-parametric continuous data, independent
t-test for parametric data, and the chi-square test for categorical data. Statistical analysis
was performed using SPSS for windows software, version 16.0 (SPSS Inc, Chicago, IL).
Results
We included 83 patients with CKD not requiring dialysis and 48 patients on haemodialysis.
Clinical and demographic characteristics of both groups and the control group are
depicted in Table 1. Median eGFR of CKD patients was 36 ml/min/1.73m² (range 6-93
ml/min/1.73m²). Causes of CKD were glomerular disease (36%), vascular disease (21%),
polycystic renal disease (12%), reflux nephropathy (9%), diabetic nephropathy (6%),
and other (16%). Patients on haemodialysis were older and showed significantly higher values of ferritin and CRP. In HD patients causes of renal disease were vascular disease (23%), glomerular disease (19%), diabetic nephropathy (17%), reflux nephropathy (13%), polycystic renal disease (4%), and other (24%).

**Chronic kidney disease**

Serum hepcidin-25 levels were not significantly increased in patients with CKD not requiring dialysis compared to 24 healthy controls (median 5.1 versus 4.2 nM, p = 0.30).

Overall, in CKD patients serum hepcidin-25 strongly correlated with ferritin (r = 0.74, p < 0.01, Figure 1A, Table 2), whereas no significant correlation was found between hepcidin-25 and serum creatinine or eGFR (r = 0.13, p = 0.26 and r = -0.12, p = 0.30 respectively, Figure 1B, Table 2). Multiple regression analysis, including the putative predictors age, ferritin, transferrin, hemoglobin, serum iron, CRP, and eGFR, showed ferritin to be the only significant, independent predictor of hepcidin-25 levels (β= 0.736, p < 0.001, R² = 0.54). Of note, eGFR had no influence on hepcidin-25 levels.

**Table 1. Clinical and demographic characteristics**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls (n=24)</th>
<th>CKD (n=83)</th>
<th>Haemodialysis (n=48)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (%)</td>
<td>46</td>
<td>60</td>
<td>62</td>
<td>0.234</td>
</tr>
<tr>
<td>Age (years)</td>
<td>39±12</td>
<td>55±17</td>
<td>61±15</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Serum creatinine (µmol/L)</td>
<td>75 [54-102]</td>
<td>151 [62-842]</td>
<td>-</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73m²)</td>
<td>84 [67-117]</td>
<td>36 [6-93]</td>
<td>-</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Proteinuria (g/10mmol creatinine)</td>
<td>n.a.</td>
<td>0.34 [0.00-16.4]</td>
<td>n.a.</td>
<td>-</td>
</tr>
<tr>
<td>Kt/V (weekly)</td>
<td>-</td>
<td>n.a.</td>
<td>0.08 [0.00-0.88]</td>
<td>-</td>
</tr>
<tr>
<td>Kt/V (weekly)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>4.1 [2.0–7.5]</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Iron (µmol/L)</td>
<td>19 [9-35]</td>
<td>14 [4-30]</td>
<td>8.5 [2-27]</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Transferrin (µmol/L)</td>
<td>56 [47-75]</td>
<td>56 [37-81]</td>
<td>44 [22-66]</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>34±7</td>
<td>26±10²</td>
<td>23±13</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Ferritin (µg/L)</td>
<td>102 [12-224]</td>
<td>111 [10-60]</td>
<td>268 [62-2167]</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>&lt;5</td>
<td>5 [5-75]</td>
<td>8 [5-104]</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hb (mmol/L)</td>
<td>8.7±0.5</td>
<td>7.6±1.1</td>
<td>6.9±0.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hepcidin 25 (nM)</td>
<td>4.2 [0.5-13.9]</td>
<td>5.1 [0.6-29.5]</td>
<td>9.4 [5.6-60.2]²</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hepcidin 20 (nM)</td>
<td>below LOD</td>
<td>1.8 [0.6-6.4]</td>
<td>2.7 [1.3-76]</td>
<td>-</td>
</tr>
<tr>
<td>Hepcidin 22 (nM)</td>
<td>below LOD</td>
<td>1.1 [0.5-2.2]</td>
<td>1.7 [0.8-2.5]</td>
<td>-</td>
</tr>
<tr>
<td>Total Hepcidin (nM)</td>
<td>4.2 [0.5-13.9]</td>
<td>6.8 [3.6-36.2]</td>
<td>11.1 [2.0-65.6]</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Median [range], mean±SD, eGFR=estimated glomerular filtration rate, CRP=C -reactive protein, Hb=hemoglobin, n.a.= not available, LOD=lower limit of detection

1 significantly different between controls and CKD (p < 0.05)
2 significantly different between CKD and haemodialysis (p < 0.05)

**Figure 1A. Relationship between serum hepcidin-25 and serum ferritin in patients with chronic kidney disease not requiring dialysis.**

**Figure 1B. Relationship between serum hepcidin-25 and estimated glomerular filtration rate in patients with chronic kidney disease not requiring dialysis.**

There was a significant relationship (r = 0.74, P < 0.01). Closed symbols represent patients treated with erythropoietin.

**Table 2. Correlation of hepcidin-25 levels with biochemical and clinical variables in CKD (with and without EPO therapy) and haemodialysis patients.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>CKD (n=83)</th>
<th>Haemodialysis (n=48)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rho</td>
<td>p-value</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>0.13</td>
<td>0.25</td>
</tr>
<tr>
<td>eGFR</td>
<td>-0.12</td>
<td>0.30</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>-0.04</td>
<td>0.73</td>
</tr>
<tr>
<td>Transferrin</td>
<td>0.14</td>
<td>0.19</td>
</tr>
<tr>
<td>Ferritin</td>
<td>0.74</td>
<td>0.00</td>
</tr>
<tr>
<td>Iron</td>
<td>-0.05</td>
<td>0.64</td>
</tr>
<tr>
<td>CRP</td>
<td>0.21</td>
<td>0.06</td>
</tr>
<tr>
<td>Hb</td>
<td>-0.04</td>
<td>0.75</td>
</tr>
<tr>
<td>EPO-dose</td>
<td>0.08⁵</td>
<td>0.45</td>
</tr>
<tr>
<td>Iron-dose</td>
<td>0.14⁴</td>
<td>0.21</td>
</tr>
<tr>
<td>Age</td>
<td>0.1¹</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Spearman’s rho was calculated because data were skewed, eGFR= estimated glomerular filtration rate, CRP=C -reactive protein, Hb=hemoglobin, n.a.= not available n=21, ¹ n=10
We specifically questioned if the use of EPO influenced the results. Twenty-one patients (25%) were treated with EPO. As expected, EPO-treated patients had a lower eGFR (median 12 versus 42 ml/min/1.73m², p< 0.001). There were no differences in serum ferritin levels (median 116 versus 106 μg/L, p= 0.42). Serum hepcidin-25 levels were not significantly higher in EPO-treated patients (median 8.0 nM versus 4.8 nM, p= 0.31). In multivariate regression analysis, EPO therapy was not a predictor of serum hepcidin-25 levels. Furthermore, limiting the analysis to EPO-naïve patients did not alter the results observed for the whole group; again, serum hepcidin-25 was strongly correlated with ferritin (r= 0.74, p< 0.01), and no significant correlation was found between hepcidin-25 and serum creatinine or eGFR (r= 0.16, p= 0.21 and r= -0.13, p= 0.32 respectively). Multiple regression analysis showed ferritin to be the only significant, independent predictor of hepcidin-25 levels (β= 0.861, p< 0.001, R²= 0.58). Serum levels of hepcidin-20 and hepcidin-22 were below the lower limit of detection in 19% and 66% of patients, respectively. In patients with hepcidin isoforms above the LLOD, hepcidin-20 and hepcidin-22 correlated with ferritin (r= 0.46, p< 0.01, and r= 0.41, p= 0.03 respectively, Figure 2) and eGFR (r= -0.49 p< 0.01 and r= -0.46, p=0.01 respectively, figure 2). Multiple regression analysis of the data including patients with concentrations of the hepcidin isoforms above the detection limit, showed eGFR to be an independent, significant predictor of hepcidin-25 and total hepcidin levels (β= -0.467, p< 0.001, and β= -0.259, p= 0.001 respectively). Furthermore, the ratio hepcidin 25/total hepcidin was significantly influenced by eGFR: the ratio was 0.80 in patients in the highest tertile of eGFR and 0.72 in patients in the lowest tertile of eGFR (p=0.044). Thus, a decrease in eGFR was associated with an increase in hepcidin-20 and total hepcidin levels. Exclusion of the patients with levels below the detection limit could have biased the results. Therefore we re-analysed the data. For this analysis, we attributed the lowest measurable value to the patients with levels below the detection limit. Also in this analysis, which thus included all patients, eGFR was an independent predictor of hepcidin-20 (β = -0.225, p= 0.018). Of note, serum ferritin proved the strongest predictor of both hepcidin-20 and total hepcidin, similar to the results described above for serum hepcidin-25 levels.

### Haemodialysis

We included 41 patients who received haemodialysis 3 times a week for approximately 4 hours and seven patients who were treated with nocturnal dialysis four times a week for approximately 8 hours. The majority of patients (n=44) were treated with intravenous iron and all but one were treated with EPO, median time on dialysis was 26 months (range 1-64 months). A low-flux membrane was used in 36 patients, whereas 12 patients were dialysed using a high-flux membrane. Median blood flow was 300 (range 130-400) ml/min, median dialysis flow was 500 (range 300-700) ml/min and median ultrafiltration volume was 2110 (range 59-3800) ml. Median hepcidin-25 levels were significantly higher compared to hepcidin-25 levels in patients with CKD (9.4 versus 5.1 nM, p< 0.001). Again, hepcidin-25 was strongly correlated with ferritin (r= 0.79, p< 0.001). Multiple regression analysis- including the putative predictors age, ferritin, transferrin, hemoglobin, serum iron, and CRP - showed ferritin and serum iron to be significant, independent predictors of hepcidin-25 levels (R² = 0.70 ).

A significant difference was observed between arterial and venous samples at the start of the dialysis procedure compatible with removal of hepcidin-25 by the artificial kidney (n=15, 15.9±15.1 versus 8.5±7.5 nM, p= 0.001). The median clearance of hepcidin-25 was 82 ml/min. Surprisingly, despite a persistent arterial-venous difference at the end of dialysis (13.6±11.5 versus 10.3±9.8, p= 0.001), only a very modest reduction in serum hepcidin-25 was observed at the end of haemodialysis (arterial values 15.9±15.1 versus 13.6±11.5 nM, p= 0.03).

Hepcidin-25 and its isoforms were present in the ultrafiltrate, but levels were too low to allow precise calculation of a sieving coefficient or to account fully for the observed arterial-venous difference. Hepcidin was also detected in significant amounts (range 0.4-2.0 nmol) after eluting the filter with acetonitril, which strongly suggests binding of hepcidin to the artificial membrane.

### Peritoneal clearance of hepcidin

We measured hepcidin-25 levels in three peritoneal dialysis patients. Hepcidin-25 was detected in the dialysate, with an average dialysate/plasma ratio of 0.22.
Discussion

We found that serum hepcidin-25 levels are not significantly increased in patients with renal dysfunction not requiring dialysis. Haemodialysis patients however, exhibited significantly higher hepcidin-25 levels than patients with CKD. Serum ferritin concentration was a significant predictor of hepcidin-25 levels in multiple regression analysis. In contrast, eGFR was not an independent predictor of hepcidin-25 levels in patients with CKD. Furthermore, serum hepcidin-25 levels decreased only slightly during haemodialysis (10-15%), despite an arterio-venous difference in hepcidin levels of > 40%. Admittedly, ultrafiltration may have resulted in more concentrated levels of hepcidin-25 and a minor underestimation of hepcidin clearance. Since blood samples were not drawn until after the initiation of haemodialysis, we cannot exclude an immediate effect of haemodialysis on serum hepcidin levels, yet it seems unlikely that hepcidin levels are substantially influenced by haemodialysis within such a short period of time.

Our data corroborate recently published studies reporting higher hepcidin levels in HD patients\cite{10,12}. There is also agreement that serum ferritin is highly correlated with hepcidin levels in patients with CKD and HD\cite{10,12,13}. However, the data seem to differ with respect to the role of the eGFR. In two studies the independent effect of eGFR on hepcidin levels was not evaluated\cite{12,13}. Ashby et al. reported that hepcidin levels in their patients were significantly correlated with eGFR, even when corrected for ferritin levels\cite{10}, whereas in the current study hepcidin-25 levels were not related to eGFR.

Instead, we found that hepcidin-20 and total hepcidin levels showed an independent significant negative relation with eGFR. The apparent discrepancies between our and Ashby et al.’s observations might therefore be explained by the use of different assays, and in particular the inability of the immunoassay used by Ashby to differentiate between the various isoforms. Alternatively, the conflicting results may be attributable to differences in the population studied. Also, the findings of Ashby et al. may reflect a type I error or we may have failed to detect a slight effect of eGFR on hepcidin levels due to a type II error. Yet, based on the data described above we must conclude that serum concentrations of hepcidin-25, the bioactive hepcidin isoform, are mainly dependent on ferritin and not on eGFR.

