Proteinuria and Progression in Glomerular Diseases

Proteinuria and Progression in Glomerular Diseases

Een wetenschappelijke proeve op het gebied van
de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de Rector Magnificus prof. dr. C.W.P.M. Blom,
volgens besluit van het College van Decanen
in het openbaar te verdedigen
op dinsdag 29 november 2005
des namiddags om 3.00 uur precies

door

Amanda Johanna Wilhelmina Branten
Geboren op 20 mei 1966
te Helmond

Promoter:	Prof. Dr. J.F.M. Wetzels
Manuscriptcommissie:	Prof. Dr. F.G.M. Russel
	Dr. C.A. Stegeman (UMC Groningen)
	Prof. Dr. J.L. Willems

ISBN: 909019875X
Printer: Printpartners Ipskamp Enschede
Ontwerp omslag en binnenwerk: Amanda Branten

The studies in this thesis were performed at the Department of Nephrology (Head: Prof. Dr. J.H.M. Berden) of the Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands, and were financially supported by The Netherlands Organisation for Scientific Research (NWO, projectno. 920-03-038).
Printing of this thesis was financially supported by the Dutch Kidney Foundation and The Netherlands Organisation for Health Research and Development (ZonMw).

Contents

Chapter 1	Introduction and outline of the thesis.	**7**
Chapter 2	Familial nephropathy differing from minimal change nephropathy and focal glomerulosclerosis. *Kidney International 2001;59:693-701*	**13**
Chapter 3	Urinary excretion of Glutathione S Transferases Alpha and Pi in patients with proteinuria: reflection of the site of tubular injury. *Nephron 2000;85:120-126*	**31**
Chapter 4	Urinary excretion of complement C3d in patients with renal diseases. *European Journal of Clinical Investigation 2003;33:449-456*	**45**
Chapter 5	Serum creatinine is a poor marker of GFR in nephrotic syndrome. *Nephrology Dialysis Transplantation 2005;20:707-711*	**61**
Chapter 6	Influence of albumin infusion on the urinary excretion of β2-microglobulin in patients with proteinuria. *Nephron 1999;81:329-333*	**73**
Chapter 7	Urinary excretion of β2-microglobulin and IgG predict prognosis in idiopathic membranous nephropathy: A validation study. *Journal American Society of Nephrology 2005;16:169-174*	**85**
Chapter 8	Oral cyclophosphamide versus chlorambucil in the treatment of patients with membranous nephropathy and renal insufficiency. *Quarterly Journal of Medicine 1998;91:359-366*	**101**
Chapter 9	Short- and long-term efficacy of oral cyclophosphamide and steroids in patients with membranous nephropathy and renal insufficiency. *Clinical Nephrology 2001;56:1-9*	**117**
Chapter 10	Summary and conclusions.	**133**
Chapter 11	Samenvatting en conclusies.	**139**
Dankwoord		**145**
Curriculum Vitae		**148**
Publicatie Lijst		**149**

Chapter 1

Introduction and Outline of the Thesis

INTRODUCTION

Proteinuria and Progression

Proteinuria is a frequently present manifestation of glomerular renal diseases. Under normal circumstances filtration of circulating proteins is limited as a consequence of the size- and charge selective properties of the glomerular basement membrane (GBM). Small molecules that still pass the GBM are reabsorbed by the renal tubular cells. For a long time the presence of proteinuria was merely considered a consequence of glomerular and/or tubular damage. More recently several data suggest that proteinuria has also a more active role: substances that pass the GBM may contribute to development of renal damage, particularly the development of tubulo-interstitial injury [1,2]. It is not known which components of the filtered molecules are the culprit, and also the underlying mechanisms that are responsible for tubulo-interstitial damage are still not elucidated. However, there is no doubt that proteinuria is associated with an unfavourable renal outcome, independent of the underlying renal disease.

Tubulo-interstitial damage

In the past decades several investigators have shown that the extent of tubulo-interstitial damage, which often accompanies primary glomerular diseases, is significantly correlated to renal function. Tubulo-interstitial damage is even more predictive for renal function deterioration than glomerular damage [3,4].

Several mediators have been mentioned to be involved in the development of tubulo-interstitial injury. For example potentially damaging antibodies, monocytes, growth factors, cytokines, and complement factors seem to play a role in the cascade that results in the generation of tubulo-interstitial inflammation and fibrosis [5,6].

The presence and severity of tubulo-interstitial injury can be examined non-invasively by measuring the urinary excretion of several marker proteins. The most studied markers are cellular enzymes like lactate dehydrogenase, N-Acetyl-β-glucosaminidase, alanine aminopeptidase and alkaline phosphatase, and low molecular weight proteins like α1-microglobulin, β2-microglobulin and retinol binding protein. Unfortunately, most of these markers mainly reflect proximal tubular cell injury [7,8]. A reliable marker for distal tubular injury is lacking.

Renal Function

In proteinuric renal diseases the risk to develop renal insufficiency is increased. The golden standard to determine the glomerular filtration rate (GFR) is to measure the clearance of inulin. However, measurement of the inulin clearance is a laborious and cumbersome procedure. In daily practice serum creatinine levels or the calculated creatinine clearance are used to determine renal function. In general creatinine clearance overestimates GFR since creatinine in not only filtered in the glomerulus but also secreted by renal tubular cells [9]. It is not known if the renal handling, particularly the tubular handling, of creatinine is changed in patients with severe proteinuria. Thus, it is not clear if serum creatinine levels or creatinine clearance are reliable markers for GFR in patients with a nephrotic syndrome.

Treatment strategy in patients with idiopathic membranous nephropathy

In the past two decennia we have focussed on the prognosis and treatment of patients with proteinuria due to idiopathic membranous nephropathy (iMN). The treatment of patients with iMN includes supportive therapy like ace-inhibitors, anti-hypertensive therapy and lipid-lowering drugs. In some patients there is a role for immunosuppressive treatment, like combinations of steroids with alkylating agents, azathioprine or cyclosporine. Consensus with regard to the preferred time point to start immunosuppression is lacking [10,11]. Since 40-50% of the patients will achieve remission of proteinuria spontaneously, we advocate to limit this kind of potential toxic treatment to patients who (will) develop renal insufficiency. The availability of reliable prognostic markers could be helpful and allow identifying patients with an unfavourable prognosis in an early phase of their renal disease. Several assumed prognostic markers such as high age, male sex, level and duration of proteinuria, and presence of tubulo-interstitial damage lack sufficient sensitivity and specificity to be useful to guide treatment [12]. In the past, two small cohort studies in our center revealed that the urinary excretion of both β2-microglobulin and IgG were useful to identify patients at risk for renal insufficiency with a sensitivity and specificity of about 90% [13,14]. If these results could be confirmed these two markers should be suitable to identify those patients in whom immunosuppressive therapy is indicated.

The efficacy of immunosuppressive treatment in idiopathic membranous nephropathy has been described in several small cohort studies. Treatment with alkylating drugs like chlorambucil or cyclophosphamide resulted in favourable effects on proteinuria and renal

function. The efficacy of such treatment was supported by a well performed randomized, controlled trial carried out by Ponticelli *et al.* [15]. These Italian investigators studied the effects of chlorambucil compared to placebo in patients with iMN, a nephrotic syndrome and a normal renal function.

OUTLINE OF THE THESIS

Persistent proteinuria in the nephrotic range (>3.5 g /day) is associated with the development of renal failure. In our studies we noticed a mother and two daughters with a remarkable familial nephropathy, characterized by longstanding proteinuria with preservation of normal renal function. To get more insight in this potential harmless proteinuria a detailed analysis of urinary proteins was carried out in these patients, which included the selectivity of the proteinuria and the presence of β2-microglobulin as marker for tubulo-interstitial damage. The results are described in chapter 2.

The above-mentioned markers for tubular function cannot differentiate between proximal or distal tubular cell injury. The alpha and pi iso-enzymes of glutathione S transferase (GST) are present in respectively proximal and distal tubular cells. We have measured the urinary excretion of both GST alpha and GST pi in patients with proteinuria in an effort to determine the site of tubular injury. The data are presented in chapter 3.

Activation of complement is suggested to play a role in the development of tubulo-interstitial injury. Activated C3 is in the center of the complement cascade. C3d is a breakdown product of activated C3, and its measurement thus could provide information on complement activation. We have examined the level and source of urinary C3d in patients with tubulo-interstitial nephritis and in patients with glomerular diseases (chapter 4).

An overload of filtered proteins will be a burden on renal tubular cells. We hypothesized that the renal handling, particularly the tubular handling, of creatinine could be changed in patients with proteinuria and questioned the reliability of serum creatinine levels and creatinine clearance as markers of GFR. In chapter 5 we have compared inulin clearance and creatinine clearance in patients with different levels of proteinuria.

We have proposed urinary β2-microglobulin as a marker of tubulo-interstitial injury and prognosis in patients with iMN. To be reliable as a marker for tubular cell dysfunction, the reabsorption of β2-microglobulin should not be influenced by the presence of other proteins.

Therefore we measured the fractional excretion of β2-microglobulin before and after the infusion of albumin in patients with proteinuric renal diseases (chapter 6).

Previous data in patients with idiopathic membranous nephropathy (iMN) revealed that the urinary excretion of β2-microglobulin and IgG could be helpful in the identification of patients at risk for development of renal insufficiency. A prospective validation study was carried out to confirm the prognostic value of these proteins in patients with iMN (chapter 7). We also tested the predictive value of other parameters such as serum albumin level and urinary α1-microglobulin excretion.

The first studies with immunosuppressive treatment in patients with proteinuria and iMN have used combinations of steroids and chlorambucil or cyclophosphamide. We also have applied both treatment options. We have analyzed the effects of chlorambucil and cyclophosphamide in patients with iMN and deterioration of renal function. As described in chapter 8 we observed a better efficacy and tolerability of cyclophosphamide compared to chlorambucil. Since this observation our standard regimen consists of cyclophosphamide with steroids. We have determined the long-term efficacy of immunosuppressive therapy by extended follow-up of a cohort of patients with iMN and renal insufficiency (chapter 9).

REFERENCES

1. Eddy AA. Experimental insights into the tubulo-interstitial disease accompanying primary glomerular lesions. J Am Soc Nephrol 1994;5:1273-1287.
2. Benigni A, Zoja C, Remuzzi G. Biology of disease. The renal toxicity of sustained of glomerular protein traffic. Lab Invest 1995;73:461-468.
3. Schainuck LI, Striker GE, Cutler RE, Benditt EP. Structural-functional correlations in renal disease. Hum Pathol 1970;1:631-641.
4. Wehrmann M, Bohle A, Bogenschutz O *et al*. Long-term prognosis of chronic idiopathic membranous glomerulonephritis. An analysis of 334 cases with particular regard to tubulo-interstitial changes. Clin Nephrol 1989;31:67-76.
5. Burton C, Harris KPG. The role of proteinuria in the progression of chronic renal failure. Am J Kidney Dis 1996;27:765-775.
6. Palmer BF. The renal tubule in the progression of chronic renal failure. J Investigative Medicine 1997;45:346-361.
7. Scherberich JE. Urinary proteins of tubular origin: basic immunochemical and clinical aspects. Am J Nephrol 1990;10 (Suppl 1):43-51.

8. Fels LM, Bundschuh I, Gwinner W *et al*. Early urinary markers of target nephron segments as studies in cadmium toxicity. Kidney Int 1994;46 Suppl 47:S81-S88.
9. Shemesh O, Golbetz H, Kriss JP, Myers BD. Limitations of creatinine as a filtration marker in glomerulopathic patients. Kidney Int 1985;28:830-838.
10. Hebert LA. Therapy of membranous nephropathy: what to do after the after (meta) analyses. J Am Soc Nephrol 1995;5:1543-1545.
11. Wasserstein AG. The more things change (editorial). Am J Kidney Dis 2001;38:406-408.
12. Reichert LJM, Koene RAP, Wetzels JFM. Prognostic factors in idiopathic membranous nephropathy. Am J Kidney Dis 1998;31:1-11.
13. Reichert LJM, Koene RAP, Wetzels JFM. Urinary excretion of β2-microglobulin predicts renal outcome in patients with idiopathic membranous nephropathy. J Am Soc Nephrol 1995;6:1666-1669.
14. Reichert LJM, Koene RAP, Wetzels JFM. Urinary IgG excretion as a prognostic factor in idiopathic membranous nephropathy. Clin Nephrol 1997;48:79-84.
15. Ponticelli C, Zuchelli P, Imbasciati E *et al*. Controlled trial of methylprednisolone and chlorambucil in idiopathic membranous nephropathy. New Engl J Med 1984;310:946-950.

Chapter 2

Familial Nephropathy differing from Minimal Change Nephropathy and Focal Glomerulosclerosis.

Amanda J.W. Branten[1]
Jacob van den Born[1]
Jan L.J. Jansen[3]
Karel J.M. Assmann[2]
and Jack F.M. Wetzels[1]

with the technical assistance of Henry B.P.M. Dijkman[2]

[1]Department of Medicine, Division of Nephrology, and [2]Department of Pathology
Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands,
[3]Department of General Internal Medicine, Bosch Medicentrum 's Hertogenbosch,
The Netherlands.

Kidney International 2001; 59:693-701

ABSTRACT

Background: Nephrotic syndrome in childhood is mainly due to minimal change nephropathy. In general, it is characterized by selective proteinuria, steroid-responsiveness, and histologically by podocytic foot process effacement. Familial presentation is rare and mainly restricted to one generation.

Methods: We describe the occurrence of a familial nephropathy in a mother and two daughters. An initial diagnosis of minimal change nephropathy was made, but subsequently unique features became apparent. During follow-up, detailed studies of renal function and urinary protein excretion were performed. Available frozen renal biopsy material was revised and processed for immunofluorescence to detect abnormalities in the expression of heparan sulfate proteoglycans. The latter results were compared with renal biopsies of a control group composed of five adult patients with minimal change nephropathy.

Results: The mother and two daughters were proteinuric since their early childhood. The mother revealed a persistent nephrotic syndrome for more than 20 years despite treatment with various immunosuppressive drugs. Likewise, treatment with prednisone was ineffective in the daughters. All three patients retained normal renal function during follow-up. Detailed measurements revealed that the proteinuria was incredibly selective (selectivity index approximately 0.01), and there was no evidence of tubulo-interstitial damage, as reflected by a normal excretion of the low-molecular weight proteins β2-microglobulin and α1-microglobulin. Renal biopsy performed in the mother and one daughter was thought to be compatible with minimal change nephropathy. However, histologically, two remarkable findings were made. By electron microscopy, there was no evidence of foot process retraction; specifically, the foot process width and slit pore diameter were normal. Furthermore, in contrast to the control patients, the expression of heparan sulfate polysaccharide side chains, as reflected by the staining with monoclonal antibody JM403, was normal.

Conclusion: We propose that this family represents a new familial nephropathy. The molecular basis of the permeability defect remains to be identified.

INTRODUCTION

Nephrotic syndrome in childhood is most commenly caused by minimal change nephropathy [1]. The term minimal change nephropathy is applied because of the absence of glomerular abnormalities in light microscopy. However, on more detailed examination by electron microscopy, alterations of the glomerular visceral epithelial cells are apparent in the form of effacement of the foot processes. It has been suggested that proteinuria in patients with minimal change nephropathy is the consequence of a loss of negative charges in the glomerular capillary wall [1]. Clinically, patients with minimal change nephropathy present with a selective proteinuria, that is, there is a preferential loss of the negatively charged albumin when compared with immunoglobulin G (IgG). Most patients quickly respond to treatment with corticosteroids, and even if left untreated spontaneous remissions of proteinuria do occur and the prognosis is good [1,2]. After years of follow-up, proteinuria disappears, and few patients, if any, develop renal insufficiency. Familial occurrence of minimal change nephropathy has been reported regularly, although in most families, the nephrotic syndrome is restricted to one generation [3,4]. The present study describes a mother and two daughters, all three of whom developed a nephrotic syndrome during early childhood. Clinical and histologic features initially suggested a minimal change nephropathy. However, several arguments favor the idea that these patients represent a new syndrome. First, the nephrotic syndrome was present in two generations; second, proteinuria was highly selective; third, the nephrotic syndrome was treatment resistant and persisted for more than 20 years without causing renal failure; fourth, there was no evidence of foot process effacement; and finally, we observed a normal expression of glomerular basement membrane heparan sulfate polysaccharide side chains, which are thought to play an important role in the charge-selective properties of the glomerular capillary wall [5,6]. We propose that this familial nephropathy represents a new entity. The pathogenetic mechanism involved in the glomerular permeability defect remain to be identified.

Case histories

Case 1: The mother.

Severe proteinuria was discovered in the mother in 1964, at the age of seven years, during investigations because of pretibial edema. Her blood pressure was normal, and the urinary sediment revealed no abnormalities. From 1966 to 1981, several efforts to reduce the proteinuria were unsuccessful. In this 15 year period, the patient was intermittently treated

with mevaquine (four months), high and low doses of prednisone and indomethacine for several years, a combination of chlorambucil and 6-mercaptopurine for three months, and two separate courses of cyclophosphamide (3 and 4 months, respectively). Throughout these years, she was regularly seen at the outpatient clinic, and the records show that the proteinuria persisted despite these treatment efforts. Urinary protein levels measured in spot urine ranged from 1.1 to 12.4 g/L, the variability most likely reflecting differences in urinary flow rate. Whenever measured in urine samples collected over 24 hours, proteinuria exceeded 3.5 g/24 h (Figure 1). Most important, hypoalbuminemia was present at all times, indicative of a persistent nephrotic syndrome (average value 23 ± 2 g/L, range 19 to 27 g/L (N=66; Figure 1).

From 1966 onward, increased levels of total cholesterol (>10 mmol/L) were reported. Because of the latter, since 1982, treatment was started with cholestyramine, and after several years this was changed to simvastatine. In 1990, she was supplemented with levothyroxine because of hypothyroidism. In the period between 1978 and 1983, she was pregnant three times. During these pregnancies she had to be treated with methyldopa and chlorthalidone because of a slight rise in blood pressure to maximum levels of 150/60 mm Hg. No increase of proteinuria was observed, and renal function remained normal. She gave birth to two daughters and one son, all born at term. Renal biopsies were performed in 1971, 1973, and 1978 (discussed later in this article).

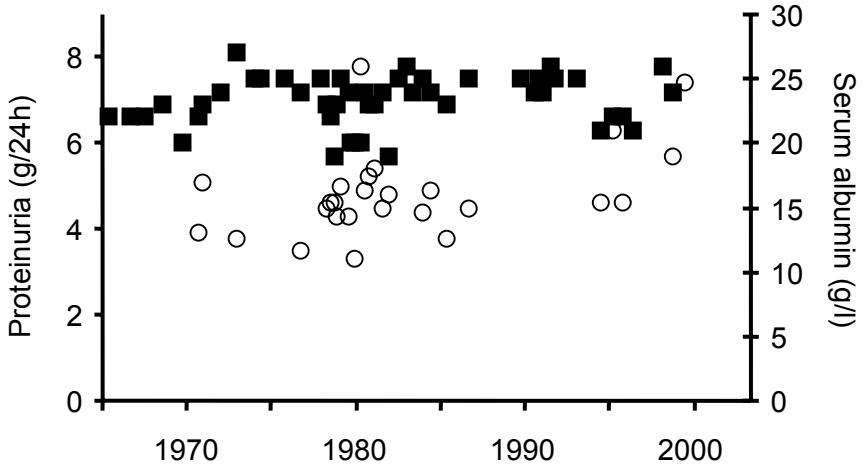

Figure 1. Time course of serum albumin (closed squares) and 24-hour urinary protein excretion (open circles) in patient 1. Serum albumin levels have been persistently below 30 g/L. Urinary protein levels measured in spot urines are not shown (discussed in the text).

Case 2: The oldest daughter.

In this girl proteinuria was diagnosed in 1979 in the first year of her life. In 1991, at the age of 12, she was sent to a pediatrician by her family doctor because of persistent asymptomatic proteinuria. The proteinuria was in the nephrotic range (4.6 to 4.9 g/L) and accompanied by hypoalbuminemia (range 27 to 31g/L) and hypercholesterolemia (> 8 mmol/L). Treatment with high-dose prednisone (45 mg daily for more than eight weeks) was started. However, the therapy was ineffective, and it was decided to perform a renal biopsy (discussed later in this article). Subsequently, in 1992, she was treated with a course of cyclophosphamide (2 mg/kg for 8 weeks). Again, the patient did not respond to this treatment. Because of the similarity in presentation between the daughter and the mother, who had a favorable course, it was decided not to do more interventions. Since 1995, the patient has been seen yearly by the same nephrologist who treated her mother. Laboratory examinations revealed average serum albumin levels of 29 g/L (range 28 to 30 g/L) and proteinuria of 2.3 g/24 h (range 2.0 to 2.6 g/24 h). Blood pressures have always been low, and edema was never noticed Detailed clinical studies were done in 1996 and 1999.

Case 3: The youngest daughter.

In this patient, born in 1982, proteinuria was also first noted in the year after her birth. In 1991, she went together with her older sister to the pediatrician. Similar to her mother and sister, she also had repeatedly elevated cholesterol levels (> 8 mmol/L) and hypo-albuminemia (28 to 30 g/L). The proteinuria averaged 4.2 g/24 h during measurements in 1991. Also, she was treated unsuccessfully with high-dose prednisone. A renal biopsy was not performed because of the known abnormalities in her mother and sister. From 1995 onward, she had visited the nephrologist yearly at the outpatient clinic. She infrequently observed edema in her hands, but most of the time she was asymptomatic. Serum albumin levels averaged 29 g/L (range 28 to 30 g/L), and proteinuria averaged 4.5 g/24 h (range 4.2 to 4.8 g/24 h). We studied this patient when she accompanied her family members in 1996 and 1999.

METHODS

Clinical studies

Clinical studies were performed according to a standard protocol. The measurements were carried out in the morning after an overnight fast. The evening before the study day the patients took 4000 mg of sodium bicarbonate to ensure an urinary pH above 6.0, which is needed to prevent degradation of β2-microglobulin. For the studies performed in 1999, the

patients also received 1200 mg of cimetidine in the morning in order to block tubular secretion of creatinine [7]. Upon arrival at the ward, the patients received 375 to 500 ml of tap water to enforce diuresis. The patients remained supine during two hours, except for voiding. Timed urine samples were collected, and in the middle of the collection period, a blood sample was drawn. Blood and urine samples were used to determine creatinine, the low-molecular weight proteins β2-microglobulin and α1-microglobulin, the enzyme β-N-acetylglycosaminidase (βNAG), and the middle- and high-molecular weight proteins albumin, transferrin, and IgG. Blood pressure was recorded using an automatic device (Dinamap), ten consecutive readings being done at five-minute intervals. In addition to the timed urine samples, the patients also collected 24-hour urine samples for determination of creatinine, sodium, and total protein.

Laboratory procedures and calculations

The concentrations of creatinine, sodium, cholesterol, and urinary total protein were measured with standard automated techniques. The concentrations of albumin, transferrin, α1-microglobulin, and IgG in serum and urine were measured by immunonephelometry using antibodies in which the specificity was checked by Ouchterlony double immunodiffusion and immunoelectrophoresis. Details of these nephelometric procedures have been described before [8,9]. Urinary β2-microglobulin was measured by an enzyme-linked immunosorbent assay (ELISA) method [10].

Creatinine clearance was estimated from the Cockcroft and Gault formula. In the 1999 studies, renal function was assessed by calculating the creatinine clearance (ECC), measured after administration of cimetidine, by using the formula Ucr*V/Pcr, where Ucr is the concentration of creatinine in the urine, V is the urine flow and Pcr is the plasma concentration of creatinine. Since cimetidine blocks the tubular secretion of creatinine, the creatinine clearance measured during cimetidine administration closely reflects the glomerular filtration rate (GFR) [11,12]. The mean arterial pressure was the average of ten registered measurements. The protein selectivity index was calculated as the clearance of IgG divided by the clearance of albumin. In cases with IgG levels in urine below the detection limit of 2 mg/L, the selectivity index and urinary excretion of IgG were calculated using this lowest measurable value of 2 mg/L, and the results are expressed as “lesser than”.

Processing of the renal biopsies

For light microscopy (LM), a portion of the kidney biopsy was fixed in Bouin’s solution, dehydrated, and embedded in paraplast (Amstelstad, Amsterdam, The Netherlands). Two micrometers sections were stained with hematoxylin-eosin, periodic acid-Schiff (PAS), and

silver methenamine. For immunofluorescence (IF), kidney fragments were snap frozen in liquid nitrogen, and 2 µm cryostat sections were incubated with fluorescein-labeled antisera directed to human IgG, IgM, IgA, C1q, C3, and fibrinogen (DAKO, Copenhagen, Denmark). For electron microscopy (EM), a small piece of the biopsy was fixed in 2.5% glutaraldehyde dissolved in 0.1 mol/L sodium cacodylate buffer, pH 7.2, for four hours at 4ºC and washed in the same buffer. The tissue fragments were postfixed in phosphate-buffered 2% OsO4 for two hours, dehydrated, and embedded in Epon 812. Ultra-thin sections were cut in an ultratome (LKB Producteer, Bromma, Sweden), and stained with 5% uranyl acetate for 45 minutes and with lead citrate for two minutes at room temperature. The sections were examined using a JEOL 1200 EX2 electron microscope (JEOL, Tokyo, Japan).

Detailed morphometric measurements of the glomerular basement membrane (GBM), the podocytic foot processes and the slit-pore diaphragm were done on high-power micrographs of EM pictures. The foot process width was determined on x75,000 power micrographs as described [13,14]. In brief, the number of foot processes overlying the capillary basement membrane was counted and divided by the length of the particular segment of the capillary basement membrane. A correction factor of $\pi/4$ was used as recommended in the literature [13]. For these measurements, five random open capillary loops were used with a total GBM length of approximately 100 µm. The thickness of the GBM was measured on x15,000 power micrographs [15]. A total of 50 measurements in five loops was done. The slit-pore diaphragm was measured on x75,000 power micrographs. The width was measured on the level of the slit membrane or at the narrowest point. An average of five to eight slit pores was used for the measurements.

Immunofluorescence studies of heparan sulfate proteoglycans

To assess heparan sulfate proteoglycan (HSPG) staining of the GBM, the following antibodies were used (Table 1): (1) goat polyclonal antibody BL31; (2) mouse IgG1 monoclonal antibody (mAb) JM72, both directed against the core protein of agrin, which is the major HSPG in the GBM [16,17]; (3) mouse IgM mAb JM403 which recognizes a low-sulfated domain within the heparan sulfate polysaccharide side chain, containing an N-unsubstituted glucosamine unit [6,18]; and (4) mouse IgG2b mAb 3G10, which is directed against heparitinase-generated HS stubs [19]. By heparitinase digestion, a terminal 4,5-unsaturated uronate residue is formed that is essential for recognition by 3G10. The staining of 3G10 can serve as a general HS marker, independent of the extent of HS modification. Indirect IF was performed as described before on 2 µm cryostat sections of kidney tissue [20]. Sections were fixed in aceton at 4ºC during 10 minutes (except for the sections that were digested by heparitinase before staining with mAb 3G10). Primary and secondary

antibodies (Table 1) were diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.05% sodium azide (IF buffer) and incubated for 30 minutes at room temperature. After incubation with antibodies, the sections were washed in PBS and embedded in Aquamount (pH 7.0; BDH, Poole, UK) and examined with a Zeiss Axioskop microscope with an epi-illuminator. For 3G10 staining, nonfixed sections were incubated with 0.25 U/mL heparitinase (heparan sulfate lyase III; Sigma, St. Louis, MO, USA) in 10 mmol/L HEPES and 2 mmol/L CaCl2, pH 7.0, for one hour at 37ºC. Negative controls include the whole IF procedure without the incubation of the primary antibodies. All controls were negative.

Table 1. Antibodies used for immunohistochemistry

Antigen	Primary Antibody			Secondary Antibody		
	Code	Ref.	Dilution	Code	Supplier	Dilution
Agrin core	BL31	[16]	400	Rabbit anti-goat IgG-FITC	De Beer Med.	500
Agrin core	JM72	[17]	400	Sheep anti-mouse IgG-FITC	Cappel	500
HS	JM403	[6,18]	800	Goat anti-mouse IgM-FITC	Nordic	75
HS stub	3G10	[19]	200	Goat anti-mouse IgG2b-FITC	Southern	100

Abbreviations are: Ref., References; HS, heparan sulfate; IgG and IgH, immunoglobulin G and H, respectively; FITC, fluorescein isothiocyanate.

RESULTS

A pedigree of the family is depicted in Figure 2. Proteinuria was only detected in the mother and her two daughters. The parents of the mother, a brother and his daughter, and a son all tested negative. In the family's history, there was no mention of marriages between family-members.

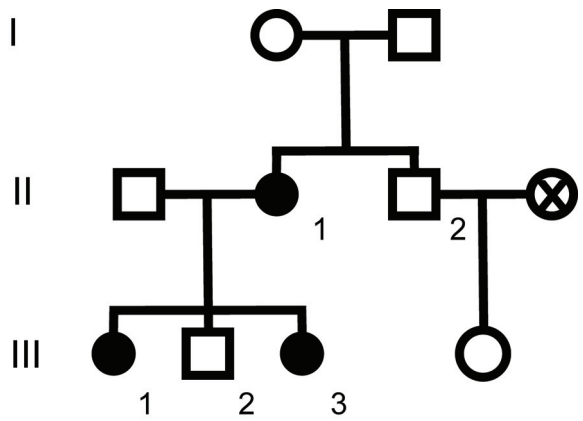

Figure 2. Pedigree of the family. The proteinuric members are depicted with closed symbols. Number II-1 represents our index patient (case 1). Numbers III-1 and III-3 are her proteinuric daughters (cases 2 and 3). The open symbols represent family members without proteinuria. The urine of one non-relative case was not tested (crossed symbol).

Table 2. Results of repeated clinical studies in the mother and her two daughters

	Mother		Daughter 1		Daughter 2		Normal
	1996	**1999**	**1996**	**1999**	**1996**	**1999**	**Range**
Age *(years)*	39	42	17	20	14	17	
Body weight *(Kg)*	48.5	56.2	47	53.7	58.9	66.3	
MAP *(mm Hg)*	90	90	81	72	83	90	
Serum levels							
Creatinine *(µmol/L)*	41	54	39	52	40	55	50-90
Albumin *(g/L)*	22	24	27	31	30	35	40-50
Cholesterol *(mmol/L)*	8.0	11.2	6.8	7.4	8.1	8.2	4.7-6.5
LDL *(mmol/L)*	5.29	7.8	4.54	5.43	5.64	5.40	<4.7
β2m *(mg/L)*	1.31	1.36	1.09	1.20	1.05	1.17	1.0-2.0
ECC (mL/min/1.73 m^2)	149	119	185	144	205	152	80-150
GFR (mL/min/1.73 m^2)		84		112		86	80-130
Urinary Excretion							
Total protein *(µg/min)*	2848	3241	250	603	1246	3211	<100
Albumin *(µg/min)*	2497	2237	184	358	1057	2248	<20
IgG *(µg/min)*	<13.2	9.1	<2.6	< 3.8	<5.4	< 10.7	<10
Transferrin *(µg/min)*	152	192	13.1	32	84	155	<2
β2m *(ng/min)*	66	50	38	41	87	91	<200
α1m *(µg/min)*	13.2	9.1	2.6	5.7	8.1	<10.7	<10
Selectivity Index	<0.02	0.01	<0.08	<0.02	<0.06	<0.02	
Proteinuria *(g/24 h)*	4.6	5.7	2.0	2.7	3.3	4.3	<0.2

Abbreviations: MAP = Mean arterial pressure, β2m = β2-microglobulin, α1m = α1-microglobulin.

Clinical studies

The results of the clinical studies performed in 1996 and 1999 are summarized in Table 2. All three patients have persistent proteinuria and a stable, normal renal function as reflected by the creatinine clearance and serum β2-microglobulin.

The urine mainly contained albumin, and in most of the urine samples the levels of IgG were below the detection limit. This precluded accurate calculations of the protein selectivity index; however, as indicated even when using the lowest detection level of 2 mg/L for urinary IgG, proteinuria was highly selective. Exact calculation of the selectivity index was possible in the 1999 samples of the mother and yielded a value as low as 0.01.

In daughter 1, we noticed a discrepancy in albuminuria in the timed urine sample compared with the proteinuria measured in the 24-hour urine. This difference in quantity suggested that the proteinuria in this patient partially was orthostatic. To exclude the presence of mere orthostatic proteinuria, all three family members subsequently collected urine separately during the day and night. Also, in the overnight urine collections, overt proteinuria was observed with protein:creatinine ratios of 6.0 g/10 mmol (versus 12.2 g/10 mmol at daytime) in the mother, 1.2 g/10 mmol (versus 3.2 g/10 mmol at daytime) in the eldest daughter, and 4.1 g/10 mmol (versus 5.3 g/10 mmol at daytime) in the youngest daughter.
We measured the urinary excretion of the low-molecular weight proteins β2-microglobulin and α1-microglobulin to ascertain tubulo-interstitial injury. As indicated in Table 2, the values were normal and did not increase over the three year period.

Histological evaluation of the renal biopsies

The first renal biopsy of the mother was performed in 1971. LM revealed 28 glomeruli that all looked normal. In the pars convoluta of the proximal tubular cells, numerous PAS-positive granules were observed, mainly at the apical site. The remaining parts of the tubuli, interstitium, and blood vessels did not demonstrate abnormalities. No tissue was processed for IF or EM. The second biopsy (22 glomeruli) and third renal biopsy (15 glomeruli) performed in 1973 and 1978, respectively, showed similar morphology as the first biopsy by LM, that is, normal-looking glomeruli and no fibrosis in the interstitium (Figure 3A page 24: in color see page 152). Minor (1+) mesangial IgM deposits were seen by IF (4 glomeruli). By EM (3 glomeruli), no retractions of the foot processes could be observed in both biopsies (Figure 3B).

The biopsy of the daughter, performed in 1991, revealed 19 glomeruli by LM examination and disclosed findings similar to the three biopsies of the mother as shown by LM, IF, and EM. Thus, in the daughter, granules in tubular cells also were present as a sign of protein reabsorption. No foot process retraction was observed by EM, and IF showed mild IgM deposits in the mesangium.
Detailed morphometric measurements of two biopsies of the mother and of the biopsy performed in the oldest daughter revealed normal values of the thickness of the GBM, and as well as normal values for both the width of the foot processes and the diameters of the slit pores (Table 3).

Table 3. Histomorphometric data

	Biopsy 1973 Mother	Biopsy 1978 Mother	Biopsy Daughter	Normal Range [14,21]
GBM thickness *(nm)*	485	411	315	221 – 437
Foot process width *(nm)*	561	764	605	511 – 963
Slit pore diameter *(nm)*	38	37	33	35 – 45

GBM is glomerular basement membrane. A description of the procedures is in the Methods section.

Expression of glomerular heparan sulfate proteoglycan

To study the expression of HSPG in renal tissue, we used various antibodies recognizing different domains of the HSPG molecule, including a mAb against the HS polysaccharide side chains. Since no renal tissue was available for the daughter, these studies could only be done on renal tissue of the mother. For comparison, we processed simultaneously renal tissue from five adult patients with a nephrotic syndrome and minimal change nephropathy. In our patient, we observed a normal, unchanged linear staining of the GBM with both antibodies against agrin core protein (BL31 and JM72). Barely any staining was observed in the mesangial regions (Figure 4B page 24: in color see page 152). These results are in agreement with our previous findings in normal human renal tissue [16,17]. Staining with mAb 3G10 revealed a linear staining of the GBM, including staining of the mesangium in accordance with earlier findings. This indicates that in this patient glycanation (the addition of HS side chains) of the HSPG core protein proceeded normally. Staining of the HS side chains using mAb JM403 revealed a linear staining of the GBM that was not discernible from normal renal tissue (Figure 4A). These data show a strictly normal distribution of the various antibodies against GBM HSPG and indicate no changes in HSPG expression in our patient.

In renal biopsies of the five control patients, normal staining with both antibodies against the agrin core protein (BL31 and JM72) as well as with 3G10 against the HS stubs was observed (Figure 4C). However, the staining for HS with mAb JM403 was almost completely absent in three patients and was reduced for about 50 and 80% in the other two patients (Figure 4D). This is in contrast to our patient who did not show any reduction in GBM HS staining.

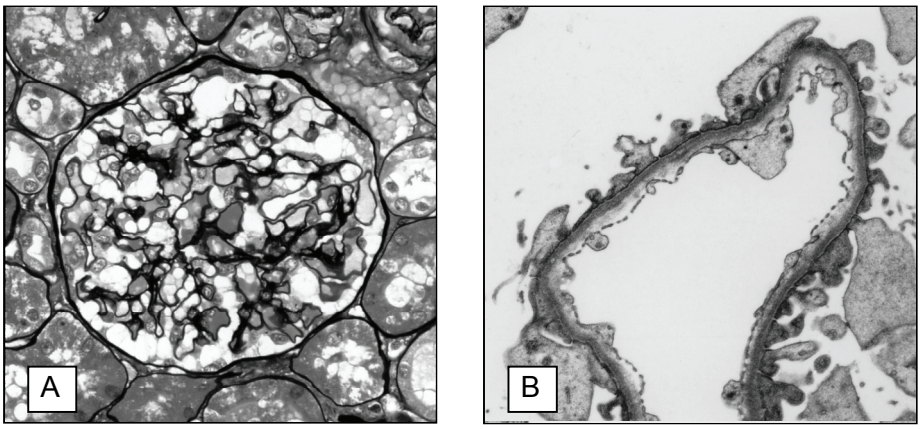

Figure 3. Light and electron microscopic pictures of the third biopsy of the mother.
(A) Normal-looking glomerulus, methenamine silver staining , x350. (B) Electron microscopy of a part of a capillary loop. The foot processes of the podocytes reveal no retraction, x12,000.

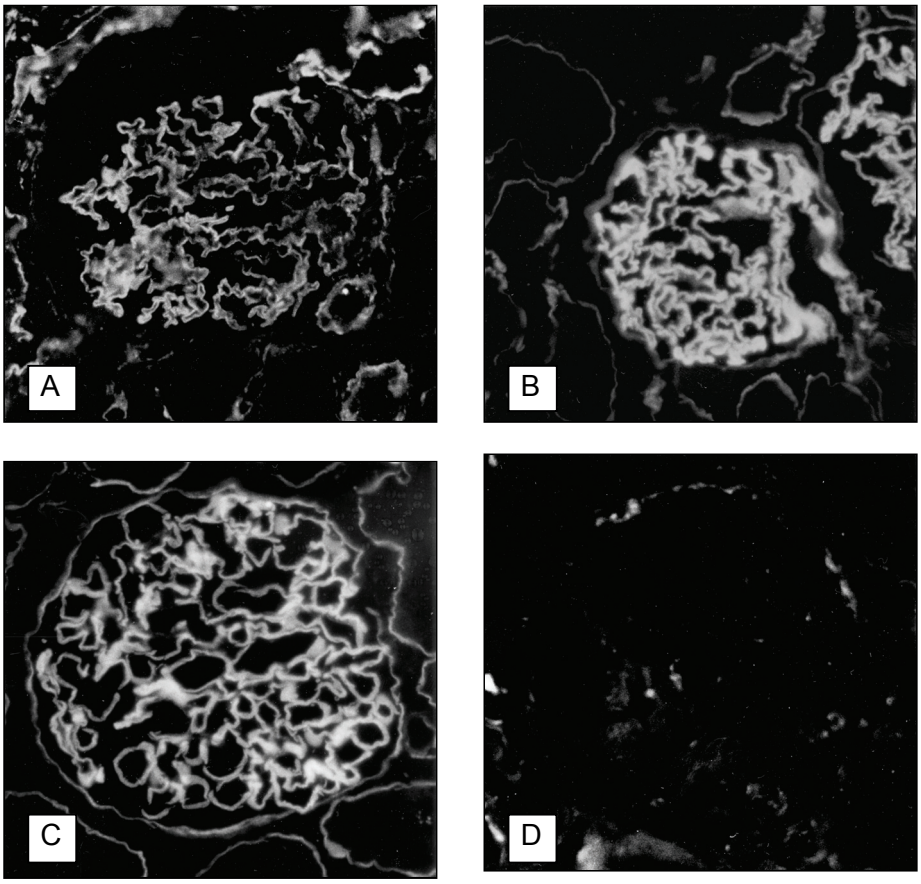

Figure 4. Immunofluorescence of the third biopsy of the mother (A and B) and a biopsy of a patient with classical minimal change nephropathy (C and D).
Unchanged presence of (A) heparan sulfate side chains as detected by monoclonal antibody (mAb) JM403, and (B) core protein as demonstrated by mAb JM72 of heparan sulfate proteoglycan (HSPG) along the capillary loops. In the biopsy of a patient with classical minimal change nephropathy staining of the core protein of HSPG is normal (C), while the heparan side chains have completely disappeared (D, x600).
The original color versions of figures 3 and 4 are depicted on page 152.

