

# Serum uromodulin—a marker of kidney function and renal parenchymal integrity

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## ABSTRACT

**Background.** An ELISA to analyse uromodulin in human serum (sUmod) was developed, validated and tested for clinical applications.

**Methods.** We assessed sUmod, a very stable antigen, in controls, patients with chronic kidney disease (CKD) stages 1–5, persons with autoimmune kidney diseases and recipients of a renal allograft by ELISA.

**Results.** Median sUmod in 190 blood donors was 207 ng/mL (women: men, median 230 versus 188 ng/mL,  $P = 0.006$ ). sUmod levels in 443 children were 193 ng/mL (median). sUmod was correlated with cystatin C ( $r_s = -0.862$ ), creatinine ( $r_s = -0.802$ ), blood urea nitrogen (BUN) ( $r_s = -0.645$ ) and estimated glomerular filtration rate (eGFR)–cystatin C ( $r_s = 0.862$ ). sUmod was lower in systemic lupus erythematosus-nephritis (median 101 ng/mL), phospholipase-A2 receptor-positive glomerulonephritis (median 83 ng/mL) and anti-glomerular basement membrane positive pulmorenal syndromes (median 37 ng/mL). Declining sUmod concentrations paralleled the loss of kidney function in 165 patients with CKD stages 1–5 with prominent changes in sUmod within the ‘creatinine blind range’ (71–106  $\mu\text{mol/L}$ ). Receiver-operating characteristic analysis between non-CKD and CKD-1 was superior for sUmod (AUC 0.90) compared with eGFR (AUC 0.39), cystatin C (AUC 0.39) and creatinine (AUC 0.27). sUmod rapidly recovered from 0 to 62 ng/mL (median) after renal transplantation in cases with immediate graft function and remained low in delayed graft function (21 ng/mL, median; day 5–9: relative risk 1.5–2.9, odds ratio 1.5–6.4). Immunogold labelling disclosed that Umod is

transferred within cytoplasmic vesicles to both the apical and basolateral plasma membrane. Umod revealed a disturbed intracellular location in kidney injury.

**Conclusions.** We conclude that sUmod is a novel sensitive kidney-specific biomarker linked to the structural integrity of the distal nephron and to renal function.

**Keywords:** kidney failure, renal biomarker, serum assay, thick ascending limb, uromodulin

## INTRODUCTION

Uromodulin (Umod) is a glycoprotein exclusively synthesized in the human kidney by epithelial cells of the thick ascending limb (TAL) of the loop of Henle and the early distal convoluted tubule [1–4]. This glycosylphosphatidylinositol (GPI)-anchored protein is predominantly localized to the apical plasma membrane, from which it is released into the tubular lumen by proteolytic cleavage [5]. Umod of urinary origin (uUmod) is a high molecular weight polymer and the most abundant urinary protein, detectable at a concentration of approximately 75 mg/24 h [6–8]. As a renal defensin, Umod protects against urinary tract infection by clotting fibrillary type-1 and type-S adhesins of bacteria [6–8]. Furthermore, uUmod prevents renal stone formation [9], is involved in handling tubular  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  transport and is suspected to contribute to arterial hypertension [10–12]. Umod exhibits immunomodulatory properties, activates granulocytes, mediates monocyte cytotoxicity and inhibits T-cell proliferation [13–15]. *In vitro* studies showed that Umod can be either pro- or anti-inflammatory [16, 17].

Epithelia of the TAL are specifically susceptible to oxidative stress, hypoxia and nephrotoxins. There, vulnerable, sublethally damaged cells may undergo apoptosis and necrosis, finally resulting in acute kidney injury (AKI) as was shown in animal models [18–23].

Up to now very little attention has been paid to the fact that uromodulin is also present in serum (sUmod; [24–26]). This might originate from a frequently overlooked basolateral release of Umod into the interstitium that was shown in a mouse model [27].

Various studies have focused on the role of uUmod in chronic kidney diseases (CKD) of different aetiologies [28–31]. Excretion of uUmod was reduced in patients with CKD or chronic graft failure and paralleled the estimated glomerular filtration rate (eGFR) [32–34]. In contrast, a case–control study reported that increased uUmod concentrations precede the development of CKD [35]. *UMOD* was confirmed as one of the most important loci associated with end-stage renal failure [36–42].

Preanalytic data showed that the urinary form impairs precision and reliability of the measurements [2, 31, 43]. It is speculated that the polymeric structure and varying degrees of polymerization hamper antibody-based detection.