Our observation that hepcidin-25 is not related to eGFR suggests that hepcidin-25 differs from other LMW proteins, such as β2-microglobulin or cystatin-C. This might be explained by binding of hepcidin to a large carrier protein that precludes it to be freely filtered. However, our additional studies are inconsistent with such a hypothesis. First, hepcidin was present in the peritoneal dialysate and when the peritoneal clearance of hepcidin was plotted on a curve depicting the relationship between the peritoneal clearance and the molecular mass of various other freely filtered marker proteins, the calculated clearance was compatible with the expected clearance of a freely filterable protein of similar molecular mass\cite{17}. Second, the arterio-venous difference in haemodialysis patients suggests that hepcidin-25 is handled similar to other LMW proteins. Admittedly, in the latter experiments we were unable to calculate the relative contribution of ultrafiltration and of sticking to the membrane of the artificial kidney.

Taken together, our findings suggest regulation of serum hepcidin-25 levels, thus attenuating any effect of eGFR. Removal of hepcidin-25 by the dialysis membrane, is partly compensated for. Studies on the immediate effects of iron administration on the increase of serum hepcidin-25 levels are consistent with a fast hepcidin regulation\cite{13,18}.

In conclusion, serum hepcidin-25 levels are not dependent on eGFR. Moreover, serum levels of hepcidin-25 decrease only slightly during dialysis, despite considerable removal of hepcidin-25 by the artificial kidney. Hepcidin isoforms accumulate in patients with renal impairment. The biological relevance of the latter finding is unknown.
Chapter 7 Hepcidin-25 levels are independent of eGFR in CKD

References

Tubular reabsorption and local production of urine

discussion-25

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ABSTRACT

Background
Hepcidin is a central regulator of iron metabolism. Serum hepcidin levels are increased in patients with renal insufficiency, which may contribute to anemia. Urine hepcidin was found to be increased in some patients after cardiac surgery, and these patients were less likely to develop acute kidney injury. It has been suggested that urine hepcidin may protect by attenuating heme-mediated injury, but processes involved in urine hepcidin excretion are unknown.

Methods
To assess the role of tubular reabsorption we compared fractional excretion (FE) of hepcidin-25 with FE of β₂-microglobulin (β₂m) in 30 patients with various degrees of tubular impairment due to chronic renal disease. To prove that hepcidin is reabsorbed by the tubules in a megalin-dependent manner, we measured urine hepcidin-1 in wild-type and kidney specific megalin-deficient mice.
Lastly, we evaluated FE of hepcidin-25 and β₂m in 19 patients who underwent cardiopulmonary bypass surgery. Hepcidin was measured by a mass spectrometry assay (MS), whereas β₂m was measured by ELISA.

Results
In patients with chronic renal disease, FE of hepcidin-25 was strongly correlated with FE of β₂m (r=0.93, P<0.01). In megalin-deficient mice, urine hepcidin-1 was 7-fold increased compared to wild-type mice (p<0.01) indicating that proximal tubular reabsorption occurs in a megalin-dependent manner. Following cardiac surgery, FE of hepcidin-25 increased despite a decline in FE of β₂m, potentially indicating local production at 12-24 hours.

Conclusions
Hepcidin-25 is reabsorbed by the renal tubules and increased urine hepcidin-25 levels may reflect a reduction in tubular uptake. Uncoupling of FE of hepcidin-25 and β₂m in cardiac surgery patients suggests local production.

Introduction
Hepcidin, a peptide predominantly produced by hepatocytes, is a major player in iron metabolism. Hepcidin decreases duodenal iron absorption and causes iron sequestration in the reticulo-endothelial system. Hepcidin expression is induced by iron storage and inflammation and suppressed by hypoxia and anemia. Serum hepcidin levels are increased in patients with renal insufficiency, and this may contribute to anemia and resistance to erythropoietin stimulating agents.

Recent studies have pointed to the relevance of urine hepcidin. In patients with lupus nephritis, changes in urine hepcidin-20 and -25 predicted renal flares. Even more striking were the findings of Ho et al., who showed that patients with increased urine hepcidin levels were at lower risk to develop acute kidney injury (AKI) after cardiac surgery. These results have recently been confirmed in a larger study that included 100 patients who had undergone cardiopulmonary bypass surgery (CABG). It was suggested that urine hepcidin may protect against AKI by attenuating heme-mediated injury.

In order to meaningfully interpret urine hepcidin as a biomarker, knowledge of renal handling is essential. Thus far, it is unclear which processes—filtration, reabsorption, local production and/or degradation—govern urine hepcidin excretion. The objective of this study was to study the role of tubular reabsorption in kidney hepcidin handling. Our data provide evidence for both tubular reabsorption and local production of hepcidin in the kidney.

Methods

Human studies
Blood and urine samples of healthy controls were collected randomly throughout the day as described before. In order to assess the role of tubular reabsorption we compared fractional excretion (FE) of hepcidin-25 with FE of β₂-microglobulin (β₂m) in patients with glomerular or tubular diseases and various degrees of impairment of tubular reabsorption. β₂m is a low molecular weight protein and an established marker of proximal tubular function. Patients were enrolled from February 2009 to October 2010 at the Department of Nephrology and Pediatric Nephrology, Radboud University Nijmegen Medical Center. Patients with biopsy proven glomerular disease (without an interstitial infiltrate) and patients with defined tubular diseases (cystinosis, Dent’s disease) were enrolled. Patients with an interstitial infiltrate as concluded from renal biopsy investigations were excluded, since monocytes may produce hepcidin.

Hepcidin-25 was also measured in patients after cardiopulmonary bypass surgery. Patients were consecutively enrolled from March 2008 to April 2008 at the Department of Intensive Care Medicine, Radboud University Nijmegen Medical Centre, and all patients
undergoing CABG were included. Serum and urine samples were obtained simultaneously either 1-2 hours after surgery (at a time the patient was admitted and stable at the ICU) or 12-24 hours after the end of surgery (morning urine collected the day after the procedure).

The study has been carried out in the Netherlands in accordance with the applicable rules concerning the review of research ethics committees and informed consent. We obtained consent from healthy volunteers. The local ethics committee waived the need to get consent from patients, as they were having blood and urine taken as part of standard care.

**Animal experiments**

In order to investigate whether hepcidin is reabsorbed in the proximal tubules in a megalin-dependent manner, we measured urine hepcidin-1 in wild type and kidney specific megalin-deficient C57BL/6 mice\(^5\). Megalin lox/lox; apoE CRE mice on a C57BL/6 background were kindly provided by Thomas E. Willnow. The creation of this kidney specific megalin-deficient mouse strain was described in detail previously\(^6\). Animals were bred locally and animals expressing the apoE CRE gene were identified by means of polymerase chain reaction (PCR) analysis. Animals that did not express the apoE CRE gene (megalinlox/lox mice) were used as wild type controls. Approximately 12 weeks old female mice were used. Their diet contained 179 mg/kg iron. To collect urine samples, mice were individually housed in metabolic cages (Techniplast\(^4\)). Mice were allowed to adapt to these cages during 2 periods of 30 min, after which 24-hour urine samples were collected. Prior to 24 h housing in metabolic cages, 2x0.5 ml salt solution was administered subcutaneously to prevent dehydration. To prevent hypothermia, room temperature was raised to 24 \(^\circ\)C with a relative humidity of 53-68%. Food and water were available ad libitum. Mice were sacrificed after blood sampling at the end of the 24-hour urine collection. Urinary protein profile of wild-type and megalin deficient mice was determined by gel electrophoresis.

Experiments were approved by the Animal Ethical Commission of the Radboud University Nijmegen Medical Centre and performed in accordance with national guidelines for the care and handling of animals.

**Laboratory measurements**

Urine and serum samples were processed, aliquoted, and stored in polypropylene tubes at −80\(^\circ\)C immediately after collection. Routine laboratory parameters and hepcidin levels were measured within 8 hours and 6 months of collection, respectively. Urinary and serum creatinine were measured with an enzymatic method. Urine \(\beta_2\) m was only measured in urine with a urinary pH >6.0, since degradation of \(\beta_2\) m may occur below this pH. Human hepcidin-25 was measured by our previously described weak cation exchange time-of-flight mass spectrometry assay\(^5\) (TOF MS). Hepcidin-1 in urine from mice was measured by surface-enhanced laser desorption ionization (SELDI) TOF MS\(^5\).

**Calculations and statistics**

Depending on its distribution, data were expressed as median (interquartile range) or mean ± SD. Fractional excretion of substance Y was defined as: (Serum Creatinine x Urine Y) / (Serum Y x Urine Creatinine) x 100%. In healthy controls, serum and urine hepcidin-25 were expressed as the mean of four samples collected at different times during the day. In mice urinary concentrations were normalized for creatinine to correct for differences in urine dilution.

Glomerular filtration rate (GFR) was estimated by the abbreviated Modification of Diet in Renal Disease equation in adults\(^11\). In children GFR was estimated using the revised Schwartz formula\(^9\).

Statistical analysis was performed using SPSS 16.0 (SPSS Inc, Chicago, IL). Correlations were assessed by linear regression, using Spearman’s rho. Mann Whitney test was used for comparison of FE of hepcidin-25 in healthy controls and patients with renal disease and for comparison of urinary concentrations in wild type and megalin-deficient mice. Statistical significance was denoted by two sided P values of <0.05.

**Results**

In 24 healthy controls fractional excretion (FE) of hepcidin-25 was 1.9 (IQR 1.0-3.2)% (table 1). We evaluated 30 patients with glomerular or tubular diseases and various degrees of impairment in tubular reabsorption. Median serum creatinine was 107 (IQR 83-147) \(\mu\)mol/l and proteinuria 4.5 (IQR 1.7-9.4) g/d (Table 1).

Renal disease consisted of idiopathic membranous nephropathy (n=13), focal segmental glomerulosclerosis (n=3), infantile nephropathic cystinosis (n=7), or other causes (n=7). There was an increased FE of hepcidin-25 in patients with renal disease compared to controls (8.0 versus 1.9%, p<0.001). We found a strong correlation between FE of hepcidin-25 and FE of \(\beta_2\) m (Spearman’s rho=0.93, p<0.01, Figure 1). Since \(\beta_2\) m is a marker of proximal tubular reabsorption, this data strongly suggest that hepcidin excretion is at least partially governed by this process.

Megalin is a multiligand endocytic receptor localized in the proximal renal tubules and plays an important role in the tubular reabsorption of various filtered proteins, amongst which \(\beta_2\) m. To prove that the bioactive mouse hepcidin-1 is reabsorbed in the proximal tubules in a megalin-dependent manner, we measured urine hepcidin in wild type and kidney specific megalin-deficient C57BL/6 mice. As expected, megalin deficient mice did excrete lower molecular weight proteins. Urine hepcidin-1 was 7-fold increased in...
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Urinary hepcidin reflects tubular dysfunction and local production

Table 1. Clinical and demographic characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls (n=24)</th>
<th>Renal disease (n=30)</th>
<th>CABG 1-2 hours (n=8)</th>
<th>CABG 12-24 hours (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (%)</td>
<td>46</td>
<td>80</td>
<td>88</td>
<td>85</td>
</tr>
<tr>
<td>Age (years)</td>
<td>39±12</td>
<td>43±24</td>
<td>65±8</td>
<td>66±10</td>
</tr>
<tr>
<td>Serum albumin (g/l)</td>
<td>n.a.</td>
<td>34 (24-35)</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Serum creatinine (μmol/l)</td>
<td>75 (68-83)</td>
<td>107 (23-147)</td>
<td>93 (74-116)</td>
<td>85 (51-102)</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73m²)</td>
<td>84 (77-108)</td>
<td>57 (34-65)</td>
<td>73 (58-95)</td>
<td>85 (17-128)</td>
</tr>
<tr>
<td>Proteinuria (g/d)</td>
<td>4.5±1.7-9.4</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Serum β2m (mg/l)</td>
<td>n.a.</td>
<td>2.9 (2.2-4.6)</td>
<td>2.1 (1.6-2.9)</td>
<td>1.8 (1.3-2.5)</td>
</tr>
<tr>
<td>Serum hepcidin-25 (nmol/l)</td>
<td>4.4 (3.2-5.8)</td>
<td>3.1 (1.1-5.7)</td>
<td>4.7 (1.5-7.1)</td>
<td>14.1 (11.1-17.3)</td>
</tr>
<tr>
<td>Urine β2m (nmol/mmol creatinine)</td>
<td>142±13-963</td>
<td>132±528-5239</td>
<td>66±21-86</td>
<td></td>
</tr>
<tr>
<td>Urine hepcidin-25 (nmol/mmol creatinine)</td>
<td>0.9 (0.4-1.7)</td>
<td>1.4 (0.3-7.1)</td>
<td>10.4 (0.8-18.7)</td>
<td>52.2 (34.0-112.1)</td>
</tr>
<tr>
<td>FE of β2m (%)</td>
<td>n.a.</td>
<td>5.1 (0.7-39.1)</td>
<td>14.1 (7.1-20.7)</td>
<td>2.7 (0.8-3.7)</td>
</tr>
<tr>
<td>FE of hepcidin-25 (%)</td>
<td>19 (10-3.2)</td>
<td>8.0 (1.9-20.3)</td>
<td>21.1 (7.2-23.1)</td>
<td>33.1 (22.2-52.9)</td>
</tr>
<tr>
<td>Duration of CPB (min)</td>
<td>-</td>
<td>-</td>
<td>101 (63-147)</td>
<td>106 (92-125)</td>
</tr>
</tbody>
</table>

Median (interquartile range), mean ± SD, eGFR = estimated glomerular filtration rate, n.a. = not available, FE = fractional excretion, CABG = coronary artery bypass grafting, CPB = cardiopulmonary bypass, ‘one patient underwent off-pump CABG.