DISCUSSION

This study describes the occurrence of a nephrotic syndrome in a mother and her two daughters. This family most likely represents a new syndrome with the following characteristics: the presence of proteinuria in two consecutive generations, normal glomeruli as shown by LM, highly selective steroid-resistant proteinuria, an absence of effacement of the podocytic foot processes, and normal expression of heparan sulfate side chains.

Initially a diagnosis of minimal change nephropathy was made in these patients. The presentation at early childhood, selective proteinuria, and the light microscopical finding of normal glomeruli were compatible with such a diagnosis. Unexpectedly, the nephrotic syndrome did not respond to treatment with steroids or other immunosuppressive drugs. Few patients, if any, with classic “minimal change nephropathy” are treatment resistant. In such cases, a diagnosis of focal glomerulosclerosis (FGS) is most likely, and the typical lesions of FGS are usually demonstrated in follow-up biopsies [22,23]. Admittedly, even the absence of such lesions in the third biopsy of our first patient does not definitely exclude the possibility of FGS; however, the clinical course in our patient clearly argues against such a diagnosis, since patients with treatment resistant FGS usually progress to renal failure. Most important, the finding of normal foot processes is a remarkable observation, as effacement of the podocytic foot processes is a characteristic finding in patients with minimal change nephropathy as well as in patients with idiopathic FGS [24,25].

The occurrence of a nephrotic syndrome within a family is not unusual. About 3.4% of European children with minimal change nephropathy have affected siblings [3]. However, in most families the disease is confined to one generation. In one of the most extended overviews of familial nephrotic syndrome, 58 affected families were studied [3]. Only one of these families had proven nephrotic syndrome in two consecutive generations, a father and daughter, who developed proteinuria at the age of seven and two years, respectively. As expected in patients with minimal change nephropathy, their nephrotic syndrome responded to treatment with steroids. Familial FGS has also been described, and recently linkage to both chromosome 19 and 11 has been suggested [26,27]. In familial FGS, two generations are more frequently involved. Clinically, familial FGS is characterized by steroid resistance and progression toward end-stage renal disease [28,29]. Finally, two families have been reported in whom IgM nephropathy occurred in two generations [30]. In both families, the disease ran a progressive course with the development of renal failure, thus bearing striking similarity with the described cases of familial FGS. Some authors consider minimal change nephropathy, FGS, and IgM nephropathy to be part of a cluster of related diseases [1,30,31].

The debate on whether these diseases are separate entities will probably continue until the pathogenesis of these glomerular disorders is unraveled at the molecular level.
In our patients, the proteinuria was highly selective; in fact, the value of 0.01 in the mother is the lowest value we have ever observed in a patient with nephrotic syndrome. Selective proteinuria is indicative of a preferential loss of albumin. Theoretically, preferential loss of albumin might be the result of an abnormal charge of the albumin. We excluded this possibility by testing urine and serum samples of the mother by agarose-electrophoresis. The increased protein loss in our patients may result from alterations in any of the components that constitute the glomerular filtration barrier. The highly selective proteinuria observed in our patients is generally considered to reflect a defect in glomerular charge selectivity [32,33]. The polyanionic coat of the glomerular epithelial cells and particularly the negatively charged heparan sulfate side chains in the GBM are the main constituents of the glomerular wall negative charge. Removal of sialic acid from the glomerular epithelial cells causes foot process fusion [34,35]. Thus, foot process effacement may reflect loss of glomerular epithelial cell polyanion. In our patients, podocytic foot processes were not retracted, suggesting that the polyanion charge of the visceral epithelial cells has remained unaltered. We also found no evidence for changes in HSPGs, which supposedly are involved in minimal change nephropathy [20].
Our patient demonstrated normal staining with JM403, a mAb directed to the negatively charged heparan sulfate polysaccharide side chain. This finding clearly separates the disease in our patient from minimal change nephropathy, since all patients in our control group showed the loss of heparan sulfate and fusion of podocytic foot processes. Overall, there was no evidence of abnormalities in the best known negatively charged components of the glomerular filter. Obviously, we cannot exclude the presence of structural abnormalities of heparan sulfate side chains or damage of other charged molecules.

Recently, major progress has been made in identifying proteins involved in maintaining the integrity of the slit pore diaphragm. One key component is nephrin, and mutations in the nephrin gene on chromosome 19 are held responsible for the congenital nephrotic syndrome of the Finnish type [36,37]. Animal studies have indicated that proteins associated with nephrin such as CD2-associated protein (CD2AP) may also play a role [38]. CD2AP knockout mice develop proteinuria and EM shows effaced foot processes [38]. The finding of a normal slit pore width and the presence of normal foot processes in our patients argues against abnormalities in nephrin, CD2AP, or other proteins involved in the slit diaphragm.
Finally, one can speculate on the role of constituents of the GBM. Knowledge of the composition of the GBM is rapidly expanding [39]. The GBM subtype of laminin (laminin 11, $\alpha 5\beta 2\ \gamma 1$) may be involved in proteinuria, since laminin-β2-deficient mice develop minimal

change like nephropathy [40]. However, these mice demonstrate foot process fusion, arguing against a defect in laminin in our patients. The exact structure of various proteins in GBM remains unknown, and probably more components of the GBM remain to be identified. Further research might help in identifying abnormalities that can cause proteinuria with retainment of the normal podocytic architecture.

Although proteinuria has been present in the mother for over 30 years now, she has retained a normal renal function. It is well known that patients with renal disease and proteinuria are prone to develop renal failure. In fact, proteinuria is a major determinant of progressive renal failure [41], and it is thought highly unlikely that patients with proteinuria will retain normal renal function for more than five years [42]. Proteinuria in itself may contribute to ongoing renal injury by causing mesangial and tubulo-interstitial injury [41,43]. No tubulo-interstitial injury was observed in the third biopsy in our patient, taken almost 14 years after presentation. This absence of tubulo-interstitial lesions seems compatible with the rather benign course. We propose that this may be explained by the characteristics of the proteinuria, that is, the almost pure loss of albumin. Although in vitro studies have suggested that albumin can cause tubular cell injury, in general, this is observed at high albumin levels that are not obtained in patients with a nephrotic syndrome [44,45]. Therefore, most likely other factors such as growth factors or cytokines are more important in causing progressive renal injury. Excretion of these inflammatory mediators may be better reflected by the excretion of IgG. In this respect, this would fit with our observations in patients with membranous nephropathy where the outcome is predicted by the urinary excretion of IgG rather than by total proteinuria [9].

In conclusion, we have described a familial nephropathy with several unique characteristics that clearly differentiate it from minimal change nephropathy or FGS. The molecular basis of the glomerular permeability defect remains to be identified.

REFERENCES

1. Glassock RJ, Cohen AH, Adler SG. Primary Glomerular Diseases. In: Brenner BM, ed. *The Kidney*. Saunders, Philadelphia: 1996: 1392-1497.
2. Tune BC, Mendoza SA. Treatment of the idiopathic nephrotic syndrome: regimens and outcomes in children and adults. J Am Soc Nephrol 1997;8:824-832.
3. White RHR. The familial nephrotic syndrome: A European survey. Clin Nephrol 1973;1:215-219.
4. Moncrieff MW, White RHR, Glasgow EF *et al*. The familial nephrotic syndrome: A clinicopathological study. Clin Nephrol 1973;1:220-229.

5. Kanwar YS, Liu ZZ, Kashihara N, Wallner EI. Current status of the structural and functional basis of glomerular filtration and proteinuria. Sem Nephrol 1991;11:390-413.
6. van den Born J, van den Heuvel LPWJ, Bakker MAH *et al*. A monoclonal antibody against GBM heparan sulfate induces an acute selective proteinuria in rats. Kidney Int 1992;41:115-123.
7. Acker van AAC, Koomen GCM, Koopman MG, Waart de DR, Arisz L. Creatinine clearance during cimetidine administration for measurement of glomerular filtration rate. Lancet 1992;340:1326-1329.
8. Elving LD, Bakkeren JAJM, Jansen MJH *et al*. Screening for microalbuminuria in patients with diabetes mellitus: frozen storage of urine samples decreases their albumin content. Clin Chem 1989;35:308-310.
9. Reichert LJM, Koene RAP, Wetzels JFM. Urinary IgG excretion as a prognostic factor in idiopathic membranous nephropathy. Clin Nephrol 1997;48:79-84.
10. Jacobs EMG, Vervoort G, Branten AJW *et al*. Atrial natriuretic peptide increases albuminuria in type I diabetic patients: evidence for blockade of tubular protein reabsorption. Eur J Clin Invest 1999;29:109-115.
11. Hilbrands LB, Artz MA, Wetzels JFM, Koene RAP. Cimetidine improves the reliability of creatinine as a marker of glomerular filtration. Kidney Int 1991;40:1171-1176.
12. Zaltzman JS, Whiteside C, Cattran DC, Lopez FM, Logan AG. Accurate measurement of impaired glomerular filtration using single-dose oral cimetidine. Am J Kidney Dis 1996;27:504-511.
13. Pagtalunan ME, Rasch R, Rennke HG, Meyer TW. Morphometric analysis of effects of angiotensin II on glomerular structure in rats. Am J Physiol 1995;268:F82-F88.
14. Pagtalunan ME, Miller PL, Jumping-Eagle S *et al*. Podocyte loss and progressive glomerular injury in type II diabetes. J Clin Invest 1997;99:342-348.
15. Osawa G, Kimmelstiel P, Seiling V. Thickness of glomerular basement membranes. J Clin Pathol 1966;45:7-20.
16. van den Heuvel LPWJ, van den Born J, van de Velden TJAM *et al*. Isolation and partial characterization of heparan sulfate proteoglycan from the human glomerular basement membrane. Biochem J 1989;264:457-465.
17. van den Born J, van den Heuvel LPWJ, Bakker MAH *et al*. Monoclonal antibodies against the protein core and glycosaminoglycan side chain of glomerular basement membrane heparan sulfate proteoglycan: Characterization and immunohistological application in human tissues. J Histochem Cytochem 1994;42:89-102.
18. van den Born J, Gunnarsson K, Bakker MAH *et al*. Presence of N-unsubstituted glucosamine units in native heparan sulfate revealed by a monoclonal antibody. J Biol Chem 1995;270:31303-31309.
19. David G, Bai XM, van der Schueren B, Cassiman J, Van den Berghe H. Developmental changes in heparan sulfate expression: In situ detection with mAbs. J Cell Biol 1992;119:961-975.

20. Raats CJI, Bakker MAH, van den Born J, Berden JHM. Hydroxyl radicals depolymerize glomerular heparan sulfate in vitro and in experimental nephrotic syndrome. J Biol Chem 1997;272:26734-26741.
21. Tryggvason K. Unraveling the mechanisms of glomerular ultrafiltration: nephrin, a key component of the slit diaphragm. J Am Soc Nephrol 1999;10:2440-2445.
22. Korbet SM, Schwartz MM, Lewis EJ. Minimal-change glomerulopathy of adulthood. Am J Nephrol 1988;8:291-297.
23. Fogo A, Hawkins EP, Berry PL *et al.* Glomerular hypertrophy in minimal change disease predicts subsequent progression to focal glomerular sclerosis. Kidney Int 1990;38:115-123.
24. D'Agati V. The many masks of focal segmental glomerulosclerosis. Kidney Int 1994;46:1223-1241.
25. McAdams AJ, Valentini R.P., Welch TR. The nonspecificity of focal segmental glomerulosclerosis. Med Baltimore 1997;76:42-52.
26. Winn MP, Conlon PJ, Lynn KL *et al*. Clinical and genetic heterogeneity in familial focal segmental glomerulosclerosis. Kidney Int 1999;55:1241-1246.
27. Winn MP, Conlon PJ, Lynn KL *et al*. Linkage of a gene causing familial focal segmental glomerulosclerosis to chromosome 11 and further evidence of genetic heterogeneity. Genomics 1999;58:113-120.
28. Conlon PJ, Butterfly D, Albers F *et al*. Clinical and pathologic features of familial focal segmental glomerulosclerosis. Am J Kidney Dis 1995;26:34-40.
29. Faubert PF, Porush JG. Familial focal segmental glomerulosclerosis: nine cases in four families and review of literature. Am J Kidney Dis 1997;30:265-270.
30. Scolari F, Scaini P, Savoldi S *et al*. Familial IgM mesangial nephropathy: A morphologic and immunogenetic study of three pedigrees. Am J Nephrol 1990;10:261-268.
31. Cohen AH, Border WA, Glassock RJ. Nephrotic syndrome with glomerular mesangial IgM deposits. Lab Invest 1978;38:610-619.
32. Guasch A, Deen WM, Myers BD. Charge selectivity of the glomerular filtration barrier in healthy and nephrotic humans. J Clin Invest 1993;92:2274-2282.
33. Kitano Y, Yoshikawa N, Nakamura H. Glomerular anionic sites in minimal change nephrotic syndrome and focal segmental glomerulosclerosis. Clin Nephrol 1993;40:199-204.
34. Kanwar YS. Biology of disease; biophysiology of glomerular filtration and proteinuria. Lab Invest 1984;51:7-21.
35. Gelberg H, Healy L, Whitely H, Miller LA, Vimr E. In vivo enzymatic removal of alpha 2--6 linked sialic acid from the glomerular filtration barrier results in podocyte charge alteration and glomerular injury. Lab Invest 1996;74:907-920.
36. Ruotsalainen V, Ljungberg P, Wartiovaara J *et al*. Nephrin is specifically located at the slit diaphragm of glomerular podocytes. Proc Natl Acad Sci USA 1999;96:7962-7967.
37. Kestila M, Lenkkeri U, Mannikko M *et al*. Positionally cloned gene for a novel glomerular protein -nephrin- is mutated in congenital nephrotic syndrome. Mol Cell 1998;1:575-582.

38. Shih N-Y, Li J, Karpitskii V *et al*. Congenital nephrotic syndrome in mice lacking CD2-associated protein. Sci 1999;286:312-315.
39. Miner JH. Renal basement membrane components. Kidney Int 1999;56:2016-2024.
40. Noakes PG, Miner JH, Gautam M *et al*. The renal glomerulus of mice lacking s-laminin/laminin ß2: nephrosis despite molecular compensation by laminin ß1. Nature Genetics 1995;10:400-406.
41. Remuzzi G, Ruggenenti P, Benigni A. Understanding the nature of renal disease in progression. Kidney Int 1997;51:2-15.
42. Mallick NP, Short CD, Hunt LP. How far since Ellis? The manchester study of glomerular disease. Nephron 1987;46:113-124.
43. Burton C, Harris KPG. The role of proteinuria in the progression of chronic renal failure. Am J Kidney Dis 1996;27:765-775.
44. Zoja C, Morigi M, Figliuzzi M *et al*. Proximal tubular cell synthesis and secretion of endothelin-1 on challenge with albumin and other proteins. Am J Kidney Dis 1995;26:934-941.
45. Zoja C, Donadelli R, Colleoni S *et al*. Protein overload stimulates RANTES production by proximal tubular cells depending on NF-kappa B activation. Kidney Int 1998;53:1608-1615.

Chapter 3

Urinary Excretion of Glutathione S Transferases Alpha and Pi in Patients with Proteinuria: Reflection of the Site of Tubular Injury.

Amanda J.W. Branten[1]
Theo P.J. Mulder[2]
Wilbert H.M. Peters[2]
Karel J.M. Assmann[3]
and Jack F.M. Wetzels[1]

Department of Medicine, Divisions of [1]Nephrology and [2]Gastroenterology, and [3]Department of Pathology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands.

Nephron 2000; 85:120-126

ABSTRACT

In patients with renal diseases, proteinuria is a major determinant of progressive renal failure, probably by causing tubular cell injury. Little is known on extent and site of tubular cell injury in patients with proteinuria. Glutathione S transferases (GST) are cytosolic enzymes. The alpha isoform is present only in proximal tubular cells, whereas the pi isoform is confined to distal tubular cells. We have studied the urinary excretion of both isoenzymes in 56 (38 male and 18 female) patients with glomerular diseases and proteinuria. The mean age was 45 ± (SD) 16 years, the median creatinine clearance was 80 (range 27-159) ml/min, and the median albuminuria was 4.2 (range 0.7-16.9) g/10 mmol creatinine. The excretions of both GST alpha (median 35.9 μg/10 mmol creatinine) and GST pi (median 24.8 μg/10 mmol creatinine) were elevated as compared with control values (upper limits 10 and 12 μg/10 mmol creatinine, respectively). The urinary excretion of GST pi, but not that of GST alpha, was inversely correlated with the creatinine clearance. The highest levels of GST alpha were found in patients with a well-preserved renal function, whereas highest levels of GST pi were found in patients with renal failure. In a small number of patients we performed immunofluorescent studies of renal tissue. An increased urinary excretion of GST alpha correlated with brush border damage and decreased staining of proximal tubules for that isoenzyme. Our data suggest that in patients with proteinuria initial injury is apparent at the proximal tubules. Measurements of GST alpha and GST pi appear useful to study longitudinal timing and site of proteinuria-induced tubular cell injury.

INTRODUCTION

Proteinuria is a major determinant of the progression of renal failure [1]. Recent studies have indicated that proteinuria is not merely a reflection of glomerular damage, but in itself contributes to further renal injury, in particular by causing tubulo-interstitial inflammation [2]. The mechanisms involved in this latter process are not fully understood. Evidence that proteins may cause tubular cell injury either directly (e.g., proximal tubular injury by lysosomal overload) or indirectly (e.g. cell injury mediated by complement activation or oxygen radical production) has been put forward in several recent reviews [3-5]. Detailed information on magnitude and site of tubular cell injury in patients with proteinuria is lacking. Thus far, several markers have been used to study tubular cell injury in humans. The best known are low molecular weight proteins such as β2-microglobulin, retinol-binding protein, and α1-microglobulin, and cellular enzymes such as β-N-acetyl-glucosaminidase (NAG), lactate dehydrogenase, and alkaline phosphatase [6-9]. Unfortunately, most of these markers mainly reflect proximal tubular cell injury. Reliable markers of distal tubular cell injury are lacking. Recent studies have suggested that measurement of the urinary excretion of glutathione S transferases (GST) alpha and pi might allow differentiation between proximal and distal tubular cell injury [10]. GSTs are cytosolic enzymes involved in the detoxification of foreign compounds by the addition of glutathione to a wide variety of xenobiotics [11,12]. Several isoforms of this enzyme exists, and in humans four main classes have been characterized: alpha, mu, pi, and theta [10]. In the kidney GST alpha and GST pi are the main isoforms present: GST alpha is highly expressed in proximal tubular cells, whereas the pi isoform is found in distal tubular and collecting duct cells [11,13-15].

In the present cross-sectional study, we have measured the urinary excretions of the alpha and pi isoforms of GST in patients with proteinuria. Our data strongly suggest that during the early phase of proteinuric renal diseases, proximal tubular cell injury predominates, whereas distal tubular cell injury supervenes as the renal function gets compromised.

PATIENTS AND METHODS

The study protocol was approved by the hospital ethics committee, and all patients gave informed consent. Patients with proven glomerular renal disease and proteinuria >1.0 g/24 h were eligible for this study. The evening before the study day, the patients took 4000 mg of sodium bicarbonate to ensure a urinary pH above 6.0, which is needed to prevent

degradation of β2-microglobulin. After an overnight fast, the patients came to the ward. Diuresis was enforced by giving 375-500 ml of tap water. The patients remained supine except for voiding. Over the next 2 h urine was collected.

Laboratory Procedures

The collected urine samples were centrifugated for 10 min at 3000 g, and the supernatant was stored at -70 ºC. Urine samples used for the analysis of the GST isoenzymes were stored in glass tubes to prevent absorption of enzymes to the wall of plastic tubes [16]. Furthermore, these urine samples were supplemented with 10% (v/v) of a storage buffer to ascertain a stable pH and to prevent bacterial growth. This storage buffer was a solution of 1 M HEPES (pH 7.5) containing 5% (w/v) bovine serum albumin, 1% (w/v) sodium azide, 1% (v/v) Tween-20 and 10% (v/v) glycerol.

GST alpha 1-1 and GST pi 1-1 were assayed by an ELISA as described recently [17]. The concentrations of albumin and IgG in the urine samples were measured by immunonephelometry using antibodies the specificity of which was checked by Ouchterlony double immunodiffusion and immunoelectrophoresis. Details of the nephelometric procedures for the measurement of albumin and IgG have been described before [18,19]. Urinary β2-microglobulin was measured by an ELISA method [20]. The antibodies used consisted of the immunoglobulin fractions of rabbit antihuman β2-microglobulin; coating: code A072, detection (horseradish peroxidase conjugated) code P174 (both Dakopatts, Copenhagen, Denmark). NAG was measured by an enzymatic assay according the instructions of the manufacturer (Boehringer Mannheim, Germany). Creatinine was measured using standard colorimetric methods.

Evaluation and Processing of Renal Biopsy Samples

We have tried to correlate results of the urinary enzyme measurements with morphological evidence of tubular damage. To this end, we have used renal biopsy tissue obtained at a time point less than 2 months apart from the day of the urine study. Light microscopical slides were reviewed, and whenever possible additional immunofluorescence studies were done (see below).

For light microscopy fragments of kidney biopsy specimens were fixed in Bouin's solution and embedded in paraplast (Amstelstad, Amsterdam, The Netherlands). Sections (2 µm) were stained with periodic acid-Schiff.

For the immunohistological detection of GST alpha, kidney fragments were snap frozen in liquid nitrogen, and 2-µm cryostat sections were initially incubated for 60 min with an anti-GST alpha monoclonal antibody, developed in our laboratory [21], diluted 1:500, followed by

a 40-min incubation with a fluorescein isothiocyanate-labeled sheep antimouse IgG, diluted 1:100 (Dakopatts). After rinsing the sections with phosphate-buffered saline for 15 min, they were additionally incubated with TRITC-labeled phalloidin diluted 1:100 (Sigma, Zwijndrecht, The Netherlands) for 15 minutes. Phalloidin stains actin and is used as a marker of proximal tubular cells due to its prominent staining of brush borders. Thus far, we have not been able to perform immunofluorescence studies with anti-GST pi antibodies. The fluorescence intensity was low when using these antibodies probably because of the low affinity.
The sections were examined in a fluorescence microscope (Leitz, Wetzlar, Germany), and the staining intensity was recorded semiquantitatively on a scale from 0 to 4+.

Calculations and Statistical Analysis

The creatinine clearance was calculated by the Cockcroft and Gault formula.
For the descriptive statistics values are given as means ± SD for parametric data or median and ranges for nonparametric data. Comparisons between groups were done using the Kruskall Wallis test and the Mann-Whitney test with Bonferroni correction. Linear regression was calculated using Spearman's correlation coëfficient. $p < 0.05$ was considered statistically significant.

RESULTS

We have studied 56 patients (38 male, 18 female) with a mean age of 45 ± 16 years, a median creatinine clearance of 80 (range 27-159) ml/min, and an albuminuria of 4.23 (range 0.72 - 16.88) g/10 mmol creatinine. The original renal diseases were focal glomerulosclerosis (n=16), minimal change nephropathy (n=6), membranous nephropathy (n=19), IgA nephropathy (n=8), membranoproliferative glomerulonephritis (n=3), and unspecified glomerulonephritis (n=4).
The overall results of urinary excretion of the parameters are summarized in Table 1. As compared with healthy controls our patients had increased urinary excretion of both NAG and β2-microglobulin. Also, the excretions of GST alpha and GST pi were increased in almost all patients. We observed weak but significant correlations between the urinary excretion of GST alpha and the excretion of NAG ($r=0.31$, $p<0.05$) and albumin ($r=0.33$, $p=0.01$), but not with β2-microglobulin, IgG or the creatinine clearance. As compared with GST alpha, the excretion of GST pi correlated stronger with the excretion of NAG ($r=0.58$, $p<0.001$) and albumin ($r=0.55$, $p<0.001$), but also with β2-microglobulin ($r=0.50$, $p<0.001$) and IgG ($r=0.58$, $p<0.001$).

Table 1. Urinary excretion of GST alpha, GST pi, β2-microglobulin, and NAG in patients with proteinuria

	Controls (n=10)	Patients (n=56)
GST alpha creatinine ratio *(μg/10 mmol)*		
Median	2.51	35.9
Range	1.17 - 7.48	7.8 – 383
GST pi creatinine ratio *(μg/10 mmol)*		
Median	5.29	24.8
Range	1.85 - 10.84	6.1 – 756
β2-microglobulin creatinine ratio *(mg/10 mmol)*		
Median	0.065	1.22
Range	0.06 - 0.08	0.08 – 125
NAG creatinine ratio *(U/10 mmol)*		
Median	1.68	17.7
Range	1.07 - 1.93	3.6 – 118

The levels are corrected for creatinine.

Table 2. Urinary excretion of proteins and enzymes in patients with proteinuria, grouped in quartiles according to the creatinine clearance

	Group 1 (n=14)	Group 2 (n=14)	Group 3 (n=15)	Group 4 (n=13)
Creatinine clearance *(ml/min)*				
Median	140	98	64	47
Range	112-159	83-111	57-77	27-55
Urinary Excretion:				
Albumin *(mg/min)*	3.54	1.68	3.67	3.77
IgG *(mg/min)*	0.13	0.05	0.20	0.30
β2m *(μg/min)*	0.25	0.18	2.76*	8.48**
β-NAG *(U/min)*	12.9	7.8	19.8	14.2
GST alpha *(ng/min)*	68.9	27.7*	21.4*	23.6*
GST pi *(ng/min)*	19.9	18.3	27.9	45.4^{+}

Values are given as medians. For all parameters the Kruskall Wallis test revealed $p<0.05$. β2m=β2-microglobuline. $^{+}$ $p = 0.07$; * $p < 0.05$;** $p < 0.01$ versus group 1.

A significant negative correlation was found between the urinary excretion of GST pi and creatinine clearance ($r= -0.37$, $p<0.001$).

In order to try to discern a pattern in the urinary excretion of the GST isoenzymes during the progression of renal disease in proteinuric patients, we have divided the patients in quartiles according to the creatinine clearance. There was no difference in albuminuria between the groups (Table 2). As expected, the urinary excretion of β2-microglobulin increased with decreasing renal function. Simultaneously, a slight increase in IgG and NAG excretions was observed. We found a clear difference in the pattern of excretion of GST alpha and GST pi; for GST alpha the excretion was highest in patients with well-preserved renal function, whereas for GST pi the excretion increased with decreasing renal function. As a result, there was a significant difference in the urinary GST alpha/GST pi ratio between the quartile groups (Figure 1).

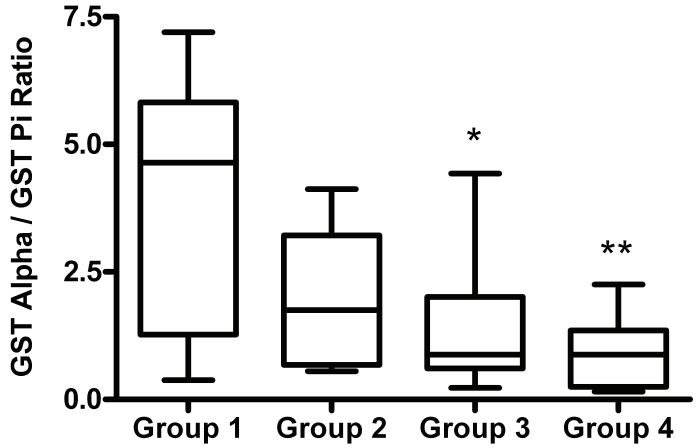

Figure 1. GST alpha and pi ratios in the urine of the patients with proteinuria, grouped in quartiles according to their endogenous creatinine clearance (ECC; see table 2). Group 1 = ECC range 112-159 ml/min, group 2 = ECC range 83-111 ml/min, group 3 = ECC range 57-77 ml/min, and group 4 = ECC range 27-55 ml/min. *$p<0.05$ compared to group 1; **$p<0.01$ compared to group 1.

We have analyzed the data according the underlying renal disease. To circumvent the effects of renal failure, we have restricted the analysis to patients with a creatinine clearance >70 ml/min. Data are given in Table 3. In patients with membranous nephropathy and minimal change nephropathy, the urinary excretion of GST alpha was higher than in patients with either IgA nephropathy or focal glomerulosclerosis; however, the differences were not statistically significant. Overall, the urinary excretion of GST alpha was positively correlated

with urinary albumin (r=0.46, p<0.01) and transferrin (r=0.50, p<0.01) excretions and negatively with the selectivity index (r= -0.49, p<0.01). It is likely that the relatively high levels of GST alpha excretion in the patients with membranous nephropathy and minimal change nephropathy are related to the higher selectivity of proteinuria in these patients.

Table 3. Urinary excretion of proteins and enzymes in patients with proteinuria and creatinine clearance >70 ml/min, grouped according to the underlying renal disease

	MCD (n=6)	**MN (n=12)**	**FGS (n=9)**	**IgA (n=4)**
Creatinine clearance *(ml/min)*				
Median	113	100	111	90
Range	83-151	73-159	73-159	75-103
Urinary Excretion :				
Albumin *(mg/min)*	4.00	2.93	3.29	1.68
GST alpha *(ng/min)*	93	54	31	29
GST pi *(ng/min)*	23	18	20	32
Selectivity index	0.15	0.19	0.24	0.23

Values are given as medians. MCD, minimal change disease; MN, membranous nephropathy; FGS, focal glomerulosclerosis; IgA, IgA-nephropathy. No statistically significant differences were noted.

In 12 patients renal biopsies have been done within two months of the urine studies. We found a clear negative correlation between the amount of tubular atrophy and the creatinine clearance. In 6 patients with normal renal function (creatinine clearance >70 ml/min) the degree of interstitial fibrosis amounted to <5% (n=5) or 5-10% (n=1), whereas the biopsy specimens of 6 patients with a creatinine clearance < 70 ml/min revealed >10% fibrosis. As expected, patients with tubular atrophy had the lowest alpha/pi ratio. In all patients with >10% interstitial fibrosis the alpha/pi ratio was below 1.0. Proximal tubular (PT) injury was best seen in periodic acid-Schiff stained slides. In patients with an increased excretion of GST alpha a more severe proximal tubular damage was seen, as indicated by changes in brush border staining and occurrence of membrane blebbing (Figure 2; in color see page 153). However, although in most patients with low levels of GST alpha, the proximal tubular injury was limited, we sometimes observed membrane blebbing in such patients too.

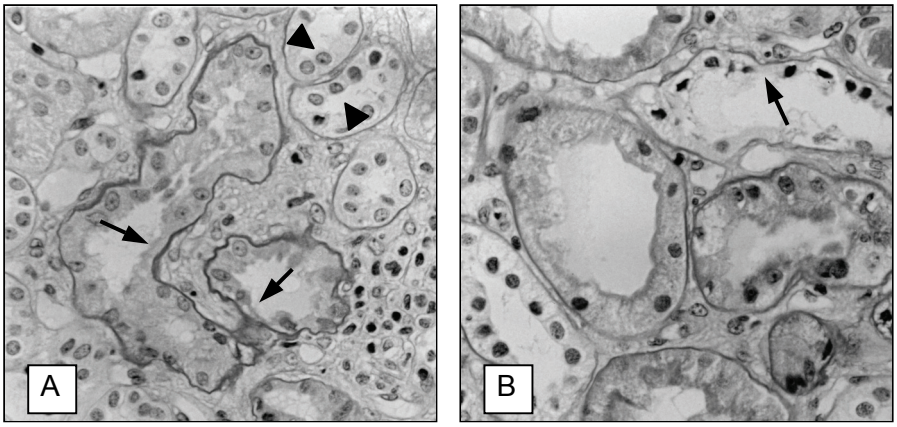

Figure 2.
Light microscopic presentation of renal biopsy specimens from 2 patients with IgA nephropathy and differing GST iso-enzyme excretion
Periodic acid-Schiff. x400
A Specimen from a patient with an increased urinary level of GST alpha (81 μg/10 mmol creatinine), and a relatively low excretion of GST pi (25 μg/10 mmol creatinine). Epithelial cells of proximal tubules are damaged (arrows) with loss of brush border, whereas distal tubular cells (arrowheads) look normal. **B** Specimen from a patient with an increased excretion of GST pi (58 μg/10 mmol creatinine) and a slightly elevated level of GST alpha (17 μg/10 mmol creatinine). In this patient the distal tubular cells are more severely damaged than the proximal tubular cells, as reflected by prominent nuclear and cellular atypia (arrow).

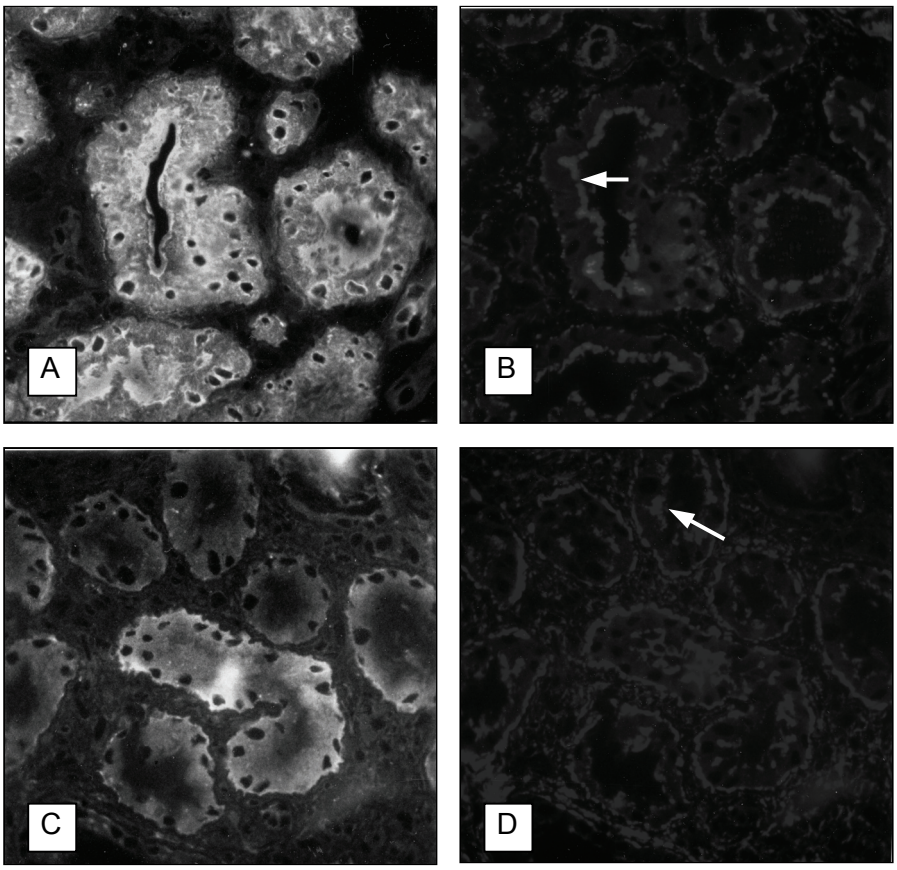

Figure 3.
Immunofluorescence double staining for GST alpha (A,C) and phalloïdin (B,D) of renal biopsy specimens from 2 patients. **A,C** Specimens from a patient with focal glomerulosclerosis, moderate proteinuria, and low levels of both GST alpha and pi (17, 14 μg/10 mmol creatinine, respectively). The proximal tubules can be easily recognized by the presence of intact brush borders. All proximal tubules show homogeneous staining of both GST alpha (A: bright green-colored cells) and of phalloïdin (B: bright red-colored brush border, arrow). **C,D** Specimens from the patient with IgA nephropathy shown in fig. 2A. GST alpha excretion was 81 μg/ 10 mmol creatinine. The proximal tubules are damaged as

indicated by the irregular and sometimes absent brush border staining (arrow in D). Staining of the proximal tubules for GST alpha revealed a variable intensity, with clear loss of staining in most proximal tubules (C). *The original color versions of figures 2 and 3 are depicted on page 153.*

We have studied the presence of GST alpha in proximal tubules by immunofluorescence using a double labeling technique. Patients with normal GST alpha and GST pi excretions showed a normal phalloïdin staining and a homogeneous staining of the proximal tubules (Figure 3). In contrast, in patients with high urinary levels of GST alpha, we frequently observed a decrease of brush border staining with phalloïdin. In these patients the intensity of staining for GST alpha was quite variable, with a decreased staining intensity occurring in many proximal tubules (Figure 3).

DISCUSSION

Although it has been suggested that proteinuria might cause tubular cell injury, the site of injury is unknown. Studies in humans are hampered by the absence of markers that can be used reliably for the detection of distal tubular injury. Recent studies have demonstrated that in the kidney GST pi is only present in distal tubules and collecting ducts, whereas GST alpha is confined to proximal tubules [11,13-15]. Furthermore, it has been shown that the urinary excretion of these isoenzymes was not increased in patients with elevated blood levels of GST nor in patients with proteinuria per se [16,22]. We could confirm the latter findings in the present study, as we have observed nine patients with proteinuria >3.5 g, in whom the excretion of GST alpha or GST pi was normal. Thus, an elevated urinary loss of either isoenzyme can only be explained by losses from damaged tubular cells.

The main goal of our study was to disclose a possible relationship between proteinuria and extent and site of tubular injury. Therefore, we have included in our study only patients with glomerular disease and proteinuria, thus excluding patients with tubulo-interstitial damage independent from proteinuria (i.e., patients with chronic pyelonephritis and those with drug-induced tubulo-interstitial nephritis).

Overall, both the excretion of GST alpha and that of GST pi were increased in our patients. We have tried to correlate the urinary excretion of GST isoenzymes with the extent of tubular injury. In agreement with observations by other investigators, we found a negative correlation between renal function and percentage of tubulo-interstitial atrophy. Using light microscopy, the evaluation of tubular damage proved difficult. Nuclear changes are often considered to

be good indicators of cell damage; however, these findings did not correlate with GST excretion. Membrane blebbing and loss of brush border seemed better markers; however, interpretation and quantitation are hampered by the occurrence of artifacts during processing. For future studies, direct fixation should be done. Most promising were the results of our immunofluorescence studies. Using a double staining technique, we were able to analyze the presence of GST alpha in brush border stained tubules. In patients with elevated levels of urinary GST alpha, we observed loss of tubular GST alpha staining.

There was a clear difference in the pattern of excretion, GST alpha being increased in patients with normal renal function and GST pi being increased in patients with renal function impairment. Based on these observations it is tempting to speculate that initially in patients with proteinuria proximal tubular cell injury prevails, resulting in an increased urinary loss of GST alpha. As a result of this initial damage, tubulo-interstitial injury progresses, causing an impairment of the renal function. At this point, distal tubular injury becomes evident. The decrease in the excretion of GST alpha at this point may reflect tubular atrophy. Since GST pi is also present in podocytes [11,13-15], the rise in urinary excretion of GST pi with decreasing renal function may reflect more advanced glomerular damage. However, it is unlikely that in renal disease podocytic injury is such that these cells lose cytosolic enzymes. Admittedly, our data are derived from a cross-sectional study. Longitudinal studies in patients with persistent proteinuria are needed to provide details on the time-related changes in proximal and distal tubular injury.