In the present work, we describe the development and application of an ELISA for the determination of sUmod, based on monoclonal antibodies (moAb). We assessed sUmod levels in healthy persons, patients with different stages of CKD and patients after kidney transplantation (KTX). Our immunohistochemical findings support the view that newly biosynthesized Umod is translocated and released bidirectionally by TAL cells, such that the basolateral pathway might contribute to sUmod levels. The observed progressive decrease of sUmod levels related to CKD stages suggests altered cell viability in the TAL segment, which mirrors a gradual and more global renal parenchymal injury.

## MATERIALS AND METHODS

### Study populations

Physiological levels of sUmod were assessed in 190 blood donors (18–60 years, Table 1) and in 443 children and adolescents (1–17 years). Thirty-three patients without kidney disease (non-CKD) and 132 patients with CKD stages 1–5 were enrolled in a nonrandomized manner. This cohort was used to determine the correlation of sUmod with cystatin C, creatinine and eGFR (Table 2). sUmod was studied in patients with autoimmune diseases classified by clinical symptoms, CKD stages and autoantibody tests (Euroimmun, Lübeck, Germany), i.e. systemic lupus erythematosus (SLE;  $n = 53$ ), anti-glomerular basement membrane (GBM)-positive pulmorenal syndromes ( $n = 10$ ), phospholipase-A2 receptor (PLA2-R) antibody (Ab)-positive glomerulonephritis ( $n = 20$ ). Forty-four kidney transplant patients [17 with delayed graft function (DGF) and 27 with immediate graft function (IGF)] were analysed for sUmod levels (Table 3). Details of immunosuppressive therapy in transplant patients have been published elsewhere [44]. All individuals underwent physical examination and completed a detailed questionnaire. Ethics approval was obtained from two ethics

committees and all participants provided informed consent, in adherence to the declarations of Helsinki and Istanbul.

### Definition of CKD stages and outcome parameters

Based on clinical and eGFR data patients were assigned to one of five stages of CKD. The underlying kidney diseases were histologically characterized by routine stains [haematoxylin & eosin (HE), periodic acid-Schiff (PAS)], immunohistology and ultrastructure of renal biopsy sections. GFR was determined by eGFR<sub>CysC</sub>. Patients were assigned to CKD stages according to Kidney Disease: Improving Global Outcomes (KDIGO) guidelines: CKD 1 with eGFR >90 mL/min, CKD 2 61–90 mL/min, CKD 3 31–60 mL/min, CKD 4 16–30 mL/min and CKD 5 0–15 mL/min. We included 33 patients without kidney disease as the control group (non-CKD).

Forty-four patients had undergone KTX between 1990 and 1995 at the Berlin-Friedrichshain Kidney Centre, Germany. Follow-up was until 2002, unless death occurred. Clinical characteristics and definitions of IGF and DGF are summarized in Table 3.

### Antibodies

Mouse moAb were generated against purified human uUmod. BALB/c mice were immunized with emulsified purified Umod in ABM-S adjuvant. After booster injections (ABM-N adjuvant) mice were anesthetized, splenectomized and spleen cells were PEG fused with mouse X63Ag8.653 myeloma cells. Hybridoma supernatants were screened for specific antibodies by ELISA with purified Umod as antigen. Positive hybridomas were subcloned by limiting dilution. Distinct hybridomas were expanded and purified. Specificity of moAb was assessed by immunocytochemistry using paraffin-embedded tissue sections from human kidney, liver, intestine, muscle and lymph nodes. Only human kidney showed a positive histochemical reaction with anti-Umod antibodies.

### Uromodulin ELISA

Purified IgG<sub>1</sub> of moAb T112A12 for coating the microtiter plates and moAb T5.G as detection antibody were used to establish an ELISA. All analytical data (sensitivity, precision, linearity, etc.) for CE labelling were determined and are available from the manufacturer (Euroimmun). All measurements were done with a 1:101 serum dilution in sample buffer. The linear measurement range was 2–400 ng/mL.

### Immunohistological and cytological analyses

Normal renal tissue was obtained from kidney resection in renal carcinoma patients and fixed in paraformaldehyde. The tissue was cut into small blocks and processed in an automatic freeze substitution unit (Reichert-Jung AFS, Leica Microsystems, Wetzlar, Germany). Umod moAb clonotypes 109F9 (IgG<sub>1</sub>), 112A12 (IgG<sub>1</sub>) and 5A2 (IgG<sub>2b</sub>) were used for staining.