Figure 1. Correlation between fractional excretion (FE) of hepcidin-25 and β2m in patients with renal disease without a tubulointerstitial infiltrate (n=30, triangles), and in patients at 1-2 hours (n=8, white circles) and 12-24 hours (n=13, black circles) after cardiopulmonary bypass grafting (CABG).

Figure 2. Hepcidin-1 in urine of C57Bl/6 Wild type mice (n=5) and mice with kidney-specific megalin deficiency (n=5).

FE of hepcidin correlates with FE of β2m, indicating tubular uptake of hepcidin. However, FE of hepcidin increased relatively more in patients 12-24 hours after CABG, suggesting local production in the kidney.

Figure 3. Serum hepcidin-25 versus estimated urinary excretion of hepcidin (or GFR*serum hepcidin vs urinary excretion of hepcidin) there was no evidence of a tubular threshold (Figure 3).

Urine was normalized for creatinine levels. Data are depicted as lower quartile, median and upper quartile (boxes), and minimum and maximum ranges (whiskers). ** P<0.01;megalin-deficient vs. wild type mice (by Mann Whitney test).

Median (interquartile range), mean ± SD, eGFR = estimated glomerular filtration rate, n.a. = not available, FE = fractional excretion, CABG = coronary artery bypass grafting, CPB = cardiopulmonary bypass, ‘one patient underwent off-pump CABG.

We next evaluated urine hepcidin-25 in patients who underwent cardiopulmonary bypass surgery (Table 1). 24 patients were initially enrolled, but five patients were excluded because of a urinary pH <6 at both time intervals, thus precluding reliable measurement of β2m. All remaining 19 patients were operated on pump, except for one patient who had pre-existing impairment of renal function. Two patients developed acute kidney injury after cardiac surgery, with AKI defined as a baseline-to-peak decrease in eGFR by 50% or more during the first five days post-operatively. pH was >6 in approximately 50% of measurements performed in these 19 patients, allowing serum and urine β2 m and hepcidin quantification in eight patients, 1-2 hours after surgery and in 13 patients 12-24 hours after surgery. In only 2 patients measurements were performed at both time intervals.

Immediately after surgery, FE of hepcidin-25 and β2m were 21 and 14%, respectively, and correlated strongly (Spearman’s rho=0.79, p=0.02). The ratio between both parameters was similar to that in patients with renal disease, and thus compatible with impairment of tubular reabsorption. At 12-24 hours after surgery FE β2m decreased (3%), indicating recovery from tubular injury. However, FE of hepcidin-25 increased further (33%). As a result, FE of hepcidin-25 did no longer correlate with FE of β2m (Spearman’s rho=0.18, p=0.55, Figure 1). When plotting serum hepcidin versus urinary excretion of hepcidin (or GFR*serum hepcidin vs urinary excretion of hepcidin) there was no evidence of a tubular threshold (Figure 3).

Median (interquartile range), mean ± SD, eGFR = estimated glomerular filtration rate, n.a. = not available, FE = fractional excretion, CABG = coronary artery bypass grafting, CPB = cardiopulmonary bypass, ‘one patient underwent off-pump CABG.

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Discussion

Our results indicate that hepcidin-25 is reabsorbed by the renal proximal tubules via megalin-mediated endocytosis. In addition, our data suggest that the increase of urine hepcidin-25 after cardiac surgery reflects local production of hepcidin in the kidney.

Urinary hepcidin has recently gained interest as a renal biomarker. However, not much is known about the renal handling of hepcidin. Because of its small size (2.8 kDa), free, unbound hepcidin is likely to be filtered by the glomeruli. After filtration, hepcidin is almost completely reabsorbed by the renal tubules. This conclusion is based on data from a small number (n=9) of healthy controls and patients with thalassemia and hemochromatosis, where FE of hepcidin was estimated to be less than 3%20;21.

In the present study, FE of hepcidin-25 in healthy controls was approximately 2%, thus confirming earlier reports and compatible with the suggested extensive tubular uptake.20,21 In patients with kidney diseases, FE of hepcidin-25 was increased and correlated strongly with FE of β2-m (n=93, p<0.01), a low molecular weight protein that is normally freely filtered by the glomerulus and almost completely reabsorbed in the proximal tubule through the megalin receptor. By using a mouse model, we confirmed the existence of megalin-dependent tubular reabsorption of hepcidin-25. Unlike humans, mice contain two related hepcidin genes, hepcidin-1 and 2, of which hepcidin-1 is almost exclusively produced in the liver. This peptide is important for iron homeostasis, and is considered the mouse equivalent of human hepcidin-25. Urine hepcidin-1 was significantly higher in megalin-deficient mice (p<0.01, Figure 2).

We observed that shortly after cardiac surgery, FE of both hepcidin-25 and β2-m were increased and correlated strongly, reflecting decreased tubular reabsorption. However, within 24 hours after surgery we observed a further increase of FE of hepcidin-25, whereas at this time point tubular injury became less severe as shown by the reduction in FE of β2-m. These findings can be explained in two ways; either by the occurrence of saturation of tubular reabsorption due to increased tubular delivery of hepcidin-25 thus exceeding reabsorption capacity or by production of hepcidin-25 locally in the kidney. Since we did not observe a threshold (Figure 3), local production is the most likely explanation.

Data on tubular reabsorption or local production are scarce. Kulaksiz et al. observed strong hepcidin expression in the thick ascending limb of the cortex and in the connecting tubules, but not in the proximal tubules. At the cellular level, hepcidin was localized to the apical cell pole of the renal epithelial cells, which is suggestive of luminoally directed release of hepcidin in the urine. Hepcidin-25 may thus be produced by the distal kidney tubules due to unknown stimuli or by inflammatory cells such as monocytes.

The origin and regulation of locally produced hepcidin-25 in patients after cardiac surgery merits further studies in view of the recent evidence that urine hepcidin may protect against the development of AKI. In a nested cohort study Ho et al. compared urine of 22 cardiac surgery patients with AKI (defined as ≥50% rise in serum creatinine during the first four postoperative days) with urine from 22 randomly selected cardiac surgery patients without AKI. They observed that hepcidin-25 was increased on the first post-operative day in patients not developing AKI. The observations of Ho et al. were corroborated by an independent observational study measuring hepcidin through ELISA in 100 cardiac surgery patients: urine hepcidin was 3-7 times higher 6 and 24 hours after surgery in AKI-free patients (n=91) compared to nine patients who developed AKI (defined as ≥50% rise in serum creatinine or urine output <0.3 ml/kg/hr during the first seven postoperative days). Additionally, FE of hepcidin increased from 8 to 40% at 24h post surgery in 93 patients exposed to cardiac surgery, and was higher in patients who did not develop AKI (AKI (n=25) 27% vs AKI-free (n=68) 37%, p=0.049).

As suggested by others, local production of hepcidin may serve to prevent oxidative damage induced by free iron and thereby protect against AKI. Some studies have reported that hepcidin binds divalent metals, amongst which Fe2+.

This study is the first to document local production of hepcidin in patients after CABG. It has several limitations. First, we included a limited number of patients. Secondly, due to a pH <6.0, β2-m could not reliably be measured in all samples. Although αβ1 microglobulin is a more stable marker of proximal tubular reabsorption and can reliably be measured in acidic urine, it is protein-bound and therefore it is impossible to calculate the fractional excretion. Third, this study is a pilot study, and our findings were not corroborated by histopathological data showing extensive proximal tubular uptake in apical endocytic vesicles, nor by data on hepcidin expression or mRNA content in the kidney or macrophages. More extensive studies are necessary to evaluate the exact timing and location of hepcidin production, and to identify possible factors influencing this process. Since increased urinary levels of hepcidin are associated with a decreased risk for post-surgical AKI, increasing local production may serve as a strategy to reduce the development of AKI.

In conclusion, our mouse study indicates that proximal tubular reabsorption of urine hepcidin-1 occurs in a megalin-dependent manner. In CKD patients FE of hepcidin-25 correlated strongly with FE of β2-m, suggesting that also in human urinary excretion of hepcidin-25 is governed by tubular reabsorption of hepcidin. Uncoupling of FE of hepcidin-25 and β2-m in cardiac surgery patients indicates local production of hepcidin-25. This local production of hepcidin-25 may be important in attenuating post-surgical AKI and merits further investigation.
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Acknowledgements

We thank Thomas E. Willnow for donating mice with kidney-specific megalin gene defect.

References


Renal proximal tubular dysfunction is a major determinant of urinary connective tissue growth factor excretion

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ABSTRACT

Connective tissue growth factor (CTGF) plays a key role in renal fibrosis. Urinary CTGF is elevated in various renal diseases and may have biomarker potential. However, it is unknown which processes contribute to elevated urinary CTGF levels. Thus far, urinary CTGF was considered to reflect renal expression. We investigated how tubular dysfunction affects urinary CTGF levels. To study this we administered recombinant CTGF intravenously to rodents. We used both full length CTGF and the amino-terminal fragment, since the N-fragment is the predominant form detected in urine. Renal CTGF extraction, determined by simultaneous arterial and renal vein sampling, was 18±3% for full length CTGF and 21±1% for the N-fragment. Fractional excretion was very low for both CTGFs (0.02±0.006% and 0.10±0.02%, respectively), indicating that more than 99% of the extracted CTGF was metabolized by the kidney. Immunohistochemistry revealed extensive proximal tubular uptake of CTGF in apical endocytic vesicles, and colocalization with megalin. Urinary CTGF was elevated in megalin/cubilin deficient mice, but not in cubilin deficient mice. Inhibition of tubular reabsorption by Gelofusine reduced renal uptake of CTGF and increased urinary CTGF. In healthy volunteers, Gelofusine also induced an increase of urinary CTGF excretion, comparable to the increase of β2-microglobulin excretion (r = 0.99). Furthermore, urinary CTGF correlated with β2-microglobulin (r = 0.85) in renal disease patients (n =108) and only β2-microglobulin emerged as an independent determinant of urinary CTGF. Thus, filtered CTGF is normally reabsorbed almost completely in proximal tubules via megalin, and elevated urinary CTGF may largely reflect proximal tubular dysfunction.

Introduction

CONNECTIVE TISSUE GROWTH FACTOR is an important profibrotic growth factor in chronic kidney disease (CKD). Elevated urinary connective tissue growth factor levels (uCTGF) are reported in diabetic nephropathy (DN), chronic allograft nephropathy (CAN), IgA nephropathy, focal segmental glomerular sclerosis and idiopathic membranous nephropathy (iMN)6,17,24. Urinary CTGF relates to markers of disease severity in CKD. In type I DN urinary CTGF excretion correlated with urinary albumin excretion and glomerular filtration rate (GFR) and in CAN with biopsy histology and proteinuria6,24. In iMN uCTGF was associated with proteinuria and GFR and uCTGF was an independent predictor of deterioration of renal function7. However, despite increasing evidence regarding the biomarker potential of uCTGF, interpretation of urinary levels is hampered by lack of understanding of the processes contributing to elevated levels.

Previously, elevated uCTGF was considered to reflect increased intrarenal production (26). In many renal diseases, overexpression of CTGF was observed in inflammatory glomerular lesions and tubulointerstitial fibrotic areas16,31. However, we hypothesized that reduced tubular reabsorption might contribute importantly to increased urinary CTGF excretion. To investigate this we studied whether CTGF is filtered and tubularly reabsorbed and whether impairment of tubular reabsorption results in increased urinary CTGF excretion. We quantified renal tubular uptake of recombinant CTGF after intravenous administration in rodents and evaluated the effect of inhibition of proximal tubular reabsorption on uCTGF in healthy rodents and humans. In addition, we examined the correlation between urinary levels of CTGF and β2-microglobulin, a sensitive marker of tubular dysfunction, in renal disease. Secondary objective was to identify the endocytic receptor involved in proximal tubular reabsorption of CTGF.

Materials and methods

Recombinant CTGF proteins and anti-CTGF antibodies. Recombinant CTGFs and anti-CTGF antibodies were supplied by Fibrogen Inc. (San Francisco, CA). The recombinant human CTGFs were produced in a baculovirus expression system in Chinese Hamster Ovary cell lines cultured in hollow-ﬁber fermentors. The proteins were purified by CTGF-affinity and cation exchange chromatography. Recombinant full length CTGF comprised all four domains (Q27-A349) and the N-terminal fragment comprised domains 1 and 2 (Q27-A178).

Gelofusine. We purchased Gelofusine (40 g/l) from Braun (Oss, The Netherlands). Gelofusine is a modified gelatin consisting of polypeptides with an average molecular weight of 30 kDa. In previous studies we have shown that Gelofusine effectively blocks tubular reabsorption of LMW proteins30.

Animal experiments. Pharmacokinetic studies were performed in mice and rats. All experiments were approved by the Animal Ethical Commission of the University of...
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Utrecht and performed in accordance with national guidelines for the care and handling of animals.

C57Bl/6J mice (12 weeks old; Harlan, Horst, The Netherlands) received recombinant human CTGF by tail vein injection (single dose), or by intraperitoneal infusion (3 days) using micro-osmotic pumps (model 1003D, ALZET, Cupertino, CA). To evaluate the plasma disappearance and uptake in the kidneys, mice were killed at various time points after bolus injection of full length CTGF or N-CTGF (12.5-50 pmol/g bodyweight). Kidneys were harvested and weighed and processed for ELISA or immunohistochemistry as described below. To evaluate the effects of inhibition of proximal tubular reabsorption, a separate group of mice received Gelofusine (40 g/l; Braun) either by tail vein injection (5 μl/g bodyweight) 1-2 min before N-CTGF injection in the other tail vein (n = 3) or by tail vein injection (10 μl/g bodyweight) during N-CTGF intraperitoneal infusion (0.6 pmol/min) by micro-osmotic pump 3 days after intraperitoneal implantation under isoflurane anesthesia (n = 3).