The finding that the highest levels of GST alpha were found in patients with minimal change nephropathy deserves further attention. It is unlikely that there are real differences between the various types of glomerular disease. Our patients with minimal change nephropathy had highest albumin and transferrin excretions and the lowest selectivity index. The excretion of GST alpha correlated with all three. The findings at least suggest that loss of GST alpha may be the result of tubular toxicity as a consequence of protein overload. This may seem unexpected in view of the fact that minimal change nephropathy in general does not cause renal failure. However, the development of tubulo-interstitial injury may depend on the presence of other filtered factors with toxic properties. Furthermore, in minimal change nephropathy proteinuria usually is not long lasting, the exposure time to potentially toxic proteins being too short to cause irreversible damage.

Our data clearly demonstrate that neither the urinary excretion of β2-microglobulin nor the excretion of NAG can be used to determine the site of tubular injury. With respect to β2-microglobulin, the increased excretion in patients with renal failure is explained by the increased tubular load, exceeding the threshold of tubular reabsorption. With respect to NAG, this lysosomal enzyme is present in proximal as well as in distal tubular cells [23].

In conclusion, the urinary excretion of the isoenzymes of GST is increased in patients with proteinuria. Our data point to the potential value of the use of urinary GST isoenzymes as markers of the site of tubular cell injury. This should be further clarified in longitudinal studies during the course of the disease in patients with proteinuria.

REFERENCES

1. Peterson JC, Adler S, Burkart JM *et al*. Blood pressure control, proteinuria, and the progression of renal disease. The modification of diet in renal disease study. Ann Intern Med 1995;123:754-762.
2. Burton CJ, Walls J. Proximal tubular cell, proteinuria and tubulo-interstitial scarring. Nephron 1994;68:287-293.
3. Burton C, Harris KPG. The role of proteinuria in the progression of chronic renal failure. Am J Kidney Dis 1996;27:765-775.
4. Remuzzi G, Ruggenenti P, Benigni A. Understanding the nature of renal disease in progression. Kidney Int 1997;51:2-15.
5. Eddy AA. Experimental insights into the tubulo-interstitial disease accompanying primary glomerular lesions. J Am Soc Nephrol 1994;5:1273-1287.
6. Sherman RL, Drayer DE, Leyland-Jones BR, Reidenberg MM. N-Acetyl-β-glucosaminidase and β2-microglobulin. Their urinary excretion in patients with renal parenchymal disease. Arch Intern Med 1983;143:1183-1185.
7. Beetham R, Newman D. Urinary albumin and low molecular weight protein excretion in the nephrotic syndrome-sequential studies during corticosteroid treatment. Ann Clin Biochem 1992;29:450-453.
8. Scherberich JE. Urinary proteins of tubular origin: basic immunochemical and clinical aspects. Am J Nephrol 1990;10 (Suppl 1):43-51.
9. Weber MH, Verwiebe R. α1-microglobulin (Protein HC): features of a promising indicator of proximal tubular dysfunction. Eur J Clin Chem Clin Biochem 1992;30:683-691.
10. Sundberg AGM, Appelkvist E-L, Backman L, Dallner G. Urinary pi-class glutathione transferase as an indicator of tubular damage in the human kidney. Nephron 1994;67:308-316.
11. Harrison DJ, Kharbanda R, Scott Cunningham D, McLellan LI. Distribution of glutathione S transferase isoenzymes in human kidney: basis for possible markers of renal injury. J Clin Pathol 1989;42:624-628.
12. Hayes PC, Bouchier IAD, Beckett GJ. Glutathione S-transferase in humans in health and disease. Gut 1991;32:813-818.
13. Sundberg AGM, Appelkvist E-L, Backman L, Dallner G. Quantitation of glutathione S transferase pi in the urine by radioimmunoassay. Nephron 1994;66:162-169.

14. Kharbanda R, Lauder J, Thomson D, Gouldesbrough DR, Harrison DJ. Heterogeneity of Glutathione S-Transferase isoenzyme expression in renal disease. Nephrol Dialysis Transplant 1991;6:695-700.
15. Sundberg AGM, Nillson R, Appelkvist E-L, Dallner G. Immunohistochemical localization of alpha and pi class glutathione transferases in normal human tissues. Pharmacol Toxicol 1993;72:321-331.
16. Sundberg AGM, Nillson R, Appelkvist E-L, Dallner G. ELISA procedures for the quantitation of glutathione transferases in the urine. Kidney Int 1995;48:570-575.
17. Mulder TPJ, Peters WHM, Court DA, Jansen JBJM. Sandwich ELISA for glutathione S-transferase Alpha 1-1: plasma concentrations in controls and in patients with gastrointestinal disorders. Clin Chem 1996;42:416-419.
18. Elving LD, Bakkeren JAJM, Jansen MJH *et al*. Screening for microalbuminuria in patients with diabetes mellitus: frozen storage of urine samples decreases their albumin content. Clin Chem 1989;35:308-310.
19. Reichert LJM, Koene RAP, Wetzels JFM. Urinary IgG excretion as a prognostic factor in idiopathic membranous nephropathy. Clin Nephrol 1997;48:79-84.
20. Jacobs EMG, Vervoort G, Branten AJW *et al*. Atrial natriuretic peptide increases albuminuria in type I diabetic patients: evidence for blockade of tubular protein reabsorption. Eur J Clin Invest 1999;29:109-115.
21. Peters WHM, Boon CEW, Roelofs HMJ *et al*. Expression of drug metabolizing enzymes and P-170 glycoprotein in colorectal carcinoma and normal tissue. Gastroenterology 1992;103:448-455.
22. Feinfeld DA, Fleischner GM, Goldstein EJ *et al*. Liganduria: an indication of tubular cell necrosis. Curr Probl Clin Biochem 1979;9:273-278.
23. Kotanko P, Gstraunthaler G, Pfaller W. Harnenzyme zur nichtinvasiven diagnostik von nierenepithelschaden in akuten nierenversagen. Wiener Klin Wochenschr 1984;96:625-629.

Chapter 4

Urinary Excretion of Complement C3d in Patients with Renal Diseases.

Amanda J.W. Branten[1]
Marjo Kock-Jansen[2]
Ina S. Klasen[2]
and Jack F.M. Wetzels[1]

[1]Department of Medicine, Division of Nephrology, and [2]Department of Clinical Chemistry, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands.

European Journal of Clinical Investigation 2003; 33:449-456

ABSTRACT

Introduction: Complement-mediated tubular injury may play an important role in the progression of renal diseases. C3d is a presumed marker of complement activation. Its precursor C3dg has been detected in the urine of patients with membranous nephropathy. However, little is known of the renal handling of C3d or its excretion in other renal diseases.
Methods: We measured the urinary excretion of albumin, IgG, β2-microglobulin (β2m), and of complement C3d in patients with tubulo-interstitial nephritis (TIN; n=8), in patients with membranous nephropathy (n=35) and in patients with nonmembranous glomerular diseases (23 nonproliferative and 21 proliferative). Fractional excretions (FE) were calculated using creatinine clearance as marker of GFR.
Results: C3d was not measurable in the urine of healthy controls, but was detectable in seven out of eight of the TIN patients (median excretion 0.11 mU/min, range 0.006-2.4 mU/min). In these patients the urinary excretion of β2m was clearly elevated (median 26.6 µg/min, range 1.0-103 µg/min). The FE of C3d correlated with the FE of β2-microglobulin (r=0.83, P=0.01), and their ratio amounted to 0.03 (range 0.003-0.06), a value in agreement with the expected sieving coefficient. Urine C3d was detectable in all but three of the patients with glomerular diseases (median excretion 0.36 mU/min, range 0.004-7.9 mU/min); C3d-excretion did not differ between the three subgroups of patients with glomerular diseases. FEC3d correlated with FEIgG (r=0.88, P<0.01). The ratio FEC3d/FE β2m was 0.78 (range 0.04-9.99). Selected patients with membranous nephropathy were re-analyzed after (partial) remission of proteinuria. Reduction of proteinuria resulted in a decrease of C3d excretion.
Conclusion: Urinary excretion of C3d is elevated in patients with TIN, most likely as a mere consequence of decreased tubular reabsorption. In patients with glomerular diseases urinary excretion of C3d is increased and related to proteinuria, independent of the underlying glomerular disease. In these patients there is evidence of increased local formation of C3d.

INTRODUCTION

There is ample evidence for the pivotal role of tubulo-interstitial damage in the progressive renal injury that occurs in patients with proteinuric renal diseases [1,2]. In experimental animal models interstitial injury has been related to complement activation. In a murine model of glomerulonephritis C3-mRNA up-regulation preceeded the development of interstitial inflammation and fibrosis [3]. Complement depletion or inactivation attenuated tubulo-interstitial injury in the puromycin aminonucleoside model in rats [4]. In the same model tubulo-interstitial injury was less severe in C6-deficient rats compared with normocomplementemic control rats [5].

Few studies have addressed the role of complement in human renal disease. In patients with SLE, an auto-immune disease with prominent complement activation, the urinary excretion of the complement degradation product C3d correlated with disease activity and predicted the presence of active lupus nephritis [6,7]. The urinary excretion of C3dg was measured in patients with primary glomerular diseases and found to be elevated in 19 of 44 patients with membranous nephropathy, and in four of 40 patients with IgA nephropathy. In three of nine patients with focal glomerulosclerosis C3dg was also detectable in the urine [8,9]. In the patients with membranous nephropathy, elevated levels of C3dg predicted a worse outcome with respect to renal function. These results suggest that the urinary excretion of complement degradation products such as C3d could reflect complement activation, related injury and renal disease activity. Thus far, the renal handling of complement C3d has received little attention. Theoretically there are several ways that could lead to an increased excretion of C3d in the urine (Figure 1). C3d is a relatively small molecule of 35 kD, which will be filtered to a certain degree. Like other low molecular weight proteins, filtered C3d may be reabsorbed by the proximal tubular cells. In such a case, urinary C3d excretion should be elevated in patients with tubulo-interstitial diseases. Urinary C3d can also be derived from filtered C3. Complement C3 has a high molecular weight (195 kD) and will only pass in the urine of patients with glomerular diseases whose glomerular permeability is increased.

Alternatively, C3 may be locally produced. C3 gene expression has been observed in both glomerular epithelial cells and in tubular cells [10-12]. In cell culture tubular cells exposed to serum proteins and IL-2 indeed produced C3 [13,14]. Finally, increased local activation of complement C3 may occur through amidation of C3 [15]. In the present study we measured the urinary excretion of C3d in patients with tubulo-interstitial diseases. The fractional excretions (FEs) of C3d and of the low molecular weight protein β2-microglobulin were compared and provided evidence for tubular reabsorption of C3d. The excretion of C3d was also measured in patients with glomerular diseases. In these patients urinary excretion

parallelled proteinuria, and was independent of the underlying glomerular disease. Furthermore, the data provided evidence for an increased local generation of C3d.

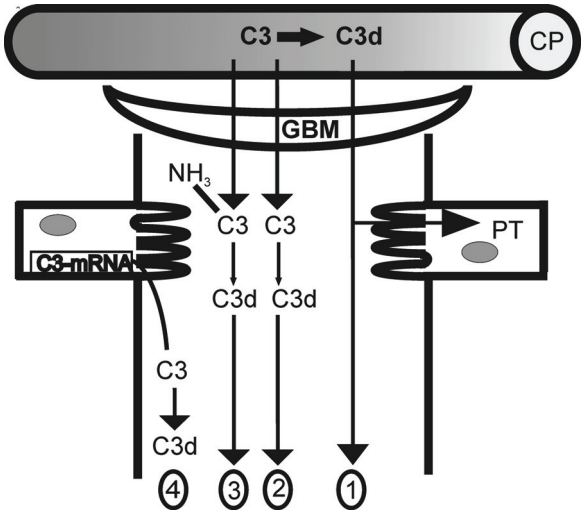

Figure 1. Possible pathways for the presence of C3d in urine. CP, Capillary; GBM, Glomerular Basement Membrane; PT, Proximale Tubular Cell. **Lane 1:** C3d in urine can be derived from circulating C3d in plasma. C3d is filtered in the glomerulus and partly reabsorbed. The fraction that escapes from reabsorption will be present in the urine. **Lane 2:** C3d can be derived from filtered C3 that is converted into C3d as in plasma. **Lane 3:** excessive formation of C3d is possible in proteinuric states. Proteinuria will lead to an increase of ammonia production (NH3). Next amidated C3 is formed, which can operate as a convertase and results in increased C3 degradation. **Lane 4:** C3d can be derived from locally produced C3.

METHODS

Patients were recruited from the population seen at our out-patient clinic. We selected patients with (suspected) tubulo-interstitial nephritis (TIN) and patients with proteinuria based on a primary glomerular disease. Patients who were treated with immunosuppressive drugs in the 6 months previous to the measurement of C3d were excluded from the baseline analysis. To determine the effect of changes in proteinuria on C3d excretion we selected a subgroup of patients with membranous nephropathy who were treated with cyclophosphamide and steroids according to our treatment schedule, as previously described [16]. In these patients measurements were carried out at baseline before the start of immunosuppressive treatment, and again 2 months later.

Measurements

All measurements were carried out in the morning, after an overnight fast and were performed according to a standard protocol. The evening before the study day the patients took 4000 mg of sodium bicarbonate to ensure an urinary pH greater than 6.0, which is

needed to prevent degradation of β2-microglobulin. In the morning the patients received 1200 mg of cimetidine, in order to block tubular secretion of creatinine [17,18]. Upon arrival at the ward the patients received 375-500 ml of tap water to enforce diuresis. The patients remained supine for 2 h except for voiding. Timed urine samples were collected, and in the middle of the collection period a blood sample was drawn. Blood and urine samples were used for the determination of creatinine, complement C3d, the low molecular weight protein β2-microglobulin, and the middle and high molecular weight proteins albumin, transferrin, and IgG. Blood pressure was recorded using an automatic device (Dinamap, Critikon, Tampa FL, USA); 10 consecutive readings being carried out at 5-min intervals.

Laboratory procedures and calculations

The concentrations of creatinine and urinary total protein were measured with standard automated techniques. The concentrations of albumin, transferrin and IgG in serum and urine were measured by immunonephelometry using antibodies whose specificity was checked by Ouchterlony double immunodiffusion and immunoelectrophoresis. Details of these nephelometric procedures have been described previously [19,20]. Urinary β2-microglobulin was measured by an ELISA method [21].

Blood- and urine samples for measurement of C3d were collected in 0.005 M EDTA tubes, transported in melting ice, and after centrifugation were stored at -70 °C. C3 and C3b molecules were precipitated from the samples by 11% PEG 6000 in 0.05 M boric acid, 0.005 M EDTA for 3 h and subsequently centrifugated at 4 °C at 2900 g. The supernatants were measured in the ELISA assay using a coat of rabbit anti-C3d (Dako A063, Glostrup, Denmark) and detection with horseradish peroxidase-labelled anti-C3d (Dako P0387). The coating antiserum and the conjugate were both tested in crossed immunoelectrophoresis. Purified C3c did not show a precipitation arch, whereas with complement-activated plasma only a C3d precipitation arch appears (data produced by Dako). The reference material serum from the healthy adults was pooled and, in the presence of sodium azide, incubated at 37 °C for 7 days. This allowed the activation and breakdown of C3 into C3d. This pooled activated serum was used as the reference standard and its value was arbitrarily set at 100 U/l. The reference curve ranged from 0.0005 to 0.01 U/l. The interassay CV was 6.5 %.

Creatinine clearance (ECC) was calculated by using the formula Ucr*V/Pcr, where Ucr is the concentration of creatinine in the urine, V is the urine flow and Pcr is the plasma concentration of creatinine. As cimetidine blocks the tubular secretion of creatinine, the creatinine clearance measured during cimetidine administration closely reflects the GFR [17,18]. The mean arterial pressure was the average of the last six consecutive registrations. Protein selectivity index was calculated as the clearance of IgG divided by the clearance of transferrin.

In patients with glomerular diseases and proteinuria urinary C3d can be derived from filtered, non-reabsorbed plasma C3d, and from activated filtered C3 (Figure 1, step 1 and step 2). In addition C3d may be formed from locally produced C3 and/or from local activation of C3 (Figure 1, step 3 and step 4). We have estimated the amount of C3d derived from the first two processes. The data obtained from the patients with TIN provided evidence for filtration and reabsorption of C3d (see Results). Fractional reabsorption of C3d parallelled the fractional reabsorption of β2-microglobulin (see Results). For calculation of the filtration of C3d we used a sieving coefficient of 0.01, based on data recently published by Norden *et al.* [22]. These authors estimated glomerular sieving coefficients (GSC) of various proteins in vivo in patients with a Fanconi syndrome related to Dent's disease [22]. The amount of filtered C3d that is not reabsorbed and will reach the distal tubules can be calculated by the formula: filtered C3d – reabsorbed C3d, in which reabsorbed C3d =(1-FEβ2m/100) * filtered C3d.

To calculate the amount of C3d formed in urine from filtered C3 we equated the ratio of C3d/C3 in urine to their plasma ratio. We assumed that the filtration of C3 was comparable to the filtration of IgG because their molecular weights are in the same range. In our laboratory, the normal plasma level of C3 is approximately 1100 mg/l; this standard value was used to calculate the C3 excretion. These statements result in the following formula: the amount of C3d formed in the urine from filtered C3 = urine C3 *(plasma C3d/plasma C3), in which urine C3 = plasma C3* (U IgG/plasma IgG).

Statistical analyses

To compare the baseline characteristics of the patient groups and to test the changes in biochemical parameters, one-way ANOVA was used for parametric data and univariate ANOVA in case of nonparametric data. When differences were significant the Bonferroni test was performed as adjustment for multiple comparisons. Spearman's bivariate correlation test was used to examine the relation between C3d excretion and proteinuria. Effects of treatment in patients with membranous nephropathy were tested with the Wilcoxon signed rank test. Results are given as means or medians when appropriate. A P-value of <0.05 was considered significant. All statistics were performed using SPSS-software, version 10.0 (SPSS Inc.,Chicago, IL).

RESULTS

We included in our study eight patients with TIN. In four patients the diagnosis was confirmed by biopsy. Two patients were not biopsied, but were diagnosed with tubular proteinuria

because of a family history of renal disease, with one member who had tubular proteinuria and a clinical picture of Dent´s disease being biopsied. The other two patients were diagnosed on clinical grounds, presenting with a drug-induced temporary deterioration of renal function and markedly elevated urinary β2-microglobulin excretion. We also included 35 patients with membranous nephropathy, 23 patients with a nonproliferative nephropathy (12 minimal change nephropathy, 10 focal glomerulosclerosis, and one Alport´s disease), and 21 patients with a proliferative glomerulonephritis (18 IgA-nephropathy, two Henoch Schönlein purpura, one membranoproliferative glomerulonephritis). The renal diagnosis was biopsy proven in all patients with membranous nephropathy, and in all but three patients with nonmembranous glomerular disease. The diagnosis Alport´s disease was made because of a positive familial history, and confirmed by DNA analysis; in the patients with Henoch Schönlein purpura the diagnosis was made on the typical clinical presentation with purpura, an active urinary sediment, and negative serology.

Urinary C3d levels measured in six normal healthy controls were undetectable (calculated excretion <0.003 mU/min). We studied eight (four male and four female) patients with TIN. The mean (±SD) age of these patients was 50 ± 17 years, serum creatinine 178 ± 98 µmol/l, serum albumin 42 ± 4 g/l, and proteinuria 0.9 ± 0.1 g/10 mmol creatinine. Renal function as measured by creatinine clearance after cimetidine was 46 ± 16 ml/min/1.73 m². By definition, urinary β2-microglobulin excretion was increased (median 26.6 µg/min, range 1.0 - 103 µg/min). C3d was detectable in the urine of seven of the eight patients, with a median excretion of 0.11 mU/min (range 0.006 – 2.4 mU/min). There was a strong correlation between the fractional excretion of C3d and the fractional excretion of β2-microglobulin (Figure 2, r=0.83, P<0.001).

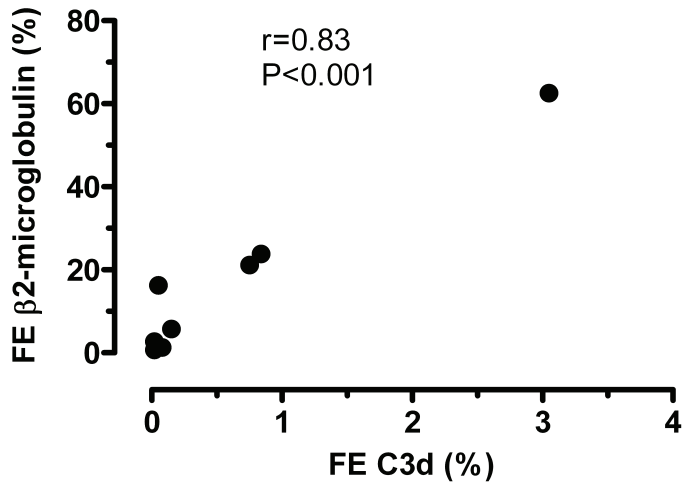

Figure 2. Fractional excretion of C3d (FE C3d) versus fractional excretion of β2-microglobulin (FE β2-microglobulin) in patients with tubulo-interstitial nephritis (n=8).

The ratio of FEC3d/FEβ2m amounted to 0.03 (range 0.003 – 0.06). This ratio, which reflects the maximal sieving coefficient of C3d, is in close agreement with the value of 0.01 derived from the sieving curve in the paper of Norden *et al.* [22]. Therefore, the data strongly suggest that C3d is filtered and reabsorbed, and reabsorption quantitatively parallels reabsorption of β2-microglobulin. In these patients there is no evidence of local production of C3d.

The baseline characteristics of the three groups of patients with glomerular disease are summarized in Tables 1 and 2. No significant differences were found with regard to age, sex, mean arterial pressure and plasma levels of C3d. Renal function was better in the patients with membranous nephropathy and other nonproliferative nephropathies compared with the patients with proliferative nephropathy. Furthermore, proteinuria was more severe and serum albumin lower in the patients with membranous nephropathy and other nonproliferative glomerular diseases (Table 2).

In 77 of the 79 patients C3d was detectable in the urine. There were no differences in urinary C3d excretion between the groups, although values were numerically lower in the group of patients with proliferative glomerular disease.

When comparing Figures 2 and 3, it is evident that in patients with glomerular diseases the urinary excretion of C3d is higher at comparable levels of β2m-excretion. The ratio of FEC3d/FEβ2m amounted to 0.78 (0.04 - 9.99) in this group ($P<0.001$ compared with the TIN patients). These data clearly suggest that altered tubular reabsorption alone cannot explain the presence of increased amounts of C3d in the urine of patients with glomerular disease. C3d may be simply derived from filtered C3. We have estimated the amount of C3d that could reach the urine as a result of filtration and reabsorption of C3d, and of filtration and conversion of C3 (see Methods).

When comparing these estimated values with the actual measured values, the median (range) excess of C3d excreted per minute amounted to 0.33 mU/min (0.0 - 7.7 mU/min) in the patients with membranous nephropathy, 0.28 mU/min (0.0 - 5.1 mU/min) in the patients with nonproliferative nephropathy, and 0.20 mU/min (0.0 - 3.6 mU/min) in the patients with proliferative nephropathy. The positive values in all three subgroups with glomerular diseases indicate increased C3d formation from activated and/or locally produced C3.

In six patients with membranous nephropathy, proteinuria decreased more than 50% during treatment with a combination of cyclophosphamide and steroids in a schedule, as we have described previously [16]. Two months after the start of treatment significant decreases of IgG-, albumin-, and C3d-excretion were observed (Table 3). The β2-microglobulin excretion decreased in five of the six patients, however, this difference was not statistically significant.

Table 1. Characteristics of patients with membranous nephropathy, nonproliferative and proliferative glomerular diseases

	MN **n=35**	**Nonproliferative** **n=23**	**Proliferative** **n=21**
Age *(years)*	46 ± 14	43 ± 17	40 ± 11
Sex *(M : F)*	26 : 9	10 : 13	14 : 7
MAP *(mm Hg)*	90 ± 14	95 ± 11	97 ± 10
Serum Creatinine *(µmol/l)*	102 ± 42**	100 ± 46**	145 ± 59
Serum Albumin *(g/l)*	27 ± 5**	26 ± 7**	38 ± 5
Plasma C3d *(U/l)*	1.59 ± 0.5	1.51 ± 0.4	1.56 ± 0.4
ECC *(ml/min/1.73 m^2)*	65 ± 22	68 ± 31*	48 ± 21

Data are mean ± SD. MN, membranous nephropathy; MAP, mean arterial pressure; ECC, endogenous creatinine clearance, measured after cimetidine pretreatment. *P<0.05 and **P<0.01 compared with proliferative nephropathy.

Table 2. Urinary protein excretion in patients with membranous nephropathy, nonproliferative and proliferative glomerular diseases

	MN **n=35**	**Nonproliferative** **n=23**	**Proliferative** **n=21**
Albumin *(mg /min)*	3.5*	4.3**	1.6
	(0.5 – 9.3)	(0.4 – 17.2)	(0.2 – 5.8)
IgG *(µg /min)*	120	99	67
	(13 – 546)	(4 – 3363)	(10 – 546)
β2m *(µg /min)*	0.70	0.30	0.42
	(0.06 – 27.1)	(0.04 – 11.7)	(0.07 – 4.33)
C3d *(mU /min)*	0.37	0.57	0.21
	(0.004 – 7.9)	(0.011 – 5.3)	(0.004 – 3.7)
Proteinuria	6.9**	6.4**	2.3
(g/10 mmol creat)	(2.2 – 17.2)	(0.8 – 13.2)	(0.3 – 7.9)
Selectivity index	0.18	0.15**	0.26
	(0.08 – 0.39)	(0.01 – 0.38)	(0.12 – 0.59)

Data are median (range). MN, membranous nephropathy; β2m, β2-microglobulin. *P<0.05 and **P<0.01 compared with proliferative nephropathy.

In the patients with glomerular disease the FE of C3d correlated significantly with the FE of β2m (Figure 3, overall r=0.80, P<0.001), and with the FE of IgG (Figure 4, r=0.88, P<0.001).

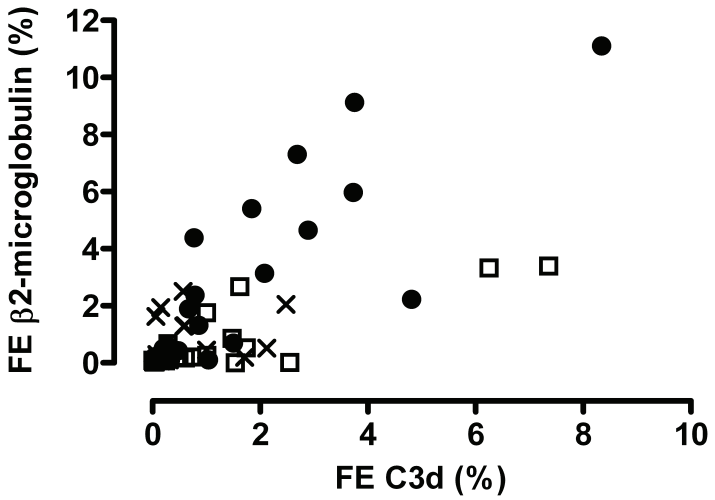

Figure 3. Fractional excretion of C3d (FE C3d) versus fractional excretion of β2-microglobulin (FE β2-microglobulin) in patients with membranous nephropathy (n=35; closed circles) nonproliferative glomerular diseases (n=23, open squares) and proliferative glomerular diseases (n=21, crosses).

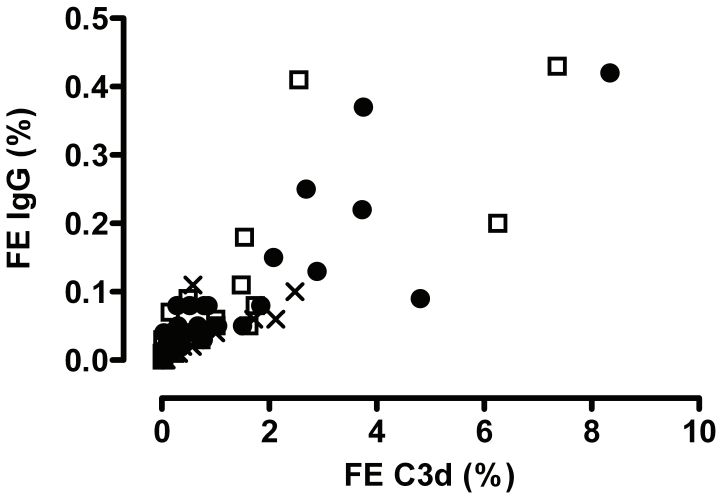

Figure 4. Fractional excretion of C3d (FE C3d) versus fractional excretion of the high molecular weight protein IgG (FE IgG) in patients with membranous nephropathy (n=35; closed circles) nonproliferative glomerular diseases (n=23, open squares) and proliferative glomerular diseases (n=21, crosses).

Table 3. Comparison of urine analysis in 6 patients with membranous nephropathy before (baseline) and after 2 months' treatment with a combination of steroids and cyclophosphamide (immunosuppression)

	Baseline	**Immunosuppression**
MAP *(mm Hg)*	93 ± 15	94 ± 13
Serum Creatinine *(µmol/l)*	152 ± 69	132 ± 43
Plasma C3d *(U/l)*	1.74 ± 0.27	1.55 ± 0.30
ECC *(ml/min/1.73 m^2)*	47 ± 17	53 ± 22
Urinary Protein Excretion:		
Albumin *(mg /min)*	6.71	3.09*
	(3.09 – 9.28)	(0.28 – 5.40)
IgG *(µg /min)*	244	55*
	(159 – 469)	(4.1 – 149)
β2m *(µg /min)*	12.9	8.3
	(3.6 – 18.0)	(0.21 – 35.4)
C3d *(mU /min)*	2.79	0.70**
	(0.46 – 7.9)	(0.04 – 4.8)
Proteinuria	8.5	3.9*
(g/10 mmol creat)	(5.3 – 15.0)	(2.1 – 10.2)
Selectivity index	0.33	0.25*
	(0.21 – 0.39)	(0.20 – 0.30)

Data are in means ± SD or median (range). MAP, mean arterial pressure; β2m, β2-microglobulin; ECC, endogenous creatinine clearance, measured after cimetidine pretreatment. *P<0.05 and **P<0.01 compared with baseline.

DISCUSSION

Our study provides more detailed insight into the renal handling of C3d, a degradation product of C3. The data obtained in patients with TIN strongly suggest that in these patients the urinary excretion of C3d is merely explained by processes of glomerular filtration and tubular reabsorption. The FE of C3d paralleled the FE of β2-microglobulin, a low molecular weight protein that is readily filtered by the glomerulus (estimated sieving coefficient >0.9) and under normal circumstances was nearly completely reabsorbed by the renal proximal tubules. For proteins that behave similarly to β2m the ratio of the FE of the protein and the FE of β2m reflects the glomerular sieving. In our patients with TIN this ratio averaged 0.03, a value that is in close agreement with the sieving coefficient for a 35 kD protein, as derived

from the sieving curve provided by Norden *et al.* [22]. Therefore, in the patients with TIN the increased urinary excretion of C3d can be readily explained by the filtration of C3d and an impairment of the tubular reabsorption. We feel that there is no evidence to consider local production or formation of C3d in these patients.

The situation is clearly more complex in patients with proteinuric renal diseases. In these patients urinary excretion of C3d was increased and correlated with the excretion of β2m and IgG. The ratio FEC3d/FEβ2m was much higher than in patients with TIN, which indicates that the increased excretion of C3d cannot be explained merely by a decrease of tubular reabsorption, but also must reflect local production of C3d most likely through activation of C3 (Figure 1). It is important to realize that we administered 4 g of sodiumbicarbonate to our patients in order to alkalinize the urine, thus allowing an accurate measurement of β2-microglobulin. We cannot exclude that this alkalinization might have affected, i.e. lowered, the urine complement C3d excretion. Morita *et al.* have demonstrated that treatment with sodiumbicarbonate for 2 weeks reduced the urinary excretion of complement activation products, at least in patients with established renal insufficiency and metabolic acidosis [23]. It is unclear if a similar effect would have occurred when using a single dose of sodiumbicarbonate in patients with less severe renal insufficiency. In vitro, lowering of pH directly influenced C3 deposition on tubular epithelial cells [24].

By using a standardized single dose of sodiumbicarbonate we eliminated one factor that could have biased our results, thus allowing an even better study of the possible relationship between proteinuria and complement excretion. Moreover, the fact that we alkalinized the urine and thus may have attenuated C3 activation strengthens further our conclusions that C3d must be produced locally in patients with proteinuria.

Theoretically, urinary C3d could be derived from filtered C3 (Figure 1, steps 2 and 3) or from locally produced C3 (Figure 1, step 4). Complement C3 is a molecule with a size comparable to IgG. C3 will be filtered in patients with a glomerular permeability defect, and indeed C3 has been detected by Western blot techniques in the urine of patients with proteinuric renal diseases [23]. The strong correlation between FEC3d and FEIgG provides supportive arguments for the formation of C3d from filtered C3. However, our calculations indicate that in such a case the formation of C3d from C3 in the urine proceeds at a much higher rate than in the plasma. This is not unexpected, as it is well known that proximal tubular cells are able to activate C3. This process is influenced by ammonia, which leads to formation of amidated C3, which operates as a C3 convertase, resulting in cleavage of C3 [15]. In patients with proteinuria the production of ammonia is further increased [25].

Complement activation may also occur at the site of the visceral epithelial cells, as has been shown in models of membranous nephropathy.

Although this schedule of events seems highly likely, we cannot exclude that urinary C3d is derived from locally produced C3. It is well established that tubular cells are able to produce C3. In cell-culture systems using human proximal tubular cells the secretion of C3 was increased after exposure to serum proteins, particularly after exposure to transferrin [13,26].

We found a definite correlation between urinary C3d and proteinuria, with no differences between the various patient groups. Thus, the level of proteinuria, rather than the type of renal disease, determines the urinary appearance of C3d.

In the literature limited data are available concerning C3d measurements in urine of patients with renal diseases. Most data are from studies in patients with lupus erythematosus (SLE). In patients with SLE, C3d levels in urine were better correlated with the disease activity score than serum C3, C4 and C3d [7]. The authors hypothesized that C3d excretion partly was the result of local production of C3d in the kidneys, as they were unable to find correlations between urine C3d and plasma C3d as well as the C3d/C3 ratio. In another study, urine C3d allowed patients with acute lupus nephritis to be distinguished from those without acute lupus nephritis [6]. However, both studies did not consider the possibility that the differences in C3d excretion between patients with and without active lupus nephritis could be explained by differences in proteinuria and/or tubular injury.

Brenchley *et al.* have suggested that tubular C3 activation is specific for patients with membranous nephropathy [8,9]. Their conclusion was based on the finding of increased urinary excretion of C3dg and C5b-9 in the urine of patients with membranous nephropathy whereas these complement products were less frequently elevated or even absent in patients with IgA nephropathy or patients with other proteinuric renal diseases. However, in the latter group of 20 patients only nine had a primary glomerular disease, i.e. focal glomerulosclerosis. In these patients with FGS urinary C3dg was present in three (33%). Furthermore, the finding of absence of C5b-9 in patients with nonmembranous glomerular disease was challenged by data of Ogrodowski *et al.* [27]. The latter investigators showed that the excretion of C5b-9 was increased, even in patients with complement-independent renal diseases, and correlated with proteinuria [27]. They hypothesized that the detection of C5b-9 in the urine was the result of leakage of complement, but also intratubular complement activation; conclusions that are in accordance with our results. In our patients with membranous nephropathy immunosuppressive treatment reduced proteinuria and urinary C3d excretion. It is generally accepted that a reduction of proteinuria predicts a better prognosis. In view of the observation that complement may contribute to the development of tubulo-interstitial injury and the relation between proteinuria and complement, it is tempting to speculate that lesser activation of complement contributes to the beneficial effects of anti-proteinuric treatment.

Of course, controlled studies are needed to determine the role of complement activation in various renal diseases and the prognostic value of complement products in the urine.
In conclusion: C3d is detectable in the urine of patients with TIN. The observation that the FE of C3d correlates with the FE of β2-microglobulin suggests a comparable renal handling of C3d, i.e. by processes of glomerular filtration and tubular reabsorption. The increased excretion of C3d in patients with proteinuric glomerular diseases cannot merely be explained by altered tubular reabsorption. In these patients C3d is formed from filtered or locally produced complement C3, a process that is independent of the underlying renal disease.

REFERENCES

1. Schainuck LI, Striker GE, Cutler RE, Benditt EP. Structural-functional correlations in renal disease. Hum Pathol 1970;1:631-641.
2. Nath KA. Tubulointerstitial changes as a major determinant in the progression of renal damage. Am J Kidney Dis 1992;20:1-17.
3. Welch TR, Frenzke M, Witte D. Evidence of a role for local complement expression in a murine model of progressive glomerulonephritis. Pediatric Research 2000;48:200-205.
4. Nomura A, Morita Y, Maruyama S *et al*. Role of complement in acute tubulointerstitial injury of rats with aminonucleoside nephrosis. Am J Pathol 1997;151:539-547.
5. Nangaku M, Pippin J, Couser WG. Complement membrane attack complex (C5b-9) mediates interstitial disease in experimental nephrotic syndrome. J Am Soc Nephrol 1999;10:2323-2331.
6. Manzi S, Rairie J, Betts Carpenter A *et al*. Sensitivity and specificity of plasma and urine complement split products as indicators of lupus disease activity. Arthritis and Rheumatism 1996;39:1178-1188.
7. Negi VS, Aggarwal A, Dayal R, Naik S, Misra R. Complement degradation product C3d in urine: marker of lupus nephritis. J Rheumatol 2000;27:380-383.
8. Brenchley PE, Coupes B, Short CD *et al*. Urinary C3dg and C5b-9 indicate active immune disease in human membranous nephropathy. Kidney Int 1992;41:933-937.
9. Coupes B, Brenchley PE, Short CD, Mallick NP. Clinical aspects of C3dg and C5b-9 in human membranous nephropathy. Nephrol Dialysis Transplant 1992;Suppl 1:32-34.
10. Montinaro V, Lopez A, Monno R *et al*. Renal C3 synthesis in idiopathic membranous nephropathy: correlation to urinary C5b-9 excretion. Kidney Int 2000;57:137-146.
11. Welch TR, Beischel L, Witte DP. Differential expression of complement C3 and C4 in the human kidney. J Clin Invest 1993;92:1451-1458.
12. Abe K, Miyazaki M, Koji T *et al*. Intraglomerular synthesis of complement C3 and its activation products in IgA nephropathy. Nephron 2001;87:231-239.