**Immunocytochemistry.** After primary antibody incubation deparaffinized sections were processed with secondary biotinylated antibody and avidin-peroxidase complex according to standard procedures. Biopsy material was obtained from patients (21 men, 11 women, mean age  $54.7 \pm 17$  years) with

**Table 1. Demographic characteristics and serum uromodulin concentration of blood donors; data are presented as mean ± SD and median, with IQR in brackets**

Parameter	Total (n = 190)	Male (n = 89)	Female (n = 101)	P-value
Age (years)	38.7 ± 11.9, 40.0 (28.0, 49.0)	37.4 ± 11.8, 38.0 (25.5, 47.5)	39.8 ± 11.9, 42.0 (29.8, 50)	0.165
Uromodulin (ng/mL)	221.5 ± 95.2, 207.4 (148.5, 275.1)	199.3 ± 78.5, 188.2 (144.4, 250.7)	241.1 ± 103.5, 229.9 (164.7, 307.6)	0.006

kidney diseases including IgA nephropathy ( $n = 12$ ), membranous glomerulonephritis (mGN) ( $n = 8$ ), ANCA-positive RPGN ( $n = 12$ ), where a minimum of two to three corticomedullary nephrons were noticed, displaying interstitial fibrosis and tubular atrophy of more than 10%. In addition, we assessed sections of non-functioning allografts of nine patients (one female, eight males). Staining included HE, Masson-Trichrom, Giemsa and PAS. Sections were examined for Umod location by two different pathologists in a blinded manner.

**Immunoelectron microscopy.** Sections were incubated with the primary antibody, followed by gold-conjugated second antibody. Stained sections were studied in a Philips/FEI CM100 transmission electron microscope. In controls, sections were incubated with secondary antibodies alone or with non-specific moAb. None of the controls showed any labelling.

### Statistical analysis

All statistical analyses were performed with SigmaPlot 13 and the language R for statistical computing. Continuous data are expressed as mean with standard deviation (SD) and median with interquartile range (IQR). Categorical variables are reported in absolute numbers and percentages. Differences concerning continuous parameters and biomarker concentrations between two groups were analysed using non-parametric Mann-Whitney *U*-test. The Kruskal-Wallis test was used for multiple group comparison. For categorical variables, possible differences between groups were examined using non-parametric chi-square test (Fisher's exact test). The association between sUmod and creatinine, cystatin C and eGFR was determined by Spearman's rank correlation analysis. Multiple linear regression modelling was conducted to evaluate the association of Umod (independent variable) and eGFR<sub>CysC</sub> (dependent variable) adjusted for gender and age. As part of the regression modelling sUmod and eGFR were logarithmically transformed. Wilcoxon-rank test and/or receiver-operating characteristic (ROC) analysis were used to evaluate diagnostic validity of sUmod, cystatin C, creatinine, BUN and eGFR<sub>CysC</sub> to differentiate between non-CKD and CKD stage 1. Relative risk (RR) and odds ratio (OR) for DGF after KTX were calculated on the basis of mean cut-off (day 7–25 post-transplant). Daily cut offs were determined according to Youden's index. The probability of error (type 1) of a two tailed *P*-value <0.05 was considered to be statistically significant.

## RESULTS

### sUmod stability

sUmod stability was analysed over 4 weeks (Supplementary data, Table S1). Serum samples from eight individuals were

stored at either  $-20^{\circ}\text{C}$ ,  $4^{\circ}\text{C}$  or  $37^{\circ}\text{C}$  before monitoring Umod levels. A high concordance in Umod concentrations was observed for all three storage conditions with mean deviations of 4% ( $-20$  versus  $4^{\circ}\text{C}$ ) and 10% ( $-20$  versus  $37^{\circ}\text{C}$ ). Even up to five freeze/thaw cycles had no impact on sUmod recoveries (Supplementary data, Table S1).

### sUmod expected normal values

sUmod was assessed in 190 blood donors (89 males, 101 females, age 18–60 years, median age 40 years). Donors were checked to be healthy by clinical examination and laboratory tests (normal controls, Table 1). Females revealed higher sUmod than males [females: median 230 ng/mL (IQR: 165, 308); males: median 188 ng/mL (IQR: 44, 251),  $P = 0.006$ ]. In adults, no correlation between age and sUmod was observed ( $r_s = -0.0197$ ,  $P = 0.787$ ). sUmod in children and adolescents ( $n = 443$ , age 1–17 years, median age 6 years) was comparable to values found in adults [median 193 ng/mL (IQR: 143, 254)]. sUmod levels did not differ between boys and girls ( $P = 0.389$ ).