To identify the endocytic receptors involved in tubular reabsorption of (endogenous) CTGF, urine was collected during a 24 hour period from mice deficient for cubilin or megalin/cubilin, obtained by crossing mice bearing floxled cubilin alleles or both cubilin and megalin floxed alleles with Mox2-Cre (MORE) mice. Surviving offspring exhibited a mosaic pattern of cubilin deficiency or megalin and cubilin deficiency11. Deletion of cubilin and megalin varied from 70% to over 95%. Age-matched wild-type mice were used as controls. Endogenous uCTGF levels were determined by ELISA as described below.

Studies in rats were performed to quantify the renal extraction and fractional excretion. Male Wistar-Kyoto rats (15-17 weeks old; Harlan) were anesthetized with intraintriperitoneal pentobarbital sodium (72 mg/kg). The trachea was intubated with a 16-G catheter (Venifiesm Abbocath-T, Abbott, Ireland). A PE-90 catheter was placed in the left jugular vein for infusion of solutions, a second PE-10 catheter was introduced for supplemental anesthetic. The left femoral artery was cannulated with PE-90 tubing for blood withdrawal and pressure monitoring. A PE-90 catheter was placed in the bladder for urine collection. The left renal vein was exposed through a ventral midline and left subcostal incision, to make it accessible for puncture. During surgery, animals received intravenous infusion of 0.9% NaCl and 6% BSA followed by 1% BSA after 20 min at a rate of 8 ml/kg/h. Following surgery, infusion rate was reduced to 6 ml/kg/h and maintained throughout the experiment. The solution also contained 30 mM Inulin (Inutest, Fresenius Pharma, Linz, Austria) and 25 mM PAH (Sigma, St. Louis, MO) for clearance measurements. After 45 min stabilization period, infusion of full length CTGF or N-CTGF was started at 54 pmol/kg/min or 7 pmol/kg/min, respectively, based on pilot clearance studies. Urine was collected at 15 min intervals. 90 Min after the start of CTGF infusion two arterial blood samples were taken at an interval of 30 min to determine fractional CTGF excretion. After a short equilibration period a third arterial blood sample was taken simultaneously with puncture of the left renal vein to determine renal extraction ratios and tubular uptake. Inulin and PAH were measured as described12.

Human studies. Effect of impairment of tubular reabsorption on urinary CTGF excretion was studied in healthy human volunteers by infusion of Gelofusine and in renal disease patients with varying degrees of tubular dysfunction. The Gelofusine protocol was approved by the Ethics Review Committee of the University Medical Center St. Radboud and informed consent was obtained from healthy volunteers. In accordance with Dutch ethical regulations, all patients consented that blood and urine samples were used for medical research. The studies were conducted according to the principles of the Declaration of Helsinki.

Three healthy volunteers received infusion of Gelofusine (40 g/l, Braun), which was started at a rate of 10 ml/min for 5 min followed by 3 ml/min during 55 min. CTGF levels were measured in urinary samples taken before the infusion and at 30, 60, and 180 min after start of the infusion, and in blood samples drawn before and at the end of Gelofusine infusion.

In a cross-sectional study, 108 patients with renal impairment due to glomerular diseases were enrolled from March 1995 through March 2004 at the Department of Nephrology, Radboud University Nijmegen Medical Center (The Netherlands). Details of the cross-sectional study have been described4. CTGF ELISA. CTGF levels in plasma, urine, and renal homogenates were determined by sandwich ELISA, using specific antibodies (FibroGen) directed against distinct epitopes in the amino-terminal fragment of CTGF, detecting both full length CTGF and the N-fragment. Frozen renal tissue (stored at -80°C) was homogenized in lysis buffer (20 mM Tris (Roche, Mannheim, Germany), 150 mM NaCl (Merck, Darmstadt, Germany), 1% Triton X-100, 10% glycerol), 1 mM EDTA (Riedel-de Haen, Seelze, Germany), 0.1% SDS (Research Organics, Cleveland, OH), 1 mM EGTA, 0.5% sodium deoxycholate, 50 mM NaF, 2 mM Na-orthovanadate (Sigma), pH 7.4 containing 5% Protease Inhibitor Cocktail (Sigma). Two ELISA assays were used: a human CTGF assay for detection of endogenous CTGF in human samples or recombinant human CTGF in rodent samples and a rodent CTGF assay for determination of endogenous CTGF in the transgenic mice. For the human CTGF assay microtiter plates (Maxisorb; Nunc, Roskilde, Denmark) were coated with capture anti-CTGF monoclonal antibody (5 μg/ml; FibroGen). Subsequently, diluted samples and standards (recombinant human CTGF; FibroGen) were added and incubated with non-cross-blocking human anti-CTGF monoclonal antibody conjugated directly to alkaline phosphatase (0.5 μg/ml; FibroGen). Para-nitrophenylphosphate (Sigma) was used as substrate for the colorimetric reaction. For the rodent CTGF assay a similar protocol was
applied: goat anti-N-CTGF polyclonal antibody (7.5 μg/ml; FibroGen) was used for coating and a non-cross-blocking human anti-CTGF monoclonal antibody was used for detection, followed by incubation with goat anti-human IgG polyclonal antibody conjugated to alkaline phosphatase (1:2000; Sigma). Recombinant mouse CTGF (FibroGen) was used for standard curves. Assay sensitivities (lower limits of detection) were 0.02 pmol/ml for the human CTGF assay and 0.04 pmol/ml for the rodent CTGF assay. The antibody used for detection in the human CTGF assay does not cross-react with rodent CTGF. Intra- and interassay coefficients of variation were within 10%. Plasma levels of endogenous CTGF in healthy rodents, healthy human subjects and renal disease patients are shown in Supplementary Table 1.

In addition to the ELISA assays detecting both full length CTGF and the N-fragment, we applied sandwich ELISA technique to specifically detect full length CTGF by using two distinct antibodies directed against the carboxy-terminal and amino-terminal fragment. We did not measure significant amounts of full length CTGF in human or rodent urine, neither in healthy situation nor in case of impaired tubular reabsorption, which is in accordance with previous reports.

Immunohistochemistry. To identify proximal tubules, staining with *Lotus tetragonolobus* agglutinin (LTA) was applied (5, 28). Deparaffinized FFPE sections (3 μm) were pretreated by boiling in citrate buffer pH 6.0 and incubated with HRP-conjugated LTA (Sigma) (1:32) for 1 h followed by incubation with NovaRed for ten minutes (Vector Laboratories). Sections were counterstained with hematoxylin and visualized by Nikon Eclipse E800 microscope (Nikon Netherlands, Lijnden, the Netherlands).

Consecutive sections were stained with LTA and anti-CTGF (see Immunofluorescence). Images of corresponding areas were merged by digital selection and coloring of LTA-positive areas using Adobe Photoshop software version 11.0 (Adobe Systems, San Jose, CA).

Measurement of creatinine. Urinary and serum creatinine were measured with enzymatic assays (J2L Elitech (Labarthe-Inard, France) for murine samples, DiaSys (Holzheim, Germany) for rat samples and Roche (Mannheim, Germany) for human samples (automated technique).

Measurement of β₂M. Urinary β₂M was measured by ELISA, as described before (18). To avoid degradation of urinary β₂M, subjects ingested 4 g sodium bicarbonate on the evening before the test. β₂M was only measured in urine with a urinary pH >6.0.

Calculations and statistics. Data, expressed as mean ± SEM, were compared with Student’s t test (two-tailed). Paired Student’s t-test was used for comparison of urinary concentrations in mice and fractional excretions in humans before and after Gelofusine administration. Statistical analysis of subsequent urinary concentrations during Gelofusine infusion in humans was performed by ANOVA for repeated measures. Correlations were assessed by linear regression. In the patient study, urinary CTGF, urinary β₂M, plasma CTGF and serum creatinine were logarithmically transformed before analysis because of their skewed distribution. Urinary concentrations were expressed in mol/g creatinine to correct for differences in urine dilution. GFR was estimated by the abbreviated Modification of Diet in Renal Disease equation. Renal clearances and fractional excretions were calculated by standard formulae. Renal extraction was calculated as follows:

\[
\text{renal extraction} = \left( \frac{\text{arterial concentration} - \text{renal vein concentration}}{\text{arterial concentration}} \right) \times 100\%
\]

Tubular uptake was expressed as percentage of the amount of N-CTGF extracted by the kidney and calculated as follows:

\[
\text{Tubular uptake} = \left( \frac{1 - \frac{\text{urine concentration}}{\text{arterial concentration} \times \text{ERPF}}}{\text{renal vein concentration} \times (\text{ERPF} - \text{V})} \right) \times 100\%
\]

where V is the urinary flow rate and ERPF the effective renal plasma flow.
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Urinary CTGF reflects tubular dysfunction

Results

Plasma CTGF is glomerularly filtered, almost completely reabsorbed by proximal tubules and only minimally excreted in urine. First, we investigated the fate of recombinant human CTGF that was administered intravenously to healthy mice. Using recombinant human CTGF and human CTGF specific antibodies we could distinguish between endogenous and exogenous CTGF and exclude contribution of locally produced CTGF to measured tissue levels. We administered both full length CTGF and the amino-terminal fragment but focused our analyses primarily on N-CTGF since this is the predominant form detected in urine6, 12, 19, 27. Intravenous bolus injection of N-CTGF led to rapid accumulation in the renal cortex (Fig. 1A). Immunofluorescent staining showed abundant N-CTGF in endocytic vesicles in renal PTCs (Fig. 1, B–C). Localization in apical vesicles suggested that circulating N-CTGF was glomerularly filtered and subsequently reabsorbed from the luminal fluid. Recombinant full length CTGF was also filtered and tubularly reabsorbed (Fig. 1D). LTA and CTGF staining of consecutive sections identified the CTGF containing segments as proximal tubules (Fig. 2). Thus, in healthy mice CTGF is glomerularly filtered and to a large extent endocytosed by PTCs.

To quantify renal extraction and fractional excretion, we performed additional experiments in anesthetized rats which received CTGF by continuous infusion. After reaching steady state, kinetic parameters were calculated from steady state plasma concentrations and urinary excretion, as summarized in Table 1. For determination of renal extraction, blood samples were drawn simultaneously from the renal vein and femoral artery. Renal N-CTGF extraction was 21±1%, which was 74% of that of inulin. For full length CTGF renal extraction was 18±3%. Tubular uptake was almost complete, resulting in fractional excretions of less than 1% (Table 1). Thus, in healthy rats renally extracted CTGF is almost completely metabolized by the kidney and minimally excreted in the urine. Inhibition of tubular reabsorption reduces renal CTGF uptake and increases urinary CTGF excretion. We next studied the effect of tubular reabsorption impairment on uCTGF. Gelofusine, a modified gelatin employed clinically applied as a plasma volume expander, was used to inhibit proximal tubular reabsorption (13, 30). In mice, intravenous injection of Gelofusine 1-2 min prior to i.v. bolus injection of N-CTGF reduced cortical N-CTGF uptake by 49±1% (P < 0.05; Fig. 3A). Pretreatment with Gelofusine resulted in a comparable decrease in cortical uptake of full length CTGF (Supplementary figure). Moreover, a single intravenous bolus dose of Gelofusine during continuous N-CTGF infusion by intraperitoneal mini-osmotic pump in mice increased urinary N-CTGF 15±1-fold (P < 0.05; Fig. 3B).

In addition to experiments with recombinant CTGFs in mice, we also studied the effect of inhibition of tubular reabsorption on urinary excretion of endogenous CTGF in healthy human volunteers. While basal levels were low, Gelofusine infusion induced

Fig. 1. Renal CTGF accumulation after single dose intravenous administration of recombinant human N-CTGF in mice.

A: estimated renal N-CTGF levels at different time points after injection, determined by ELISA. Each dot represents one animal. Curve fitting, performed with GraphPad Prism, was by one-phase exponential decay model. B-D: immunofluorescence of C57Bl/6J kidney by using human anti-CTGF (green) and TO-PRO 3 Iodide for nuclear staining (red), visualized by confocal laser scanning microscopy. B: N-CTGF treated animal. Magnification × 630. C: N-CTGF treated animal at 30 min showing abundant N-CTGF positive apical endocytic vesicles in proximal tubular cells. Magnification × 1000. D: CTGF treated animal at 10 min showing abundant N-CTGF in proximal tubules, intensely staining the brush border. Magnification × 630. E: saline treated animal. Magnification × 630.
a 23±1-fold increase in uCTGF (P < 0.01), which was paralleled by an increase in urinary β2-microglobulin (uβ2M) (r = 0.99) (Fig. 4, A and B). Fractional excretion of CTGF increased from 0.47±0.1% to 13±3% (P < 0.05). These results demonstrate that, in healthy subjects, diminished tubular reuptake of CTGF from the luminal fluid is sufficient to induce a significant increase of uCTGF.

In renal disease, elevated urinary CTGF is tightly associated with tubular dysfunction. To investigate whether uCTGF in renal disease correlated with urine levels of uβ2M as a marker of tubular dysfunction, we analyzed urines of 108 patients with renal impairment due to glomerular diseases. The patients had varying degrees of tubular dysfunction, as indicated by a wide range of uβ2M levels. General characteristics are summarized in the Supplementary Table 2. We observed a tight correlation between uCTGF and uβ2M levels, suggesting that uCTGF can be used as a marker for tubular dysfunction.