13. Tang S, Sheerin NS, Zhou W, Brown Z, Sacks SH. Apical proteins stimulate complement synthesis by cultured human proximal tubular epithelial cells. J Am Soc Nephrol 1999;10:69-76.
14. Brooimans RA, Stegmann APA, van Dorp WT *et al*. Interleukin 2 mediates stimulation of complement C3 biosynthesis in human proximal tubular epithelial cells. J Clin Invest 1991;88:379-384.
15. Nath KA, Hostetter MK, Hostetter TH. Pathophysiology of chronic tubulo-interstitial disease in rats. Interactions of dietary acid load, ammonia, and complement component C3. J Clin Invest 1985;76:667-675.
16. Branten AJW, Reichert LJM, Koene RAP, Wetzels JFM. Oral cyclophosphamide versus chlorambucil in the treatment of patients with idiopathic membranous nephropathy and renal insufficiency. Q J Med 1998;91:359-366.
17. Hilbrands LB, Artz MA, Wetzels JFM, Koene RAP. Cimetidine improves the reliability of creatinine as a marker of glomerular filtration. Kidney Int 1991;40:1171-1176.
18. Zaltzman JS, Whiteside C, Cattran DC, Lopez FM, Logan AG. Accurate measurement of impaired glomerular filtration using single-dose oral cimetidine. Am J Kidney Dis 1996;27:504-511.
19. Elving LD, Bakkeren JAJM, Jansen MJH *et al*. Screening for microalbuminuria in patients with diabetes mellitus: frozen storage of urine samples decreases their albumin content. Clin Chem 1989;35:308-310.
20. Reichert LJM, Koene RAP, Wetzels JFM. Urinary IgG excretion as a prognostic factor in idiopathic membranous nephropathy. Clin Nephrol 1997;48:79-84.
21. Jacobs EMG, Vervoort G, Branten AJW *et al*. Atrial natriuretic peptide increases albuminuria in type I diabetic patients: evidence for blockade of tubular protein reabsorption. Eur J Clin Invest 1999;29:109-115.
22. Norden AGW, Lapsley M, Lee PJ *et al*. Glomerular protein sieving and implications for renal failure in Fanconi syndrome. Kidney Int 2001;60:1885-1892.
23. Morita Y, Ikeguchi H, Nakamura J *et al*. Complement activation products in the urine from proteinuric patients. J Am Soc Nephrol 2000;11:700-707.
24. Peake PW, Pussell BA, Mackinnon B, Charlesworth JA. The effect of pH and nucleophiles on complement activation by human proximal tubular epithelial cells. Nephrol Dialysis Transplant 2002;17:745-752.
25. Rustom R, Jackson MJ, Critchley M, Bone JM. Tubular metabolism of aprotinin 99mTc and urinary ammonia: effects of proteinuria. Miner Electrolyte Metab 1992;18:108-112.
26. Tang S, Lai KN, Chan TM *et al*. Transferrin but not albumin mediates stimulation of complement C3 biosynthesis in human proximal tubular epithelial cells. Am J Kidney Dis 2001;37:94-103.
27. Ogrodowski JL, Hebert LA, Sedmak D *et al*. Measurement of SC5b-9 in urine of patients with the nephrotic syndrome. Kidney Int 1991;40:1141-1147.

Chapter 5

Serum Creatinine is a Poor Marker of GFR in Nephrotic Syndrome.

Amanda J.W. Branten
Gerald Vervoort
and Jack F.M. Wetzels

Department of Medicine, Division of Nephrology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands.

Nephrology Dialysis Transplantation 2005; 20:707-711

ABSTRACT

Background: In daily clinical practice creatinine clearance is used as marker of glomerular filtration rate (GFR). As a result of the tubular secretion process endogenous creatinine clearance (ECC) overestimates glomerular filtration rate, particularly in patients with impaired renal function. It has been suggested that the tubular handling of creatinine is altered in patients with a nephrotic syndrome.

Methods: Inulin clearance (GFR) and creatinine clearance (ECC) have been simultaneously measured in a cohort of 42 patients with proteinuria and 45 healthy controls. The clearance of creatinine by tubular secretion (TScreat) can be estimated by ECC–GFR. TScreat was calculated in both groups. Regression analysis was performed to identify factors that independently influence tubular creatinine secretion.

Results: The mean age (±SD) of the patients was 41±13 years, serum albumin 26±9 g/l, median (IQR) proteinuria 4.5 (3.6-8.2) g/10 mmol creatinine, serum creatinine 103 (84-143) µmol/l, ECC 85 (69-118) ml/min/1.73m^2, and GFR 54 (36-83) ml/min/1.73m^2. Median TScreat amounted to 29 (21-36) ml/min/1.73m^2. In the healthy controls serum creatinine was 75 (70-81) µmol/l, ECC 118 (109-125) ml/min/1.73m^2, GFR 106 (102-115) ml/min/1.73m^2, and TScreat 11 (3.5-19) ml/min/1.73m^2. By regression analysis serum albumin was identified as an independent predictor of tubular creatinine secretion. We divided the patients in two subgroups based on serum albumin levels. TScreat was 24 (14-29) ml/min/1.73m^2 in patients with serum albumin levels >25.8 g/l, and 36 (28-54) ml/min/1.73m^2 in patients with serum albumin levels <25.8 g/l (P<0.01).

Conclusion: Serum albumin levels influence tubular creatinine secretion. As a result, the endogenous creatinine clearance as well as estimated GFR using a modified MDRD equation more pronouncedly overestimate glomerular filtration rate in nephrotic syndrome.

INTRODUCTION

Patients with a nephrotic syndrome are at risk to develop renal failure. Accurate assessment of renal function is important in the care of these patients. The glomerular filtration rate (GFR) can be reliably measured by using filtration markers such as inulin. Since GFR measurements with exogenous markers are generally time demanding and costly, in daily clinical practice the endogenous creatinine clearance (ECC) is often used. It is well known that in normal man the ECC overestimates the GFR as a result of the renal tubular secretion of creatinine [1]. In case of a decreased GFR the contribution of tubular secretion to total excretion of creatinine is enhanced, and as a result the overestimation of the GFR by the ECC will be more pronounced in patients with impaired renal function [2]. It has been suggested that the tubular handling of creatinine is also altered in patients with a nephrotic syndrome [2,3]. Berlyne *et al.* have reported four patients with a nephrotic syndrome, in whom creatinine clearance markedly overestimated GFR. However, since renal function was impaired in some of these patients, firm conclusions cannot be drawn [3]. The study of Carrie and colleagues also suggested that the creatinine clearance greatly exceeded the GFR in patients with a nephrotic syndrome [2]. However, in the latter study patients with heart failure were used as controls.

Based on the data of the Modification of Diet in Renal Disease (MDRD) study prediction equations have been developed that allow a better estimate of GFR in patients with renal diseases [4]. Recently, a simplified MDRD equation was published, which used serum creatinine as the only serum assay, and which had similar predictive ability as the original MDRD equation [5,6]. This simplified equation has already been used in large studies [7]. However, the performance of this formula in patients with hypoalbuminaemia is unknown.

In the present study we have examined the relationship between ECC and GFR in patients with proteinuria. Our data indicate that tubular handling of creatinine is dependent on serum albumin levels.

METHODS

For clinical studies in patients with renal diseases we regularly measured GFR using inulin clearances, according to a standardized protocol as described before [8]. All GFR measurements were approved by the hospital ethics committee, and all subjects gave their informed consent. For the present study we have analysed the data of all patients with a glomerular renal disease, and proteinuria >0.5 g/10 mmol creatinine. GFR was measured

under baseline conditions. Creatinine and inulin concentrations were measured in all urine and blood samples. For comparison we have used data on ECC and GFR measured in a group of 45 healthy controls. These healthy controls were recruited from the local population and before renal measurements screened for the absence of hypertension, cardiovascular disease, renal dysfunction, and microalbuminuria. In the controls medication was not allowed except for oral anticonceptives [1].

Laboratory Measurements

In blood and urine samples creatinine was determined according to a modified Jaffé method on a Hitachi 747 autoanalyser (Roche, Almere, The Netherlands). Inulin concentrations were determined in duplicate by a semi-automatic technique (centrifugal analysis, Multistat) using enzymatic degradation of inulin [9]. Albumin was measured in serum by immunonephelometry on a BNII nephelometer (Behring, Marburg, Germany) using antibodies whose specificity was checked by Ouchterlony double immunodiffusion and immunoelectrophoresis (Dako, Gloostrup, Denmark). Urinary protein was measured in 24 h urine samples using a turbidimetric method with trichloracetid acid.

Calculations and Statistics

The creatinine clearance was calculated according to the standard formula Ucr*V/Pcr in which Ucr is the creatinine concentration in the timed urine portion, V is the volume of the timed urine portion, and Pcr is the plasma concentration of creatinine measured in the same time period. The GFR was calculated with the same formula, but now using inulin concentrations instead of creatinine concentrations. The clearance of creatinine by tubular secretion (tubular clearance of creatinine; TScreat) was calculated from ECC-GFR. The simplified MDRD equation was used as follows: estimated GFR=186*[Plasma creatinine]-1.154 *[Age] -0.203 *[0.742 if patient is female] (plasma creatinine in mg/dL). Because all patients were Caucasian the correction factor for black people in the formula was eliminated [5]. Since serum urea was not regularly measured in our patients we were not able to use the extensive MDRD equation [4]. Total proteinuria as measured in 24 h urine samples was expressed as grams per 10 mmol creatinine to correct for errors in urine collection.

Results are given as means (±SD) or medians (interquartile range; IQR) when appropriate. For comparisons of means and medians the Student T-test or the Mann-Whitney U-test were used respectively. The Spearman correlation test was used to identify individual factors that are related to the TScreat. Next, linear regression analysis was carried out, using a forward stepwise procedure, to determine which of these individual factors independently influenced

the TScreat. To allow regression analysis non-parametric parameters were transformed. TScreat showed a normal distribution after square root transformation, serum creatinine and proteinuria after log transformation. The transformed TScreat was defined as the dependent variable and the identified factors as result of the univariate analysis were introduced as possible predicting variables. A P value <0.05 was considered significant. All statistics were performed using SPSS software, version 11 (SPSS, IL, Chicago, USA).

RESULTS

Data of 42 patients with proteinuria were available for analysis. The original, biopsy proven renal diseases were: membranous nephropathy (n=23), focal glomerulosclerosis (n=9), IgA-nephropathy (n=4), unspecified glomerulonephritis (n=3), membranoproliferative glomerulonephritis (n=2), and M. Alport (n=1). Data about medication was lacking in one patient. None of the remaining 41 patients was treated with drugs that interfere with creatinine secretion such as cimetidine or trimethoprim. No patient used steroids. Two patients were treated with NSAIDs. Twelve patients were treated with an ACE inhibitor or an AT1 receptor blocker. Clinical characteristics of the patients are summarized in Table 1. The mean age (±SD) of the 45 healthy controls (23 males, 22 females) was 28 ± 6 years, serum albumin 44 ± 4 g/l, the median (IQR) serum creatinine 75 (70-81) µmol/l, ECC 118 (109-125) ml/min/1.73m^2, simplified MDRD 113 (104-125) ml/min/1.73 m^2, and GFR 106 (102-115) ml/min/1.73m^2.

In the controls TScreat was 11 (3.5-19) ml/min/1.73m^2. TScreat was independent from GFR. In the patients median (IQR) TScreat was 29 (21-36) ml/min/1.73m^2 (P<0.001 vs controls) and was also not correlated with GFR (r=0.01, P=0.9). In univariate analysis TScreat was significantly correlated with serum albumin (r= -0.52, P<0.001). Weak correlations were found with serum creatinine (r= -0.27, P=0.08), and proteinuria (r=0.25; P=0.12). Linear regression analysis was performed using the square root transformed TScreat as dependent factor and serum albumin, log transformed proteinuria, and log transformed serum creatinine as variables. Serum albumin proved the only independent predictor. The relation between serum albumin and TScreat and between proteinuria and TScreat are depicted in Figure 1.

The use of an ACE inhibitor or AT1 blocker did not influence the results. If we restricted the analysis to patients not using an ACE inhibitor (n=29) serum albumin proved an independent predictor of TScreat. To illustrate the potential magnitude of the effect we have analysed the data for patients divided in subgroups based on the median serum albumin level (25.8 g/l).

Table 1: Baseline characteristics of all patients and of groups of patients, ranked according to their serum albumin level

		Serum Albumin	
	All Patients (n=42)	**<25.8 g/l (n=21)**	**>25.8 g/l (n=21)**
Age *(years)*	41 ± 13	42 ± 14	40 ± 13
Sex *(M :F)*	35 : 7	17 : 4	18 : 3
NSAID *(N)*	2	1	1
ACEi / AT1B *(N)*	11 / 1	3 / 0$^{\#}$	8 / 1
Serum albumin *(g/l)*	26 ± 9	19 ± 5*	33 ± 6
Serum creatinine *(µmol/l)*	103 (84-143)	95 (83-156)	108 (92-125)
ECC *(ml/min/1.73m^2)*	85 (69-118)	82 (63-125)	86 (74-111)
GFR *(ml/min/1.73m^2)*	54 (36-83)	43 (33-77)	63 (46-89)
MDRD-GFR *(ml/min/1.73 m^2)*	68 (49-83)	68 (46-90)	67 (56-78)
TScreat *(ml/min/1.73 m^2)*	29 (21-36)	36 (28-54)*	24 (14-29)
Proteinuria *(g/10 mmol creat)*	4.5 (3.6-8.2)	6.0 (4.3-10.5)*	4.0 (2.7-4.9)

Data are given as means ± SD or median (IQR). NSAID, number of patients treated with an NSAID; ACEi/AT1B, number of patients treated with an angiotensin converting enzyme inhibitor or angiotensin II type 1-receptor blocker; ECC, creatinine clearance; GFR, glomerular filtration rate; MDRD-GFR, GFR calculated by the Modification of Diet in Renal Disease formula, simplified version [5]; TScreat, creatinine clearance by tubular secretion. $^{\#}$P=0.062 and *P≤0.01 compared to patients with serum albumin >25.8 g/l.

Characteristics of the two subgroups are summarized in Table 1. TScreat was highest and thus overestimation of the GFR by the ECC was most pronounced in the subgroup of patients with the lowest serum albumin level, thus confirming the results of the linear regression analysis.

The relationship between the ECC and GFR for the two subgroups is depicted in Figure 2. It is evident that the regression lines are different. To illustrate the consequences of this difference for clinical practice, we calculated GFR for a typical patient with a measured ECC of 80 ml/min. In healthy controls an ECC of 80 ml/min/1.73m^2 represents a GFR of 60 ml/min/1.73m^2; in patients with proteinuria and a serum albumin level >25.8 g/l it represents a GFR of 57 ml/min/1.73m^2; in patients with a serum level <25.8 g/l an ECC of 80 reflects a GFR of 42 ml/min/1.73m^2. As expected, we observed a similar difference between the patient groups when considering the relationship between calculated GFR using the simplified MDRD formula and true GFR (inulin clearance) (Figure 3).

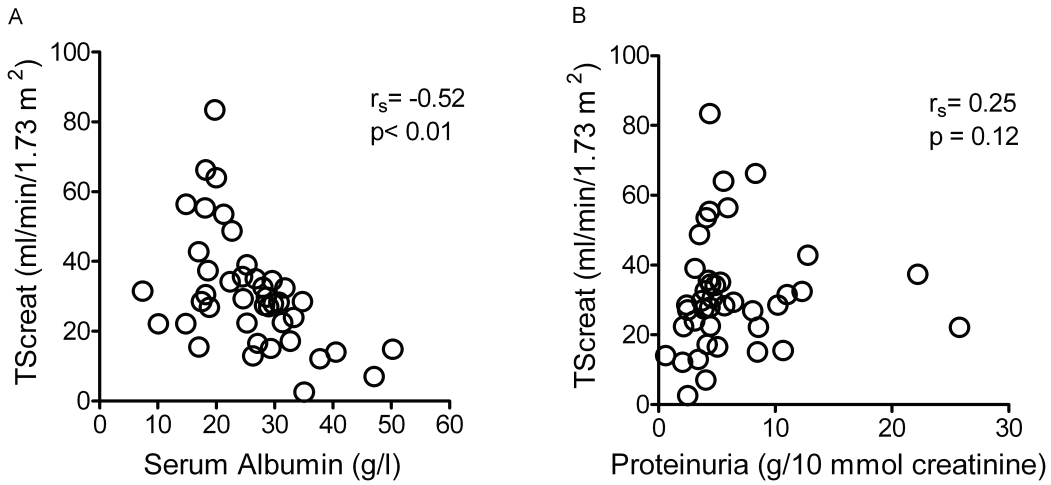

Figure 1. The clearance of creatinine by tubular secretion (TScreat) versus serum albumin (panel A), and versus proteinuria (panel B) in patients (n=42) with proteinuria. A significant correlation was only observed between TScreat and serum albumin.

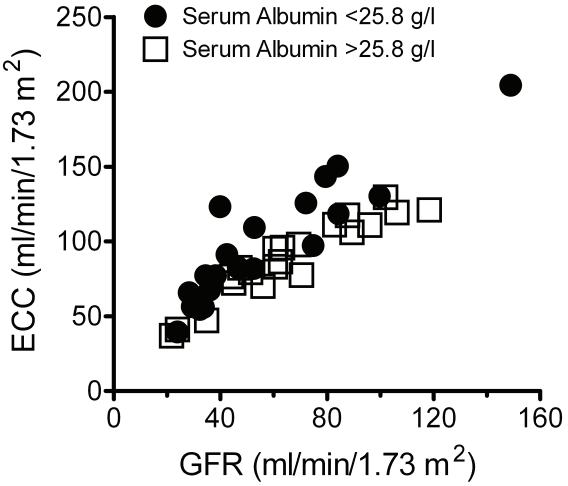

Figure 2. Glomerular filtration rate (GFR) versus endogenous creatinine clearance (ECC) in patients with proteinuria and a serum albumin level <25.8 g/l (closed circles), and in patients with proteinuria and a serum albumin level >25.8 g/l (open squares). The overestimation of GFR by ECC was more pronounced in patients with low serum albumin levels.

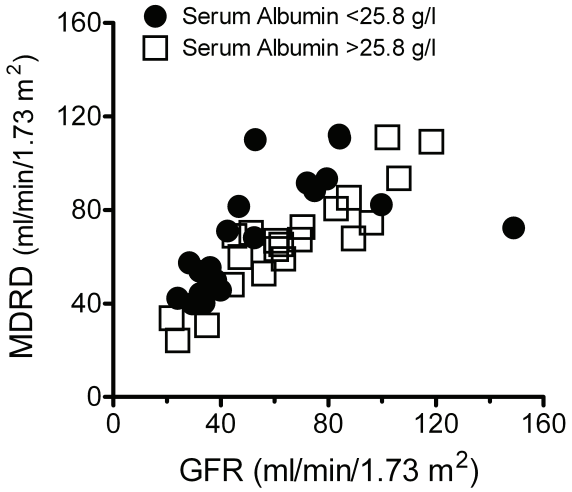

Figure 3. Glomerular filtration rate (GFR) versus MDRD-GFR in patients with proteinuria and a serum albumin level <25.8 g/l (closed circles), and in patients with proteinuria and a serum albumin level >25.8 g/l (open squares). In patients with low serum albumin levels the GFR was overestimated by the MDRD-GFR.

DISCUSSION

Our study shows that serum albumin is an independent determinant of the clearance of creatinine by tubular secretion. As a consequence overestimation of GFR by ECC is more pronounced in patients with a nephrotic syndrome. This conclusion also holds when using the simplified MDRD formula instead of ECC as predictor of GFR.

It is well known that ECC overestimates GFR, due to fact that creatinine is not only filtered but also secreted by the renal proximal tubules. Under normal circumstances tubular secretion contributes approximately 10-15% to renal creatinine clearance, the ratio of ECC/GFR amounting 1.15 in healthy volunteers [1,10]. In patients with a decreased renal function the relative contribution of tubular secretion to renal creatinine clearance increases, which explains the widely recognized fact that ECC increasingly overestimates GFR at lower GFR [10].

Previous investigators have already pointed to the sometimes marked discrepancies between ECC and GFR in patients with a nephrotic syndrome, however, our study is the first to demonstrate the independent association between serum albumin levels and tubular creatinine handling resulting in a more pronounced overestimation of GFR. Berlyne *et al.* described four patients with a nephrotic syndrome and ECC/GFR ratios ranging form 1.24 to 2.37 [3]. However, the two patients with the highest ratio had markedly impaired GFR (inulin clearance). Carrie *et al.* studied 38 patients with a nephrotic syndrome. The mean ECC/GFR ratio was 1.70 ± 0.11. In these nephrotic patients inulin clearance was impaired. The impairment of renal function could not fully explain the increased ratio of ECC/GFR since a significantly lower ratio (1.22 ± 0.14) was observed in patients with a comparable GFR. The latter group of “control” patients suffered form heart failure, and therefore it remained undetermined if tubular creatinine handling was altered in the nephrotic patients or in the patients with heart failure [2]. In a study in diabetic patients cimetidine, an inhibitor of creatinine transport, more pronouncedly reduced creatinine clearance in patients with macroproteinuria [11]; however, again GFR (inulin clearance) was lowest in patients with macroproteinuria. In contrast, Anderson *et al.* did not observe a difference in the ratio ECC/GFR between nephrotic and non-nephrotic subjects [12].

Our observations suggest that alterations in tubular creatinine handling take place as consequence of hypoalbuminaemia which can lead to major errors in the estimation of renal function in patients with a nephrotic syndrome. Our calculations indicate that in patients with a nephrotic syndrome roughly a 25% decrease of GFR may occur without any change in ECC or serum creatinine. This means that in such patients a fall in GFR will not be noticed, even by slight increases of serum creatinine.

Our study explains some discrepancies in the literature with respect to renal function parameters in patients with proteinuria. Experimental data have unequivocally shown that a reduction of albumin causes a decrease in the ultrafiltration coefficient Kf [13]. As a consequence GFR and filtration fraction are decreased when measured by precise techniques (inulin clearance or comparable methods) in patients with a nephrotic syndrome. In contrast, serum creatinine and creatinine clearance are reported as normal in most patients with minimal change nephropathy [14,15].

In recent years new equations have been developed for the estimation of GFR. Based on the MDRD data a new formula was developed, which has been validated in patients with renal failure [4]. In the MDRD formula parameters included were age, sex, race, serum creatinine, serum urea, and serum albumin. Levey *et al.* have subsequently published a simplified formula that included only serum creatinine as serum parameter [5]. Serum albumin and urea were excluded since these variables supposedly only contributed <1% to the observed variance of the calculations [6]. This simplified formula has been tested also in patients without renal diseases [6], and has been applied in recent studies [7]. It is clear from our study that the performance of this simplified formula is also dependent on serum albumin levels, overestimation of GFR being more prominent in patients with severe hypoalbuminaemia. Thus, a formula solely based on serum creatinine as the only serum marker should not be used in studies that include patients with severe proteinuria.

Unfortunately, serum urea levels were only available in 11 of our patients. Application of the original MDRD formula in this subgroup suggested a better performance of the original formula (data not shown). However, the paucity of data does not allow firm conclusions and larger studies are needed to validate the original MDRD formula particularly in patients with proteinuria.

It is difficult to speculate on the mechanism that may cause the altered tubular handling of creatinine in patients with low serum albumin levels. Apparently, tubular creatinine secretion is increased in patients with low serum albumin levels. Of note, serum albumin and not proteinuria was independently related to tubular creatinine handling. One mechanism could be that low serum albumin levels reflect lesser transport of albumin bound molecules that normally compete with creatinine for tubular transport.

We would like to point out an alternative explanation. We feel that our findings may be compatible with a decrease in creatinine reabsorption. In patients with a nephrotic syndrome and severe hypoalbuminaemia proximal sodium reabsorption is reduced [16]. As such, creatinine reabsorption as a passive process will be influenced by changes in water and sodium reabsorption. The findings of Carrie *et al.* with low ECC/GFR ratio in patients with heart failure also are compatible with such an idea, since in these patients creatinine

reabsorption will be increased. Admittedly, creatinine reabsorption has only been demonstrated in some animal species [17]. There are data available in human studies suggesting the presence of tubular reabsorption of creatinine, particularly in patients with low rates of urine flow, however proof is lacking [18-20]. Although studies in humans are difficult, we feel that such a process cannot be excluded.

In conclusion, the present data indicate that serum albumin levels influence tubular handling of creatinine. As a result GFR is more pronouncedly overestimated by ECC in patients with a nephrotic syndrome. In patients with a nephrotic syndrome a normal serum creatinine should not be regarded as evidence of a normal GFR.

REFERENCES

1. Vervoort G, Willems HL, Wetzels JFM. Assessment of glomerular filtration rate in healthy subjects and normo-albuminuric diabetic patients: validity of a new (MDRD) prediction equation. Nephrol Dialysis Transplant 2002;17:1909-1913.
2. Carrie BJ, Golbetz HV, Michaels AS, Myers BD. Creatinine: an inadequate filtration marker in glomerular diseases. Am J Med 1980;69:177-182.
3. Berlyne GM, Varley H, Nilwarangkur S, Hoerni M. Endogenous creatinine clearance and glomerular filtration rate. Lancet 1964;II:874-876.
4. Levey AS, Bosch JP, Breyer Lewis J *et al*. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Ann Intern Med 1999;130:461-470.
5. Levey AS, Greene T, Kusek JW, Beck GJ, MDRD Study Group. A simplified equation to predict glomerular filtration rate from serum creatinine. J Am Soc Nephrol 2000;11:Abstract A0828.
6. Lin J, Knight EL, Hogan M-L, Singh AK. A comparison of prediction equations for estimating glomerular filtration rate in adults without kidney disease. J Am Soc Nephrol 2003;14:2573-2580.
7. Tonelli M, Moye L, Sacks FM *et al*. Effect of pravastatin on loss of renal function in people with moderate chronic renal insufficiency and cardiovascular disease. J Am Soc Nephrol 2003;14:1605-1613.
8. Branten AJW, Wetzels JFM. Influence of albumin infusion on the urinary excretion of beta-2-microglobulin in patients with proteinuria. Nephron 1999;81:329-333.
9. Degenaar CP, Frenken LA, van Hooff JP. Enzymatic method for determination of inulin. Clin Chem 1987;33:1070-1071.
10. Shemesh O, Golbetz H, Kriss JP, Myers BD. Limitations of creatinine as a filtration marker in glomerulopathic patients. Kidney Int 1985;28:830-838.

11. Kemperman FAW, Silberbusch J, Slaats EH *et al*. Estimation of the glomerular filtration rate in NIDDM patients from plasma creatinine concentration after cimetidine administration. Diabetes Care 1998;21:216-220.
12. Anderson CF, Jaecks DM, Ballon HS, Palma de JR, Cutler RE. Renal handling of creatinine in nephrotic and non-nephrotic patients. Clin Sci 1970;38:555-562.
13. Baylis C, Ichikawa I, Willis WT, Wilson CB, Brenner BM. Dynamics of glomerular ultrafiltration. IX. Effects of plasma protein concentration. Am J Physiol 1977;232:F58-F71.
14. Korbet SM, Schwartz MM, Lewis EJ. Minimal-change glomerulopathy of adulthood. Am J Nephrol 1988;8:291-297.
15. Ponticelli C, Edefonti A, Ghio L *et al*. Cyclosporin versus cyclophosphamide for patients with steroid-dependent and frequently relapsing idiopathic nephrotic syndrome: a multicentre randomized controlled trial. Nephrol Dialysis Transplant 1993;8:1326-1332.
16. Grausz H, Lieberman R, Earley LE. Effect of plasma albumin on sodium reabsorption in patients with nephrotic syndrome. Kidney Int 1972;1:47-54.
17. Namnun P, Insogna K, Baggish D, Hayslett JP. Evidence for bidirectional net movement of creatinine in the rat kidney. Am J Physiol 1983;244:F719-F723.
18. Miller BF, Leaf A, Mamby AR, Miller Z. Validity of the endogenous creatinine clearance as a measure of glomerular filtration rate in the diseased human kidney. J Clin Invest 1952;30:309-313.
19. Burcht H. On the tubular excretion of thiosulphate and creatinine under the influence of caronamide. Scand J Clin Lab Invest 1949;1:270-276.
20. Perrone RD, Madias NE, Levey AS. Serum creatinine as an index of renal function: new insights into old concepts. Clin Chem 1992;38:1933-1953.

Chapter 6

Influence of Albumin Infusion on the Urinary Excretion of β2-microglobulin in Patients with Proteinuria.

Amanda J.W. Branten
Jack F.M. Wetzels

Department of Medicine, Division of Nephrology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands.

Nephron 1999; 81:329-333

ABSTRACT

Most filtered proteins are reabsorbed by the renal proximale tubule by a mechanism that involves binding to the brush border membrane and endocytosis. Under normal conditions the low molecular weight protein β2-microglobulin (β2M), which is used to detect tubular injury, is reabsorbed almost completely. However, in proteinuric patients an increased urinary excretion of β2M may not simply reflect tubular damage but might also result from a decreased tubular reabsorption due to competitive mechanisms. To examine the magnitude of such an effect we have studied the renal effects of albumin infusion (40 g in 2 h of a 20% solution) in 10 patients with a glomerular disease and proteinuria >3.5 g/24 h. Before, during and after albumin infusion the GFR (inulin clearance), RPF (PAH clearance), blood pressure and the urinary excretion of albumin, IgG, transferrin, and β2M were measured. Albumin infusion resulted in a slight decrease of the GFR (72±11 ml/min before and 67±10 ml/min after infusion), an increase of the RPF (379±66 ml/min before and 445±83 ml/min after), a decrease of the filtration fraction (0.20 before and 0.17 after), and hemodilution. After infusion the urinary excretion of albumin increased from 4.5±0.7 to 8.4±1.6 mg/min ($p<0.05$). The urinary excretion of IgG and transferrin increased, probably reflecting a change in glomerular size-selectivity. In contrast, the urinary excretion of β2M did not change significantly (baseline 12±5 µg/min, end 13±6 µg/min, percentage change 16.8±11%). To correct for changes in tubular load we calculated the fractional reabsorption of β2M. The initial rise in albuminuria during infusion did not affect fractional tubular reabsorption (Δ%: 0.72±0.52%, median 0.005%). In the period after infusion a slight decrease was noted (median -0.33%, $p<0.01$). A decrease in the fractional reabsorption was particularly observed in patients with pre-existing tubular damage.

In conclusion: infusion of albumin in proteinuric patients has no clinically relevant effect on the tubular reabsorption of β2M. Therefore, β2M is useful as a parameter to detect tubular injury and alterations in tubular handling of proteins in patients with proteinuria and glomerular diseases.

INTRODUCTION

Proteinuria is a common finding in renal diseases. An increased excretion of proteins can result from a loss of the glomerular barrier function, a decrease of the tubular protein reabsorption, or a combination of both. In general, glomerular injury is characterized by urinary losses of high molecular weight (HMW) proteins such as albumin, transferrin, and IgG. In contrast, tubular disorders are characterized by increased urinary losses of low molecular weight (LMW) proteins like β2-microglobulin (β2M). β2M is a 11,800-kD protein which is nearly completely filtered through the glomerulus and very efficiently reabsorbed by the proximal tubules. Because of these properties the urinary excretion of β2M has been found very useful in the detection of tubular injury [1,2].

Already 20 years ago, Schainuck *et al.* [3] demonstrated that the development of renal insufficiency in patients with renal diseases correlated better with tubulo-interstitial changes on renal biopsy than with glomerular changes. In line with these observations, we have recently demonstrated that an increased excretion of β2M predicted the future development of renal insufficiency in patients with membranous nephropathy [4]. Similar observations were done by Bazzi *et al.* [5]. These investigators used SDS-PAGE electrophoresis to analyze urinary proteins. The presence of LMW proteins of 10 kD (i.e. β2M) predicted the development of chronic renal failure in their patients with focal glomerulosclerosis, membranous nephropathy or membranoproliferative glomerulonephritis. Furthermore, the presence of increased levels of β2M in the urine is also suggestive for treatment failure in patients with an idiopathic nephrotic syndrome [6]. Therefore, measurement of urinary β2M might be of particular value in predicting prognosis and in guiding treatment of patients with proteinuric renal diseases.

However, it is unclear if the urinary excretion of β2M is merely dependent on the extent of tubulo-interstitial injury. It has been suggested that HMW proteins may interfere with the tubular reabsoption processes of LMW proteins. Intravenous injection of albumin in rats abruptly increased the urinary excretion of β2M, most likely the result of a competitive inhibition [7]. Thus far there are no convincing data in the literature on the direct effects of albumin on the tubular reabsorption of β2M in man. Therefore, we have studied the effects of an intravenous infusion of albumin on the urinary excretion of β2M in patients with nephrotic range proteinuria (>3.5 g/24 h). Our data demonstrate that such an increased load of albumin has no major impact on tubular β2M reabsorption.

PATIENTS AND METHODS

The study protocol was approved by the hospital ethics committee. All participants gave informed consent before study entrance. Ten patients with proteinuria >3.5 g/24 h and stable renal function were selected from our outpatient clinic. The patients were instructed to refrain from smoking and coffee drinking during the last 8 h and from alcohol during the last 24 h before the study. On the day of investigation the patients came to the ward at 08:30 h. Venous catheters were inserted in both forearms; one for continuous infusion of an inulin (Poly-fructosan, Inutest, Laevosan Ges., Linz, Austria) and PAH solution, as described before [8], and the other catheter for blood sampling. The patients remained supine except for spontaneous voiding. Blood pressure (BP) was monitored every 30 min with a sphygmomanometer. During the measurements, diuresis was maintained by an oral water load of 110 ml every hour and a continuous infusion of a 5% glucose solution, 100 ml/h. The administered albumin solution contained 145 mmol/l sodium. To achieve a constant sodium intake during the whole measurement, an 1.5% NaHCO3 solution was infused, 81.5 ml/h, in the periods before and after the albumin infusion. The NaHCO3 infusion also contributed to an urinary pH >6.0. This is a requirement to determine β2M in a reliable way, because ß2M is unstable in acid urine. After a 90-min equilibration period, two 45-min clearance periods were scheduled for baseline measurements. Thereafter, an infusion of 200 ml of a 20% solution of human albumin (total dose 40 g) was given over a 120-min period. Four clearance periods were scheduled from the start of the albumin infusion; two periods of 60 min during, and two periods of 45 min after albumin infusion, respectively. Blood and urine samples were collected at the beginning and at the end of each clearance period. The freshly voided urine samples were centrifuged and stored at -70°C. In blood and urine samples the concentrations of albumin, IgG and transferrin were measured by immunonephelometry using antibodies whose specificity was checked by Ouchterlony double immunodiffusion and immunoelectrophoresis. Details of the nephelometric procedures for the measurement of albumin, transferrin and IgG have been described before [9,10]. Serum and urinary β2M were measured by RIA (Pharmacia, Uppsala, Sweden). Inulin, PAH and creatinine were measured using standard colorimetric methods.

Calculations and statistical analysis

The glomerular filtration rate (GFR) was estimated by inulin clearance and effective renal plasma flow (RPF) by PAH clearance. The filtration fraction (FF) was calculated by

GFR/RPF. The percentage of tubular reabsorption of β2-microglobulin (β2M) was calculated as:

$$\frac{\text{(Serum level } \beta\text{2M x GFR) - Absolute urinary excretion of } \beta\text{2M}}{\text{(Serum level } \beta\text{2M x GFR)}} \times 100$$

Unless otherwise indicated, results are expressed as mean ± SEM. The values obtained during and after albumin infusion were compared with baseline data using a two-tailed paired Wilcoxon test with Bonferroni correction. A probability value <0.05 was considered statistically significant. Exponential trendlines to determine the relationship between the urinary excretion of albumin and β2M were generated using Excel software (Excel 5.0, Microsoft Corp.).

RESULTS

We have studied 10 patients (9M, 1F) with a mean (±SD) age of 33 ± 14 years. The original renal disease was primary focal glomerulosclerosis (n=4), IgA nephropathy (n=2), membranous nephropathy (n=1), membranoproliferative glomerulonephritis (n=1), amyloidosis (n=1), and Alport's disease (n=1). Mean serum creatinine was 133 ± 49 µmol/l, mean serum albumin 27 ± 3.7 g/l, and proteinuria 7.1 ± 2.3 g/24 h. The baseline urinary β2M excretion was normal (< 0.340 µg/min) in three, slightly increased (ranging from 0.51 to 1.16 µg/min) in three, and highly elevated in four patients (range 12.8 - 38.1 µg/min).

Infusion of albumin increased plasma volume as reflected by a change in hematocrit from 0.37 ±0.02 l/l at baseline to 0.35 ± 0.02 l/l during, and 0.34 ±0.02 l/l after the infusion ($p<0.01$). The systemic and renal hemodynamic parameters as measured before, during, and after albumin infusion are given in Table 1. The infusion of albumin did not significantly alter blood pressure or pulse rate. Also, urinary flow rate and fractional sodium excretion remained unchanged. After the infusion of albumin a slight decrease of the GFR was observed, simultaneously with an increase of the RPF, resulting in a significant decrease of the filtration fraction.

As expected, serum albumin increased (Table 2). As a result of the increased plasma volume, serum levels of the high molecular weight proteins IgG and transferrin decreased, thus mimicking the change in hematocrit. In contrast, the serum levels of β2M did not change during the study period (Table 2).

Table 1. Systemic and renal hemodynamic effects of albumin infusion in patients with proteinuric renal diseases (n=10)

		Albumin Infusion	
	Baseline	**During**	**After**
SBP *(mm Hg)*	132 ± 10	133 ± 9	137 ± 9
DBP *(mm Hg)*	84 ± 4	85 ± 4	87 ± 5
HR *(beats/min)*	61 ± 3	60 ± 3	63 ± 4
GFR *(ml/min)*	72 ± 11	69 ± 10	67 ± 10
RPF *(ml/min)*	379 ± 66	401 ± 70	445 ± 83*
FF	0.20 ± 0.02	0.18 ± 0.02	0.17 ± 0.02*
Urine Flow *(ml/min)*	7.3 ± 1.3	6.1 ± 0.8	7.2 ± 0.6
FE Na *(%)*	3.1 ± 0.9	2.8 ± 0.6	3.7 ± 0.8

Abbreviations: SBP = systolic blood pressure; DBP = diastolic blood pressure; HR = heart rate; GFR = glomerular filtration rate; RPF = renal plasma flow; FF = filtration fraction; FE Na = fractional excretion of sodium. Values are given as means ± SEM, * $p < 0.05$ compared to baseline.
Blood pressure measurements were available in 8 subjects.

Table 2. Effects of albumin infusion on serum protein levels and urinary protein excretion

		Albumin Infusion	
	Baseline	**During**	**After**
Serum			
Albumin *(g/l)*	27 ± 3.7	34 ± 3.6*	35 ± 3.6*
IgG *(g/l)*	5.7 ± 0.8	5.1 ± 0.8*	4.9 ± 0.8*
Transferrin *(g/l)*	1.9 ± 0.2	1.7 ± 0.1*	1.6 ± 0.1*
β2M *(mg/l)*	3.4 ± 0.5	3.3 ± 0.5	3.3 ± 0.5
Urinary excretion			
Albumin *(mg/min)*	4.5 ± 0.7	5.5 ± 1.1*	8.4 ± 1.6*
IgG *(µg/min)*	311 ± 74	282 ± 65	351 ± 87
Transferrin *(µg/min)*	369 ± 82	333 ± 73	384 ± 69
β2M *(µg/min)*	12 ± 5	10 ± 4	13 ± 6

Values are given as means ± SEM, * $p < 0.01$ compared to baseline values.

Values of the urinary excretion of the various proteins at baseline and in response to the infusion of albumin are given in Table 2. We observed an immediate increase in the urinary excretion of albumin already apparent in the period during the infusion of albumin, with a further rise occurring in the period thereafter. The absolute urinary excretion of IgG and transferrin did not change significantly. However, fractional excretions of IgG and transferrin both increased significantly (IgG: 0.18 ± 0.07% before vs. 0.23 ± 0.09% after infusion of albumin; transferrin: 0.54 ± 0.19% before vs. 0.71 ± 0.25 % after infusion; both $p<0.01$).

Overall, there was no change in the absolute urinary excretion of β2M (Table 2). Compared to baseline values, the median change in the β2M excretion was +5.3% after albumin infusion. The increase amounted to > 30% in only two (71 and 79%, respectively) of the ten patients. To account for changes in the tubular load of β2M, we next calculated the fractional tubular reabsorption of β2M. At baseline the fractional tubular reabsorption of β2M ranged from 73.1 to 99.89% (median 99.52%). Tubular reabsorption of β2M was approximately normal in 6 patients in whom fractional reabsorption averaged 99.73% (range 99.39 to 99.89%), and abnormal in four with values ranging from 73.06 to 92.32%. Fractional reabsorption of β2M remained unchanged during the infusion of albumin, at a time point that the urinary albumin excretion had increased from 4.5 ±0.7 to 5.5 ±1.1 µg/min. In the period after the infusion of albumin, the fractional reabsorption of β2M decreased slightly but significantly with a median percentage decrease of -0.33% ($p<0.01$). A closer look at the data revealed that the fractional tubular reabsorption of β2M hardly decreased in the six patients with normal baseline values (from 99.73 to 99.59%), whereas an apparent decrease was observed in the four patients with evidence of a disturbed tubular reabsorption of β2M at baseline (percentage decrease: -1.12 to -10.76%). To better assess the amount of albumin present at the site of the putative transport in the individual tubules, we have analyzed the data after factoring the albumin excretion by the GFR. These data, expressed as mg albumin per 100 ml GFR, and their relationship with the fractional β2M excretion are depicted in Figure 1. At baseline, highest values for urinary excretion of β2M were seen in patients with the highest levels of albuminuria, a finding compatible with an interference of albumin with the tubular reabsorption of β2M. However, from Figure 1 it is evident that when the tubular load of albumin is increased by the infusion, this does not result in an increase in the fractional excretion of β2M.