### sUmod in renal failure

sUmod levels and the filtration markers creatinine and cystatin C were analysed in 165 patients (110 males, 55 females, age 22–94 years, median age 65 years) with various diseases potentially affecting the kidney. sUmod ranged from 1 to 667 ng/mL with a median of 85 ng/mL (Table 2). In univariate analysis, sUmod was significantly correlated with cystatin C, creatinine and all eGFR calculations. Scatter plots demonstrate an inverse hyperbolic relationship of sUmod with cystatin C ( $r_s = -0.862$ ,  $P < 0.001$ ), creatinine ( $r_s = -0.802$ ,  $P < 0.001$ ) and blood urea nitrogen (BUN,  $r_s = -0.645$ ,  $P < 0.001$ ) and, accordingly, a positive relationship with cystatin C-based eGFR (eGFR<sub>CysC</sub>, Hoek's equation) ( $r_s = 0.862$ ,  $P < 0.001$ ) and eGFR (CKD-EPI) ( $r_s = 0.842$ ,  $P < 0.001$ ) (Figure 1). The creatinine-sUmod ratio was not superior compared with assessing sUmod levels alone. The data disclose that sUmod parallels the progressive deterioration of kidney function and behaves oppositely to the filtration markers. In a multiple linear regression model, sUmod was adjusted for age and gender, which did not significantly influence the regression model with eGFR<sub>CysC</sub> (log) as a dependent variable. The correlation between sUmod (log) and eGFR<sub>CysC</sub> (log) was  $r^2 = 0.735$  ( $P < 0.001$ ).

To study the relationship between sUmod and GFR, sUmod levels were assigned to the stages of renal failure CKD 1–5, based on the eGFR<sub>CysC</sub> data (Figure 2). sUmod decreased with progressive CKD (non-CKD: median 228 ng/mL; stage 1: 153 ng/mL, stage 2: 107 ng/mL; stage 3: 52 ng/mL; stage 4: 30 ng/mL; stage 5: 13 ng/mL). Differences in sUmod were shown for all pairs of CKD stages ( $P < 0.001$ ), except for stages 1 and 2 ( $P = 0.051$ ). The most prominent changes, i.e. the

**Table 2. Demographic characteristics and laboratory blood parameters; data are presented as mean  $\pm$  SD and median, with IQR in brackets, or as absolute numbers (n) with percentage (%) of the referring group) in brackets**