Table 1. Renal handling parameters of recombinant human CTGF in healthy rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N-fragment (N = 4)</th>
<th>Full length CTGF (N = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>323 ± 28</td>
<td>309 ± 11</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>92 ± 7</td>
<td>101 ± 2</td>
</tr>
<tr>
<td>GFR (ml/kg/min; inulin)</td>
<td>7.8 ± 0.5</td>
<td>9.0 ± 0.1</td>
</tr>
<tr>
<td>Renal plasma flow (ml/kg/min; PAH)</td>
<td>30 ± 4</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>Renal extraction (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inulin</td>
<td>29 ± 2</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>CTGF</td>
<td>21 ± 0.7</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>Tubular uptake of CTGF</td>
<td>99.9 ± 0.02</td>
<td>99.96 ± 0.004*</td>
</tr>
<tr>
<td>(% of extracted amount)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractional excretion of CTGF (%)</td>
<td>0.10 ± 0.02</td>
<td>0.025 ± 0.006*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.01

A: estimated renal levels of recombinant human N-CTGF 10-15 min after intravenous N-CTGF administration in mice. Gelofusine administration did not affect plasma N-CTGF levels. B: urinary N-CTGF levels during continuous infusion of recombinant human N-CTGF by intraperitoneal mini-osmotic pump in 3 mice before (16h urine collection) and within 1 hour after intravenous administration of Gelofusine bolus injection. Urinary N-CTGF increased from 0.22±0.03 to 3.4±0.7 nmol/g creatinine (P = 0.04; paired t test).

A: Gelofusine infusion for 60 min in 3 healthy volunteers induced a 23±1-fold increase in urinary CTGF (uCTGF) (P = 0.004; repeated measures ANOVA) which was paralleled by an increase in urinary β2-microglobulin (uβ2M). B: uCTGF and uβ2M were tightly correlated before, during and after Gelofusine infusion.
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Correlation between log transformed urinary CTGF (uCTGF) and urinary β2-microglobulin (uβ2M) in 108 chronic kidney disease patients.

(r = 0.85; P < 0.001, Fig. 5). Urinary β2M emerged as the only independent determinant of uCTGF (β = 0.81; P < 0.001) in a multiple linear regression model containing age, eGFR (or serum creatinine) and plasma CTGF, which were all significantly associated with uCTGF in univariate analysis. This is strong evidence that tubular dysfunction is a major determinant of uCTGF increase in renal disease.

Megalin is involved in proximal tubular reabsorption of CTGF. To identify the endocytic receptor involved in the proximal tubular reabsorption of CTGF, we studied uCTGF in transgenic mice with deficiencies of the endocytic receptors megalin and/or cubilin. These multiligand receptors are known to be responsible for tubular reabsorption of most of the filtered proteins. In Mox2-Cre transgenic mice with a mosaic expression pattern of megalin and cubilin in the kidney, exhibiting combined deficiency of both receptors, uCTGF was very high (14±1.5 nmol/g creatinine), while in cubilin KO and wild type mice uCTGF levels were below level of quantification. There were no differences in plasma CTGF between these various mouse strains. Immunofluorescent staining after intravenous administration of CTGF in healthy C57Bl/6J mice revealed colocalization of megalin and CTGF in the cortical (proximal) tubules (Fig. 6). These results show that proximal tubular endocytosis of CTGF is mediated by the multiligand receptor megalin.

Discussion

The main finding of our studies is that failure of proximal tubular reabsorption is a major determinant of increased urinary CTGF. In healthy animals, intravenously administered CTGF was filtered in the glomerulus, but only minimal excretion in the urine was observed. This was found to be due to almost complete reabsorption in proximal tubular cells by megalin-mediated endocytosis.

Our data show that both full length and the N-CTGF fragment are similarly handled by the kidney. For both CTGFs we observed extensive proximal tubular uptake in apical endocytic vesicles, very low fractional excretions (< 1%) and almost complete tubular uptake of extracted CTGF (> 99%). We focused our analyses on the amino-terminal fragment of CTGF since this is the predominant CTGF peptide in urine and because the biomarker potential of CTGF has been studied most extensively for this proteolytic fragment.

After intravenous administration in rats, renal extraction of circulating N-CTGF was 74% of that of inulin. Since we found no immunohistochemical evidence for basolateral uptake in proximal tubular epithelial cells, we assume that most of the extracted N-CTGF was filtered into the primary urine by glomerular sieving, which would imply a sieving coefficient of approximately 0.7. This is consistent with the observation that proteins smaller than 20 kDa have sieving coefficients greater than 0.5. The high degree of tubular reabsorption of filtered N-CTGF and low fractional excretion (FE) are also in accordance with previously observed values for other LMW proteins, e.g. β2M (fractional reabsorption 99.97%, FE 0.03%) and lysozyme (FE 0.4%).

Impairment of proximal tubular reabsorption, either induced by Gelofusine administration or in human renal disease, was consistently associated with an increase in urinary CTGF excretion in close correlation with that of β2-microglobulin. Tubular...
dysfunction is an important parameter in the assessment of renal damage. In CKD, the degree of tubulointerstitial damage and the amount of LMW proteinuria are better predictors of functional outcome than the severity of glomerular damage or the total amount of proteinuria\(^8, 9\). In diabetic nephropathy, LMW proteinuria was found even before the occurrence of microalbuminuria, probably representing a sign of incipient DN\(^9, 15\). Our results imply that uCTGF, reflecting proximal tubular dysfunction, may be a valuable prognostic marker in renal disease. Moreover, in contrast to known proximal tubular dysfunction markers such as the biologically inert β\(_2\)M and α\(_1\)-microglobulin, luminal accumulation of CTGF and its fragments might contribute actively to the pathogenic process\(^16, 23\). Consistent with such a concept, uCTGF predicted progression of microalbuminuria in diabetic patients\(^29\) and in another study uCTGF independently predicted deterioration of renal function in patients with iMN (1). However, the true clinical value of uCTGF in renal disease still needs to be established.

Although we show that uCTGF is highly dependent on tubular function and tightly associated with uβ\(_2\)M, the correlation between the two proteins is not absolute (R\(^2\) = 0.72). This might reflect disease associated regulation of CTGF production, either systemically or locally in different segments of the nephron\(^16, 31\). As expected for a filtered solute, plasma CTGF was correlated with uCTGF in univariate analysis. However, it did not emerge as an independent determinant in multivariate analysis. The relative contribution of increased local CTGF production could not be determined because the filtered load was unknown. Considering the virtually complete proximal reabsorption from the primary urine in the healthy kidney, CTGF produced in the glomeruli and released into the luminal fluid will have limited impact on uCTGF as long as the proximal endocytic apparatus is intact. On the other hand, luminal secretion of CTGF expressed in distal tubules would be expected to considerably affect urinary CTGF levels, but the linearity of the relationship between uβ\(_2\)M and uCTGF indicates that tubular dysfunction is the major determinant of uCTGF in the renal diseases included in our survey. Yet, the relative contribution of increased local synthesis on uCTGF in specific renal diseases remains to be elucidated in future studies.

In conclusion, CTGF is reabsorbed almost completely from the glomerular filtrate in proximal tubules via megalin-mediated endocytosis in the healthy kidney, and elevated uCTGF may largely reflect proximal tubular dysfunction. This should be taken into account when using uCTGF as a biomarker and in experimental work addressing its pathogenic involvement in chronic kidney disease.

Supplementary Table 1. Plasma CTGF levels in healthy rodents, healthy human subjects and renal disease patients

<table>
<thead>
<tr>
<th>Species</th>
<th>Plasma CTGF (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>0.23 (0.19-0.29)</td>
</tr>
<tr>
<td>Rats</td>
<td>0.27 (0.18-0.37)</td>
</tr>
<tr>
<td>Humans</td>
<td></td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>0.097 (0.06-0.16)</td>
</tr>
<tr>
<td>Renal disease patients</td>
<td>0.21 (0.11-0.39)</td>
</tr>
</tbody>
</table>

Values are median (interquartile range)

Supplementary Table 2. Characteristics of 108 patients with renal disease

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N = 108</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male:female</td>
<td>73:35</td>
</tr>
<tr>
<td>Age (yr; median (range))</td>
<td>46 (16 to 82)</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl; median (range))</td>
<td>1.1 (0.5 to 3.3)</td>
</tr>
<tr>
<td>eGFR (ml/min per 1.73 m(^2); median (range))</td>
<td>63 (19 to 145)</td>
</tr>
<tr>
<td>Proteinuria (g/10 mmol creatinine; median (range))</td>
<td>4.6 (0.1 to 18.2)</td>
</tr>
<tr>
<td>Renal disease (N)</td>
<td></td>
</tr>
<tr>
<td>Idiopathic membranous nephropathy</td>
<td>52</td>
</tr>
<tr>
<td>IgA nephropathy</td>
<td>43</td>
</tr>
<tr>
<td>Focal glomerular sclerosis</td>
<td>13</td>
</tr>
</tbody>
</table>

Acknowledgments

We thank Marcel Fens, Dionne van der Giezen, Willemsk Kassing–van der Ven, Arianne van Koppen, Jan Willem Leeuwis, Paula Martens, Pia Nielsen, Ebel Pieters and Kevin van der Ven for technical assistance and support.
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References


Summary and general discussion
In the first part of this chapter we summarize our findings. In the second part we discuss our findings, provide information on the relevance of biomarkers for current practice, and provide perspectives for future research.

**Part 1a. Biomarkers to predict outcome in IgA nephropathy**

In chapter 2, we evaluated whether the urinary excretion of the low molecular weight (LMW) proteins α-microglobulin (α₁m) and β₂-microglobulin (β₂m) predicted renal outcome in a cohort of 70 patients with histologically proven IgAN. These patients were mostly referred for advice regarding treatment. The majority of patients (57%) had significant proteinuria (>2 g/d), and impaired kidney function (eGFR < 60 ml/min/1.73m²). Twenty-five patients (36%) developed ESRD, and 46 patients (66%) had an increase in serum creatinine of >50% after a median follow-up of 75 months. Nineteen patients (27%) received immunosuppressive treatment. Mean urinary excretion of α₁m and β₂m was increased above normal values. However, only baseline serum creatinine and total proteinuria proved significant independent predictors of ESRD or progression of renal disease. Of note, patients that received immunosuppressive therapy were less likely to develop ESRD. We concluded that measuring urinary excretion of α₁m and β₂m has no added value in predicting renal outcome over established clinical predictors such as baseline proteinuria and eGFR.

In recent years novel urinary biomarkers for acute and chronic kidney injury were described. We selected two biomarkers of early tubular damage, kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) and studied their accuracy in predicting renal outcome in patients with IgAN. This study, described in chapter 3, included most of the abovementioned patients who had been evaluated in our hospital for treatment advice, with available stored urine samples. Sixty-five patients were evaluated. During a median follow-up of 76 months, 23 patients (35%) developed ESRD, and KIM-1 excretion predicted ESRD specifically in the subgroup of patients with normal or mildly impaired kidney function.

The diagnosis IgAN requires a kidney biopsy to identify mesangial IgA in the glomeruli. For many years pathologists have developed grading systems for IgAN with the expectation that scoring of histological variables would allow risk-stratification. Thus far, the severity of tubulointerstitial fibrosis is the best-validated and most potent histologic prognostic marker. Since inflammation, characterized by recruitment of T-lymphocytes and macrophages, precedes fibrosis, it was hypothesized that detailed analysis of inflammatory cells would allow predicting outcome. Indeed, in 2008 a Dutch study that included 50 patients with IgAN showed that the presence of GMP-17 positive T-lymphocytes in the kidney tubules predicted progression of kidney disease. In this study, immunophenotyping and localization of interstitial infiltrate was determined by using a subset of monoclonal antibodies against a variety of leukocytic markers. Statistical analysis revealed that of the morphologic changes and immunophenotypic parameters the presence of GMP-17+/CD8+ intraepithelial lymphocytes in the tubules was the most sensitive marker with the highest likelihood ratio. Risk prediction was limited to the subgroup of 31 patients with no or mildly impaired renal function.

In chapter 4, we aimed to validate the prognostic value of this parameter. This study included 24 patients with IgAN and available stored kidney biopsies. The presence of GMP-17 positive T-lymphocytes was scored semi-quantitatively. In 9 patients (38%) GMP-17 positive T-lymphocytes were found. At the time of the biopsy, GMP-17 positive patients had more severely impaired kidney function, and higher urinary levels of LMW proteins and KIM-1 than GMP-17 negative patients. During a median follow-up of 102 months, eight patients (33%) developed ESRD, whereas 13 patients (54%) had an increase in serum creatinine of >50%. There was no difference regarding ESRD or progression of kidney disease between GMP-17 positive and GMP-17 negative patients: three out of nine (33%) vs. five out of 15 (30%) and four out of nine (44%) vs. nine out of 15 (60%), respectively. Limiting our analysis to patients with an eGFR ≥ 60 ml/min/1.73m² left only nine patients. Two of these were GMP-17 positive, none developed ESRD, and of the three patients who showed progression of renal disease one was GMP-17 positive. Despite its limitations, our small retrospective study certainly puts the previously reported prognostic value into question. Notably, the presence of GMP-17 positive T-lymphocytes was significantly correlated with higher levels of α₁m, and in our previous study (chapter 2), α₁m did not predict progression of renal disease. In three out of six patients with idiopathic membranous nephropathy GMP-17 positive T-lymphocytes were observed in the tubules, so the presence of these lymphocytes is not restricted to patients with IgAN.