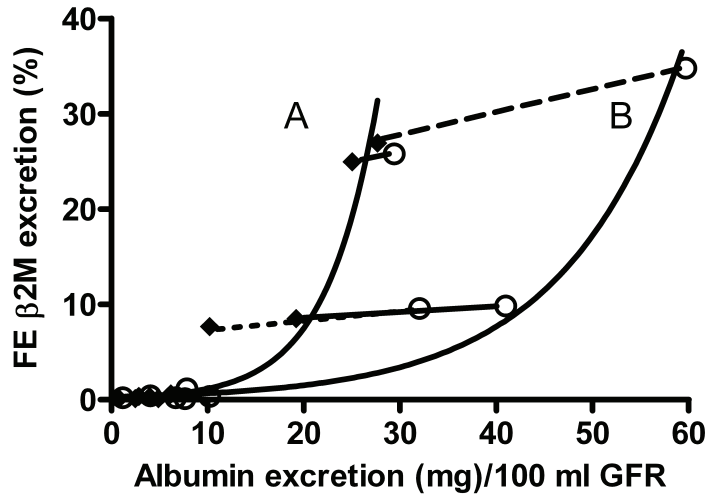

Figure 1. The relationship between the urinary excretion of albumin expressed per 100 ml GFR and the fractional excretion of β2M at baseline (♦), and after (O) infusion of albumin. Lines A and B represent the respective trendlines (exponential fit, Excel Software). The paired values before and after albumin infusion of the 4 patients with the highest excretion of both albumin and β2M are connected with lines.

DISCUSSION

Most filtered proteins are reabsorbed by the proximal tubules. This process of reabsorption is governed by binding of the proteins to the brush border membrane and subsequent endocytosis [11]. Based on data derived from animal experiments it has been suggested that albumin could interfere with the tubular reabsorption of low molecular weight proteins such as β2M or lysozyme [7,12]. To address this particular question in humans, we increased the tubular load of albumin in patients with proteinuria, and studied the effects on the urinary excretion of β2M. As expected, after the infusion of albumin an increase of the albuminuria was observed. This increase is not only due to the increased serum levels of albumin. The infusion of albumin and the resulting volume expansion also causes an enhancement of the defect in the glomerular size-selectivity as has been demonstrated by Shemesh *et al.* [13]. In accordance with such a defect in glomerular permeability, we observed an increase in the fractional excretions of IgG and transferrin.

Since the sieving coëfficient of β2M is close to 1.0, the tubular reabsorption of this low molecular weight protein can be precisely calculated, and corrected for changes in GFR and serum levels. The infusion of albumin and the ensuing albuminuria slightly attenuated the fractional reabsorption of β2M. At first sight these findings might seem compatible with an interference of albumin with the tubular reabsorption of β2M via a competitive mechanism. In such a case, the magnitude of this effect is rather small and clinically irrelevant. Admittedly, we cannot exclude that an effect of albumin on the reabsorption of β2M would become apparent at levels of albumin in the tubular fluid that exceed the maximaum capacity of the transporter protein in the brush border. Our data indicate that this might be the case in situations when the quotient albumin/100 ml GFR exceeds 50, a value which reflects a daily proteinuria of 80 g, a value not readily obtainable in patients.

Our data argue against a direct competitive effect of albumin on the tubular reabsorption of β2M. First, the initial increase of albuminuria did not attenuate the tubular reabsorption of β2M. A decrease of the fractional reabsorption of β2M was observed in the post-infusion period and occurred predominantly in patients with evidence of tubular injury. In these patients the increased excretion of β2M might reflect protein-induced lysosomal and tubular damage of already vulnerable tubules. Secondly, we did not observe a relationship between (the increase of) albuminuria and the urinary excretion of β2M (Figure 1).

Data in the literature also argue against an important effect of albumin on the tubular reabsorption of low molecular weight proteins. Several authors have reported normal urinary excretion of β2M in patients with minimal change nephropathy and profound albuminuria [2,6,14]. Harrison *et al.* [15] found that in patients with glomerulonephritis and heavy proteinuria, lysozyme excretion was not necessarily elevated. Also in another study [16], no relationship was observed between the excretion of high molecular weight proteins and the presence in the urine of low molecular weight proteins as analyzed by SDS-PAGE electrophoresis.

In animal studies different approaches have been used to study the interaction between albumin and the tubular reabsorption of low molecular weight proteins. Results have been contradictory. The strongest support for the concept that albumin interferes with the tubular reabsorption of β2M via a competitive mechanism comes from two studies [17,18]. In in vitro experiments, Simonnet *et al.* [17] demonstrated that the binding of β2M to brush border membranes decreased upon increasing the concentration of albumin in the incubation medium. Bernard *et al.* [18] infused albumin in rats and observed an increase in the excretion of β2M [18]. However, it should be recognized that the results of the above-mentioned studies may not be applicable to the human situation. In fact both Bernard *et al.* [18] and

Simonnet *et al.* [17] have seen the effects mentioned in particular when using bovine serum albumin in rats, whereas the effects of human and rat albumin were less clear. It seems quite likely that the specific characteristics of bovine albumin determine the potency to inhibit tubular protein reabsorption. This might explain the limited effects of albumin on the urinary excretion of β2M in humans.

In conclusion: our data indicate that there is no major effect of albumin on the tubular reabsorption of β2M. Therefore, the urinary excretion of β2M can be used as a reliable parameter to detect tubular damage in patients with proteinuria. Also, measurements of the urinary excretion of β2M can be used to determine if agents influence proteinuria by altering glomerular protein leakage, tubular protein reabsorption or both.

REFERENCES

1. Schardijn GH, Statius van Eps LW. Beta-2-microglobulin: its significance in the evaluation of renal function. Kidney Int 1987;32:635-641.
2. Portman RJ, Kissane JM, Robson AM, Peterson LJ, Richardson A. Use of β2-microglobulin to diagnose tubulo-interstitial renal lesions in children. Kidney Int 1986;30:91-98.
3. Schainuck LI, Striker GE, Cutler RE, Benditt EP. Structural-functional correlations in renal disease. Hum Pathol 1970;1:631-641.
4. Reichert LJM, Koene RAP, Wetzels JFM. Urinary excretion of β2-microglobulin predicts renal outcome in patients with idiopathic membranous nephropathy. J Am Soc Nephrol 1995;6:1666-1669.
5. Bazzi C, Petrini C, Rizza V *et al*. Characterization of proteinuria in primary glomerulonephritides. SDS-PAGE patterns: clinical significance and prognostic value of low molecular weight (tubular) proteins. Am J Kidney Dis 1997;29:27-35.
6. Sesso R, Santos AP, Nishida SK *et al*. Prediction of steroid responsiveness in the idiopathic nephrotic syndrome using urinary retinol-binding protein and beta-2-microglobulin. Ann Intern Med 1992;116:905-909.
7. Bernard A, Ouled Amor A, Viau C, Lauwerys R. The renal uptake of proteins: a nonselective process in conscious rats. Kidney Int 1988;34:175-185.
8. Sluiter HE, Wetzels JFM, Huysmans FThM, Koene RAP. The natriuretic effect of the dihydropyridine calcium antagonist felodipine: A placebo-controlled study involving intravenous angiotensin II in normotensive volunteers. J Cardiovasc Pharmacol 1987;10 (suppl 10):S154-S161.
9. Elving LD, Bakkeren JAJM, Jansen MJH *et al*. Screening for microalbuminuria in patients with diabetes mellitus: frozen storage of urine samples decreases their albumin content. Clin Chem 1989;35:308-310.

10. Reichert LJM, Koene RAP, Wetzels JFM. Urinary IgG excretion as a prognostic factor in idiopathic membranous nephropathy. Clin Nephrol 1997;48:79-84.
11. Maack T, Hyung Park C, Camargo M-JF. Renal filtration, transport, and metabolism of proteins. In: Seldin DW, Giebisch G, eds. *The kidney: Physiology and Pathophysiology.* Raven Press, New York: 1992: 3005-3038.
12. Hutchison FN, Kaysen GA. Albuminuria causes lysozymuria in rats with Heymann nephritis. Kidney Int 1988;33:787-791.
13. Shemesh O, Deen WM, Brenner BM, McNeely E, Myers BD. Effect of colloid volume expansion on glomerular barrier size-selectivity in humans. Kidney Int 1986;29:916-923.
14. Beetham R, Newman D. Urinary albumin and low molecular weight protein excretion in the nephrotic syndrome-sequential studies during corticosteroid treatment. Ann Clin Biochem 1992;29:450-453.
15. Harrison JF, Lunt GS, Scott P, Blainey JD. Urinary lysozyme, ribonuclease, and low-molecular-weight protein in renal disease. Lancet 1968;1:371-375.
16. Alt JM, Hacke M, von der Heyde D *et al*. Urinary protein excretion in interstitial and tubular kidney disease as characterized by gradient electrophoresis. Klin Wochenschr 1983;61:641-648.
17. Simonnet H, Gauthier C, Vincent C, Revillard J-P. The binding of beta-2-microglobulin to renal brush-border membrane: affinity measurement, inhibition by serum albumin. Biochim Biophys Acta 1988;940:247-254.
18. Bernard A, Viau C, Ouled A, Lauwerys R. Competition between low- and high-molecular weight proteins for renal tubular uptake. Nephron 1987;45:115-118.

Chapter 7

Urinary Excretion of β2-microglobulin and IgG Predict Prognosis in Idiopathic Membranous Nephropathy: A Validation Study.

Amanda J.W. Branten[1]
Peggy W. du Buf-Vereijken[1,3]
Ina S. Klasen[2]
Frank H. Bosch[4]
Geert W. Feith[5]
Daan A. Hollander[6]
and Jack F.M. Wetzels[1]

[1]Department of Medicine, Division of Nephrology and [2]Department of Clinical Chemistry, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands, [3]Department of Internal Medicine Amphia Hospital, Breda, The Netherlands, [4]Department of Internal Medicine Hospital Rijnstate, Arnhem, The Netherlands, [5]Department of Internal Medicine Hospital Gelderse Vallei, Ede, The Netherlands, [6]Department of Internal Medicine Jeroen Bosch Hospital, 's-Hertogenbosch, The Netherlands.

Journal American Society of Nephrology 2005; 16:169-174

ABSTRACT

An accurate prediction of the prognosis of patients with idiopathic membranous nephropathy (iMN) should allow restriction of immunosuppressive treatment to patients who are at highest risk for ESRD. On the basis of retrospective studies, it has previously been suggested that the urinary excretions of β2-microglobulin (Uβ2m) and IgG (UIgG) are useful predictors of renal insufficiency in patients with iMN. The threshold values of 0.5 μg/min (Uβ2m) and 250 mg/24 h (UIgG) have been validated in a new and larger patient cohort.

From 1995 onward, 57 patients with iMN (38 men,19 women; age 48±16 yr), a nephrotic syndrome, and a serum creatinine level ≤1.5 mg/dl were studied prospectively. At baseline, a standardized measurement was carried out to determine renal function and protein excretion. The end point renal death was defined as a serum creatinine exceeding 1.5 mg/dl or a rise of serum creatinine of >50%. Mean (±SD) follow-up was 53 ± 23 mo. Thus far, 25 (44%) of the patients have reached the end point renal death. Multivariate analysis confirmed Uβ2m as the strongest independent predictor for the development of renal insufficiency. Sensitivity and specificity were 88% and 91%, respectively, for Uβ2m, and both were 88% for UIgG. When the excretions of both proteins were combined, specificity improved to 97%. It is concluded that the present data validate the accuracy of Uβ2m and of UIgG in predicting renal outcome in patients with iMN. These markers can be used to guide decisions on the start of immunosuppressive treatment.

INTRODUCTION

Idiopathic membranous nephropathy (iMN) is one of the most frequent causes of the nephrotic syndrome in adults [1]. If left untreated, up to 40% of patients will progress to end stage renal disease (ESRD) [2-4]. The efficacy of immunosuppressive therapy has been demonstrated in a randomized, controlled trial [4]. Although this study provided arguments to treat all patients with iMN and a nephrotic syndrome, most authors advocate restricting immunosuppressive treatment to patients who are at highest risk for developing ESRD [5,6]. It is well established that deterioration of renal function is a powerful predictor of ESRD [7,8]. Therefore, a trial of immunosuppressive therapy is warranted in patients with iMN and established renal insufficiency. However, it is evident that immunosuppressive treatment started at a relatively late time point may be less effective in attaining normal renal function [9]. Moreover, we and others have noted that the use of immunosuppressive agents in patients with renal insufficiency was associated with more frequent and more severe side effects than in patients who are treated in an earlier phase of their disease [10-12]. Therefore, it would be ideal if treatment could be optimized by identifying high-risk patients at an earlier time point. In patients with iMN, various risk factors for the development of renal failure have been identified [13]. However, the sensitivity and specificity of most of these factors (e.g. age, gender, glomerular injury, tubular interstitial fibrosis) are too low to justify their use to guide decisions on the start of immunosuppressive therapy. Thus far, the level and the duration of proteinuria are the best predictive factors in a model introduced by the Toronto Glomerulonephritic Registry [14]. This model requires a minimal observation period of 6 to 18 mo.

On the basis of data derived from small patient cohorts, we demonstrated previously that the urinary excretion of β2-microglobulin (Uβ2m) and IgG (UIgG), assessed in a single urine sample, independently predicted the development of renal insufficiency in patients with iMN [15,16]. Our data suggested high sensitivities and specificities, which ranged from 80 to 90%. We now have validated these results in a prospectively studied, new and larger patient cohort.

METHODS

In our center, patients with proteinuria are evaluated using a standard protocol. In all of these patients, standardized urine and blood measurements are carried out as described below. For the validation study, we prospectively studied patients with biopsy-proven iMN, evaluated

from 1995 onward. In the analysis, we included only patients with a baseline serum creatinine ≤1.5 mg/dl and proteinuria ≥2.7 g/g creatinine and/or serum albumin ≤3.0 g/dl. We excluded patients who had been treated with immunosuppressive drugs other than oral prednisone. Patients were also excluded when the interval between renal biopsy and the baseline measurement exceeded 3 years.

Standardized Measurement of Urinary Proteins

Patients come to the ward after an overnight fast. Patients are instructed to take 4000 mg of sodium bicarbonate on the evening before to ensure that urinary pH exceeds 6.0, which is mandatory for the measurement of Uβ2m. On the morning of the measurement, patients are not allowed to take diuretics. Upon arrival, 375 to 500 ml of tap water is given to enforce diuresis. The patients remain supine during 2 h except for voiding. Blood pressure measurements are done using an automatic device, and 10 consecutive readings are registered with an interval of 5 minutes (DINAMAP, Criticon, Tampa FL). Timed urine samples are collected, and in the middle of the collection period, a blood sample is drawn. In addition, two 24-h urine samples are collected for assessment of daily excretion of total protein and creatinine.

Laboratory Measurements

In the blood samples, we assessed the following parameters: creatinine, cholesterol, β2m, albumin, IgG, and transferrin. In the timed urine samples, we measured creatinine, β2m, α1-microglobulin, albumin, IgG, and transferrin. The concentrations of serum creatinine, serum cholesterol, urinary total protein, and urinary creatinine were measured with standard automated techniques. The concentrations of albumin, transferrin, α1-microglobulin, and IgG in serum and urine were measured by immunonephelometry on a BNII nephelometer (Behring, Marburg, Germany) using antibodies whose specificity was checked by Ouchterlony double immunodiffusion and immunoelectrophoresis (Dako, Glostrup, Denmark). Urinary and serum β2m were measured by ELISA as described before [17].

Calculations

Endogenous creatinine clearance (ECC) was calculated according to the formula Ucr x V/Pcr, where Ucr is the concentration of creatinine in the urine, V is the urine flow, and Pcr is the plasma concentration of creatinine, and was corrected for body surface area. Because 24-h urine samples were not collected regularly during follow-up, we estimated creatinine clearances using the Cockcroft and Gault formula. In addition, we calculated GFR for patients who reached the end point renal death by applying the recently developed

Modification of Diet in Renal Disease (MDRD) formula using serum creatinine, age, gender, race, serum albumin and serum urea [18]. The mean arterial pressure was the average of the last six out of 10 registered measurements.

The amounts of β2m, α1-microglobulin, IgG, transferrin, and albumin in the timed urine samples are expressed as excretion per unit time (minute or 24 h).

Protein selectivity index was calculated as the clearance of IgG divided by the clearance of transferrin. The total protein excretion in the 24-h urine samples was expressed as g / g creatinine to correct for sampling errors.

Statistical Analysis

For the validation study, we calculated renal survival using Kaplan-Meier statistics. Renal death was defined as an increase of serum creatinine >50% or an increase of serum creatinine >1.5 mg/dl. Survival was calculated using the date of the baseline study at t=0. We compared renal survival using log-rank test for patients with low and high Uβ2m and UIgG. We used the threshold values established in our previous studies [15,16]. The threshold level for β2m excretion was 0.5 µg/min and for IgG was 250 mg/24 h. Using these threshold levels, we calculated sensitivity, specificity, true positive predictive value, and true negative predictive value.

Because the use of a fixed serum creatinine value as end point, irrespective of the baseline value, might have introduced a bias (a subtle increase in serum creatinine could have been defined as failure), we performed a subanalysis in a group of patients with a baseline serum creatinine <1.2 mg/dl.

Using the data of the present patient cohort, we also studied the effect of other parameters in predicting renal outcome. Univariate analysis and multivariate analysis using the Cox proportional hazard model with a forward stepwise procedure was performed to identify independent predictive parameters. Receiver operating characteristics (ROC) curves were made to determine the area under the curve (AUC), and to calculate the sensitivity and specificity using the most discriminative thresholds. The following parameters were plotted into ROC curves: β2m excretion, IgG excretion, α1-microglobulin excretion, transferrin excretion, albumin excretion, selectivity index, ECC, serum creatinine, serum albumin, and total proteinuria per 24 h. The parameters with the highest AUC were selected and used as covariates in the Cox regression analysis. All values are given as means (±SD) or medians (range) when appropriate. All statistics were performed using SPSS software, version 11.0 (Chicago, IL). $P < 0.05$ was considered significant.

RESULTS

From 1995 to 2002, we studied 57 patients who had iMN and fulfilled the inclusion criteria. In 90% of the patients, the baseline measurement was performed within 1 yr after renal biopsy. Baseline characteristics are given in Table 1. Two patients had been treated with prednisone. Patients have been followed for 53 ± 23 mo.

Table 1. Baseline characteristics of patients with iMN (n=57)

Gender *(M/F)*	38/19
Age *(yr)*	48 ± 16
MAP *(mm Hg)*	98 ± 16
ECC 24 h *(ml/min/1.73 m^2)*	88 ± 26
Serum creatinine *(mg/dl)*	1.00 ± 0.23
Serum β2m *(mg/l)*	2.8 ± 1.1
Serum albumin *(g/dl)*	2.4 ± 0.5
Cholesterol *(mg/dl)*	329 ± 76
Interval Bx – Measurement *(mo)*	2 (0–33)
Follow-up[a] *(mo)*	53 ± 23
Timed urine sample:	
Albumin excretion *(mg/min)*	3.8 (0.3–16)
IgG excretion *(mg/24h)*	197 (18–3597)
β2m excretion *(µg/min)*[b]	0.38 (0.05–68.4)
α1m excretion *(µg/min)*	29 (4–418)
Transferrin excretion *(µg/min)*	283 (17–1455)
Selectivity Index	0.18 (0.06–0.39)
Proteinuria (*g/g creatinine*)	5.8 (1.7–13.3)

Data are means ± SD or medians (range). iMN, idiopathic membranous nephropathy; MAP, mean arterial pressure; ECC 24 h, creatinine clearance, calculated from 24 h urine; β2m, β2-microglobulin; Bx, renal biopsy. [a]From baseline measurement until end of follow-up. [b]In case of β2m excretion: n=56; in one patient, β2m was not measurable because pH urine was too low (<6.0).

Thus far, 25 (44%) patients have reached the predefined end point of renal death. The reason for renal death was a serum creatinine >1.5 mg/dl in 21 patients and a rise of >50% of serum creatinine in 4 patients. Overall renal survival was 81% at 6 mo, 68% at 1 yr, and 54% at 3 yr. Thus, in most patients progressive disease was apparent within 3 years after the baseline study. In this new patient cohort, the use of the previously established threshold

values of Uβ2m and UIgG excretion allowed an accurate prediction of renal outcome. Renal survival curves are depicted in Figures 1 and 2.

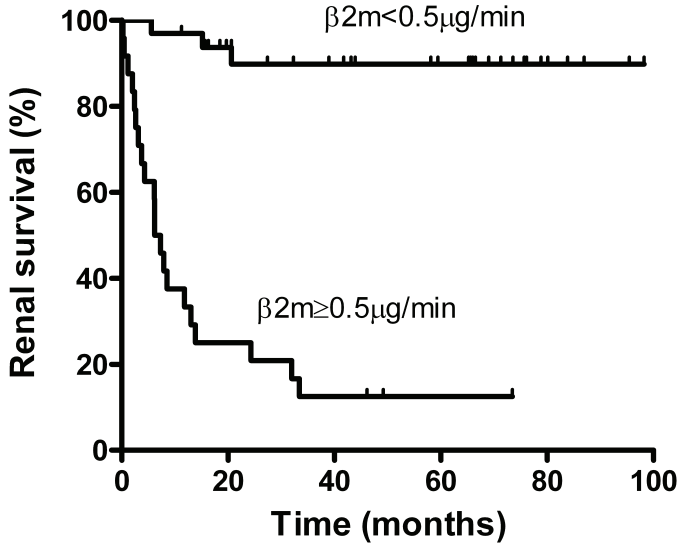

Figure 1. Renal survival in patients with idiopathic membranous nephropathy with urinary β2-microglobulin excretion (β2m) <0.5 μg/min and ≥0.5 μg/min. Renal death was defined as an increase of serum creatinine to values >1.5 mg/dl or an increase of serum creatinine >50%.

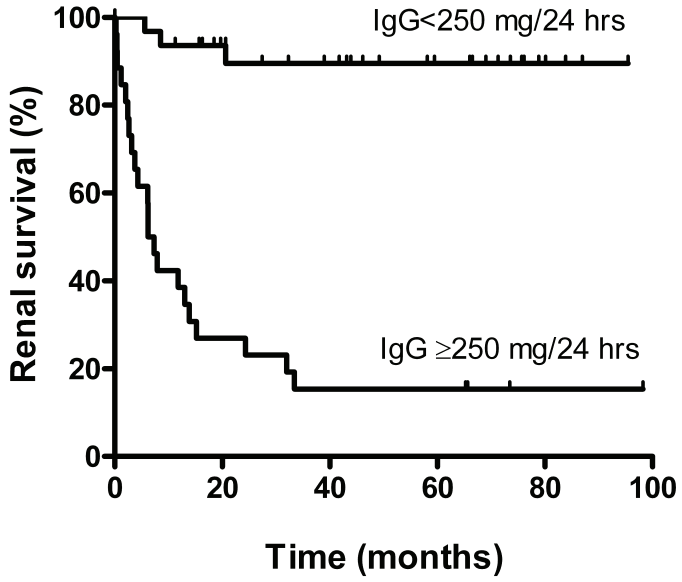

Figure 2. Renal survival in patients with idiopathic membranous nephropathy and an IgG excretion <250 mg/24 h versus patients with an IgG excretion ≥250 mg/24 h.

Our calculations confirmed the high sensitivity and specificity (Table 2). We evaluated the possible bias of using the fixed serum creatinine value of 1.5 mg/dl as end point. To this end,

we assessed the extent of the deterioration of renal function. In the 25 patients who reached the predefined end point of renal death, serum creatinine had increased by an average of 46% from 1.15±0.2 to 1.65±0.24 mg/dl. Calculated creatinine clearance (Cockcroft and Gault formula) was 76±22 ml/min/1.73m^2 at baseline and 52±13 ml/min/1.73m^2 at the end point. The absolute decrease of creatinine clearance averaged 45 ml/min/1.73m^2/yr. For comparison, in the nonfailure group, the average change of calculated creatinine clearance was 1.7 ml/min/1.73m^2/yr. When we estimate GFR using the recently developed MDRD formula, the severity of renal dysfunction is even more manifest: the MDRD GFR at the predefined end point (and thus at the start of immunosuppressive therapy) was 37±9 ml/min/1.73m^2. Of note, because we did not calibrate serum creatinine values against the standard of the MDRD reference laboratory, our calculated MDRD GFR may underestimate true GFR by 5 ml/min/1.73m^2.

The difference in course of renal function between patients with high and low Uβ2m can be appreciated by comparing the slopes of 1/serum creatinine: in patients with low Uβ2m, the slope was –0.012 dl/mg per yr (interquartile range, –0.04 to 0.014); in patients with high Uβ2m, the slope was –0.42 dl/mg per yr (interquartile range, -0.91 to -0.16; P<0.01).

A subgroup analysis limited to 44 patients with an initial serum creatinine <1.2 mg/dl resulted in similar conclusions: renal survival was 93% at 6 mo, 79% at 1 yr, and 67% at 3 yr. In this subgroup, 14 (32%) patients reached the end point of renal death; at baseline, their serum creatinine was 1.00±0.14 mg/dl and increased by 63% to 1.64±0.31 mg/dl before start of immunosuppressive therapy. In this subgroup analysis, both Uβ2m and UIgG predicted prognosis. Renal survival was 33% at 1 yr in patients with high Uβ2m and 97% in patients with low Uβ2m. Calculated sensitivity and specificity were 79 and 97% for the Uβ2m and 79 and 90% for the IgG excretion. The specificity improved to 100% when the β2m and IgG excretion were combined.

We also explored our data using all available parameters. In the initial multivariate analysis, α1-microglobulin was not included in view of the very high correlation between Uβ2m and urinary α1-microglobulin. In univariate analysis, the following parameters were significantly related to renal outcome: serum creatinine (P<0.001), serum albumin (P<0.001), ECC (P<0.01), proteinuria (P<0.001), selectivity index (P<0.001), and urinary excretion of albumin, β2m, α1-microglobulin, transferrin and IgG (all P <0.001). Multivariate analysis revealed that Uβ2m was the strongest independent predictive factor (relative risk, 1.030; 95% confidence interval, 1.017 to 1.043; P<0.001), indicating that the risk for renal insufficiency increased by 3.0% for every 0.1 µg/min increase of Uβ2m.

Table 2: Sensitivity, specificity, PPV and NPV of the most discriminative threshold levels of urinary proteins and creatinine clearance in the prediction of renal failure

Parameter	AUC	Threshold	Sensitivity	Specificity	PPV	NPV
Uβ2m	0.947	0.5 µg/min	88%	91%	88%	91%
UIgG	0.876	250 mg/24 h	88%	88%	85%	90%
Uα1m	0.956	40 µg/min	84%	94%	91%	88%
Uexc albumin	0.896	2.8 mg/min	92%	69%	70%	92%
Uexc transferrin	0.906	350 µg/min	80%	84%	80%	84%
Proteinuria	0.898	8 g/24 h	88%	72%	71%	89%
SI	0.687	0.16	76%	50%	54%	73%
ECC 24h	0.741	80 ml/min/1.73m^2	64%	81%	73%	74%
Serum creatinine	0.833	1 mg/dl	76%	81%	76%	81%
Serum albumin	0.913	2.2 g/dl	80%	97%	95%	86%
Combinations:						
High Uβ2m + High UIgG		0.5 µg/min + 250 mg/24 h	83%	97%	95%	89%
High Uβ2m + Low serum albumin		0.5 µg/min + 2.2 g/dl	75%	100%	100%	84%
High Uα1m + High UIgG		40 µg/min + 250 mg/24 h	76%	94%	91%	83%
High Uα1m + Low serum albumin		40 µg/min + 2.2 g/dl	72%	100%	100%	82%

Uexc, urinary excretion; β2m, β2-microglobulin; α1m, α1-microglobulin; SI, selectivity index; PPV, positive predictive value; NPV, negative predictive value.

After Uβ2m, serum albumin was identified as the second independent predictive factor (relative risk, 0.786; 95% confidence interval, 0.691 to 0.894 P<0.01).

We calculated sensitivity and specificity for the various parameters (Table 2). When combining parameters, specificity can be somewhat increased (Table 2). ROC curves, as depicted in Figure 3, confirmed the best performance of Uβ2m, as reflected by the AUC.

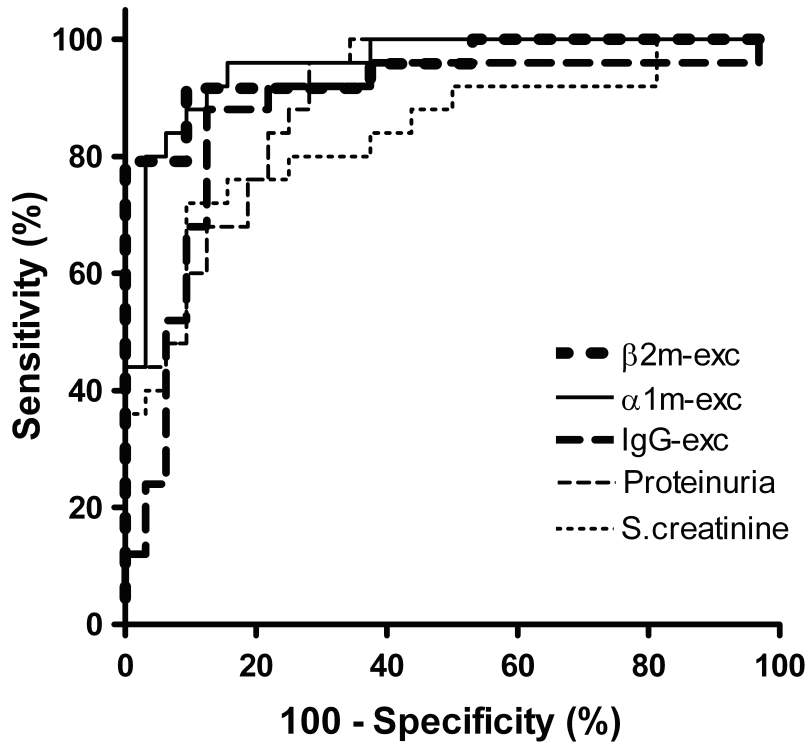

Figure 3. Comparative efficacy of serum creatinine and the urinary excretion of several proteins for predicting renal death in patients with iMN (n=57). Receiver operating characteristics curves of β2-microglobulin excretion (β2m-exc; area under the curve (AUC) 0.947), α1-microglobulin excretion (α1m-exc; AUC 0.956), IgG excretion (IgG-exc; AUC 0.876), proteinuria per day (AUC 0.898), and serum creatinine concentration (s.creatinine; AUC 0.833).

We specifically evaluated urinary α1-microglobulin excretion in comparison with β2m excretion. There was a high correlation between these parameters (r=0.80, P<0.001). In fact, it is evident from Table 2 and Figure 3 that urinary α1-microglobulin excretion and Uβ2m give comparable results.

DISCUSSION

We have validated the performance of Uβ2m and UIgG as predictors for renal insufficiency in patients with iMN. To this end, we tested the threshold values developed in our previous studies in a new, prospectively studied patient cohort. Our data clearly demonstrate that Uβ2m and UIgG predict with high accuracy renal outcome in patients with iMN. In fact, the calculated sensitivities and specificities are nearly identical to the values obtained in our previous studies [13]. Thus, our data indicate that the model parameters are robust.

Our study may be criticized because we used a fixed value of serum creatinine of 1.5 mg/dl as end point for defining renal death. However, it is evident from calculated creatinine clearance and MDRD GFR that renal function was severely disturbed at the end point. The slope of 1/serum creatinine proved that there was a clear loss of renal function. Adopting a doubling of serum creatinine or 50% decrease of GFR as end point would have resulted in even longer withholding of immunosuppressive treatment.

We used a restrictive treatment policy in our patients, initiating immunosuppressive treatment as renal failure was evident. On the basis of the results of the randomized study conducted by Ponticelli *et al.* [4], one might ask whether delay of treatment is justified especially in patients with a nephrotic syndrome. Our treatment policy was based on our preliminary findings that immunosuppressive treatment with cyclophosphamide is effective in patients with established renal failure. We recently extended these observations and also demonstrated that a restrictive treatment policy results in excellent patient and renal survival rates [9,12].

In our previous study, we noted that the UIgG was the only variable that was independently associated with renal function deterioration. This superiority of UIgG over Uβ2m was explained by one patient in whom results of UIgG and Uβ2m did not concur. In this patient, who developed renal insufficiency, UIgG exceeded the threshold value of 250 mg/day whereas Uβ2m was below the threshold [16]. In our present, larger study cohort, Uβ2m was the most significant independent predictive factor. It has been well established that Uβ2m reflects the severity of tubulo-interstitial injury [19,20]. Thus, our findings are in good agreement with studies that have unequivocally shown that in patients with glomerular diseases, renal outcome is more related to the presence and extent of tubulo-interstitial injury than to glomerular pathology. In general, there was a good agreement between Uβ2m and UIgG. When both parameters were combined, specificity even increased to a value of 97%.

How can we explain that UIgG and Uβ2m accurately predict renal failure? We propose that UIgG reflects the severity of glomerular damage, whereas Uβ2m is a marker of tubulo-

interstitial injury. It has been suggested that IgG or other high molecular weight proteins cause tubular cell activation or injury that results in tubulo-interstitial inflammation, the final step toward renal insufficiency.

Thus far, only one model for the identification of patients who have iMN and are at risk for the development of chronic renal failure has been validated. The model was developed with data derived from the Toronto Glomerulonephritis Registery. In the first study, the duration and the level of proteinuria proved to be fairly accurate predictive factors. The best performance was found using a level of proteinuria >8 g/day for >6 mo. Calculated sensitivity was 66%, and specificity was 88% [13,14]. In the validation study, roughly similar figures were reported with a sensitivity of 58% and a specificity of 93% [21]. In addition, the Toronto group extended the model by calculating a risk score on the basis of the data of a selected 6-mo interval with the worst sustained proteinuria. In this model are included the minimum amount of proteinuria in that 6-mo interval, the initial creatinine clearance, and the slope of the creatinine clearance during the 6-mo period. The risk score model was validated in three different populations and proved quite good with sensitivities varying from 60 to 89%, specificities from 86 to 92%, and an overall accuracy of 79 to 87% [21]. Obviously, this model has a very good performance. However, there are several disadvantages, particularly the need to have an observational period that exceeds a period of 6 mo and the necessity of multiple, accurate 24-h urine collections. Our model is based on the collection of a single timed urine sample collected in the morning period.

Furthermore, it is unproved whether the Toronto model can be applied to patients with newly diagnosed iMN. The model has been validated and applied to a group of patients with well-defined follow-up. This suggests that a long observation period was used to define the 6-mo period with the worst sustained proteinuria. In more than one quarter of the patients, the 6-mo period started >12 mo after renal biopsy. Therefore, the model may not be applicable to patients with a follow-up after biopsy of <12 to 18 mo.

In the present study, we specifically analyzed the value of urinary α1-microglobulin, a low molecular weight protein like β2m. In routine clinical practice, measurement of urinary α1-microglobulin is easier in view of its relative stability at pH<6.0. We observed a very high correlation between Uβ2m and urinary α1-microglobulin. Sensitivities and specificities were also comparable, although, admittedly, the threshold values used for α1-microglobulin should be validated in a second population. Our data confirm and strengthen the conclusion of Bazzi *et al.* [22]. In a small cohort of 19 untreated patients with iMN, a nephrotic syndrome, and normal renal function, these authors found that urinary α1-microglobulin predicted the development of chronic renal failure with a sensitivity and specificity of 100%. We have applied their threshold value of 33.5 mg/g creatinine to our study cohort of 57 patients and

calculated a sensitivity of 88% and a specificity of 78%. Bazzi *et al.* also reported the predictive value of UIgG. Using a threshold value of 110 mg/g creatinine, sensitivity was 100% and specificity was 69%. Applying this threshold value to our study cohort, we calculated a sensitivity of 92% and a specificity of 63%. We used a higher cutoff value (250 mg/day, approximately 180 mg/g creatinine), thereby increasing specificity. We believe that a high specificity should be pursued to be able to avoid unnecessary immunosuppressive therapy in patients with iMN.

The data of our secondary analysis suggest that serum albumin may have added value as prognostic marker. Admittedly, this needs confirmation in another patient cohort. Can we avoid unnecessary immunosuppressive treatment by using Uβ2m and UIgG as prognostic markers? From our data, it can be calculated that when used in the present population with a failure rate of 44% (which is in close agreement with literature data), our established threshold values would have resulted in the unnecessary treatment of one patient (1.8% overall, 4.8% of all treated patients), whereas 31 patients rightly would not have received treatment.

Conclusion: We have validated the performance of Uβ2m and UIgG as prognostic markers in patients with iMN. Urinary α1-microglobulin can replace Uβ2m. Use of these markers will allow identification of high-risk patients at an early stage. We propose that these markers may help to guide the time of start of immunosuppressive treatment in individual patients.

Acknowledgments

A.J.W. Branten is supported by a grant from the Dutch Science Foundation (NWO-MW 920-03-038). P.W.G. du Buf-Vereijken is supported by a grant from the Dutch Kidney Foundation (NSN:PC152). We thank Drs. J.J. Beutler and J.L.J. Jansen, Jeroen Bosch Hospital 's-Hertogenbosch; Dr. P.A. Bleeker, Hospital Rivierenland Tiel; Dr. M.A.G.J. ten Dam, Canisius Wilhelmina Hospital Nijmegen; Dr. P.G.G. Gerlag, Maxima Medical Center Veldhoven; Drs. W. Grave and J.J.J.M. Wirtz, St. Laurentius Hospital Roermond; Dr. M. den Hartog Hospital Gelderse Vallei, Ede; Dr. L.H. Hulsteyn, Bernhoven Veghel; Drs. R. van Leusen and K.J. Parlevliet Hospital Rijnstate Arnhem; Dr. A.J. Luik, Hospital VieCuri Venlo; Drs. T.C. Noordzij and H.P.C. van Roermund, St. Franciscus Hospital Roosendaal; Dr. J.J.G.Offerman, Isala Klinieken Zwolle; Dr. P.L. Rensma, St. Elisabeth Hospital Tilburg; Dr. G. Schrijver, Hospital Rode Kruis Beverwijk; Dr. R.E.H. Smeets, St. Anna Hospital Geldrop; Dr. P.F.M.J. Spooren, TweeSteden Hospital Tilburg; and Dr. P.J. Stijnen, Amphia Hospital Breda, for participation in this study.