	Total (n = 165)	Non-CKD (n = 33, 20.0%)	CKD 1 (n = 21, 12.7%)	CKD 2 (n = 27, 16.4%)	CKD 3 (n = 43, 26.1%)	CKD 4 (n = 38, 23.0%)	CKD 5 (n = 3, 1.8%)	P-value
Age (years)	61.9 $\pm$ 14.4, 65.0 (51.0, 73.0)	60.8 $\pm$ 14.2, 66.0 (62.0, 67.0)	56.0 $\pm$ 11.3, 55.0 (45.0, 63.0)	60.5 $\pm$ 12.4, 62.0 (48.0, 72.0)	64.0 $\pm$ 17.6, 71.0 (52.5, 77.0)	63.9 $\pm$ 12.1, 66.0 (56.3, 73.0)	63.3 $\pm$ 16.0, 53.0 (52.0, 69.5)	0.159
Gender (m/f in %)	110/55 (66.7/33.3)	20/13 (60.6/39.4)	15/6 (71.4/28.6)	18/9 (66.7/33.3)	28/15 (65.1/34.9)	26/12 (68.4/31.6)	3/0 (100/0)	0.888
Hypertension	74 (44, 8%)	1 (3%)	12 (57%)	17 (60, 7%)	19 (44%)	23 (60, 5%)	3	
NIDDM	27	0	6 (28, 5%)	5 (17, 8%)	8 (18, 6%)	7 (18, 4%)	1	
IDDM	13	0	0	0	5 (11, 6%)	8 (21%)	0	
GN, others		1 former GN in complete remission	mGN (1) SLE (1) ANCA (1)	GN 7 (25%), pHPT (2), sarkoidosis (3), myeloma (2)	GN 7 (16, 2%), PN 2 (4, 6%), myeloma 6 (13, 9%)	GN 8 (21%), toxN 6 (15, 7%), obstrN 3 (7, 9%)	mGN (1)	
Proteinuria <1 g/g creat		1	9 (42%)	13 (46%)	12 (28%)	15 (39%)	0	
Proteinuria >1 g/g creat		0	1 (4, 7%)	2 (7, 1%)	8 (18, 6%)	15 (31, 8)	3	
Uric acid ( $\mu$ mol/L)	375.3 $\pm$ 93.9, 369.0 (315.5, 424.1)	316.0 $\pm$ 79.7, 315.5 (267.9, 381.0)	353.4 $\pm$ 81.0, 330.4 (293.2, 431.5)	332.5 $\pm$ 76.6, 333.3 (273.8, 378.0)	391.9 $\pm$ 64.2, 372.0 (345.2, 422.6)	469.0 $\pm$ 92.8, 422.6 (401.8, 523.8)	333.3 $\pm$ 0.0, 333.3 (333.3, 333.3)	<0.001
C-reactive protein (mg/dL)	1.3 $\pm$ 1.9, 0.9 (0.4, 1.5)	0.6 $\pm$ 0.2, 0.5 (0.4, 0.6)	0.8 $\pm$ 0.5, 0.7 (0.6, 0.9)	1.4 $\pm$ 0.2, 1.4 (1.3, 1.5)	0.7 $\pm$ 0.4, 0.7 (0.4, 1.1)	2.5 $\pm$ 3.2, 1.7 (1.2, 2.0)	1.4 $\pm$ 0.0, 1.4 (1.4, 1.4)	<0.001
Urea (mmol/L)	11.9 $\pm$ 8.0, 8.4 (5.9, 16.7)	5.9 $\pm$ 1.6, 5.7 (4.9, 6.8)	5.1 $\pm$ 1.2, 4.9 (4.6, 5.9)	7.3 $\pm$ 3.4, 6.2 (6.0, 7.5)	11.9 $\pm$ 4.4, 11.8 (7.9, 15.5)	21.8 $\pm$ 6.5, 20.2 (16.7, 26.7)	28.4 $\pm$ 4.3, 29.5 (26.1, 31.2)	<0.001
Sodium (mmol/L)	138.0 $\pm$ 12.1, 139.0 (138.0, 141.0)	135.1 $\pm$ 19.9, 139.0 (138.0, 140.0)	138.2 $\pm$ 3.8, 138.0 (137.0, 141.0)	139.4 $\pm$ 2.9, 139.0 (139.0, 140.0)	139.1 $\pm$ 4.0, 139.0 (137.5, 141.0)	137.4 $\pm$ 17.0 (140.0, 139.0, 142.8)	141.7 $\pm$ 0.5, 142.0 (141.5, 142.0)	0.088
Potassium (mmol/L)	4.4 $\pm$ 0.6, 4.3 (4.0, 4.7)	4.2 $\pm$ 0.4, 4.1 (3.9, 4.5)	4.2 $\pm$ 0.9, 4.0 (3.9, 4.3)	4.3 $\pm$ 0.5, 4.3 (4.0, 4.6)	4.5 $\pm$ 0.5, 4.4 (4.2, 4.8)	4.7 $\pm$ 0.7, 4.7 (4.2, 5.1)	4.7 $\pm$ 0.2, 4.7 (4.6, 4.9)	<0.001