In chapter 5, we studied the effect of immunosuppressive therapy in 19 patients with IgAN at high risk for progression of kidney disease (median eGFR 33 ml/min/1.73m²,
median urine protein-creatinine ratio 3.8 g/10mmol). This study specifically aimed at identifying a biomarker predicting response to therapy. Most patients were treated with cyclophosphamide and prednisone, treatment duration was 19 (range 3-81) months. During follow-up (median 35 months after start of treatment), 10 patients showed progression of kidney disease, whereas eGFR remained stable in 9 patients. Based on the clinical characteristics progression would have been expected in more than 80% of patients. Thus, our study supports the efficacy of immunosuppressive therapy in patients with IgAN. More important was our observation that a reduction of proteinuria ≥ 50% at 6 months after start of treatment predicted a favourable long-term response.

Part 1b. Renal handling of hepcidin and CTGF

Our studies in patients with IgAN clearly demonstrated the almost negligible role of established biomarkers such as low molecular weight proteins, KIM-1, and histological markers in predicting outcome in patients with IgAN. Therefore, we searched for other potentially useful biomarkers. In this thesis we focused on hepcidin and CTGF. Before assessing the role of these novel biomarkers in IgAN, we performed studies to evaluate relevant items such as variability of assays and renal handling.

The interest in hepcidin as biomarker for kidney disease was stimulated by studies that showed that in patients with lupus nephritis, alterations in urinary hepcidin predicted renal flares. Furthermore, elevated urinary hepcidin levels predicted a lower risk for acute kidney injury (AKI) in patients after cardiac surgery. In chapter 6, we evaluated the intra-individual variability of serum hepcidin and its determinants in a cohort of 63 hemodialysis patients. Serum hepcidin was measured using mass spectrometry (MS) and ELISA assays. With both assays there was a considerable intra-individual variability of serum hepcidin levels, with 60% of patients having a coefficient of variance (CV, i.e. standard deviation divided by mean) >20%. Surprisingly, administration of iron and degree of inflammation failed to support this hypothesis: the calculated clearance of hepcidin-25 by peritoneal dialysis, serum levels of hepcidin decreased only slightly during dialysis. This might be due to rapid increased synthesis of hepcidin or due to release of hepcidin-25 from available stores.

Solid data defining whether the appearance of hepcidin in the urine reflects filtration, tubular reabsorption, or local production are not available, but essential. In chapter 8, we studied the role of tubular reabsorption in kidney handling of hepcidin by comparing fractional excretion (FE) of hepcidin-25 with FE of β2m in 30 patients with various degrees of impairment in tubular reabsorption (median eGFR 57 ml/min/1.73m², median proteinuria 4.5 g/d). FE of hepcidin-25 was higher in patients than in 24 controls (8.0 vs 1.9 %, p < 0.001) and strongly correlated with FE of β2m (r = 0.93, p < 0.01), the latter being an established marker of proximal tubular reabsorption. To prove that hepcidin is reabsorbed in the proximal tubule by binding to megalin, we measured urine hepcidin-1 in wild type and kidney specific megalin-deficient mice. Indeed, urine hepcidin-1 was increased in megalin-deficient mice compared to wild-type mice (p < 0.01) indicating that reabsorption is dependent on megalin-mediated endocytosis. We also measured hepcidin in 19 patients after cardiopulmonary surgery, two of these patients developed acute kidney injury (AKI). Immediately after surgery FE of hepcidin and β2m were 21 and 14% (r = 0.79, p = 0.02), indicating impairment of tubular reabsorption. At 12-24 hours after surgery FE of β2m decreased to 3% indicating an improvement of tubular damage, but FE of hepcidin increased further to 33%. These findings suggest that the kidney locally produces hepcidin-25. Local production of hepcidin-25 may protect against AKI by attenuating oxidative injury due to free iron. The results of this pilot study should be corroborated with histopathological data showing proximal tubular uptake of hepcidin and hepcidin expression by the kidney or macrophages. Moreover, the exact timing and the possible factors influencing local production should be evaluated in future studies, since upregulation of urinary hepcidin might protect against AKI. It is suggested by
several investigators that kidney damage in patients with IgAN is partly explained by hematuria, and the consequence of haem-induced tubular injury. Since hepcidin may protect against iron-mediated injury, local production of hepcidin in the kidney may be beneficial in IgAN. Studies of urinary hepcidin in IgAN and other hematuric glomerular diseases are needed.

Connective tissue growth factor (CTGF) is a profibrotic peptide and may be another potential biomarker for early tubulointerstitial fibrosis. Elevated urinary CTGF levels have been reported in patients with diabetic glomerulopathy and other glomerular diseases such as IgAN. Elevated urinary CTGF was considered to reflect increased intrarenal production. In chapter 9, we studied glomerular and tubular handling of CTGF. In healthy subjects FE of CTGF was low (<1%), and impairment of tubular reabsorption by gelofusin administration, which competitively blocks protein reabsorption, induced a significant increase in urinary CTGF. Urinary CTGF was also elevated in megalin-deficient mice. Lastly, we observed that CTGF strongly correlated with β₂m. Thus, we conclude that CTGF is filtered and almost completely reabsorbed via megalin-mediated endocytosis in the kidney tubules, and elevated urinary CTGF is mainly explained by tubular dysfunction. This should be considered when using urinary CTGF as a biomarker. In the context of predicting progression in patients with IgAN, CTGF does not seem to be an accurate marker, since it is strongly correlated with β₂m, which failed to predict prognosis.

Part 2. General discussion

In this thesis we have studied the role of biomarkers in predicting prognosis in patients with IgAN. Overall, our studies may seem disappointing: the urinary excretion of low molecular weight proteins did not predict renal outcome; although urinary excretion of KIM-1 proved a statistically significant, independent predictor of outcome, its value in clinical practice is doubtful; we could not confirm the predictive value of the presence of GMP-17 positive lymphocytes in the kidney tubuli. The most promising finding was that the level of proteinuria after 6 months of immunosuppressive therapy could reliably predict long-term response to therapy. Monitoring proteinuria, a rather old-fashioned traditional biomarker, thus may help in guiding therapy.

In recent years many investigators have evaluated biomarkers in IgAN and promising results have been reported. This may seem contradictory to our findings. Yet, it is important to carefully evaluate the weaknesses and strengths of these biomarker studies. It is too early to draw too optimistic conclusions.

1 Why do we need biomarkers in IgAN?

IgAN is a disease with a very heterogeneous course. Although IgAN is defined by the presence of IgA deposits in the mesangium, the mere presence of IgA in the mesangium does not suffice to define IgAN as a disease. In fact, IgA deposits can be observed in many apparently healthy individuals\(^1\). The pathogenesis of IgAN involves a multistep process starting with the initial phase of producing galactose deficient IgA, and ending with progressive kidney failure\(^2\). The various steps are illustrated in Figure 1 and Table 1.

Clinically, the disease manifests with microscopic or sometimes bouts of macroscopic hematuria. This phase cannot clinically be discerned from thin basement membrane nephropathy, a disorder that is even more benign. A minority of patients who present with

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**Table 1. Multistep pathogenesis of IgAN and potential biomarkers**

<table>
<thead>
<tr>
<th>Proposed phases in the pathogenesis of IgAN</th>
<th>Traditional biomarkers</th>
<th>Novel biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Increased circulating galactose-deficient IgA1 (gd-IgA1)</td>
<td>Genetic markers</td>
<td>Galactose-deficient IgA1 levels in serum</td>
</tr>
<tr>
<td>2. Generation of galactose-deficient IgA1 antibodies (IgG or IgA)</td>
<td>Galactose-deficient IgA1 specific antibodies in serum</td>
<td></td>
</tr>
<tr>
<td>3. Mesangial deposition of galactose-deficient IgA1 containing immune complexes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Activation, proliferation and damage of mesangial cells</td>
<td>Hematuria</td>
<td>MicroRNA profiling in blood and urine (e.g. miR-148b)</td>
</tr>
<tr>
<td>5. Glomerulosclerosis and tubulointerstitial fibrosis</td>
<td>Serum creatinine/ eGFR</td>
<td>Complement in urine and blood (e.g. mannose binding lectin, resp IgA/C3 ratio)</td>
</tr>
<tr>
<td></td>
<td>Proteinuria</td>
<td>Urine and serum proteomics</td>
</tr>
<tr>
<td></td>
<td>Interstitial fibrosis on biopsy</td>
<td>Markers of oxidative stress (e.g. AOPPs)</td>
</tr>
<tr>
<td></td>
<td>Clinical risk score</td>
<td>Urine cytokines (e.g. MCP-1)</td>
</tr>
<tr>
<td></td>
<td>Proteinuria during follow-up</td>
<td>Immunohistochemistry for fibroblasts (e.g. FSP1)/ mesangial matrix markers on biopsy</td>
</tr>
<tr>
<td></td>
<td>Isolated macroscopic</td>
<td>Low-molecular-weight proteins in urine (e.g. hemopexin)</td>
</tr>
<tr>
<td></td>
<td>hematuria</td>
<td>MIEST score on biopsy</td>
</tr>
<tr>
<td></td>
<td>Persistent microscopic</td>
<td>Complement in urine and blood (e.g. mannose binding lectin, resp IgA/C3 ratio)</td>
</tr>
<tr>
<td></td>
<td>hematuria</td>
<td>Glomerular density measurement on biopsy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine and serum proteomics</td>
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<td>Markers of oxidative stress (e.g. AOPPs)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine cytokines (e.g. MCP-1)</td>
</tr>
</tbody>
</table>

FSP1: fibroblast specific protein 1-positive, AOPP: advanced oxidation protein product, MCP: monocyte attractant protein
Unfortunately, most studies do not allow specific evaluation of the various outcome measures. This is evident from Table 2, which summarizes the characteristics of the patients that were included in recent predictor studies\textsuperscript{3-6}. It is evident that these studies all included a very heterogeneous patient population, as reflected by the wide range in proteinuria, eGFR, and histological damage. Furthermore, 25-55\% of patients in these studies received immunosuppressive therapy during follow-up. Thus, at best, these studies can evaluate associations between a biomarker and overall outcome. Confounding by indication, collinearity of parameters etcetera, will bias the analyses.

2 Limitations of (studies of) novel biomarkers

In general, biomarker studies evaluate the association between the presence or level of the relevant biomarker and outcome. It is not sufficient to find a significant association in univariate analyses, since a relevant biomarker should provide additional value and prove an independent predictor in multivariate analysis. Moreover, novel biomarkers should be superior to traditional biomarkers such as proteinuria and their incorporation in prediction models should improve overall prediction. The role of a biomarker is often suggested by studies in an initial (incipient) cohort, but the value of a biomarker must be confirmed in a validation study. Finally, a statistical significant effect may not be clinically relevant.

2a: Novel versus traditional biomarkers

Traditional biomarkers predict prognosis with reasonable accuracy. Proteinuria, eGFR, and hypertension are well known predictors of outcome in IgAN. In a recent study Xie et al. improved the performance of these clinical parameters by combining them in a risk score based on eGFR, systolic blood pressure, haemoglobin and serum albumin\textsuperscript{4}. The ROC curve for predicting ESRD after 10 yr was 0.85, reflecting reasonable accuracy. Although sensitivity and specificity are not mentioned, an overall accuracy of 85\% may reflect a sensitivity of 85\% and a corresponding specificity of 85\%.

The study of Xie et al. also included a heterogeneous patient group (see Table 2) and conclusions cannot be generalised to untreated patients with normal renal function. Still, it is clear that in patients with established IgAN it will be difficult to find novel biomarkers that improve prediction over and beyond risk prediction by traditional risk factors.

Our study is an example (chapter 3). Both serum creatinine and proteinuria predicted outcome. A model based on serum creatinine predicted outcome with reasonable accuracy (AUC 0.82). In multivariate analysis urinary excretion of KIM-1 proved a statistically significant independent predictor of outcome. However, the ROC AUC increased only from 0.82 to 0.86, indicating that the use of KIM-1 only improved accuracy slightly.

For many years investigators have tried to use kidney biopsy findings to predict outcome. Glomerulosclerosis, interstitial fibrosis and vascular hyalinosis all have been associated with outcome. However, the value of histology over clinical risk variables has been
difficult to prove. In 2009, an international study identified mesangial hypercellularity (M), endocapillary proliferation (E), segmental sclerosis (S) and tubular atrophy/interstitial fibrosis (T) as independent predictors of renal outcome. Recently, the predictive value of this classification was validated in the large, multicentre European VALIGA study that included 1147 patients. Again, clinical characteristics were quite variable (Table 2), and the study included patients with minimal proteinuria as well as patients with severe renal insufficiency (eGFR < 30 ml/min/1.73 m^2). Overall 46% of patients received immunosuppressive therapy. A close look at the results provides interesting information, relevant for the interpretation of all biomarker studies.

The authors observed a close association between the E score and proteinuria, whereas the M, S, and T scores were associated with proteinuria, eGFR and blood pressure. The M, S, and T score predicted outcome, the E score was not associated with outcome. Of note, the predictive value of the pathology score was no longer present in the patients who received immunosuppressive therapy. Thus, clearly the use of immunosuppressive therapy affected the performance of biomarkers.