REFERENCES

1. Haas M, Meehan SM, Karrison TG, Spargo BH. Changing etiologies of unexplained adult nephrotic syndrome: A comparison of renal biopsy findings from 1976-1979 and 1995-1997. Am J Kidney Dis 1997;30:621-631.
2. Donadio JJV, Torres VE, Velosa JA *et al*. Idiopathic membranous nephropathy: The natural history of untreated patients. Kidney Int 1988;33:708-715.
3. Schieppati A, Mosconi L, Perna A *et al*. Prognosis of untreated patients with idiopathic membranous nephropathy. New Engl J Med 1993;329:85-89.
4. Ponticelli C, Zuchelli P, Passerini P *et al*. A 10-year follow-up of a randomized study with methylprednisolone and chlorambucil in membranous nephropathy. Kidney Int 1995;48:1600-1604.
5. Muirhead N. Management of idiopathic membranous nephropathy: evidence-based recommendations. Kidney Int 1999;55 (Suppl. 70):S-47-S-55.
6. Cattran DC. Idiopathic membranous glomerulonephritis. Kidney Int 2001;59:1983-1994.
7. Davison AM, Cameron JS, Kerr DN, Ogg CS, Wilkinson RW. The natural history of renal function in untreated idiopathic membranous glomerulonephritis in adults. Clin Nephrol 1984;22:61-67.
8. Honkanen E, Tornroth T, Gronhagen-Riska C, Sankila R. Long-term survival in idiopathic membranous glomerulonephritis: can the course be clinically predicted? Clin Nephrol 1994;41:127-134.
9. du Buf-Vereijken PWG, Feith GW, Hollander D *et al*. Restrictive use of immunosuppressive treatment in patients with idiopathic membranous nephropathy: high renal survival in a large patient cohort. Q J Med 2004;97:353-360.
10. Branten AJW, Reichert LJM, Koene RAP, Wetzels JFM. Oral cyclophosphamide versus chlorambucil in the treatment of patients with idiopathic membranous nephropathy and renal insufficiency. Q J Med 1998;91:359-366.
11. Mathieson PW, Turner AN, Maidment CG, Evans DJ, Rees AJ. Prednisolone and chlorambucil treatment in idiopathic membranous nephropathy with deteriorating renal function. Lancet 1988;2:869-872.
12. du Buf-Vereijken PWG, Branten AJW, Wetzels JFM, for the Membranous Nephropathy Study Group. Cytotoxic therapy for membranous nephropathy and renal insufficiency; improved renal survival but high relapse rate. Nephrol Dialysis Transplant 2004;19:1142-1148.
13. Reichert LJM, Koene RAP, Wetzels JFM. Prognostic factors in idiopathic membranous nephropathy. Am J Kidney Dis 1998;31:1-11.
14. Pei Y, Cattran DC, Greenwood C. Predicting chronic renal insufficiency in idiopathic membranous glomerulonephritis. Kidney Int 1992;42:960-966.

15. Reichert LJM, Koene RAP, Wetzels JFM. Urinary excretion of β2-microglobulin predicts renal outcome in patients with idiopathic membranous nephropathy. J Am Soc Nephrol 1995;6:1666-1669.
16. Reichert LJM, Koene RAP, Wetzels JFM. Urinary IgG excretion as a prognostic factor in idiopathic membranous nephropathy. Clin Nephrol 1997;48:79-84.
17. Jacobs EMG, Vervoort G, Branten AJW *et al*. Atrial natriuretic peptide increases albuminuria in type I diabetic patients: evidence for blockade of tubular protein reabsorption. Eur J Clin Invest 1999;29:109-115.
18. Levey AS, Bosch JP, Breyer Lewis J *et al*. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Ann Intern Med 1999;130:461-470.
19. Lai KN, Mac-Moune Lai F, Vallance-Owen J. The clinical use of serum beta-2-microglobulin and fractional beta-2-microglobulin excretion in IgA nephropathy. Clin Nephrol 1986;25:260-263.
20. Portman RJ, Kissane JM, Robson AM, Peterson LJ, Richardson A. Use of β2-microglobulin to diagnose tubulo-interstitial renal lesions in children. Kidney Int 1986;30:91-98.
21. Cattran DC, Pei Y, Greenwood CMT *et al*. Validation of a predictive model of idiopathic membranous nephropathy: its clinical and research implications. Kidney Int 1997;51:901-907.
22. Bazzi C, Petrini C, Rizza V *et al*. Urinary excretion of IgG and alpha1-microglobulin predicts clinical course better than extent of proteinuria in membranous nephropathy. Am J Kidney Dis 2001;38:240-248.

Chapter 8

Oral Cyclophosphamide versus Chlorambucil in the Treatment of Patients with Membranous Nephropathy and Renal Insufficiency.

Amanda J.W. Branten
Louis J.M. Reichert
Robert A.P. Koene
and Jack F.M. Wetzels

Department of Medicine, Division of Nephrology,
Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands.

Quarterly Journal of Medicine 1998; 91:359-366

ABSTRACT

We treated patients with idiopathic membranous nephropathy (iMN) and renal insufficiency, using: (I) (n=15) monthly cycles of steroids (1 g methylprednisolone i.v. on three consecutive days, followed by oral prednisone 0.5 mg/kg/day months 1, 3, and 5) and chlorambucil (0.15 mg/kg/day month 2, 4, and 6); or (II) (n=17) oral cyclophosphamide (1.5 - 2.0 mg/kg/day for 1 year) and steroids in a comparable dose. The groups were comparable in age, renal function and levels of proteinuria. During the 6 months preceding treatment, serum creatinine levels increased from 148±50 to 219±73 µmol/l in the chlorambucil group and from 164±86 to 274±126 µmol/l in the cyclophosphamide group. Median (range) follow-ups were: chlorambucil 38 months (8-71); cyclophosphamide 26 months (5-68) (NS). Renal function improved in both groups, but the improvement was short-lived in the chlorambucil group; 12 months after starting treatment, mean serum creatinine was 6.3 µmol/l lower in the chlorambucil group and 121 µmol/l lower in the cyclophosphamide group ($p<0.01$). Four chlorambucil-treated patients developed ESRD, and five needed a second course of therapy, whereas only one cyclophosphamide-treated patient developed ESRD ($p<0.05$). Remissions of proteinuria occurred more frequently after cyclophosphamide treatment (15/17 versus 5/15; $p<0.01$). Side-effects necessitated interruption of treatment in six patients on cyclophosphamide and in 11 on chlorambucil ($p<0.05$).

In our patients, oral cyclophosphamide was better tolerated than oral chlorambucil. The suggested greater efficacy of the oral cyclophosphamide regimen needs to be ascertained by longer follow-up.

INTRODUCTION

In the majority of patients with idiopathic membranous nephropathy (iMN), the renal disease runs a benign course; about 50% of nephrotic patients with iMN will spontaneously enter a partial or complete remission. However, up to 40% of the patients will develop end-stage renal failure [1]. There is no consensus about the treatment of patients with iMN [2]. Most clinicians tend to follow the recommendations of Cattran, who recently suggested that treatment with immunosuppressive drugs should be considered only in patients with longstanding proteinuria and/or evidence of deteriorating renal function [3]. Both chlorambucil and cyclosporine were mentioned as treatment options. However, information on the tolerability and efficacy of immunosuppressive treatment in patients with iMN and renal insufficiency is limited to small, non-randomized studies. Positive effects have been described for chlorambucil [4,5], oral cyclophosphamide [6,7], azathioprine [8,9], and cyclosporine [10]. Formal comparisons of immunosuppressive drug regimens have not been reported. Over the past 10 years, we have used immunosuppressive therapy in patients with iMN and renal insufficiency [11,12]. We developed treatment schedules with chlorambucil and oral cyclophosphamide, both combined with corticosteroids. At the end of 1996, we analysed the long-term outcome of all patients treated with chlorambucil [13]. The results were less favourable than expected. Most remarkable was the high incidence of side-effects, often necessitating interruption or premature withdrawal of chlorambucil treatment. These results prompted us to analyse the results of treatment with oral cyclophosphamide and to compare them with those for chlorambucil treatment.

PATIENTS AND METHODS

From 1986 onwards, we have used immunosuppressive therapy in patients with membranous glomerulopathy. To be eligible for such treatment, patients were required to have biopsy-proven membranous nephropathy, nephrotic syndrome and deteriorating renal function. Patients younger than 18 years or with evidence of secondary types of membranous nephropathy were excluded. In 1986, we treated one patient with chlorambucil and prednisone in a pilot phase. From 1989 onwards, patients were randomized for treatment with either chlorambucil and corticosteroids (n=9) or intravenous boluses of cyclophosphamide and methylprednisolone. Intravenous cyclophosphamide proved an ineffective treatment modality [12]. Therefore, we conducted a pilot study in which we treated all eligible patients with oral cyclophosphamide and prednisone (n=7). From 1994 till 1996,

patients were then asked to participate in a randomized trial in which we compared chlorambucil and corticosteroids with oral cyclophosphamide and corticosteroids. Patients unwilling to participate in this study were treated with the oral cyclophosphamide treatment regimen. Our interim analysis revealed a particularly high frequency of side-effects with chlorambucil treatment [13]. Therefore, we decided in 1996 to stop this randomized study prematurely. Meanwhile, five patients were included in each treatment group and in parallel with the study, another five patients were treated with oral cyclophosphamide. At start of the present analysis the cumulative number of patients that had been treated with chlorambucil and prednisone was 15, whereas 17 patients had received oral cyclophosphamide and prednisone.

Treatment regimens

Patients assigned to chlorambucil treatment were treated according to the scheme originally described by Ponticelli *et al*. [14], although we used a lower dose of chlorambucil in view of the more severe side-effects of this drug in patients with renal insufficiency [4,11]. In brief, the patients received three cycles of steroids consisting of intravenous pulses of methylprednisolone, 1 g on 3 consecutive days, followed by oral prednisone 0.5 mg/kg of body weight per day for 27 days. Each cycle was followed by 1 month of treatment with oral chlorambucil (0.15 mg/kg per day). The total duration of treatment was six months. Patients treated with oral cyclophosphamide received this drug in a daily dose of 1.5 to 2 mg/kg body weight for 1 year. In the pilot phase (in which seven patients were treated) concomitant treatment consisted of oral prednisone 60 mg/day or 125 mg every other day for at least 8 weeks (mean cumulative dose 8400 mg, range 4200-13650 mg). The last 10 patients received intravenous pulses of methylprednisolone, 1 g each on 3 consecutive days at the beginning of the first, third and fifth month, and oral prednisone 0.5 mg/kg every other day for 6 months. The latter schedule precisely matches the corticosteroid dose given to chlorambucil treated patients.

All patients received diuretics and antihypertensive drugs if required. Clinical examinations, biochemical profiles and full blood counts were done every one or two weeks during the first months of treatment, and at regular intervals thereafter. The end-point was defined as deterioration of renal function requiring a second course of immunosuppressive therapy or the development of end-stage renal disease. A second course of immunosuppressive therapy was offered to patients who experienced a rise of serum creatinine of >50 % over the lowest value reached after the first immunosuppressive treatment. Otherwise patients were followed until July 1997.

For calculations of renal survival, the time of renal death was defined as the time of the start of a second course of immunosuppressive treatment or the time of start of renal replacement therapy. We have used the reciprocal of serum creatinine (1000/serum creatinine level) to assess the effects of treatment on the progression of renal insufficiency. Changes in this ratio parallel changes in endogenous creatinine clearance. For the calculations, 1000 µmol/l was used as the serum creatinine level in patients on renal replacement therapy. To correct for inappropriate 24-hr urine collections, the amount of urinary protein was adjusted for the amount of urinary creatinine (protein-creatinine index). A complete remission of proteinuria was defined as a reduction of the protein-creatinine index to less then 0.2 g/10 mmol creatinine, and a partial remission as a protein-creatinine index of between 0.2 and 2.0 g/10 mmol creatinine.

Statistics

Changes in biochemical parameters were analysed with repeated measures ANOVA, and post-test according to Newman-Keuls. Comparisons between groups were done by Fisher's test, Mann-Whitney U test, or unpaired t-test where appropriate. Probabilities of survival were calculated by the Kaplan-Meier method and for comparison of survival curves the log rank test was used. Results are given as means ± SD, or medians and range when appropriate. A p value < 0.05 was considered significant.

RESULTS

The baseline characteristics of both treatment groups are summarized in Table 1. All but two patients were male. Both treatment groups were comparable with respect to age, blood pressure, renal function, proteinuria, and the interval between renal biopsy and start of the immunosuppressive treatment. In four patients of the chlorambucil group and in five of the cyclophosphamide group, this interval was more than 2.5 years. All patients had evidence of renal function deterioration. In the 6 months before the start of treatment, serum creatinine levels of patients in the chlorambucil group increased from 148±50 µmol/l to 219±73 µmol/l; in the cyclophosphamide group, serum creatinine increased from 164±86 µmol/l to 274±126 µmol/l (chlorambucil versus cyclophosphamide: p=NS). Six patients in the chlorambucil group had received prednisone therapy in an earlier phase of the disease, whereas in the cyclophosphamide group, eight patients had been treated previously (four with prednisone and four with prednisone and chlorambucil). Seven patients in the chlorambucil group and nine patients in the cyclophosphamide group were treated with an angiotensin-converting enzyme inhibitor.

Table 1. Baseline characteristics

	Chlorambucil	Cyclophosphamide
Number of patients	15	17
Sex *(M/F)*	15/0	15/2
Age *(years)*	51 ± 12	53 ± 14
Time from kidney biopsy to start of treatment *(months)*	14 (1 - 120)	11 (1 - 157)
Blood pressure *(mm Hg)*		
Systolic	145 ± 18	151 ± 29
Diastolic	85 ± 9	89 ± 10
Proteinuria *(g/10 mmol creat)*	9 ± 2.6	11 ± 5.3
ECC *(ml/min)*	46 ±17	43 ± 23

Values are given as means (±SD) or medians (range).

During the course of the follow-up the blood pressures of the patients in the chlorambucil group did not differ significantly from the blood pressures in the cyclophosphamide treated patients (systolic blood pressure at month 12; 140±11 versus 135±20 mm Hg (NS), at month 24; 136±17 versus 137±18 mm Hg, diastolic blood pressure at month 12; 89±8 versus 83±8 mm Hg, at month 24; 87±17 versus 85±16 mm Hg.

Short-term effects of treatment are given in Table 2. Both treatment regimens reversed the deterioration of renal function, as evidenced by the decrease of serum creatinine. In most patients, improvement of renal function was already apparent after 1 month of therapy.

Table 2. Short-term effects of immunosuppressive treatment on renal function and proteinuria

		0 months	3 months	6 months	12 months
Serum creatinine	CA	219 ± 73	165 ± 56**	166 ± 54**	216 ± 99
(µmol/l)	CP	274 ± 126	171 ± 82***	165 ± 80***	174 ± 78***
Serum albumin	CA	22 ± 5.6	26 ± 6.0**	31 ± 6.2***	32 ± 6.8***
(g/l)	CP	22 ± 6.0	29 ± 5.1***	34 ± 5.2***	40 ± 4.7***
Proteinuria	CA	9.1 ± 2.6	8.3 ± 5.9	6.5 ± 3.9*	6.8 ± 4.4
(g/10 mmol creat)	CP	11.2 ± 5.3	4.9 ± 2.3***	3.0 ± 2.3***	2.0 ± 3.0**

CA, Chlorambucil group; CP, Cyclophosphamide group. Values are means ± SD.

Treatment was started at 0 months. *p<0.05, ** p<0.01, *** p<0.001 versus 0 months.

Overall, renal function improved or stabilized in 13/15 patients on chlorambucil, and in all patients on cyclophosphamide. The improvement of renal function was also evident from significant changes in the slope of 1000/serum creatinine. In the chlorambucil group the slope of 1000/Screat changed from -0.38 (95%CI -0.48 to -0.29) in the 6 months before start of treatment to 0.29 (95%CI 0.13 to 0.44) in the 6 months after start of treatment. In the cyclophosphamide group, values were -0.53 (95%CI -0.75 to -0.30) and 0.40 (95%CI 0.21 to 0.58), respectively. However, in the chlorambucil treated patients the improvement in renal function was short-lasting. In these patients a decline in renal function was already apparent at 12 months after start of treatment, which contrasts with the findings in the cyclophosphamide treated patients: at 12 months serum creatinine levels had changed by -6.3 µmol/l (95%CI: -65 to 52 µmol/l) in the chlorambucil group and by -121 µmol/l (95%CI: -166 to -76 µmol/l) in the cyclophosphamide group ($p<0.01$). This difference is also reflected in the slope of 1000/Screat from 6 to 12 months (chlorambucil -0.16, 95%CI -0.24 to -0.08 versus cyclophosphamide 0.00, 95%CI -0.11 to 0.11, $p<0.05$).

Long-term follow-up

The median duration of follow-up was 26 months (range 5-68) in the cyclophosphamide group and 38 months (range 8-71) in the chlorambucil group (NS). Eleven of the cyclophosphamide treated patients and 14 of the chlorambucil treated patients were followed for at least 24 months (NS). Pertinent data for the individual patients of both treatment groups are given in Tables 3 and 4. In the chlorambucil group, four patients progressed to ESRD, whereas in 5 other patients a second course of therapy was given because of deterioration of renal function. Thus far, only one of the cyclophosphamide treated patients has developed ESRD. The difference in renal survival between the groups is significant ($p<0.05$).

There was a striking difference in the cumulative incidence of the occurrence of a complete or partial remission of proteinuria (Figure 1). Overall, a partial remission of proteinuria was observed in 5 (33%) patients after chlorambucil treatment and in 15 (92%) patients after cyclophosphamide treatment ($p<0.01$). Of these latter patients, six developed a complete remission, whereas none of the chlorambucil treated patients did ($p<0.01$). At the end of follow-up, two patients in the chlorambucil group and 11 patients in the cyclophosphamide group were still in remission. The median interval between start of treatment and development of partial remission was 6 months (range 1 - 24) in the chlorambucil treated patients and 12 months (range 1 - 24) in the cyclophosphamide treated patients. Four patients in the cyclophosphamide group had previously been treated with chlorambucil. Exclusion of these patients did not alter the results, as remissions still occurred more frequently in the cyclophosphamide group (11/13 versus 5/15, $p<0.01$).

Table 3: Characteristics of patients and effects of treatment in the chlorambucil group

Patient	Sex	Age	Previous	FU	S. creatinine (µmol/l)			Proteinuria[+]		Side-effects
(No)		(Years)	therapy#	(months)	Start	Min.	End	Start	End	
1	M	43	Y	36	304	248	ESRD	13.8	14.1	*, respiratory tract infection,
2	M	34	Y	38	408	211	313**	9.6	8.7	*, leukopenia,
3	M	57	N	40	197	132	512**	7.0	10.2	*, respiratory tract infection,
4	M	66	N	39	287	232	ESRD	6.1	na	*, respiratory tract infection, renal artery stenosis
5	M	47	N	71	143	82	124	9.7	3.4	*, leukopenia
6	M	54	Y	55	176	125	441	8.7	11.3	*, axillary abscess,leuko- and trombocytopenia
7	M	32	Y	35	231	175	ESRD	6.4	11.6	
8	M	32	Y	38	237	165	ESRD	11.9	3.1	*, mycoplasma pulmonary infection, varicella infection, osteonecrosis, leuko- and trombocytopenia
9	M	46	N	46	176	109	88	6.7	2.6	nausea, leukopenia
10	M	62	N	25	208	108	113	9.7	1.6	*, leukopenia
11	M	59	N	12	269	146	319 **	7.8	9.3	herpes zoster
12	M	67	N	23	165	102	109	6.8	1.7	*, leukopenia
13	M	43	N	8	176	119	229 **	12.2	9.8	*, leuko- and trombocytopenia, anaemia, respiratory tract infection
14	M	59	N	11	183	211	390	13.2	13.7	anaemia necessitating blood transfusions
15	M	57	Y	66	126	135	446**	7.6	11.6	*, leukopenia

S. creatinine, serum creatinine; ESRD, end-stage renal disease; NA, not analysed; [+]Proteinuria is given as g/10 mmol creatinine; *patients in whom chlorambucil dose was reduced, temporarily interrupted or prematurely stopped. #Previous therapy consisted of treatment with short-term high-dose oral prednisone , shortly after the onset of the disease; Min, minimum value of serum creatinine within the first 12 months after start of treatment; **because of deteriorating renal function, five patients received a second course of therapy consisting of oral cyclophosphamide and prednisone (patients 2,3,11,15) or azathioprine and prednisone (patient 13).

Table 4: Characteristics of patients and effects of treatment in the cyclophosphamide group

Patient (No)	Sex	Age (Years)	Previous therapy#	Follow-up (months)	S.creatinine (µmol/l)		Proteinuria+		Side-effects
					Start	End	Start	End	
2	M	37	Y	49	313	215	8.7	0.86	
3	M	61	Y	48	512	274	10.2	0.40	*, respiratory tract infection
11	M	60	Y	23	319	207	9.3	0.13	
15	M	62	Y	68	446	202	11.6	4.1	
16	M	32	Y	49	492	ESRD	19.3	2.4	*, respiratory tract infection
17	M	45	Y	13	162	129	6.6	0.68	respiratory tract infection
18	M	59	N	41	196	88	5.3	5.4	malaise
19	M	60	N	33	323	135	12.9	0.08	
20	M	28	N	27	215	95	11.7	0.18	
21	F	43	N	25	210	169	9.6	0	
22	M	53	N	24	142	126	6.9	0	*, leukopenia, respiratory tract infection
23	M	70	Y	14	386	330	23	1.7	*, leukopenia, anaemia, respiratory tract infection, nausea
24	M	37	Y	8	185	138	5.3	2.0	*, nausea
25	M	70	N	5	195	144	14.1	3.1	
26	M	51	N	12	106	91	4.5	2.3	
27	M	51	N	8	149	82	8.8	1.9	
28	F	72	N	26	305	134	19.4	0.10	*, leukopenia

S. creatinine, serum creatinine; ESRD, end-stage renal disease; +Proteinuria is given as g/10 mmol creatinine; *patients in whom cyclophosphamide dose was reduced, temporarily interrupted or prematurely stopped. #Previous therapy in the earlier phase of the disease consisted of treatment with prednisone (patients 16,17,23,24) or prednisone and chlorambucil (patients 2,3,11,15). Patients 17 and 20-28 received methylprednisolone pulses.

To further exclude as much as possible any bias because of a difference in the year of treatment start, we have analysed separately the data of patients treated from 1992 onward, and excluding the patients who received cyclophosphamide as retreatment. Results are given in Table 5. This analysis confirmed the superiority of cyclophosphamide treatment.

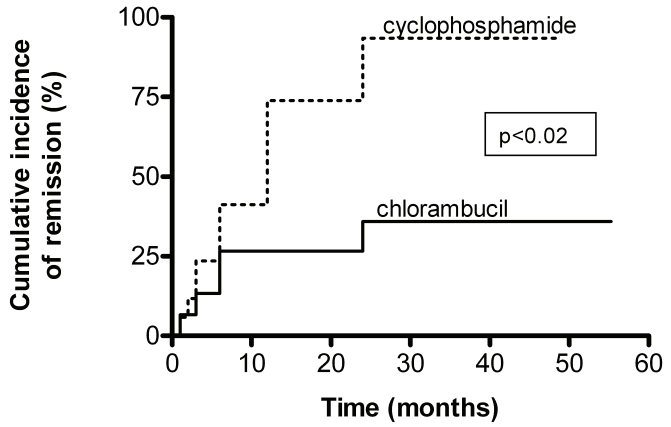

Figure 1. Cumulative incidence of partial remissions of proteinuria (i.e. proteinuria < 2 g/10 mmol creatinine) in patients treated with either chlorambucil or cyclophosphamide.

From Tables 3 and 4, it is evident that side-effects were observed regularly. Side-effects included leukopenia, anaemia, thrombocytopenia, infectious complications and nausea, and occurred more frequently in the chlorambucil group. Overall, only one patient in the chlorambucil group did not experience side-effects as compared to nine patients in the cyclophosphamide group ($p<0.01$). It is unlikely that the differences in side-effects are related to the use of methylprednisolone. As indicated in Table 4, the incidence of infectious complications was similar in patients treated with or without pulses methylprednisolone.

Table 5. Analysis of data of patients treated from 1992 onward

	Chlorambucil (n=8)	**Cyclophosphamide (n=12)**	**p**
Follow-up duration *(months)*	25 ± 14	19 ± 11	
Serum creatinine month 0	170 ± 45	215 ± 83	
Rise of creatinine >50%	5	0	<0.01
Proteinuria month 0	9.3 ± 2.8	10.7 ± 5.9	
Partial Remission	3	11	<0.02
Complete Remission	0	5	0.055

Treatment had to be reduced, temporarily interrupted, or prematurely stopped in 11/15 chlorambucil treated patients and in 6/17 cyclophosphamide treated patients ($p<0.05$).
As a result, patients have used a lower cumulative dosage (9.8±4.1 mg/kg) of chlorambucil than initially scheduled (13.5 mg/kg). For cyclophosphamide, this difference is less clear; the median daily dose amounting to 1.56 mg/kg.

DISCUSSION

In iMN, immunosuppressive therapy should be reserved for patients at high risk for developing ESRD [3]. Thus far, a steady rise in serum creatinine is the best predictor of future development of ESRD [15,16]. Therefore, it has been recommended that immunosuppressive therapy should be delayed until renal insufficiency becomes apparent. However, little is known on the efficacy of immunosuppressive treatment when it is initiated at this stage of the disease, and comparisons between the various immunosuppressive drugs are lacking. We have compared oral chlorambucil- and oral cyclophosphamide-based regimens, which have been used successfully in previous, smaller studies [4,7]. Our study confirms that immunosuppressive treatment is indeed effective, and able to preserve or even improve renal function when initiated in patients with moderately to severely impaired renal function. Furthermore, our data suggest that oral cyclophosphamide is more effective than chlorambucil, in preserving renal function as well as in inducing remissions of proteinuria.
Admittedly, in the cyclophosphamide treated patients a longer follow-up is needed to ascertain that renal function will remain stable for a longer time period. However, we observed a very high rate of remissions of proteinuria in this group, and it is generally accepted that the development of remissions of proteinuria is associated with a good prognosis [17]. Treatment with cyclophosphamide was reasonably well tolerated. In contrast, chlorambucil caused side-effects more frequently, often necessitating interruption of therapy.

Although our study was not fully randomized, it seems unlikely that the observed differences between both drugs are caused by a selection bias. All patients were treated prospectively; the majority of patients as part of a randomized study, or in parallel with one of the randomized studies. Moreover, both groups had similar baseline characteristics, in particular with respect to risk factors such as baseline serum creatinine, rate of renal function deterioration and the amount of proteinuria. Furthermore, the cumulative dose of steroids was comparable in both treatment groups. Subgroup analysis also suggested a higher efficacy of cyclophosphamide treatment, thus confirming and strenghtening our overall conclusions.

It is quite possible that the better efficacy of cyclophosphamide is fully explained by the longer duration of cyclophosphamide therapy (12 versus 6 months) and the lesser need to interrupt treatment. However, it is also possible that cyclophosphamide is a more effective drug than chlorambucil. A review of the available literature provides support for this latter explanation. Thus far, six other studies have addressed the effects of cyclophosphamide or chlorambucil in patients with iMN and deteriorating renal function [4,7,18,19]. The results of these non-randomized, small studies and the current study are summarized in Table 6. From this table, it is evident that remissions of proteinuria are more frequent during treatment with cyclophosphamide, a complete remission occurring in 16/41 patients after cyclophosphamide and in 2/39 patients on chlorambucil ($p<0.001$). A similar significant difference is observed when counting the number of complete and partial remissions (Table 6). The differences remain present after exclusion of the data of the present study, complete remissions occurring in 11/24 patients on cyclophosphamide and in 2/24 patients on chlorambucil ($p<0.01$). Admittedly, with respect to the effects on renal function, the differences are not significant.

The available literature data do not allow meaningful conclusions on the efficacy of oral cyclophosphamide in comparison with immunosuppressive drugs such as azathioprine and cyclosporine. We are aware of only one study in which patients with iMN and renal failure were treated with cyclosporine [10]. In this study, only 9 patients were included, and although renal function was preserved, the results are somewhat disappointing since neither improvement of renal function nor sustained remissions of proteinuria were observed. Two groups of investigators have reported on the effects of azathioprine [8,9]. The initial data, on only 10 and 6 patients, respectively, showed an improvement of renal function after start of azathioprine. In the short term, sustained remissions of proteinuria were rare, occurring in only 3/16 patients. However, with longer follow-up, results seem more favourable. Bone and colleagues recently reported 10-year follow-up data for 21 patients treated with azathioprine [20]. In most patients there was a permanent improvement of renal function, and a partial or complete remission of proteinuria occurred in up to two third of patients [20]. These data show that treatment with azathioprine has favourable effects. It should be noted however, that these results were obtained with continued, possibly lifelong treatment with low-dose azathioprine and prednisone. Longer follow-up of our cyclophosphamide treated patients is needed to see whether limited duration of treatment with this drug has similar effects in the long run.

Table 6: Summary of therapeutic trials

Reference	N	Sex (M/F)	S.creatinine (μmol/l)	Follow-up (months)	Proteinuria				Renal Function		
					Cumulative		Final		Im	S	ESRD
					CR	PR	CR	PR			
Cyclophosphamide											
[6]	11	9/2	198 (159-173)	33 (12-54)	4	5	3	4	7	4	0
[7]	9	7/2	222 (130-300)	83 (13-144)	4	4	3	4	4	1	4
[18]	4	NA	>200	NA	3	1	3	1	3	1	0
This study	17	15/2	274 (106-492)	26 (5-67)	6	9	6	5	13	3	1
Total	*41*				*17*	*19*	*15*	*14*	*27*	*9*	*5*
Chlorambucil											
[4]	8	7/1	194 (122-312)	21 (16-42)	1	4	1	3	7	0	1
[5]	7	6/1	247 (190-360)	32 (17-59)	1	4	1	3	4	1	2
[19]	9	6/3	227 (115-420)	20 (12-24)	0	3	0	3	4	3	2
This study	15	15/0	219 (126-408)	37 (10-70)	0	5	0	2	4	2	9
Total	*39*				*2*	*16*	*2*	*11*	*19*	*6*	*14*

S. creatinine, serum creatinine; CR, complete remission; PR, partial remission; FU, follow-up; Im, improved; S, stabilized; ESRD, end-stage renal disease, for this analysis including patients who died or had evidence of progressive renal failure.

For chlorambucil therapy, we have adapted the treatment protocol developed by Ponticelli *et al.* for patients with iMN and normal renal function [14]. In their patients, side-effects were uncommon (occurring in 10% of patients). Although we have used a lower dosage, we observed a very high incidence of side-effects. Similar observations were made by Mathieson and Warwick [4,5]. This suggests that patients with renal insufficiency are more sensitive to the side-effects of chlorambucil. An even lower dose might have been better tolerated [19], but the efficacy of such low doses has not been proven. A formal comparison of the efficacy of oral chlorambucil and cyclophosphamide can best be performed in patients with normal renal function.

In conclusion, immunosuppressive treatment preserves renal function and can result in complete remission of proteinuria in patients with iMN and renal insufficiency. In view of its efficacy and tolerability, we prefer oral cyclophosphamide over chlorambucil for the treatment of these patients.

Acknowledgments

The authors thank Drs F. Bosch, Hospital Rijnstate Arnhem; R. Brouwer, Medisch Spectrum Twente; G. Feith, Hospital Gelderse Vallei Wageningen; L. Frenken, Hospital De Wever Heerlen; I. Go, Canisius Wilhelmina Hospital Nijmegen; D. de Gooyer and H. van Roermund, St. Franciscus Hospital Roosendaal; P. Hillen and A. Apperloo, Hospital St. Elisabeth Tilburg; J. Jansen and M. Koolen, Bosch Medicentrum ‘s Hertogenbosch; R. Smeets, St. Anna Hospital Geldrop, and A. van den Wall Bake, St. Joseph Hospital Veldhoven for participation in this study.

REFERENCES

1. Ponticelli C, Zuchelli P, Passerini P *et al*. A 10-year follow-up of a randomized study with methylprednisolone and chlorambucil in membranous nephropathy. Kidney Int 1995;48:1600-1604.
2. Lewis EJ. Idiopathic membranous nephropathy: to treat or not to treat? New Engl J Med 1993;329:85-89.
3. Cattran DC. Cytotoxics, cyclosporine and membranous nephropathy. Curr Opin Nephrol Hypertens 1996;5:427-436.
4. Mathieson PW, Turner AN, Maidment CG, Evans DJ, Rees AJ. Prednisolone and chlorambucil treatment in idiopathic membranous nephropathy with deteriorating renal function. Lancet 1988;2:869-872.

5. Warwick GL, Geddes CG, Boulton-Jones JM. Prednisolone and chlorambucil therapy for idiopathic membranous nephropathy with progressive renal failure. Q J Med 1994;87:223-229.
6. Bruns FJ, Adler S, Fraley DS, Segel DP. Sustained remission of membranous glomerulonephritis after cyclophosphamide and prednisone. Ann Intern Med 1991;114:725-730.
7. Jindal K, West M, Bear R, Goldstein M. Longterm benefits of therapy with cyclophosphamide and prednisone in patients with membranous glomerulonephritis and impaired renal function. Am J Kidney Dis 1992;19:61-67.
8. Williams PS, Bone JM. Immunosuppression can arrest progressive renal failure due to idiopathic membranous glomerulonephritis. Nephrol DialysisTransplant 1989;4:181-186.
9. Baker LRI, Tucker B, Macdougall IC. Treatment of idiopathic membranous nephropathy. Letter. Lancet 1994;343:290-291.
10. Cattran DC, Greenwood C, Ritchie S *et al*. A controlled trial of cyclosporine in patients with progressive membranous nephropathy. Kidney Int 1995;47:1130-1135.
11. Wetzels JFM, Hoitsma AJ, Koene RAP. Immunosuppression for membranous nephropathy. Lancet 1989;I:211-211(letter).
12. Reichert LJM, Huysmans FThM, Assmann K, Koene RAP, Wetzels JFM. Preserving renal function in patients with membranous nephropathy: daily oral chlorambucil compared with intermittent monthly pulses of cyclophosphamide. Ann Intern Med 1994;121:328-333.
13. Wetzels JFM, Reichert LJM. Efficacy of immunosuppressive treatment in patients with membranous nephropathy and renal insufficiency. Kidney Int 1997;52 Suppl 61:S63-S66.
14. Ponticelli C, Zuchelli P, Imbasciati E *et al*. Controlled trial of methylprednisolone and chlorambucil in idiopathic membranous nephropathy. New Engl J Med 1984;310:946-950.
15. Davison AM, Cameron JS, Kerr DN, Ogg CS, Wilkinson RW. The natural history of renal function in untreated idiopathic membranous glomerulonephritis in adults. Clin Nephrol 1984;22:61-67.
16. Honkanen E, Tornroth T, Gronhagen-Riska C, Sankila R. Long-term survival in idiopathic membranous glomerulonephritis: can the course be clinically predicted? Clin Nephrol 1994;41:127-134.
17. Passerini P, Pasquali P, Cesana B, Zucchelli P, Ponticelli C. Long-term outcome of patients with membranous nephropathy after complete remission of proteinuria. Nephrol DialysisTransplant 1989;4:525-529.
18. Faedda R, Satta A, Bosincu L, Pirisi M, Bartoli E. Immune suppressive treatment of membranous glomerulonephritis. J Nephrol 1995;8:107-112.
19. Brunkhorst R, Wrenger E, Koch KM. Low-dose prednisolone/chlorambucil therapy in patients with severe membranous glomerulonephritis. Clin Invest 1994;72:277-282.
20. Bone JM, Rustom R, Williams PS. "Progressive" versus "indolent" idiopathic membranous glomerulonephritis. Q J Med 1997;90:699-706.

Chapter 9

Short- and Long-term Efficacy of Oral Cyclophosphamide and Steroids in Patients with Membranous Nephropathy and Renal Insufficiency.

Amanda J.W. Branten
and Jack F.M. Wetzels
for the Study group.

Department of Medicine, Division of Nephrology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands.

Clinical Nephrology 2001; 56:1-9

ABSTRACT

Background: Up to half of the patients with idiopathic membranous nephropathy (iMN) will develop renal failure. Preferably, immunosuppressive treatment should be restricted to patients at risk for the development of end-stage renal disease. However, the evidence that immunosuppressive treatment is effective in patients with iMN and renal insufficiency is weak and based on few studies with short follow-up in a limited number of patients.

Methods: We have analyzed the efficacy of immunosuppressive treatment in a large number of patients with membranous nephropathy and renal insufficiency. Since 1991, we have prospectively treated 39 patients (31 M, 8 F) with membranous nephropathy and evidence of deterioration of renal function. Treatment consisted of oral cyclophosphamide, 1.5-2.0 mg/kg body weight for 12 months, and corticosteroids for 6 months. At regular intervals blood pressure, serum creatinine, serum albumin, and proteinuria were measured. Adverse events were recorded.

Results: Average follow-up is 32 months (range 6-104), 18 patients have been followed for more than three years. Mean age of the patients was 55±12 years. In the six months before start of therapy, serum creatinine increased from 150±74 to 226±108 µmol/l. After start of treatment renal function rapidly improved, serum creatinine at 12 months averaging 143±62 µmol/l. Proteinuria decreased from 10.3±4.9 g/10 mmol creatinine at baseline to 2.2±2.4 g/10 mmol creatinine at month 12. These initial favorable effects have persisted. Overall, 12 patients have developed a complete remission of proteinuria (persistent in 11), and an additional 19 have developed a partial remission of proteinuria (persistent in 15). Thus far, only one treated patient has developed end-stage renal disease. Side effects are a major drawback of the treatment, with 7 patients being admitted, mainly for the treatment of infectious complications.

Conclusions: Cyclophosphamide is effective in the treatment of patients with idiopathic membranous nephropathy and deterioration of renal function. The favorable effects are maintained well beyond the one-year treatment period. Therefore, we propose that in patients with iMN immunosuppressive therapy can be restricted to patients at high risk for end-stage renal disease.

INTRODUCTION

About 40% of patients with idiopathic membranous nephropathy (iMN) and nephrotic range proteinuria will finally develop end-stage renal disease (ESRD). In their large placebo-controlled, randomized study, Ponticelli *et al.* showed that treatment with a combination of chlorambucil and steroids improved renal survival and increased the number of complete and partial remissions of proteinuria [1]. This favorable outcome was still present after a follow-up of 10 years [2]. More recently, the same investigators have documented that cyclophosphamide is as effective as chlorambucil [3]. In these Italian studies, treatment was initiated in patients with iMN at an early stage. Most patients had normal renal function and a short duration of the disease. Since 33% of their untreated patients spontaneously developed a complete of partial remission of proteinuria, the treatment policy as adopted by the Italian investigators can been criticized for being too aggressive. Several recently published reports recommend restricting immunosuppressive treatment to patients with iMN who are at high risk for the development of ESRD [4,5]. However, the long-term efficacy of immunosuppressive treatment in patients with iMN and renal insufficiency remains to be proven.

The short-term efficacy of immunosuppressive therapy has been suggested in a number of small, non-randomized studies, in which various (combinations of) drugs were used such as chlorambucil, cyclophosphamide, cyclosporine or azathioprine [6-16]. Over the last 15 years we have adopted a policy restricting immunosuppressive drugs to patients with iMN and evidence of deterioration of renal function. We initially have used corticosteroids combined with either chlorambucil for 6 months or cyclophosphamide for 12 months. When comparing these drug regimens we observed that cyclophosphamide was better tolerated and on the short-term more effective than chlorambucil [17]. Ever since, a cyclophosphamide based regimen has become our treatment of choice in patients with iMN and renal insufficiency. Patients eligible for this therapy have been prospectively studied. Therefore, we are now able to present data on the long-term efficacy of cyclophosphamide treatment in our patient group. Our data indicate that cyclophosphamide treatment is indeed effective in patients with iMN and renal insufficiency.

METHODS

Since 1986, we have prospectively treated patients with biopsy-proven iMN and renal insufficiency with immunosuppressive drugs. From 1986 till 1993, we have used a

combination of chlorambucil and corticosteroids [15]. In 1991 we started to use a combination of oral cyclophosphamide and steroids, and this treatment has been the treatment of choice after a comparison of the data indicated that cyclophosphamide was more effective and better tolerated [17].

Our treatment regimen has been described in detail before [17]. Briefly, cyclophosphamide was administered orally during one year in a target dose of 1.5 to 2 mg/kg body weight per day. The majority of patients received intravenous pulses of methylprednisolone, 1 g each on 3 consecutive days at the beginning of the first, third, and fifth month, and oral prednisone 0.5 mg/kg bodyweight every other day for 6 months. As methylprednisolone was initially not included in the treatment regimen the first 7 patients only received oral prednisone in a different schedule of 125 mg every other day, or 60 mg/day for at least 8 weeks [17].
Eligible patients had to present with evidence of deterioration of renal function defined as a serum creatinine of >130 µmol/l, an increase of the serum creatinine >50%, or a calculated Ccr<70 ml/min. Previous immunosuppressive treatment was not an exclusion criterion. Renal biopsies were not repeated before start of therapy. Clinical examinations, biochemical profiles and full blood counts were done every one or two weeks during the first months of treatment, and at regular intervals thereafter. Blood pressure was repeatedly measured by use of a mercury sphygmomanometer. The presence of hypertension was defined as a systolic blood pressure ≥140 mm Hg and/or a diastolic blood pressure ≥90 mm Hg. The use of antihypertensive drugs, diuretics, ACE inhibitors, angiotensin II receptor blockers and lipid lowering drugs was allowed during the study period as required.
In the present study we have analyzed the effects of cyclophosphamide therapy in patients who have been treated and followed for at least 6 months.