The VALIGA study also showed that clinical predictors such as eGFR, and time-averaged proteinuria were more accurate than histological predictors (AUC 0.72 vs 0.65). Adding histological parameters to clinical parameters improved prediction, but only in untreated patients. The authors write that “the added value of pathology was evident”. Still, the AUC improved from 0.72 to only 0.75, a minor increase, with limited clinical relevance.

Although the VALIGA study is a cross-sectional study in a heterogeneous patient group, there are several other interesting findings. As already mentioned, the use of immunosuppressive therapy had a major impact on the relevance of the histological biomarkers, indicating that in future studies we must differentiate between biomarkers that predict outcome in untreated patients, and biomarkers that are relevant for treated patients.

Even more important is the observation that time-averaged proteinuria during follow-up is a good predictor of outcome. This may not be surprising, and is a confirmation of other studies.

In view of the low risk of ESRD in a 10 yr period, it is more revealing to consider the risk of proteinuria progression. After 10 years of follow-up approximately 40% of patients with initial proteinuria <0.5 g/day had progressed to proteinuria > 1 g/day. Of note, in this subgroup of patients (n=219) the E score proved a significant predictor, with a Hazard ratio of developing proteinuria > 1 g/day or > 2 g/day being 2.3 and 3.5 respectively.
2b. Novel biomarkers should not be used to predict ESRD

As discussed, IgAN involves a multistep process. In Table 1 we have tried to dissect the various steps involved in this process (column 1). Next, we have added traditional and novel biomarkers, in relation to the pathogenic pathways (column 2). This table clearly illustrates why most biomarker studies fail: most biomarkers only reflect one step in the multifactorial process of progressive IgAN. The final outcome is the consequence of the combination of these factors. Therefore, one biomarker will never be able to predict final outcome with sufficient accuracy. Obviously, biomarkers that reflect the final stage of process (i.e. severe kidney injury) are more likely to be better predictors of ESRD. This explains the predictive value of biomarkers of severe kidney injury such as proteinuria, eGFR, and severe tubular atrophy.

3 Change in current practice and prospects for future research

Traditional clinical parameters such as eGFR, proteinuria, and blood pressure are good predictors of outcome, certainly when considering outcome after 10 years. It has become evident that regular measurement of proteinuria during follow-up adds valuable information. Time-averaged proteinuria should be our main guide in the management of patient with IgAN. The recent studies show that time-averaged proteinuria should be below 0.5 g/day. This founding should influence current practice, and in particular should stimulate performing a kidney biopsy in an earlier stage (Figure 2). Thus far, most nephrologists would wait until proteinuria exceeds 1-2 g/day.

The VALIGA study suggested benefits of immunosuppressive therapy even in patients with eGFR < 50 ml/min/1.73m². Unfortunately late treatment is not the most effective: renal survival after 10 yr approximates 90% in patients with initial eGFR > 50 ml/min/ min/1.73m² and only 60% in patients with initial eGFR 50 ml/min/1.73m² ¹⁰. Thus, it is important to start treatment in an early stage of the disease, although this obviously will increase the number of patients that are treated unnecessarily. Here is the unmet need for predictive biomarkers (see below).

For now, it would be important to predict responsiveness to immunosuppressive therapy. As such, a Japanese study suggested that the number of fibroblasts in the interstitium could serve as an excellent predictor of steroid response¹¹.

These investigators counted the number of cells that stained positive for fibroblast specific protein 1. The number of FSP positive cells correlated with the degree of tubulointerstitial fibrosis and negatively with eGFR.

A higher number of cells decreased the likelihood of stabilisation of eGFR and reduction of proteinuria with steroid therapy. Using the optimal cut-off value of 32.6 cells/ high power field sensitivity was 0.75% and specificity 0.87%. Although the accuracy of this method seems reasonable, we argue that introduction of this methodology (which would often require a new kidney biopsy) would lead to undertreatment: 25% of patients who are considered treatment-resistant based on the test results would respond to steroids and thus maintain eGFR for a longer period.

Our finding that a reduction of proteinuria at 6 months after start of therapy is a good predictor therefore is relevant. In this way patients that do respond will be selected for continuous treatment. In non-responders treatment can be stopped, and the period of unnecessary therapy is at least reduced. Hopefully, the increased knowledge of the multistep pathogenesis of IgA nephropathy and its genetic and environmental modifiers will result in novel potent therapies with less adverse effects in the near future (Figure 1).

Future studies should focus on patients with early stage IgAN. Predator studies should only include patients with normal eGFR and limited proteinuria, and progression of proteinuria could be the optimal outcome measure. In these studies combinations of the various biomarkers should be tested for their predictive value. Also, it is important to evaluate changes in biomarkers during follow-up. IgAN is a dynamic disease and a single baseline measurement probably will never suffice.
Figure 2. Suggested therapeutic approaches in patient with IgA nephropathy based on KDIGO Clinical Practice Guideline for Glomerulonephritis 2012, recent literature and current thesis.

<table>
<thead>
<tr>
<th>eGFR ≤60 ml/min</th>
<th>Progressive loss of eGFR and/or proteinuria &gt;1g/d after optimal supportive therapy during at least 3-6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFR 25-60 ml/min and proteinuria &gt;1g/d after optimal supportive therapy during at least 3-6 months</td>
<td></td>
</tr>
<tr>
<td>Protein restriction Sodium restriction</td>
<td></td>
</tr>
<tr>
<td>Consider kidney biopsy and/or early therapy</td>
<td></td>
</tr>
<tr>
<td>Immunosuppressive therapy consisting of combined therapy with prednisone and cyclophosphamide</td>
<td></td>
</tr>
<tr>
<td>Continuation of supportive therapy, no immunosuppressive treatment</td>
<td></td>
</tr>
<tr>
<td>Immunosuppressive therapy consisting of combined therapy with prednisone and cyclophosphamide</td>
<td></td>
</tr>
<tr>
<td>Prednisone monotherapy</td>
<td></td>
</tr>
</tbody>
</table>

Normal eGFR, proteinuria ≤0.5 g/d: no hypertension

Normal eGFR, proteinuria ≤0.5 g/d: no hypertension

Supportive therapy: Control of blood pressure (≤130/80 mmHg), ACEI or ARB therapy (even if normotenstive), sodium restriction, and/or kidney biopsy.

Failure to respond to therapy: consider immunosuppressive therapy consisting of combined therapy with prednisone and cyclophosphamide.

Immunosuppressive therapy consisting of combined therapy with prednisone and cyclophosphamide.

Immunosuppressive therapy consisting of combined therapy with prednisone and cyclophosphamide.

Immunosuppressive therapy consisting of combined therapy with prednisone and cyclophosphamide.

Supportive therapy, most importantly: Control of blood pressure (≤125/75 mmHg), ACEI or ARB therapy. Protein restriction.

Exclusion of acute kidney injury due to prolonged macroscopic hematuria or other causes of progression of renal insufficiency than IgAN.

Rapid decline of renal function and/or nephrotic syndrome.

Nephrotic syndrome (selective proteinuria, and normally appearing glomeruli, apart from IgA deposits in the mesangium, with foot process effacement).

References

Samenvatting

Deel 1a. Biomarkers om de uitkomst van IgA nefropathie te voorspellen

In hoofdstuk 2 gaan we in op de voorspellende waarde van urinemarkers voor proximale tubulus dysfunctie ($q_1$-microglobuline ($q_m$) en $\beta_2$-microglobuline ($\beta_2m$)) in een cohort van 70 patiënten met IgA nefropathie (IgAN) bewezen middels een nierbiopsie. Deze patiënten werden verwezen voor een behandeldivisie. De meerderheid had een forse proteinurie (>2g/d), en een gestoorde nierfunctie (eGFR <60ml/min/1,73m²). Na ruim 6 jaar, was bij 25 van hen sprake van nierfalen en maar liefst twee derde had een nierfunctieverlies met >50%. Immunosuppressieve behandeling werd gegeven aan 19 patiënten. De gemiddelde uitscheiding van $q_m$ en $\beta_2m$ was meer dan normaal. Maar alleen het serum creatinine en de mate van proteinurie bleken onafhankelijke voorspellers van eindstadium nierfalen of verslechtering van de nierfunctie. Opmerkelijk genoeg hadden patiënten die een immunosuppressieve behandeling ondergingen een kleinere kans op nierfalen. Het meten van de uitscheiding middels de urine van $q_m$ en $\beta_2m$ helpt dus niet om verslechtering van de nierfunctie te voorspellen.

De laatste jaren is melding gemaakt van nieuwe urine biomarkers voor acute en chronische nierschade. Wij selecteerden twee biomarkers van beginnende schade aan de tubuli, te weten kidney injury molecule-1 (KIM-1) en neutrophil gelatinase-associated lipocalin (NGAL) en bestudeerden hun waarde om het beloop van de nierfunctie te voorspellen bij patiënten met een IgA nefropathie. Deze studie, die wordt beschreven in hoofdstuk 2, bevat vrijwel alle hierboven genoemde patiënten van wie opgeslagen urine voorradig was. Zesenvijftig patiënten werden ruim 6 jaar gevolgd, 23 van hen ontwikkelden nierfalen, wederom was bij 19 patiënten immunosuppressieve behandeling gestart. De uitscheiding via de urine van KIM-1 en NGAL was veel hoger bij patiënten met IgAN was hoger dan bij gezonde vrijwilligers. KIM-1 en NGAL correlerden met elkaar ($r=0.53, p<0.01$) en met proteinurie ($r=0.40$ en $0.34$, respectievelijk, $p<0.01$), maar niet met eGFR. Ook was er geen correlatie tussen uitscheiding van KIM-1 en $q_m$ en $\beta_2m$. Univariate analyse liet zien dat eGFR, proteinurie, urine uitscheiding van KIM-1, NGAL, $q_m$, $\beta_2m$, IgG en albumine, en immunosuppressieve therapie allen geassocieerd waren met nierfalen. Middels multivariate analyse bleken alleen serum creatinine, urine uitscheiding van KIM-1 en behandeling met immunosuppressieve medicatie onafhankelijke voorspellers van nierfalen. Wanneer dit wordt uitgezet in een zogenaamde receiver operator characteristics curve (ROC) lijkt dit model een redelijk voorspellende waarde te hebben (area under the curve (AUC) 0.86). Aanvullende analyse doet vermoeden dat urine uitscheiding van KIM-1 nierfalen vooral voorspelt in een subgroep van patiënten met IgAN en een slechts minimaal gestoorde nierfunctie.

Voor het stellen van de diagnose IgAN is een nierbiopt absoluut noodzakelijk. Al jaren worden door pathologen scoringssystemen ontwikkeld om zo een risico inschatting te
Deel 1b. Verwerking van hepcidine en CTGF door de nieren

De hierboven beschreven onderzoeken in patiënten met IgAN laten zien dat er geen of slechts een zeer bescheiden rol is voor nieuwe biomarkers zoals α1m en β2m, KIM-1 en histologische variabelen wanneer het gaat om het voorspellen van de kans op progressie van nierfalen. Aldus zijn we op zoek gegaan naar andere biomarkers. We hebben ons daarbij beperkt tot hepcidine en CTGF. Alvoens de prognostische waarde van deze nieuwe biomarkers in IgAN na te gaan, hebben we onderzocht hoe betrouwbaar gebruikte assays zijn waarmee deze biomarkers worden gemeten en hoe deze biomarkers door de nieren worden verwerkt.

Hepcidine kwam in beeld als een biomarker voor nierziekte nadat verschillende onderzoeken lieten zien dat opvlaming van lupus nefritis kunnen worden voorspeld door verandering in urine hepcidine. Daarnaast werd beschreven dat hoge urine hepcidine concentraties gepaard gaan met een lager risico op acute nierschade in patiënten die een hertchirurgie hebben ondergaan. In hoofdstuk 6 bespreken we de intra-individuele variabeliteit van serum hepcidine en factoren die het serum hepcidine beïnvloeden in 63 chronische hemodialyse patiënten. Serum hepcidine werd gemeten middels massa spectrometrie (MS) en ELISA. Bij beide methodes was varieerden de serum hepcidine waarden aanzienlijk binnen een individu; 60% van de patiënten had een coefficient van variatie (CV) te weten standaard deviatie gedeeld door het gemiddelde) > 20%. Verrassenderwijs hadden toediening van intraveneus ijzer en het CRP gehalte geen grote invloed op deze variabiliteit. Deze forse en grotendeels onverklaarde variabiliteit doet vermoeden dat serum hepidin-25 waarden niet geschikt zijn om een anemie behandeling voor een individuele patiënt op te baseren. Met het oog op urine hepcidine als biomarker, is aannemelijk dat variaties in het serum eveneens zullen leiden tot variaties in de urine, tenzij hepcidine eiwitgebonden is en daardoor niet of nauwelijks gefilterd wordt door de glomerulus of indien hepcidine vrijwel volledig wordt gereabsorbeerd door de renale tubuli.

Gezien het feit dat hepcidine-25 een zeer klein eiwit is, ligt het voor de hand dat het vrij gefilterd wordt door de glomerulus en vervolgens tubulair wordt gereabsorbeerd. In hoofdstuk 7 beschreven we de invloed van de eGFR op serum hepcidine-25, maar ook hepcidine-20 en -22 waarden gemeten in 83 patiënten met chronisch nierfalen die niet dialyse afhankelijk waren. Serum hepcidine-25 waarden waren niet significant hoger dan bij controles (4,2 versus 5,1nM, p=0,30). De geschatte glomerulaire filtratie (eGFR) was niet van invloed op serum hepcidine-25 spiegels, ferritine was de enige onafhankelijk variabele voor een individuele patiënt op te baseren. Met het oog op urine hepcidine als biomarker, is aannemelijk dat variaties in het serum eveneens zullen leiden tot variaties in de urine, tenzij hepcidine eiwitgebonden is en daardoor niet of nauwelijks gefilterd wordt door de glomerulus of indien hepcidine vrijwel volledig wordt gereabsorbeerd door de renale tubuli.

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de eGFR en serum hepcidine waarden. Onze bevinding dat hepcidine-25 niet afhankelijk is van de eGFR kan erop duiden dat hepcidine eiwit gebonden is. Echter, dit wordt tegengesproken door data verkregen van patiënten die peritoneaal dialyse ondergaan; de berekende klaring kwam daarbij overeen met de te verwachten klaring op basis van het molecuulgewicht. Bij 48 patiënten die behandeld werden met hemodialyse vonden we duidelijk hogere hepcidine-25 concentraties dan in patiënten met nierschade die niet dialyse afhankelijk waren (mediaan 94 versus 5,2 nM, p<0,001), hepcidine-25 was sterk gecorreleerd met ferritine. Hepcidine waarden afgenomen tijdens de dialyse waren veel hoger uit de arteriële lijn dan in de veneuze lijn, hetgeen wijst op verwijdering van hepcidine-25 door de kunstnier (klaring 82 ml/min). Opmerkelijk genoeg was er desondanks slechts een geringe daling van hepcidine-25 tijdens de dialyse sessie. Mogelijk momt dit door een snelle synthese van hepcidine danwel het vrijkomen van hepcidine uit een of andere opslag.

Concrete gegevens over hoe de nier met hepcidine omgaat, te weten of hepcidine vrij wordt gefiltreerd, of hepcidine tubulair wordt gereabsorbeerd, of lokaal wordt geproduceerd, ontbreken, maar zijn essentieel. In hoofdstuk 8 rapporteren we de tubulaire reabsorptie van hepcidine door de fractionele excretie (FE) van hepcidine te vergelijken met de FE van β₂m (wordt normaliter vrijwel volledig gereabsorbeerd door de tubuli) gemeten in 30 patiënten met verschillende gradaties van tubulaire dysfunctie. (mediaan eGFR 57 ml/min/1,73 m², mediane proteïnurie 4,5 g/d). De FE van hepcidine-25 was hoger in patiënten dan in 24 controles (8,0 versus 1,9%, p<0,001) en sterk gecorreleerd met de FE van β₂m (r=0,93, P<0,001). Om te bewijzen dat hepcidine wordt gereabsorbeerd in de proximale tubulus door binding aan megaline, hebben we urine hepcidine-1 gemeten in wild-type en megaline deficiënte muizen. Inderdaad bleek urine hepcidine-1 significant verhoogd bij de muizen zonder megaline in de nier, waarmee waarschijnlijk is dat hepcidine reabsorptie afhankelijk is van megaline-medieerde endocytose. In 19 patiënten die cardiopulmonale chirurgie hebben ondergaan en waarvan er 2 acute nierschade ontwikkelden, hebben we eveneens hepcidine bepalingen uitgevoerd. Direct na de operatie was de FE van hepcidine en β₂m 21 en 14% (r=0,79, p=0,02), daarmee aangevend dat er verminderde tubulaire reabsorptie was. Na 12-24 uur was de FE van β₂m gedaald naar 3%, duidend op een verbetering van de tubulaire reabsorptie, maar de FE van hepcidine steeg verder door tot 33%. Deze gegevens wijzen erop dat de nier hepcidine-25 produceert. Lokale productie van hepcidine-25 zou beschermend kunnen zijn tegen acute nierschade door het verminderen van oxidatieve schade ten gevolge van vrij ijzer. De resulaten van dit onderzoek zouden moeten worden onderbouwd door histopathologische data waarbij inzichtelijk wordt gemaakt dat hepcidine tubulair wordt opgenomen en dat hepcidine tot expressie wordt gebracht door de nier en/of macrofagen. Bovendien zou verder onderzoek moeten plaatsvinden naar de exacte timing en de factoren die de lokale productie beïnvloeden, aangezien verhoging van urine hepcidine mogelijk beschermt tegen acute nierschade. Verschillende onderzoekers suggereren dat nierschade bij patiënten met IgAN deels wordt veroorzaakt door hematurie, en haem geïnduceerd tubulaire schade. Indien hepcidine beschermt tegen ijzer gemedieerde nierschade, zou lokale aanmaak van hepcidine in de nier gunstig kunnen zijn in IgAN. Onderzoek in naar urine hepcidine-25 en andere glomerulaire ziekten met hematurie zijn noodzakelijk.

Connective tissue growth factor (CTGF) is een profibrotisch eiwit en een andere potentieel biomarker voor vroege tubulointerstitiële schade. Verhoogde urine CTGF waarden zijn beschreven bij patiënten met een diabetische nefropathie en overige glomerulaire ziekten zoals IgAN. Een verhoogde CTGF werd gezien als een uiting van verhoogde productie van de nier. In hoofdstuk 9 beschrijven we de glomerulaire en tubulaire verwerking van CTGF. In gezonde personen was de FE van CTGF laag (<1%). Blokkade van de tubulaire reabsorptie door gelofusine infusie gaf aanleiding tot een significant hogere urine CTGF concentratie. Urine CTGF was eveneens verhoogd in megaline deficiënte muizen. Tot slot zagen we dat CTGF sterk gecorreleerd was met β₂m. We concluderen aldus dat CTGF wordt gefiltreerd en vrijwel compleet wordt geresorbeerd via megaline-gemedieerde endocytose in de tubuli van de nier. Een verhoogd urine CTGF wordt met name verklaard door tubulaire dysfunctie. Dit moet in acht worden wanneer CTGF als biomarker wordt ingezet. In het kader van het voorspellen van progressie in patiënten met IgAN, lijkt CTGF geen goede marker, het is immers sterk gecorreleerd met β₂m en dit eiwit bleek eerder geen voorspellende waarde te hebben.
Dit proefschrift was niet mogelijk geweest zonder de hulp en ondersteuning van velen. Iedereen die op enigerlei wijze heeft bijgedragen wil ik heel hartelijk bedanken. Een aantal mensen wil ik in het bijzonder noemen.

Prof. dr. J. F.M. Wetzels, beste Jack, jij bent de drijvende kracht geweest achter dit proefschrift. Ik ben je enorm dankbaar dat je dit promotietraject voor me mogelijk hebt gemaakt. Je was er altijd voor me als ik hulp nodig had en tot op het laatst toe was het kortdurend van gedachten wisselen tussen de stapels op jouw kamer voldoende om met frisse moed en een nieuwe invalshoek weer aan de slag te gaan. Ik heb gedurende de jaren veel geleerd van je heldere, consequente manier van redeneren en bewonder je integriteit en de wijze waarop je in staat bent onderzoeksresultaten te vertalen naar de dagelijkse patiëntenzorg. Het mooiste wat dit promotietraject me heeft gebracht is jouw vriendschap.

Prof. dr. D.W. Swinkels, beste Dorine, je hebt me vol enthousiasme wegwijs gemaakt in de wereld van hepcidine. Jouw oprechte interesse voor hepcidine in het kader van nierziekten, maakte het mogelijk om op prettige en soepele wijze samen een aantal studies te voltooien, waarbij jouw optimisme, inzichten en contacten onontbeerlijk waren.

Prof. dr. H. Van Goor, beste Harry, dankzij Anne-Roos hebben we tijdens een ASN lunch in San Diego de basis gelegd voor een vruchtbare samenwerking op het gebied van KIM-1 en NGAL. Jouw gedrevenheid heeft indruk op me gemaakt, evenals het gemak waarmee je zeer uiteenlopende onderwerpen ter sprake kon brengen tijdens de lunches die volgden.

Prof. dr. L. Van Es, beste Bob, je hebt me zeer gastvrij ontvangen in Leiden en me in de gelegenheid gesteld om onder begeleiding van Annemieke van der Wal nierweefsel te bewerken en te kleuren.

Prof. dr. R. Goldschmeding, beste Roel, in de beginfase van mijn promotie heb ik met je mogen samenwerken. Ik ben je erkentelijk voor je hulp en wijze adviezen.

De manuscript commissie bestaande uit prof. dr. J.W.M. Lenders, prof. dr. J.G.J. Hoenderop en dr. M.G. Vervloet, wil ik bedanken voor hun kritische beschouwing en goedkeuring van het manuscript.

Coby Laarakkers, jij was mijn rots in de branding bij het hepcidine onderzoek. Geduldig, deskundig, nauwgezet en altijd bereid mee te denken. Ik ben je zeer dankbaar.
Dankwoord

Dr. Roos Maasereeuw, dankzij jouw hulp bij de dierexperimenten kon er een brug worden geslagen tussen het klinisch onderzoek en de basale studies naar hepcidine.

Dr. Jan van den Brand, je hebt me tijdens dit proefschrift enorm geholpen, allereerst door samen middels statusonderzoek data te verzamelen, later als steun en toeverlaat als het aankwam op statistiek. Waarbij ik de propensity score als een absoluut hoogtepunt beschouw.

Dr. Julia Hofstra, lieve Julia, met Barack in de barak. We werkten samen in het Rijnstate, begonnen tegelijkertijd (met Judith in de rol van NIO) op de afdeling nierziekten in het UMCN en hebben samen heel wat uren in de Zeldenrustbarak aan onderzoek besteed. Ik ben blij met zo’n fantastische collega in de academie die ik altijd om raad kan vragen en ben er trots op dat je vandaag mijn paranimf wilt zijn.

Dr. Eric Steenbergen, een serie IgA biopten scoren volgens de MEST classificatie, deed je gemoedelijk maar efficiënt. Dank voor al je hulp, ik hoop in de toekomst nog veel met je samen te werken.

Dr. Femke Waanders en dr. Esther Meijer, dank voor het verrichten van de bepalingen van KIM-1 en NGAL en jullie waardevolle bijdrage aan dit manuscript.

Dr. Ingeborg Bajema, hartelijk dank voor het beoordelen van de diverse niercoupes op de aanwezigheid van GMP-17 positieve T-lymfocyten.

Dr. Stefan Berger, dank voor de patiënt data uit het LUMC en voor je nuttige kritiek.

Dr. Karin Gerritsen en dr. Tri Nguyen, dank voor alle hulp op het gebied van CTGF, ik heb veel bewondering voor het basale onderzoek dat jullie beiden hebben verricht.

Secretariaat, staf en research verpleegkundigen van de afdeling nierziekten in het UMCN, bedankt voor jullie betrokkenheid en toewijding bij mijn opleiding tot nefroloog en bij dit onderzoek.

Secretariaat en verpleegkundigen van de afdeling hemodialyse in het UMCN, jullie hulp bij het stickeren, afnemen en verzamelen van alle buizen bij hemodialyse en peritoneaal dialyse patiënten op de meest uiteenlopende momenten, was onmisbaar.

Alle mede auteurs van de artikelen in dit proefschrift, dank voor jullie bijdrage en voor de prettige samenwerking.

Sectiegenoten: Joop, Sabine, Karlijn, Judith, Hans en Joan, jullie hebben op vele manieren een waardevolle bijdrage geleverd aan dit manuscript. Op belangrijke momenten maakten jullie het mogelijk dat ik me op dit onderzoek kon concentreren, door een aantal taken als vanzelfsprekend over te nemen en dat maakte het verschil. Ik voel me een bevoorrecht mens om deel uit te mogen maken van dit team en hoop nog vele jaren met jullie te mogen samenwerken.

Judith, lieve Judith, het is ons gelukt! En wat geweldig dat we dit vandaag samen kunnen afronden en vieren, dat maakt het extra speciaal. Je bent een fantastische collega en hebt me zo ontzettend geholpen de afgelopen tijd.

Vrienden en familie, ik heb jullie niet altijd de aandacht gegeven die jullie verdienen, maar ben ontzettend blij met jullie in mijn leven.

Anne-Roos Frenay, al jaren volg je mij op de voet. Heel bijzonder dat onze paden zich zo vaak kruisen. Ik heb er alle vertrouwen in dat we elkaar in de toekomst blijven zien (uiternaar bij jouw promotie) en ben ontzettend blij met jou als paranimf.

Lieve mama en papa, door de jaren heen hebben jullie me altijd gesteund tijdens studie en werk. De afgelopen tijd is jullie hulp bij de zorg voor Charlotte heel belangrijk geweest, ik kan altijd op jullie terugvallen, en dat biedt geborgenheid.

Lieve Loes-Sophie, lieve zus, als ik weer eens vergeet of een cadeau te kopen voor een verjaardag of andere gelegenheid, help jij me altijd uit de brand, je herinnert me zelfs aan mijn eigen trouwdag. Ik ben trots op de manier waarop jij zorgt voor Joep, Daan en Roos.

Lieve Frances, jij geeft me naast liefde ook alle ruimte om me te ontwikkelen en hebt er altijd op vertrouwd dat we samen een plek zouden vinden voor een thuis. Nu het eindelijk zover is, hoop ik dat er meer tijd zal zijn om met zijn drieën te genieten.

Lieve Charlotte, jij hebt geen enkele boodschap aan dit manuscript en leest veel liever met mama ‘Het Walvisje’, waarbij je nooit nalaat het laatste woord van de laatste zin vol vuur aan te vullen: ‘Hier is het heerlijk, het water is blauw. Jij hoort bij mij. En ik hoort bij jou.’
Publications


Curriculum vitae


Hilde is getrouwd met Frances Dethmers en samen hebben zij een dochter, Charlotte.