Calculations and statistics

The Ccr was calculated according to the standard formula Ucr x V/Pcr, in which Ucr reflects the urinary creatinine concentration and V the volume of a 24-hour urine sample, and Pcr the serum creatinine level. The reciprocal of serum creatinine (1000/serum creatinine level) has been used to assess the effects of treatment on the progression of renal insufficiency. Changes in this ratio parallel changes in Ccr. For the calculations, 1000 µmol/l was used as the serum creatinine level in patients on renal replacement therapy. To correct for incomplete 24-hour urine collections during the follow-up, the amount of urinary protein was adjusted for the amount of urinary creatinine (protein-creatinine index). A complete remission of proteinuria was defined as a reduction of the protein-creatinine index to less then 0.2 g/10 mmol creatinine, and a partial remission as a protein-creatinine index between 0.2 and 2.0

g/10 mmol creatinine. Patients with a stable remission were defined as those having a remission at the end of follow-up.

For descriptive statistics, results are given as means ±SD, or medians and range when appropriate. Changes in biochemical parameters were analyzed with repeated measures ANOVA, with adjustment for multiple comparisons by Bonferroni correction. Probabilities of survival were calculated by the Kaplan-Meier method. A Cox regression analysis was performed to examine if a specific set of variables could predict the occurrence of remission of proteinuria. To select variables worth including as co-variates in the regression analysis, initially variables were tested univariate using Kaplan Meier survival curves. The log-rank test was used to compare the survival curves. The following variables were considered relevant: sex, age, the slope of the plotted reciprocal of serum creatinine in the 6 months previous to start of treatment, previous immunosuppressive treatment, serum creatinine level, serum albumin level, and proteinuria at start of treatment, blood pressure during the year of treatment, use of an ACE inhibitor or angiotensin II receptor blockers during at least 80% of the time period before reaching a partial remission of proteinuria, and total dose of cyclophosphamide. Cox regression analysis was restricted to variables with a P-level <0.20. For the performance of the test a forward-step procedure was used, in which the cut-off value to be entered in the equation was defined as <0.10. A P-value <0.05 was considered significant. All statistical procedures were done using SPSS software (SPSS version 9.0, Chicago, Illinois).

RESULTS

From January 1991 until April 1999, 46 patients with iMN and renal failure came to our attention. Two patients refused immunosuppressive treatment. Five patients were treated with chlorambucil in the period 1991-1993 as part of a controlled study [17]. The remaining 39 patients that have received cyclophosphamide form the basis of this report. In 18 patients the duration of the follow-up has exceeded 36 months. Baseline characteristics of the patients are summarized in Table 1. Five patients were previously treated with steroids only, another 4 with a combination of chlorambucil and steroids, one patient with cyclosporine A, and one patient was previously treated with a short course of cyclophosphamide because of severe proteinuria with absence of renal failure. In all these 11 patients, deterioration of renal function occurred that led us to consider cyclophosphamide treatment. The median interval between the end of the previous treatment and the start of cyclophosphamide therapy was 30 months (range 8 – 133). At baseline, 27 patients were hypertensive, and 26 patients were on treatment with an ACE inhibitor or angiotensin II receptor antagonist. All patients had

evidence of deterioration of renal function. In the 6 months before the start of treatment, serum creatinine levels increased from 150±74 µmol/l to 226±108 µmol/l. In all patients but one, proteinuria exceeded the nephrotic level of 3.5 g/10 mmol creatinine.

Table 1. Baseline characteristics of the patients

	All patients	**Patients followed for >36 months**
Number *(N)*	39	18
Sex *(M/F)*	31/8	16/2
Age *(yrs)*	55 ± 12	53 ± 13
Time from kidney biopsy to start of treatment *(months)*	11 (0 – 158)	14 (1 – 158)
Previous immunosuppressive treatment *(N)*	11	9
Blood pressure *(mm Hg)*		
Systolic	149 ± 26	149 ± 25
Diastolic	84 ± 10	87 ± 10
Proteinuria *(g/10 mmol creatinine)*	10.3 ± 4.9	10.7 ± 5.9
Ccr *(ml/min*)	45 ± 21	42 ± 23
Follow-up *(months)*	32 (6 - 104)	49 (37 - 104)

Values are given as means ± SD or median (range).

After start of the immunosuppressive treatment the serum creatinine level decreased (n=37) or stabilized (n=2) in all patients (Table 2 and Figure 1). The decrease of serum creatinine became notable within one month after start of treatment and the lowest level was reached within the first 6 months in 35 patients.

Table 2. Short-term effects of cyclophosphamide on renal function and proteinuria

	0 months (n=39)	**3 months (n=39)**	**6 months (n=39)**	**12 months (n=35)**
Serum creatinine *(µmol/l)*	226 ± 108	145 ± 66*	142 ± 64*	143 ± 62*
Serum albumin *(g/l)*	23 ± 7	29 ± 6*	33 ± 6*#	38 ± 5*#
Proteinuria *(g/10 mmol creatinine)*	10.3 ± 4.9	4.9 ± 3.9*	3.2 ± 2.8*#	2.2 ± 2.4*#

Values are means ± SD. Treatment was started at 0 months. *P<0.01 vs. 0 months,
#P<0.01 vs. month 3.

The median decrease in serum creatinine was 38% (9-67%). Subanalysis in patients with a follow up of > 36 months revealed a stable improvement of renal function on the long-term as depicted in Figure 1. Serum creatinine levels in this subgroup amounted 277±125, 168±75, 183±87, and 187±95 µmol/l at month 0, 12, 24, and 36, respectively. Thus far, serum creatinine has increased by more than 50% over the lowest value in 4 cyclophosphamide-treated patients, and only one patient has developed end-stage renal disease.
Treatment also resulted in a significant decline in proteinuria, and an increase of the serum albumin level (Table 2). The effects were stable during prolonged follow up (Figure 2).

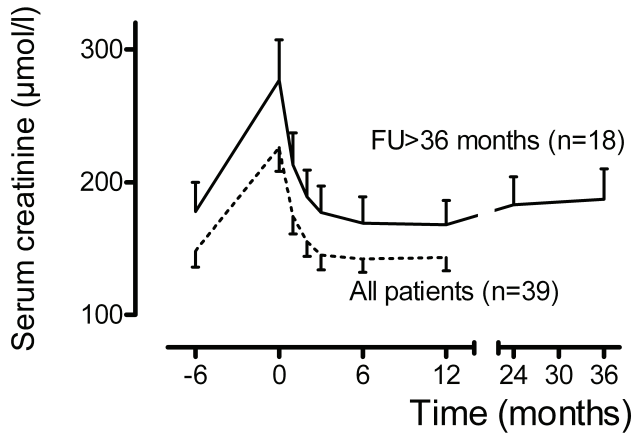

Figure 1. Serum creatinine levels before, during and after immuno-suppressive treatment of all patients (n=39, dashed line), and of the subgroup of patients with a follow-up >36 months (n=18, straight line). Cyclophosphamide was administered from month 0 to month 12. In both groups the creatinine levels from month 3 onward significantly differ from the levels at month 0 (P<0.01)

In the 18 patients with follow up >36 months, levels of proteinuria and serum albumin at month 36 amounted to 1.6±2.1 g/10 mmol creatinine and 40±7 g/l, respectively.

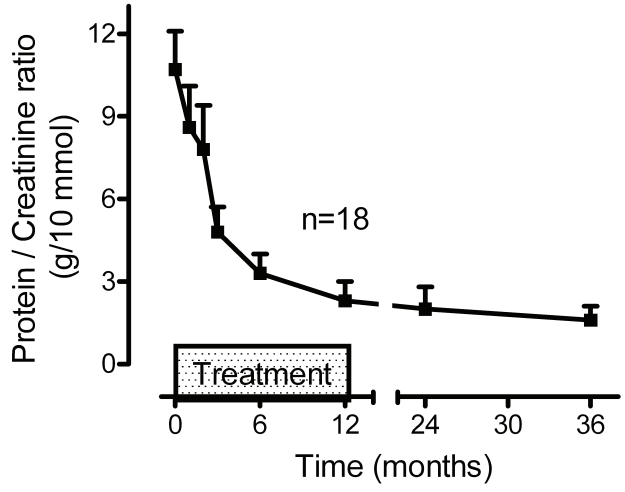

Figure 2. The effects of immunosuppressive treatment of proteinuria in patients with a follow-up of >36 months (n=18). Proteinuria is corrected for creatinine levels in the urine and expressed as g per 10 mmol creatinine. All levels from month 3 onward are significantly lower than baseline levels (P<0.01).

Partial remission of proteinuria occurred in 31 of the treated patients, and 12 patients further improved to complete remission. The cumulative remission rates are depicted in Figure 3.

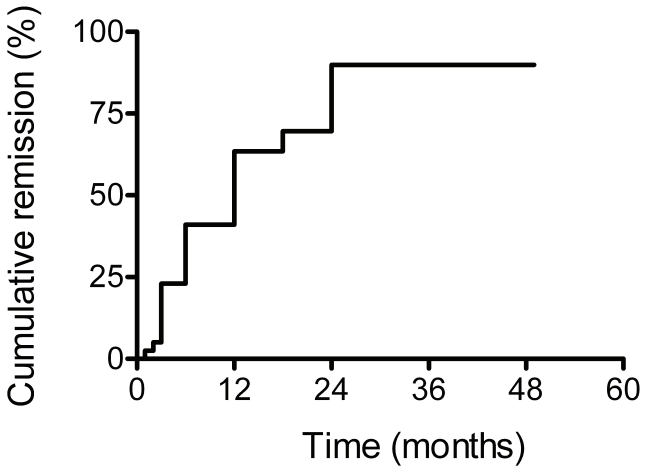

Figure 3. Cumulative incidence of partial remission of proteinuria (i.e. proteinuria ≤2g / 10 mmol creatinine) in patients treated with cyclophosphamide.

The median interval between start of treatment and development of partial or complete remisson was 12 months (range 1-24) and 21 months (range 2-36), respectively. In all patients complete remission was reached within one year after the onset of partial remission. At the end of follow-up 26 patients were still in remission, including 11 patients with a complete remission. In 7 patients relapses of proteinuria (defined as proteinuria > 2.0 g/10 mmol creatinine) were noticed (overall relapse rate 23%); two of these came in remission for a second time. In general, in patients who relapsed, remissions had been of short duration (<1 year). The occurrence of a relapse was not related to the length of the interval from start of therapy to the time when remission was reached: relapses were noticed in patients with early as well as late remissions.

We have evaluated whether the outcome with respect to the development of remissions could be predicted. All variables tested in univariate analysis are listed in Table 3. The likelihood of developing a partial remission of proteinuria tended to be higher in patients with lower levels of proteinuria and lower diastolic blood pressure. In the Cox regression analysis no factor really proved significant. In patients with a stable remission the likelihood of renal failure was significantly less than in patients with persistent or relapsing proteinuria (0% vs 33% $p<0.01$).

Table 3. Results of univariate survival analysis: the influence of several parameters on remission of proteinuria

	Values	N	Remission at 36 months (%)	P
Sex	M	30	95	0.83
	F	8	75	
Age *(years)*	< 59	18	91	0.30
	≥ 59	20	92	
1000/S.creatinine *(µmol.l^{-1}.yr^{-1})*	< -1.7	17	87	0.21
	≥ -1.7	17	92	
Previous immunosuppressive therapy (*N)*	Yes	10	90	0.99
	No	28	93	
S.creatinine at start of therapy *(µmol/l)*	< 184	19	100	0.22
	≥ 184	19	89	
S.albumin at start of therapy *(g/l)*	< 23	19	86	0.86
	≥ 23	19	94	
Proteinuria *(g /10 mmol creat)*	< 10	21	100	0.09
	≥ 10	17	78	
SBP year 1 *(mm Hg)*	< 134	19	89	0.70
	≥ 134	16	92	
DBP year 1 *(mm Hg)*	< 80	17	100	0.06
	≥ 80	18	83	
ACE-inhibitor *(N)*	Yes	24	94	0.16
	No	14	88	
Dosage cyclophosphamide *(mg/Kg BW/day)*	< 1.35	20	83	0.73
	≥ 1.35	18	100	

One patient is not included because he had a proteinuria at start of treatment of < 2.0 g/10 mmol creatinine. 1000/S.creatinine represents deterioration of renal function in the 6 months preceding start of treatment. S=serum, SBP=systolic blood pressure, DBP=diastolic blood pressure. Systolic and diastolic blood pressures are the means of the measurements during the first year of treatment. ACE-inhibitor is the use of an ACE-inhibitor / AIIA receptor blocker during >80% of the time untill the remission of proteinuria is achieved.

During treatment all adverse events were registered. The majority of patients experienced side effects, which resulted in therapy interruption in 12 patients (Table 4).

Table 4. Therapy related side-effects

	Number of patients (N)
Without side effects	15
> 1 side effect	8
Leukopenia	10 (7 / 0)
Anemia	5 (0 / 0)
Infections: Respiratory	7 (3 / 5)
Oral candidiasis	1 (0 / 0)
Herpes Zoster	1 (1 / 0)
Bursitis	1 (0 / 0)
Campylobacter	1 (0 / 0)
Febris e.c.i.	1 (0 / 1)
Nausea/Vomiting/Malaise	6 (6 / 1)

Numbers in brackets represent patients in which the side effect necessitated interruption of treatment / hospital admission.

Therefore, the calculated daily dose of cyclophosphamide used throughout the treatment period was slightly lower than the target dose, and the median dose amounted 1.4 mg/kg/day (range 0.5 – 2.2). Seven patients were admitted mostly for treatment of infections; however no serious, life-threatening events were observed. Thus far, malignancies have not been observed in any of the patients.

DISCUSSION

Our descriptive study is the largest reported thus far on immunosuppressive treatment in patients with idiopathic MN and renal insufficiency. The data clearly indicate that treatment with cyclophosphamide and steroids is effective in inducing a remission of proteinuria, and a stable improvement of renal function in the majority of such patients. Thus far, only one patient has developed end-stage renal disease and calculated renal survival at 5 years after start of treatment is 89%. Although our study was not controlled, we feel that the data strongly suggest that patients with iMN and renal failure can benefit from immunosuppressive

treatment. This conclusion is supported by literature data that indicate that patients with iMN who present with renal function deterioration have an unfavorable prognosis [12,18-24]. In such patients, development of end-stage renal disease is the rule, with less than 10% of the patients demonstrating a spontaneous improvement [18,24].

Average blood pressures in our patients exceeded the value of 125/75 mm Hg, the current target value for patients with overt proteinuria. Nowadays we strongly advocate such blood pressures in order to attenuate deterioration of renal function. However, we feel that it is highly unlikely that the improvement of renal function that we observed in our patients could have been obtained merely by more aggressive antihypertensive treatment. Moreover, in about a quarter of our patients renal function decreased despite blood pressure values below 125/75 mmHg.

The present data support and extent our previous observations on the short-term efficacy of cyclophosphamide in the treatment of patients with iMN [17]. In our latter study we observed that the cyclophosphamide schedule was more effective and better tolerated than the chlorambucil regimen. However, the number of patients included in our previous study was small and follow-up was limited. Likewise, most data on the effects of immunosuppressive treatment in patients with iMN and renal insufficiency in the literature come from small, non-randomized studies (reviewed in [17]). Most studies have suggested that both cyclophosphamide and chlorambucil can attenuate deterioration of renal function [6-11,15-17]. When analyzing these literature data, we observed a significant difference in the remission rate of proteinuria in favor of the use of cyclophosphamide. Also, the use of chlorambucil was associated with a higher incidence of side effects [15,17].

The efficacy of cyclosporine in the treatment of patients with iMN and renal insufficiency has been evaluated in one controlled study [12]. Cyclosporine significantly decreased the rate of renal function deterioration. However, the number of treated patients was small (n=9), and in none of the patients was a sustained remission of proteinuria or real improvement of renal function observed. The latter findings certainly are in contrast with our experience with cyclophosphamide. Also, the lack of data in larger patients groups and the well-known nephrotoxicity of cyclosporine argue against the routine use of cyclosporine in patients with iMN and renal insufficiency.

Data on the efficacy of azathioprine in patients with iMN and renal insufficiency are equivocal. In 3 studies, positive effects of azathioprine on renal function and proteinuria in patients with moderate to severe renal impairment were reported [14,25,26]. In most patients an improvement of renal function was observed, and at the end of the follow-up few patients still had proteinuria in the nephrotic range. These favorable effects of azathioprine have – in one recent study - not been confirmed [27]. In the latter study a comparison was made between treated and untreated patients. However, patients were not randomized, and the

control group had better renal function and lower proteinuria (although no significant differences were noted). Most importantly, therapy was not restricted to patients with deterioration of renal function. The inclusion of patients with a good prognosis, even if left untreated, certainly makes it difficult to find differences in small sized studies.

To determine if immunosuppressive therapy can be restricted to patients with evidence of renal function deterioration, our data must be compared to the results obtained by the Italian group of investigators, who have unequivocally demonstrated that immunosuppressive therapy improves renal survival in patients with iMN [1,2,24]. As noted before, in the Italian studies all patients with iMN were treated at a time when their renal function was still normal. In the initial studies, immunosuppressive treatment consisted of a combination of chlorambucil and prednisone [1]. Recently, similar results were obtained with a combination of cyclophosphamide and prednisone [3]. Duration of treatment was 6 months, and the cumulative dose of cyclophosphamide amounted 17 g. Notably, in the latter study the total number of side effects was less in the cyclophosphamide-treated group, thus supporting our data in patients with renal impairment [17]. In this study, 43 patients with iMN were treated with cyclophosphamide and prednisone and followed for 42 months (range 12 – 72 months) [3]. The cumulative incidence of a first partial remission of proteinuria amounted 93%, with 37% of patients entering a complete remission. The relapse rate was 24%. Two patients (5%) have developed end-stage renal disease. Data after a 10-year follow-up are available for patients treated with chlorambucil and prednisone [2]. Cumulative incidence of remission was 88%, and 10-year renal survival was 92%. We feel that our figures in patients with renal insufficiency compare favorably, with a calculated remission rate of 92% at 5 years and a renal survival of 89%. Although we have no formal renal survival data of 10 years' follow-up, we feel that the prospects are good since the majority of patients has developed a stable remission of proteinuria. Our present data as well as other studies indicate that achievement of a stable remission is associated with a good prognosis [28,29]. Side effects are a major draw-back of our cyclophosphamide therapy. Obviously, the most dreaded side effect is development of malignancies, in particular bladder carcinoma. The development of such malignancies is dose-dependent and has been mainly reported in patients with Wegener's granulomatosis who had received a cumulative dose of more than 100 g, or were treated for more than a year with a daily dose of 2.0 mg/kg BW [30,31]. In patients with non-Hodgkin's disease, an increased risk for bladder malignancies has been observed with a lower cumulative dosage of 20-50 g. However, these patients were simultaneously treated with other chemotherapeutics and radiation [32]. In our cyclophosphamide schedule we remain far below the dosage of 100 g (e.g. bodyweight 75 kg; $\leq$55 g). The most noted side effects were leukopenia, anemia and infections. In many patients it was necessary to reduce the cyclophosphamide dose or to temporarily interrupt treatment. Also, several patients needed

in-hospital treatment for infectious problems. Apparently, side-effects are encountered more frequently when using immunosuppressive drugs in patients with renal insufficiency [15]. Several approaches can be taken to reduce the number of side-effects. First, we currently have introduced once daily cotrimoxazole as prophylaxis for infectious complications. Second, one could consider reducing the dosage of cyclophosphamide treatment in line with the above mentioned schedule designed by Ponticelli *et al.* [3]. Future studies need to assess the efficacy of a lower dose cyclophosphamide in patients with iMN and renal failure. Third, side effects may occur less frequently if treatment is started earlier. Patients at risk for the development of ESRD can be identified at an early stage by using the proteinuria criteria of Pei *et al.* [33], or by quantitating urinary IgG or β2-microglobulin excretion [34,35].

In conclusion: combination therapy of oral cyclophosphamide and steroids is effective in patients with iMN and renal insufficiency. Treatment results in persistent improvement of renal function and remission of proteinuria. We recommend using immunosuppressive treatment only in patients with iMN and evidence of renal dysfunction.

Acknowledgements

A. Branten is supported by a grant from the Dutch Science Foundation (NWO-MW 920-03-038).

The authors thank all colleagues who have participated in this study: Drs P. Bleeker, Hospital Rivierenland Tiel; F. Bosch, R. van Leusen and K. Parlevliet, Hospital Rijnstate Arnhem; M. ten Dam and I. Go, Canisius Wilhelmina Hospital Nijmegen; G. Feith and M. den Hartog, Hospital Gelderse Vallei Wageningen; L. Frenken, Hospital De Wever Heerlen; P. Gerlag and A. van den Wall Bake, St Joseph Hospital Veldhoven; D. de Gooyer and H. van Roermund, St. Franciscus Hospital Roosendaal; P. Hillen, A. Apperloo and W. Rensma, St. Elizabeth Hospital Tilburg; D. Hollander, J. Jansen and M. Koolen, Bosch Medicentrum 's Hertogenbosch; A. Lieverse, Diaconessen Hospital Eindhoven; R. Smeets, St. Anna Hospital Geldrop; P. Spooren, TweeSteden Hospital Tilburg; R. Stuyver, Hospital Velp; J. Wirtz and W. Grave, St. Laurentius Hospital Roermond; J. Berden and H. van Hamersvelt, University Medical Center Nijmegen.

REFERENCES

1. Ponticelli C, Zuchelli P, Imbasciati E *et al*. Controlled trial of methylprednisolone and chlorambucil in idiopathic membranous nephropathy. New Engl J Med 1984;310:946-950.
2. Ponticelli C, Zuchelli P, Passerini P *et al*. A 10-year follow-up of a randomized study with methylprednisolone and chlorambucil in membranous nephropathy. Kidney Int 1995;48:1600-1604.

3. Ponticelli C, Altieri P, Scolari F *et al*. A randomized study comparing methylprednisolone plus chlorambucil versus methylprednisolone plus cyclophosphamide in idiopathic membranous nephropathy. J Am Soc Nephrol 1998;9:444-450.
4. Cattran DC. Cytotoxics, cyclosporine and membranous nephropathy. Curr Opin Nephrol Hypertens 1996;5:427-436.
5. Muirhead N. Management of idiopathic membranous nephropathy: evidence-based recommendations. Kidney Int 1999;55 (Suppl. 70):S-47-S-55.
6. Warwick GL, Geddes CG, Boulton-Jones JM. Prednisolone and chlorambucil therapy for idiopathic membranous nephropathy with progressive renal failure. Q J Med 1994;87:223-229.
7. Mathieson PW, Turner AN, Maidment CG, Evans DJ, Rees AJ. Prednisolone and chlorambucil treatment in idiopathic membranous nephropathy with deteriorating renal function. Lancet 1988;2:869-872.
8. Brunkhorst R, Wrenger E, Koch KM. Low-dose prednisolone/chlorambucil therapy in patients with severe membranous glomerulonephritis. Clin Invest 1994;72:277-282.
9. Bruns FJ, Adler S, Fraley DS, Segel DP. Sustained remission of membranous glomerulonephritis after cyclophosphamide and prednisone. Ann Intern Med 1991;114:725-730.
10. Jindal K, West M, Bear R, Goldstein M. Longterm benefits of therapy with cyclophosphamide and prednisone in patients with membranous glomerulonephritis and impaired renal function. Am J Kidney Dis 1992;19:61-67.
11. Faedda R, Satta A, Bosincu L, Pirisi M, Bartoli E. Immune suppressive treatment of membranous glomerulonephritis. J Nephrol 1995;8:107-112.
12. Cattran DC, Greenwood C, Ritchie S *et al*. A controlled trial of cyclosporine in patients with progressive membranous nephropathy. Kidney Int 1995;47:1130-1135.
13. Williams PS, Bone JM. Immunosuppression can arrest progressive renal failure due to idiopathic membranous glomerulonephritis. Nephrol Dialysis Transplant 1989;4:181-186.
14. Brown JH, Alistair FD, Murphy BG *et al*. Treatment of renal failure in idiopathic membranous nephropathy with azathioprine and prednisolone. Nephrol Dialysis Transplant 1998;13:443-448.
15. Wetzels JFM, Reichert LJM. Efficacy of immunosuppressive treatment in patients with membranous nephropathy and renal insufficiency. Kidney Int 1997;52 Suppl 61:S63-S66.
16. Reichert LJM, Huysmans FThM, Assmann K, Koene RAP, Wetzels JFM. Preserving renal function in patients with membranous nephropathy: daily oral chlorambucil compared with intermittent monthly pulses of cyclophosphamide. Ann Intern Med 1994;121:328-333.
17. Branten AJW, Reichert LJM, Koene RAP, Wetzels JFM. Oral cyclophosphamide versus chlorambucil in the treatment of patients with idiopathic membranous nephropathy and renal insufficiency. Q J Med 1998;91:359-366.
18. Davison AM, Cameron JS, Kerr DN, Ogg CS, Wilkinson RW. The natural history of renal function in untreated idiopathic membranous glomerulonephritis in adults. Clin Nephrol 1984;22:61-67.

19. Noel LH, Zanetti M, Droz D. Long-term prognosis of idiopathic membranous glomerulonephritis. Am J Med 1979;66:82-90.
20. Erwin DT, Donadio JV, Holley KE. The clinical course of idiopathic membranous nephropathy. Mayo Clin Proc 1973;48:697-712.
21. Murphy BF, Fairley KF, Kincaid-Smith PS. Idiopathic membranous glomerulonephritis: long-term follow-up in 139 cases. Clin Nephrol 1988;30:175-181.
22. Donadio JJV, Torres VE, Velosa JA *et al*. Idiopathic membranous nephropathy: The natural history of untreated patients. Kidney Int 1988;33:708-715.
23. Collaborative study of the adult idiopathic nephrotic syndrome. A controlled study of short-term prednisone treatment in adults with membranous nephropathy. New Engl J Med 1979;301:1301-1306.
24. Ponticelli C, Zuchelli P, Passerini P *et al*. A randomized trial of methylprednisolone and chlorambucil in idiopathic membranous nephropathy. New Engl J Med 1989;320:8-13.
25. Bone JM, Rustom R, Williams PS. "Progressive" versus "indolent" idiopathic membranous glomerulonephritis. Q J Med 1997;90:699-706.
26. Baker LRI, Tucker B, Macdougall IC. Treatment of idiopathic membranous nephropathy. Letter. Lancet 1994;343:290-291.
27. Ahuja M, Goumenos D, Shortland JR, Gerakis A, Brown CB. Does immunosuppression with prednisolone and azathioprine alter the progression of idiopathic membranous nephropathy? Am J Kidney Dis 1999;34:521-529.
28. Passerini P, Pasquali P, Cesana B, Zucchelli P, Ponticelli C. Long-term outcome of patients with membranous nephropathy after complete remission of proteinuria. Nephrol Dialysis Transplant 1989;4:525-529.
29. Hunt LP, Short CD, Mallick NP. Prognostic indicators in patients presenting with the nephrotic syndrome. Kidney Int 1988;34:382-388.
30. Talar-Williams C, Hijazi YM, Walther MM *et al*. Cyclophosphamide-induced cystitis and bladder cancer in patients with Wegener Granulomatosis. Ann Intern Med 1996;124:477-484.
31. Westman KWA, Bygren PG, Olsson H, Ranstam J, Wieslander J. Relapse rate, renal survival, and cancer morbidity in patients with Wegener's granulomatosis or microscopic polyangiitis with renal involvement. J Am Soc Nephrol 1998;9:842-852.
32. Travis LB, Curtis RE, Glimelius B *et al*. Bladder and kidney cancer following cyclophosphamide therapy for non-hodgkin's lymphoma. J Natl Cancer Inst 1995;87:524-530.
33. Pei Y, Cattran DC, Greenwood C. Predicting chronic renal insufficiency in idiopathic membranous glomerulonephritis. Kidney Int 1992;42:960-966.
34. Reichert LJM, Koene RAP, Wetzels JFM. Urinary excretion of β2-microglobulin predicts renal outcome in patients with idiopathic membranous nephropathy. J Am Soc Nephrol 1995;6:1666-1669.
35. Reichert LJM, Koene RAP, Wetzels JFM. Urinary IgG excretion as a prognostic factor in idiopathic membranous nephropathy. Clin Nephrol 1997;48:79-84.

Chapter 10

Summary and Conclusions

SUMMARY AND CONCLUSIONS

Proteinuria is a strong predictor of progressive renal insufficiency. The precise mechanisms to explain this relationship are still unknown, although many experimental studies have shown that proteinuria may induce tubulo-interstitial injury. Many investigators have observed that tubulo-interstitial injury better reflects renal insufficiency than glomerular injury. Indeed, in pilot-studies we have demonstrated that the urinary excretion of the low molecular weight protein β2-microglobulin, a marker of tubulo-interstitial injury, predicts the development of chronic renal insufficiency in patients with idiopathic membranous nephropathy. It remains unknown which components in proteinuric urine are responsible for the induction of tubulo-interstitial injury. Identification of these components should enable to develop more specific therapeutic interventions and to identify patients at risk for chronic renal insufficiency with higher specificity and at an earlier stage. Therefore, we have initiated studies in patients with proteinuria to evaluate tubulo-toxic injury and possible mediators. We specifically have focussed on patients with membranous nephropathy, the most common cause of the nephrotic syndrome in adults. In these patients we also have evaluated the efficacy of immunosuppressive therapy.

In chapter 2 we describe the course of a familial nephropathy in a mother and her two daughters. In all three family members the proteinuria was discovered in early childhood. The mother had a persistent proteinuria, exceeding the nephrotic level of 3.5 g/day for more than 20 years despite treatment with several immunosuppressive drugs. Likewise, in both daughters treatment with prednisone was ineffective. In view of the relation between proteinuria and tubular cell damage we expected that the persistent nephrotic range proteinuria should have led to the development of tubulo-interstitial damage and loss of renal function. Remarkably, in these patients there was no evidence of tubulo-interstitial injury, as expressed by the low excretion of β2-microglobulin, and all maintained normal renal function. Analysis of the proteinuria revealed that the patients had a very selective proteinuria, albumin being the main constituent of urinary proteins. Based on the observations in this family we can conclude that urinary albumin per se does not cause renal tubular cell damage.

In chapter 3 data are presented of a study that was aimed at analysing the site of tubular injury in patients with a nephrotic syndrome. Most markers that have been used to assess tubular cell injury provide solely information on proximal tubular cells or do not allow

differentiating between various tubular segments. Glutathione S transferases (GST) are cytosolic enzymes involved in the metabolism of glutathione. Several iso-forms are known, with different distribution in the kidney. GST alpha is mainly present in proximal tubular cells, whereas GST pi is found in distal tubular cells and collecting ducts. We have measured the urinary excretion of both GST alpha and GST pi in 56 patients with glomerular diseases and proteinuria. The excretion of both enzymes was elevated compared to values measured in healthy controls. The urinary excretion of GST pi was inversely correlated with the creatinine clearance, a measure of renal function. The highest levels of GST alpha were found in patients with well-preserved renal function. Histological studies confirmed that in these patients the GST alpha staining in the proximal tubules was decreased. The data suggest that in patients with proteinuria initial injury is localized in the proximal tubules as reflected by the increased excretion of GST alpha. However, loss of renal function is characterized by increased injury of the distal tubules, as reflected by increased excretion of GST pi. Longitudinal studies are needed to confirm this time course of tubular cell injury.

Based on experimental data we hypothesized that complement activation may have a prominent role in proteinuria-induced tubular cell injury. We questioned if measurement of C3d, a degradation product of complement C3, could provide a valuable marker of complement activation. As described in chapter 4 we performed a study to evaluate the urinary excretion of C3d in patients with various renal diseases. Theoretically, C3d in the urine can be derived from locally activated C3. However, urinary C3d excretion may also be governed by glomerular filtration of circulating C3d and decreased tubular reabsorption. To evaluate the tubular reabsorption of C3d we have measured urinary C3d in 8 patients with tubulo-interstitial nephritis (TIN). Patients with TIN have markedly injured tubular cells, which results in a decreased reabsorption of proteins. We have used urinary β2-microglobulin as a marker of the tubular reabsorption process. We observed that urinary C3d was highly correlated with urinary β2-microglobulin, thus indicating that C3d is indeed reabsorbed by the proximal tubular cells. We next measured urinary C3d in 79 patients with glomerular diseases and proteinuria. In these patients urinary C3d levels were clearly elevated. A mathematical analysis suggested that the increased C3d excretion in patients with proteinuria was the result of both an increased filtration as well as an increased local production of C3d.

In the next study, chapter 5, we evaluated the reliability of the creatinine clearance as marker of GFR in patients with nephrotic syndrome. In normal daily practice the creatinine clearance

is routinely used as marker of GFR. However, creatinine is not only filtered in the glomeruli, but also secreted by renal tubular cells. As a consequence creatinine clearance usually overestimates glomerular filtration, particularly in patients with impaired renal function. It is not known if the tubular handling of creatinine is changed in patients with nephrotic syndrome. Therefore we compared in 42 patients with proteinuria the creatinine clearance with the clearance of inulin, the golden standard of GFR. The data show that in our patients tubular secretion of creatinine was independently related to serum albumin levels. As a consequence, creatinine clearance overestimated GFR more markedly in patients with the lowest serum albumin levels. Thus, in patients with a recurrent nephrotic syndrome a decrease of GFR is not always reflected by an increase of serum creatinine.

In chapter 6 we describe the effect of albumin on the tubular reabsorption of β2-microglobulin (β2M). Most filtered proteins are reabsorbed by the renal proximal tubular cells after binding to ligands in the brush border membrane. The low molecular weight protein β2M is normally completely reabsorbed by the proximal tubular cells. Even subtle tubular cell injury is reflected by a decreased reabsorption and thus an increased urinary excretion of β2M. As such, urinary β2M excretion was proposed as marker of tubulo-interstitial injury and predictor of prognosis in patients with proteinuric renal diseases. However, it was undetermined if other urinary proteins could affect tubular reabsorption of β2M merely by competitive inhibition at the level of the brush border membrane. We have evaluated the possible role of albumin. To increase the tubular load, we administered 40 g albumin intravenously in 10 patients with a nephrotic syndrome and measured urinary β2M excretion. Although albumin excretion increased considerably, we did not observe major changes in urinary β2M excretion. Thus, in patients with a nephrotic syndrome urinary β2M can be reliably used as a marker of tubular injury.

Based upon data from pilot experiments we proposed urinary β2M and IgG as predictors of progressive renal failure in patients with idiopathic membranous nephropathy (iMN). Previously, high sensitivities and specificities, ranging from 80 to 90% were found, using a threshold level of 500 ng/min for β2M-excretion (Uβ2M), and 250 mg/day for the IgG-excretion (UIgG). In a validation study (chapter 7) we prospectively tested the accuracy of Uβ2m and UIgG to predict the renal outcome. We measured Uβ2M and UIgG in 57 patients with iMN, proteinuria and normal renal function, and followed these patients prospectively. At the end of follow-up 44% of the patients had reached the end point “renal death” which was

defined as a rise of serum creatinine of >50%, or a serum creatinine >135 mmol/l (1.5 mg/dl). Multivariate analysis confirmed that Uβ2M was the strongest independent factor to predict the development of renal insufficiency. Sensitivity and specificity were 88% and 91%, respectively, for Uβ2M, and both were 88% for UIgG. When the excretions of both proteins were combined, specificity improved to 97%. We conclude that these markers can be used to guide decisions on the start of immunosuppressive treatment.

There is no consensus with regard to the optimal treatment schedule in patients with idiopathic membranous nephropathy (iMN). In chapter 8 we have compared the efficacy of two treatment strategies in patients with iMN and renal insufficiency. Fifteen patients were treated with oral prednisone (0.5 mg/kg/day, month 1, 3 and 5) in combination with chlorambucil (0.15 mg/kg/day, month 2, 4 and 6), and 17 patients were treated with prednisone (0.5 mg/kg every other day during 6 months) and oral cyclophosphamide (1.5-2.0 mg/kg/day during 1 year). In both groups also 3 cycles of methylprednisolone were administered intravenously. The groups were comparable in age, renal function, and level of proteinuria at start of therapy. Renal function improved in both treatment groups, however the improvement was less sustained in the chlorambucil-group. One year after start of treatment mean serum creatinine was only 6.3 mmol/l below baseline in the chlorambucil-group as compared to 121 mmol/l lower in the cyclophosphamide-group ($P<0.01$). Four chlorambucil- and one cyclophosphamide-treated patients developed end-stage renal disease. Remissions of proteinuria (<2.0 g/10 mmol creatinine) were observed in 15 of the cyclophosphamide treated patients and in 5 chlorambucil treated patients ($P<0.01$). Of course, the better efficacy of the cyclophosphamide regimen could be explained by the longer duration of treatment. However, elongation of the treatment period with chlorambucil is hardly an option since in 11 of the 15 treated patients chlorambucil had to be stopped prematurely because of side effects. From these data we concluded that cyclophosphamide was better tolerated and more effective than oral chlorambucil.

In chapter 9 we present our extended experience with prednisone and cyclophosphamide treatment in 39 patients with idiopathic membranous nephropathy, proteinuria and renal insufficiency. The average follow-up was 32 months. Eighteen patients have been followed for more than three years. As described in detail above, all patients were treated with prednisone during 6 months and cyclophosphamide during 1 year. After start of treatment renal function rapidly improved: serum creatinine decreased from 226±108 mmol/l at baseline to 143±62 mmol/l after 12 months of treatment. This favourable effect was

maintained during the extended follow-up. In 31 patients a partial remission of proteinuria (<2.0 g/10 mmol creatinine) was noted, which in 12 patients further improved to a complete remission (<0.2 g/10 mmol creatinine). Thus far a relapse of proteinuria occurred in 7 patients. Side effects of immunosuppressive treatment occurred in the majority of the patients, however no serious life-threatening events were observed. Based on the results of this study we concluded that treatment with cyclophosphamide and steroids is effective both on the short- and long-term in patients with idiopathic membranous nephropathy and renal insufficiency. The high incidence of side effects supports our opinion to restrict this treatment to patients at risk for end-stage renal disease.

Chapter 11

Samenvatting en Conclusies

SAMENVATTING EN CONCLUSIES

Proteïnurie is een goede voorspeller van progressief nierfunctieverlies. Het hoe en waarom van deze relatie is niet geheel verklaard, alhoewel meerdere experimentele studies hebben aangetoond dat proteïnurie tubulo-interstitiële schade kan induceren. Diverse onderzoekers hebben vastgesteld dat tubulo-interstitiële schade een betere maat is voor nierinsufficiëntie dan glomerulaire schade. Daarmee in overeenstemming zijn de bevindingen van onze pilotstudies waaruit bleek dat de excretie van het laag moleculaire eiwit β2-microglobuline, een marker voor tubulo-interstitiële schade, de ontwikkeling van nierinsufficiëntie voorspelde bij patiënten met een idiopathische membraneuze nefropathie. Het is nog onbekend welke specifieke componenten in eiwitbevattende urine verantwoordelijk zijn voor het induceren van tubulo-interstitiële schade. Identificatie van deze componenten zou kunnen leiden tot de ontwikkeling van een meer gerichte behandeling. Ook wordt het dan mogelijk om al in een vroege fase van de ziekte met grote specificiteit die patiënten te identificeren die een verhoogd risico hebben om chronische nierinsufficiëntie te ontwikkelen. Daarom zijn we gestart met onderzoeken bij patiënten met proteïnurie met als doel tubulo-interstitiële schade te kwantificeren en de mogelijke mediatoren te identificeren. We hebben ons met name gericht op patiënten met een membraneuze nefropathie, de meest voorkomende oorzaak van het nefrotisch syndroom bij volwassenen. Bij deze patiënten hebben we tevens de effectiviteit van immunosuppressieve therapie geëvalueerd.

In hoofdstuk 2 beschrijven we het beloop van een familiaire nefropathie bij een moeder en haar twee dochters. Bij allen werd proteïnurie ontdekt op de vroege kinderleeftijd. De moeder had gedurende meer dan 20 jaar een proteïnurie van meer dan 3.5 g/dag ondanks behandeling met diverse immunosuppressieve middelen. Ook bij beide dochters bleek behandeling met prednison niet effectief. Vanwege de sterke relatie tussen proteïnurie en tubuluscelschade hadden we verwacht dat de persisterende proteïnurie zou hebben geleid tot de ontwikkeling van tubulo-interstitiële schade en verlies van nierfunctie. Opmerkelijk is echter dat deze patiënten geen tekenen hadden van tubulo-interstitiële schade, getuige de lage uitscheiding van β2-microglobuline. De nierfunctie was volstrekt normaal gebleven. Bij nadere analyse bleek dat er sprake was van een zeer selectieve proteïnurie, met albumine als de belangrijkste component van het eiwit in de urine. Op grond van onze bevindingen in deze familie kunnen we concluderen dat albumine in de urine niet zonder meer renale tubuluscelschade veroorzaakt.

In hoofdstuk 3 worden de gegevens gepresenteerd van een studie die als doel had het analyseren van de plaats van de tubulusschade bij patiënten met een nefrotisch syndroom. De meeste markers die worden gebruikt om tubulusschade te beoordelen geven alleen informatie over de proximale tubuluscellen, of maken het niet mogelijk om te differentiëren tussen verschillende tubulussegmenten. Glutathione S transferases (GST) zijn cytosolische enzymen die betrokken zijn bij het metabolisme van glutathion. Er zijn diverse isovormen bekend, met verschillende distributie in de nier. GST alpha is hoofdzakelijk aanwezig in de proximale tubuluscellen, terwijl GST pi beperkt is tot distale tubuluscellen en de verzamelbuizen. Wij hebben de uitscheiding in de urine van zowel GST alpha als GST pi gemeten bij 56 patiënten met glomerulaire nierziekten en proteïnurie. Zowel van GST alpha als GST pi was de uitscheiding verhoogd. De excretie van GST pi was omgekeerd evenredig gecorreleerd met de creatinine klaring, een maat voor de nierfunctie. De hoogste GST alpha waarden werden waargenomen bij patiënten met een goede nierfunctie. Histologisch onderzoek bevestigde dat bij deze laatstgenoemde patiënten de GST alpha kleuring in de proximale tubuluscellen was verminderd. De bevindingen suggereren dat in de beginfase bij patiënten met proteïnurie de schade is gelocaliseerd in de proximale tubuluscellen, met als gevolg een verhoogde uitscheiding van GST alpha. Echter, bij verlies van nierfunctie blijkt er meer sprake van schade van distale tubuluscellen, zoals gereflecteerd door de verhoogde uitscheiding van GST pi. Er zijn longitudinale studies nodig om dit beloop van tubuluscelschade in de tijd te bevestigen.

Op basis van gegevens verkregen uit experimenteel onderzoek lijkt het logisch te veronderstellen dat complementactivatie een prominente rol speelt bij proteïnurie-geïnduceerde tubuluscelschade. Wij stelden de vraag of C3d, een degradatieproduct van complement C3, in de urine een goede marker zou zijn voor complementactivatie. We hebben de uitscheiding van C3d in de urine bepaald bij patiënten met verschillende nierziekten (hoofdstuk 4). Theoretisch kan C3d in de urine terechtkomen door activatie van locaal aanwezig C3. C3d kan echter ook in de urine terecht komen door glomerulaire filtratie van C3d en/of een verminderde tubulaire reabsorptie. Om een indruk te krijgen van de tubulaire reabsorptie hebben we C3d gemeten in de urine van 8 patiënten met een tubulo-interstitiële nefritis (TIN). Patiënten met een TIN hebben evidente tubuluscelschade hetgeen leidt tot een verminderde tubulaire reabsorptie van eiwitten. Als marker voor het tubulaire reabsorptie proces hebben we de uitscheiding van β2-microglobuline gebruikt. De uitscheiding van C3d was sterk gecorreleerd met de uitscheiding van β2-microglobuline hetgeen suggereert dat C3d inderdaad wordt gereabsorbeerd door de tubuluscellen.

Vervolgens hebben we C3d gemeten in de urine van 79 patiënten met glomerulaire nierziekten en proteïnurie. In deze patiënten was de concentratie van C3d in de urine duidelijk verhoogd. De resultaten van een mathematische analyse suggereerden dat de toegenomen uitscheiding van C3d in de urine van patiënten met proteïnurie het resultaat was van zowel toegenomen filtratie als toegenomen locale productie van C3d.

In het volgende onderzoek, beschreven in hoofdstuk 5, hebben we de betrouwbaarheid van de creatinine-klaring als maat voor de GFR bestudeerd in patiënten met een nefrotisch syndroom. In de normale dagelijkse praktijk wordt de creatinine-klaring routinematig gebruikt als maat voor de GFR. Creatinine wordt echter niet alleen gefiltreerd door de glomeruli, maar ook gesecreteerd via de tubuluscellen. Het gevolg hiervan is dat de creatinine-klaring in het algemeen de GFR overschat, met name bij patiënten met een gestoorde nierfunctie. Het is niet bekend of het tubulaire transport van creatinine is veranderd in patiënten met een nefrotisch syndroom. Daarom hebben we bij 42 patiënten met proteïnurie de creatinine-klaring vergeleken met de inuline-klaring, de gouden standaard voor de GFR. De tubulaire secretie van creatinine bleek op onafhankelijke wijze gerelateerd aan het serum albumine, met als gevolg dat de overschatting van de GFR door de creatinine-klaring meer uitgesproken was bij patiënten met het laagste serum albumine. Een afname van de GFR bij patiënten die een nefrotisch syndroom ontwikkelen wordt dus niet zonder meer weerspiegeld door een gelijktijdige stijging van het serum creatinine.

In hoofdstuk 6 beschrijven we het effect van albumine op de tubulaire reabsorptie van β2-microglobuline (β2M). De meeste gefiltreerde eiwitten worden, na binding aan een ligand in de brush border membraan, gereabsorbeerd door de renale proximale tubuluscellen. Het laag moleculaire eiwit β2M wordt onder normale omstandigheden volledig gereabsorbeerd via de proximale tubuluscellen. Zelfs minimale schade aan de tubuluscellen wordt al weerspiegeld door een verminderde reabsorptie en dus een verhoogde excretie van β2M. Als zodanig wordt de uitscheiding van β2M in de urine beschouwd als een marker voor tubulo-interstitiële schade en een voorspeller ten aanzien van de prognose bij patiënten met nierziekten en proteïnurie. Het was echter niet duidelijk of andere eiwitten in de urine in staat waren de tubulaire reabsorptie van β2M via een competitief mechanisme op het niveau van de brush border membraan te beïnvloeden. We hebben de mogelijke rol van albumine onderzocht. Om het tubulaire aanbod te vergroten, hebben we intraveneus 40 g albumine toegediend aan 10 patiënten met een nefrotisch syndroom en het effect hiervan op de

uitscheiding van β2M onderzocht. Ondanks een aanzienlijke toename van de uitscheiding van albumine, zagen we geen grote veranderingen in de uitscheiding van β2M. Uit deze resultaten kunnen we concluderen dat bij patiënten met een nefrotisch syndroom urine β2M kan worden gebruikt als betrouwbare maat voor tubulusschade.

Gebaseerd op de resultaten van pilotstudies hebben wij gesuggereerd dat β2-microglobuline en IgG in de urine voorspellende factoren zijn voor de ontwikkeling van progressief nierfalen bij patiënten met idiopathische membraneuze nefropathie (iMN). In deze eerdere studies bedroegen de sensitiviteit en specificiteit 80 tot 90%, uitgaande van een drempelwaarde van 500 ng/min voor β2-microglobuline (Uβ2m), en 250 mg/24 uur voor IgG (UIgG). In een validatiestudie, beschreven in hoofdstuk 7, hebben we de betrouwbaarheid van de voorspellende waarde van Uβ2m en UIgG voor nierfunctieverlies getest. We hebben Uβ2m en UIgG gemeten in 57 patiënten met iMN, proteïnurie en normale nierfunctie en deze patiënten prospectief gevolgd. Aan het einde van de follow-up had 44% van de patiënten het eindpunt van nierfalen, gedefinieerd als een stijging van het serum creatinine >50% of een serum creatinine >135 µmol/l, bereikt. Multivariaat analyse bevestigde dat Uβ2m de sterkste onafhankelijke voorspellende factor voor de ontwikkeling van nierinsufficiëntie was. De sensitiviteit en specificiteit bedroegen respectievelijk 88% en 91%. Indien de uitscheiding van β2m en IgG werden gecombineerd, verbeterde de specificiteit tot 97%. Op basis van deze studie concluderen wij dat Uβ2m en UIgG kunnen worden gebruikt voor het bepalen van de noodzaak tot het starten van immunosuppressieve therapie.

Er bestaat geen consensus over de optimale behandeling van patiënten met een idiopathische membraneuze nefropathie (iMN). In hoofdstuk 8 hebben we de effectiviteit vergeleken van twee behandelschema's toegepast bij patiënten met iMN en nierinsufficiëntie. Vijftien patiënten werden behandeld met oraal prednison (0.5 mg/kg/dag, maand 1, 3 en 5), in combinatie met chloorambucil (0.15 mg/kg/dag, maand 2, 4 en 6), en 17 patiënten werden behandeld met prednison (0.5 mg/kg om de dag, gedurende 6 maanden) en oraal cyclofosfamide (1.5-2.0 mg/kg/dag, gedurende 1 jaar). Beide groepen ontvingen daarnaast methylprednisolon intraveneus gedurende 3 cycli. De patiënten groepen waren vergelijkbaar wat betreft leeftijd, nierfunctie en ernst van de proteïnurie op het moment van de start van de behandeling. De nierfunctie verbeterde in beide groepen, echter de verbetering was minder uitgesproken in de chloorambucil-groep. Eén jaar na start van de behandeling was het gemiddelde serum creatinine slechts 6.3 µmol/l lager dan de

uitgangswaarde in de chloorambucil-groep en 121 µmol/l lager in de cyclofosfamide-groep (P<0.01). Bij vier van de met chloorambucil behandelde patiënten en één met cyclofosfamide behandelde patiënt ontwikkelde zich een terminale nierinsufficiëntie. Een remissie van proteïnurie (<2 g/10 mmol creatinine) werd waargenomen bij 15 van de met cyclofosfamide behandelde patiënten en bij 5 van de met chloorambucil behandelde patiënten (P<0.01). Natuurlijk kan het zo zijn dat de betere effectiviteit van cyclofosfamide is toe te schrijven aan de langere duur van deze behandeling. Verlenging van de behandeling met chloorambucil is echter nauwelijks een optie aangezien bij 11 van de 15 patiënten de behandeling met chloorambucil voortijdig moest worden gestopt wegens bijwerkingen. Op grond van deze gegevens concluderen wij dat cyclofosfamide beter wordt verdragen en effectiever is dan chloorambucil.

In hoofdstuk 9 beschrijven we onze langere termijn ervaringen met de combinatietherapie prednison en cyclofosfamide gebaseerd op gegevens van 39 patiënten met een idiopathische membraneuze nefropathie, proteïnurie en nierinsufficiëntie. De gemiddelde follow-up duur was 32 maanden. Achttien patiënten hadden een follow-up van meer dan 3 jaar. Zoals in detail in het voorgaande beschreven, werden alle patiënten behandeld met prednison gedurende 6 maanden en cyclofosfamide gedurende 1 jaar. Na start van de behandeling verbeterde de nierfunctie al snel: het serum creatinine daalde van 226±108 µmol/l bij het begin tot 143±62 µmol/l na 12 maanden behandeling. Dit gunstige effect bleef aanwezig gedurende de follow-up. Bij 31 patiënten ontstond een partiële remissie van de proteïnurie (<2.0 g/10 mmol creatinine), en bij 12 uiteindelijk een complete remissie (<0.2 g/10 mmol creatinine). Tot nu toe namen wij bij 7 patiënten een recidief van de proteïnurie waar. De meeste patiënten ontwikkelden bijwerkingen als gevolg van de immunosuppressieve behandeling, al was de ernst hiervan meestal beperkt. Gebaseerd op de resultaten van deze studie mogen we concluderen dat behandeling met cyclofosfamide en prednison effectief is, zowel op de korte als de langere termijn, voor patiënten met een idiopatische membraneuze glomerulopathie en nierinsufficiëntie. De hoge incidentie van bijwerkingen ondersteunt onze mening dat deze behandeling beperkt moet blijven tot die patiënten met een verhoogd risico op ontwikkeling van een terminale nierinsufficiëntie.

DANKWOORD

Na een lange weg is het er toch van gekomen: het proefschrift is klaar. Dit was niet mogelijk geweest zonder de hulp en inspanning van vele mensen, inclusief alle patiënten die hebben meegewerkt. Het is onmogelijk om alle betrokkenen in dit dankwoord te vermelden; daarom aan allen die mijn pad de afgelopen jaren hebben gekruist en een bijdrage hebben geleverd aan het volbrengen van mijn onderzoekstaken en opleiding hierbij mijn welgemeende dank.

Een aantal mensen wil ik natuurlijk toch nog expliciet met naam noemen. Ik ervaar dit dankwoord als bijzonder omdat de eerst genoemde persoon ook de laatst genoemde zal zijn.
Jack (Prof. Wetzels), met respect en bewondering heb ik jou als onderzoeksleider jaren kunnen volgen. Je bent enorm gedreven in je werkzaamheden. Je (literatuur)kennis, inzicht en snelheid van werken zijn door weinigen te overtreffen. Dank voor je steun en begeleiding.
Beste **Rob** (Prof. Koene), toen ik in 1992 bij de afdeling nierziekten begon, was je het hoofd van deze afdeling. Ik had een moeizame start bij het verwezenlijken van mijn doelen. Je hebt in de loop der jaren een enorme sleutelrol gespeeld zodat ik toch de route kon volgen om internist te worden en promotieonderzoek te doen; ik ben je veel dank verschuldigd voor alle steun en vertrouwen.
Beste **Jo** (Prof. Berden), in 1992 was jij degene die naar Mierlo belde met de vraag of ik interesse had in een baan als AGNIO. Jij was het startpunt van mijn “nefro-loopbaan” en hebt me tot aan het einde van het onderzoek de mogelijkheid geboden het werk af te maken, mijn dank hiervoor.
Overige (ex-)stafleden van de afdeling nierziekten (**Roland, Andries, Frans, Henk, Ine, Luuk, Ruud, Gerald, inmiddels ook Heinrich**): jullie hebben mij in de loop der jaren begeleid bij onderzoek en patiëntenzorg. Ik dank jullie voor de inhoudelijke begeleiding, maar met name ook voor de gemoedelijke, prettige sfeer die de afdeling nierziekten al jarenlang zo karakteriseert.
Alle meewerkende internist-nefrologen: de hieronder genoemde collegae hebben in de afgelopen jaren patiënten naar Nijmegen verwezen voor proteïnurie analyse en deelname aan een van de onderzoeken in dit proefschrift :

Amsterdam, St. Lucas Andreas Ziekenhuis: **Dr B. Potter van Loon**
Apeldoorn, Gelre Ziekenhuizen locatie Lucas: **Dr J. Barendregt**
Arnhem, Rijnstate Ziekenhuis: **Dr F. Bosch**, **Dr R. van Leusen**, **Dr K. Parlevliet**, **Dr L. Reichert**
Beverwijk, Rode Kruis Ziekenhuis: **Dr G. Schrijver**

Breda, Amphia Ziekenhuis: **Dr P. Stijnen, Dr G. Verburg**
Doetinchem, Slingeland Ziekenhuis: **Drs G. Bruinings**, **Dr E. Muller**
Ede, Ziekenhuis Gelderse Valei: **Dr G. Feith**, **Drs M. den Hartog**
Eindhoven, Maxima Medisch Centrum: **Dr A. Lieverse**
Enschede, Medisch Spectrum Twente: **Drs R. Brouwer**
Geldrop, St. Anna Ziekenhuis: **Drs R. Smeets**
Heerlen, Atrium Medisch Centrum: **Dr L. Frenken**
's-Hertogenbosch, Jeroen Bosch Ziekenhuis: **Dr J. Beutler**, **Dr A. Hollander**, **Dr J. Jansen**, **Dr M. Koolen**
Nijmegen, Canisius Wilhelmina Ziekenhuis: **Dr M. ten Dam**, **Dr I. Go**, **Dr M. Schuurmans**
Roermond, Laurentius Ziekenhuis: **Drs W.Grave**, **Dr J. Wirtz**
Roosendaal, Ziekenhuis Franciscus: **Drs D. de Gooyer**, **Drs T. Noordzij**, **Drs H. van Roermund**
Tiel, Ziekenhuis Rivierenland: **Dr P. Bleeker**
Tilburg, St. Elisabeth Ziekenhuis: **Dr A. Apperloo**, **Dr P. Rensma**
Tilburg, TweeSteden Ziekenhuis: **Dr P. Spooren**
Veghel, Ziekenhuis Bernhoven: **Dr L. van Hulsteijn**
Veldhoven, Maxima Medisch Centrum: **Dr P. Gerlag**, **Dr A. van den Wall Bake**
Venlo, VieCuri Medisch Centrum: **Dr W. van Kuijk**, **Dr A. Luik**, **Dr V. Verstappen**
Zwolle, Isala Klinieken: **Dr G. Kolsters**, **Dr J. Offerman**

De jarenlange studies bij patiënten met proteïnurie konden alleen worden uitgevoerd dankzij de medewerking van U/jullie allen. Het verwijzen van patiënten voor de "2-uurs metingen", het doorsturen van de benodigde data en de gastvrije ontvangst als ik data wilde komen verzamelen zijn van essentieel belang geweest om te komen tot het huidige resultaat. Ik hoop dat deze goede werkrelatie tussen de perifere centra en de academische afdeling nefrologie te Nijmegen een lange toekomst heeft, naar tevredenheid van alle partijen.
Beste **Peggy** (du Buf-Vereijken), destijds als nefroloog in opleiding raakte je betrokken bij het proteïnurie-onderzoek bij patiënten met membraneuze glomerulopathie. Je enthousiasme lijkt vaak nauwelijks te temperen. Met bewondering heb ik gezien hoe je de drukke weg naar de promotie hebt afgelegd. Ik heb onze samenwerking erg gewaardeerd en het is plezierig om de promotiedag samen met jou en Gerard te kunnen vieren. Beste **Gerard** (du Buf), je bent zeer waardevol en onmisbaar geweest bij het verwezenlijken van een wens die ik al jaren had: een Accessbestand voor de data van het proteïnurieonderzoek. Je hebt daar op vrijwillige basis (samen met Peggy!) erg veel tijd ingestoken, en het resultaat mag er zijn. Ook ben je behulpzaam geweest bij het drukklaar maken van "het (dit) boekje". Mijn dank hiervoor. Beste **Iris** (Kremer) en **Marijke** (Rodermond), jullie staan aan de basis van de

uitvoering van de “2-uurs urinemetingen”. Jullie hebben veel werk verricht bij het begeleiden van de patiënten tijdens de vele metingen en de registratie/opslag van al het materiaal. Als ik weer eens iets in de procedure wilde wijzigen hielpen jullie mij zonder gemopper; dank voor jullie goede inzet.

Beste **Annemiek** (Wellesen-Polman), **Simone** (Mooren) en **Yvonne** (Hooghof), jullie hebben de uitvoering van de metingen sinds een aantal jaren overgenomen. In deze laatste jaren was ik zelf steeds minder op de functiekamer en verliep het overleg regelmatig per e-mail. Vanaf het begin heb ik er het volste vertrouwen in gehad dat de metingen en alles wat daar bij komt bij jullie in goede handen was en is.

Vanaf 1995 zijn er vele honderden bloed- en urinemonsters naar de laboratoria gegaan voor diverse eiwit-, enzym, en complement bepalingen. Voor al het werk dat daaraan verbonden was (en is) wil ik **alle medewerkers van het voormalige immunochemisch laboratorium onder leiding van Ina Klasen** hartelijk bedanken. **Wilbert** Peters, **Theo** Mulder en **Hennie** Roelofs van het laboratorium MDL wil ik bedanken voor hun inzet bij de verwezenlijking en uitvoer van de bewerkelijke GST-bepalingen.

Voor diverse onderzoeken was het nodig om PA van nierbiopten opnieuw te beoordelen en en/of voor publicaties tot mooie afbeeldingen te verwerken. Van de afdeling pathologie wil ik voor hun vakkundige inzet en prettige contact danken **Karel** Assmann en **Henry** Dijkman.

Jaap van den Born wil ik bedanken voor zijn belangrijk aandeel in het onderzoek naar de expressie van heparan sulfaat proteoglycanen zoals beschreven in hoofdstuk 2.

In de loop der jaren heb ik diverse **kamergenoten** gehad, **collega-onderzoekers** en **collega arts-assistenten**: aan allen mijn dank voor het delen van ervaringen en leuke contacten.

Beste **Ilse en Camiel**, al meer dan 10 jaar zijn jullie door dik en dun goede vrienden. Jullie zijn een enorme steun, alleen al doordat ik weet dat ik altijd bij jullie aan kan kloppen. Ik hoop op nog vele jaren van goede vriendschap. **Ilse en Erik**, het is fijn dat jullie als mijn paranimfen naast mij staan op deze voor mij zo bijzondere dag; dank voor jullie aanwezigheid en inzet.

Lieve pap en mam, als kleuter zei ik al dat ik “dokteres” wilde worden. Een droom die jullie altijd hebben gesteund. Ik ben me altijd bewust geweest van jullie betrokkenheid, die zelfs tot uiting kwam door brandende kaarsjes op spannende (examen)momenten. Een goed thuisfront, zoals jullie (met Arthur) aan mij hebben gegeven, is van onschatbare waarde.

Lieve Jack, zoals ik aan het begin al aankondigde, sluit ik dit dankwoord met jou af. Aan het einde van een dankwoord is vaak te lezen dat het t.a.v. het werk nu wel rustiger zal worden. Voor ons zal dit laatste voorlopig niet opgaan, maar dat staat een fijne, goede toekomst voor ons samen niet in de weg.

CURRICULUM VITAE

Amanda Branten werd geboren op 20 mei 1966 te Helmond en groeide op in Mierlo. In 1984 werd het VWO examen behaald aan het Strabrecht College te Geldrop. In dat zelfde jaar begon zij met de studie Geneeskunde aan de Katholieke Universiteit te Nijmegen (inmiddels Radboud Universiteit Nijmegen). Het doctoraal examen werd behaald in 1988 en het artsexamen in 1991. In 1991 heeft zij via een toegekende subsidie van de Nederlandse Hartstichting een onderzoeksstage in het universiteitsziekenhuis van Gent kunnen verrichten waarbij ervaring werd opgedaan met de capillair microscoop. In 1992 begon zij als AGNIO (arts-assistent geneeskundige niet in opleiding) op de verpleegafdeling nierziekten van het St. Radboudziekenhuis te Nijmegen. Vanaf medio 1993 is het accent van de werkzaamheden meer naar onderzoeksactiviteiten verschoven. In juli 1997 kon zij in het kader van een AGIKO-constructie starten met de opleiding tot internist, gecombineerd met het promotieonderzoek waaruit het huidige proefschrift is voortgekomen. De opleiding tot internist werd grotendeels gevolgd in het Universitair Medisch Centrum St Radboud te Nijmegen (opleider Prof. Dr J.W.M. van der Meer) en vanaf juli 2002 in het Canisius Wilhelmina Ziekenhuis te Nijmegen (opleider Dr. A.S. Dofferhoff). Sinds januari 2004 staat zij geregistreerd als internist. In september 2004 startte zij met de opleiding reumatologie in het Universitair Medisch Centrum St. Radboud te Nijmegen (opleider Prof. Dr. P.L.C.M. van Riel). Zij woont samen met Jack Wetzels.

PUBLICATIE LIJST

1. Wollersheim HCH, Branten AJW, Duprez D. Het fenomeen van Raynaud. Tijdschrift voor Cardiologie 1992;4:39-44.
2. Branten AJW, Assman KJM, Koene RAP. Matrix stones and acquired renal cysts in a non-dialysed patient with chronic renal failure. Nephrol Dialysis Transplant 1995;10:123-125.
3. Branten AJW, Beckers PJA, Tiggeler RGW, Hoitsma AJ. Pneumocystis carinii pneumonia in renal transplant recipients. Nephrol Dialysis Transplant 1995;10:1194-1197.
4. Branten AJW, Smits P, Jansen TLThA, Wollersheim HCH, Thien Th. Effects of atrial natriuretic factor on skin microcirculation versus skeletal muscle blood flow. J Cardiovasc Pharmacol 1996;27:303-306.
5. van de Ven AJAM, Rieger A, Branten AJ *et al*. Cutaneous reactions to trimethoprim-sulphametrole in AIDS patients treated for Pneumocystis carinii pneumonia. AIDS 1996;1996:341-342.
6. Branten AJW, Hilbrands LB, van Hamersvelt HW, Koene RAP, Huysmans FThM. Renal and systemic effects of atenolol and tertatolol in renal transplant recipients on cyclosporine A. Nephrol Dialysis Transplant 1998;13:423-426.
7. Branten AJW, Wetzels JFM, Weber AM, Koene RAP. Hyponatremia due to sodium valproate. Ann Neurol 1998;43:265-267.
8. Branten AJW, Reichert LJM, Koene RAP, Wetzels JFM. Oral cyclophosphamide versus chlorambucil in the treatment of patients with idiopathic membranous nephropathy and renal insufficiency. Q J Med 1998;91:359-366.
9. Branten AJW, Wetzels JFM. Immunosuppressieve therapie van idiopathische membraneuze glomerulopathie. Ned Tijdschr Geneeskd 1998;142:767-771.
10. Branten AJW, Wetzels JFM. Immunosuppressieve behandeling van patiënten met een nefrotisch syndroom op basis van "minimal change" nefropathie. Ned Tijdschr Geneeskd 1998;142:2832-2838.
11. Kloke HJ, Branten AJ, Huysmans FT, Wetzels JF. Antihypertensive treatment of patients with proteinuric renal diseases: risks or benefits of calcium channel blockers? Kidney Int 1998;53:1559-1573.
12. De Keijzer MH, Klasen IS, Branten AJW, Hordijk W, Wetzels JFM. Infusion of plasma expanders may lead to unexpected results in urinary protein assays. Scand J Clin Lab Invest 1999;59:133-138.
13. Hordijk W, Klasen IS, De Keijzer MH, Branten AJW, Wetzels JFM. Pseudoproteinuria following gelatin infusion. Nephrol Dialysis Transplant 1999;14:2782-2783 (letter).

14. Jacobs EMG, Vervoort G, Branten AJW *et al*. Atrial natriuretic peptide increases albuminuria in type I diabetic patients: evidence for blockade of tubular protein reabsorption. Eur J Clin Invest 1999;29:109-115.
15. Branten AJW, Wetzels JFM. Influence of albumin infusion on the urinary excretion of beta-2-microglobulin in patients with proteinuria. Nephron 1999;81:329-333.
16. Branten AJW, Mulder TPJ, Peters WHM, Assmann KJM, Wetzels JFM. Urinary excretion of glutathione S-transferases alpha and pi in patients with proteinuria: reflection of the site of tubular injury. Nephron 2000;85:120-126.
17. Ten Dam MAGJ, Branten AJW, Klasen IS, Wetzels JFM. The gelatin-derived plasma substitute gelofusine causes low-molecular-weight proteinuria by decreasing tubular protein reabsorption. Journal of Critical Care 2001;16:115-120.
18. Branten AJW, Wetzels JFM. Short- and long-term efficacy of oral cyclophosphamide and steroids in patients with membranous nephropathy and renal insufficiency. Clin Nephrol 2001;56:1-8.
19. Branten AJW, van den Born J, Jansen JLJ, Assmann KJM, Wetzels JFM. Familial nephropathy differing from minimal change nephropathy and focal glomerulosclerosis. Kidney Int 2001;59:693-701.
20. Branten AJW, Klasen IS, Wetzels JFM, for the Study Group. Short-term effects of fish oil treatment on urinary excretion of high- and low-molecular weight proteins in patients with IgA nephropathy. Clin Nephrol 2002;58:267-274.
21. Branten AJ, Kock-Jansen M, Klasen IS, Wetzels JF. Urinary excretion of complement C3d in patients with renal diseases. Eur J Clin Invest 2003;33:449-456.
22. Branten AJW, Swinkels DW, Klasen I, Wetzels JFM. Serum ferritin levels are increased in patients with glomerular diseases and proteinuria. Nephrol Dialysis Transplant 2004;19:2754-2760.
23. du Buf-Vereijken PWG, Branten AJW, Wetzels JFM, for the Membranous Nephropathy Study Group. Cytotoxic therapy for membranous nephropathy and renal insufficiency; improved renal survival but high relapse rate. Nephrol Dialysis Transplant 2004;19:1142-1148.
24. Branten AJW, Vervoort G, Wetzels JF. Serum creatinine is a poor marker of GFR in nephrotic syndrome. Nephrol Dialysis Transplant 2005;20:707-711.
25. Branten AJW, du Buf-Vereijken PWG, Klasen IS *et al*. Urinary excretion of β2-microglobulin and IgG predict prognosis in idiopathic membranous nephropathy: a validation study. J Am Soc Nephrol 2005;16:169-174.
26. du Buf-Vereijken PWG, Branten AJW, Wetzels JFM. Idiopathic membranous nephropathy:outline and rationale of a treatment strategy. Am J Kidney Dis 2005;In Press.

ADDENDUM CHAPTER 2 : Figure 2 and 3 (page 24) in color.

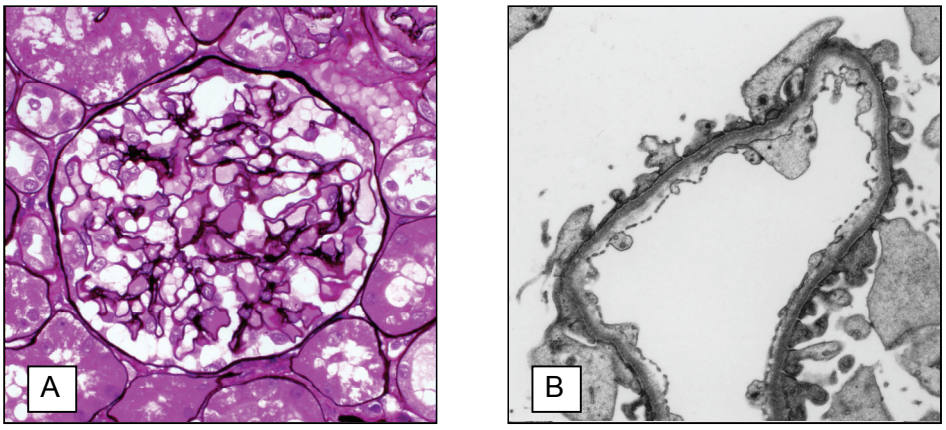

Figure 3. Light and electron microscopic pictures of the third biopsy of the mother.
(A) Normal-looking glomerulus, methenamine silver staining , x350. (B) Electron microscopy of a part of a capillary loop. The foot processes of the podocytes reveal no retraction, x12,000.

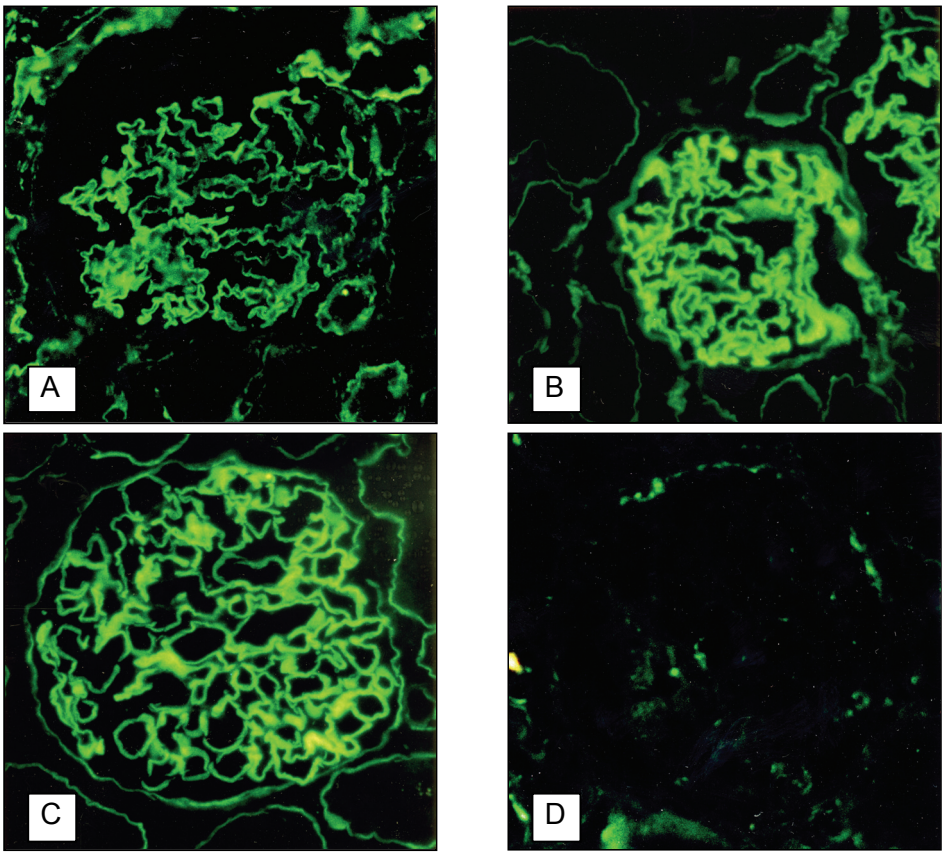

Figure 4. Immunofluorescence of the third biopsy of the mother (A and B) and a biopsy of a patient with classical minimal change nephropathy (C and D).
Unchanged presence of (A) heparan sulfate side chains as detected by monoclonal antibody (mAb) JM 403, and (B) core protein as demonstrated by mAb JM 72 of heparan sulfate proteoglycan (HSPG) along the capillary loops. In the biopsy of a patient with classical minimal change nephropathy staining of the core protein of HSPG is normal (C), while the heparan side chains have completely disappeared (D, x600).

ADDENDUM CHAPTER 3 : Figure 2 and 3 (page 39) in color.

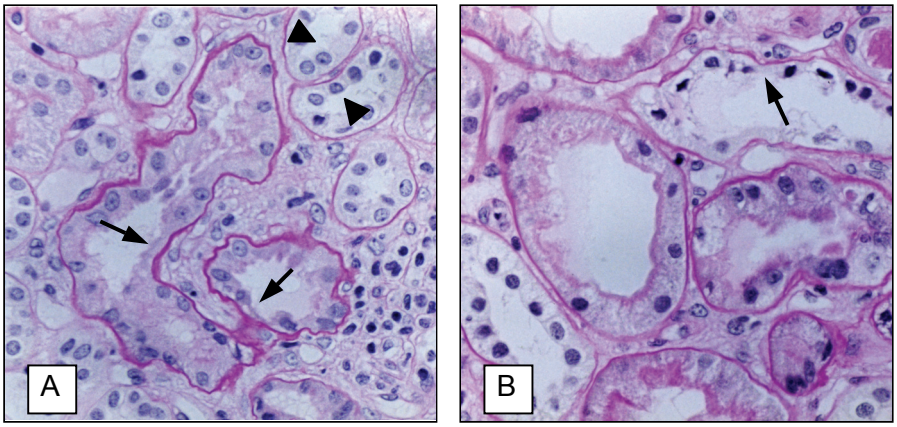

Figure 2.
Light microscopic presentation of renal biopsy specimens from 2 patients with IgA nephropathy and differing GST iso-enzyme excretion
Periodic acid-Schiff. x400
A Specimen from a patient with an increased urinary level of GST alpha (81 μg/10 mmol creatinine), and a relatively low excretion of GST pi (25 μg/10 mmol creatinine). Epithelial cells of proximal tubules are damaged (arrows) with loss of brush border, whereas distal tubular cells (arrowheads) look normal. **B** Specimen from a patient with an increased excretion of GST pi (58 μg/10 mmol creatinine) and a slightly elevated level of GST alpha (17 μg/10 mmol creatinine). In this patient the distal tubular cells are more severely damaged than the proximal tubular cells, as reflected by prominent nuclear and cellular atypia (arrow).

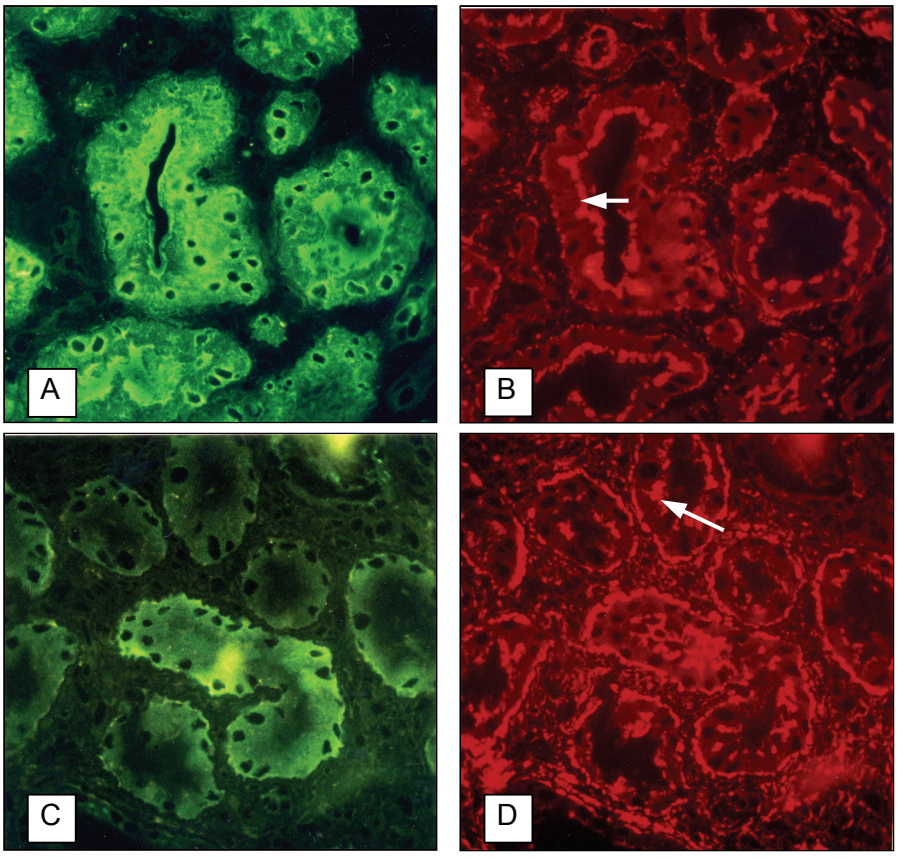

Figure 3.
Immunofluorescence double staining for GST alpha (A,C) and phalloïdin (B,D) of renal biopsy specimens from 2 patients. **A,C** Specimens from a patient with focal glomerulosclerosis, moderate proteinuria, and low levels of both GST alpha and pi (17, 14 μg/10 mmol creatinine, respectively). The proximal tubules can be easily recognized by the presence of intact brush borders. All proximal tubules show homogeneous staining of both GST alpha (A: bright green-colored cells) and of phalloïdin (B: bright red-colored brush border, arrow). **C,D** Specimens from the patient with IgA

nephropathy shown in fig. 2A. GST alpha excretion was 81 μg/ 10 mmol creatinine. The proximal tubules are damaged as indicated by the irregular and sometimes absent brush border staining (arrow in D). Staining of the proximal tubules for GST alpha revealed a variable intensity, with clear loss of staining in most proximal tubules (C).