Calcium (mmol/L)	2.3 $\pm$ 0.2, 2.4 (2.3, 2.4)	2.4 $\pm$ 0.1, 2.4 (2.3, 2.5)	2.4 $\pm$ 0.1, 2.4 (2.3, 2.4)	2.4 $\pm$ 0.1, 2.4 (2.3, 2.4)	2.3 $\pm$ 0.2, 2.3 (2.3, 2.4)	2.36 $\pm$ 0.2, 2.3 (2.2, 2.4)	2.3 $\pm$ 0.1, 2.3 (2.3, 2.4)	0.096
Phosphate (mmol/L)	1.2 $\pm$ 0.3, 1.1 (1.0, 1.3)	1.1 $\pm$ 0.2, 1.1 (1.0, 1.2)	1.0 $\pm$ 0.2, 1.1 (0.9, 1.1)	1.0 $\pm$ 0.2, 1.0 (0.9, 1.2)	1.1 $\pm$ 0.2, 1.1 (0.9, 1.3)	1.4 $\pm$ 0.5, 1.3 (1.1, 1.5)	1.6 $\pm$ 0.6, 1.6 (1.3, 2.0)	<0.001
Total protein (g/L)	75 $\pm$ 28, 73 (67, 76)	73 $\pm$ 5, 73 (69, 77)	73 $\pm$ 6, 74 (67, 77)	73 $\pm$ 6, 74 (69, 76)	74 $\pm$ 10, 73 (70, 76)	84 $\pm$ 63, 67 (65, 73)	73 $\pm$ 0, 73 (73, 73)	0.325
Cholesterol (mmol/L)	5.7 $\pm$ 1.6, 5.8 (4.6, 7.0)	6.6 $\pm$ 1.0, 6.6 (5.8, 7.4)	6.0 $\pm$ 1.3, 6.1 (5.3, 7.2)	5.5 $\pm$ 1.3, 5.1 (4.8, 6.4)	4.8 $\pm$ 1.8, 4.4 (3.7, 5.0)	6.0 $\pm$ 1.3, 5.9 (5.1, 6.9)	4.9 $\pm$ 0.0, 4.9 (4.9, 4.9)	<0.001
HDL (mmol/L)	1.5 $\pm$ 0.4, 1.4 (1.2, 1.7)	1.6 $\pm$ 0.3, 1.6 (1.4, 1.8)	1.4 $\pm$ 0.5, 1.3 (1.0, 1.7)	1.4 $\pm$ 0.5, 1.2 (1.1, 1.7)	1.4 $\pm$ 0.4, 1.4 (1.2, 1.6)	1.5 $\pm$ 0.6, 1.3 (1.2, 1.6)	1.1 $\pm$ 0.0, 1.1 (1.1, 1.1)	0.112
LDL (mmol/L)	4.0 $\pm$ 1.3, 4.0 (3.0, 5.0)	4.6 $\pm$ 0.8, 4.6 (4.0, 5.1)	4.4 $\pm$ 1.2, 4.4 (3.9, 5.0)	3.7 $\pm$ 0.8, 3.7 (3.1, 4.5)	3.2 $\pm$ 1.4, 2.7 (2.4, 3.2)	4.0 $\pm$ 1.3, 4.1 (3.1, 4.9)	3.6 $\pm$ 0.0, 3.6 (3.6, 3.6)	<0.001
Triglyceride (mmol/L)	1.9 $\pm$ 1.4, 1.5 (1.0, 2.2)	1.5 $\pm$ 0.5, 1.5 (1.1, 1.9)	1.8 $\pm$ 0.8, 1.7 (1.3, 1.9)	2.4 $\pm$ 2.1, 1.7 (1.1, 2.6)	1.7 $\pm$ 1.2, 1.2 (0.9, 2.2)	2.4 $\pm$ 1.7, 2.1 (1.1, 3.0)	1.9 $\pm$ 0.0, 1.9 (1.9, 1.9)	0.256
Haemoglobin (mmol/L)	8.3 $\pm$ 1.6, 8.7 (7.9, 9.2)	8.9 $\pm$ 0.5, 8.9 (8.4, 9.2)	8.8 $\pm$ 1.5, 9.2 (8.7, 9.5)	8.2 $\pm$ 1.8, 8.6 (7.9, 9.2)	8.0 $\pm$ 2.1, 8.1 (7.0, 9.0)	7.7 $\pm$ 1.4, 8.2 (6.8, 8.9)	7.8 $\pm$ 0.0, 7.8 (7.8, 7.8)	0.006
Leucocyte count ( $10^9$ /L)	8.0 $\pm$ 7.4, 6.9 (5.9, 8.2)	8.1 $\pm$ 11.1, 6.4 (5.4, 7.1)	10.0 $\pm$ 10.0, 6.6 (6.1, 9.5)	7.6 $\pm$ 2.6, 8.0 (6.8, 8.6)	7.5 $\pm$ 4.6, 6.9 (6.1, 7.8)	7.2 $\pm$ 1.3, 7.4 (6.2, 8.4)	9.2 $\pm$ 0.0, 9.2 (9.2, 9.2)	0.057
Kidney biomarker level								
Uromodulin (ng/mL)	115.6 $\pm$ 101.0, 84.8 (37.0, 180.0)	260.4 $\pm$ 101.6, 227.6 (194.4, 277.8)	150.7 $\pm$ 54.8, 153.4 (121.5, 180.7)	116.7 $\pm$ 44.5, 111.8 (80.9, 139.4)	70.5 $\pm$ 47.1, 51.8 (36.1, 86.2)	29.1 $\pm$ 13.2, 30.3 (19.2, 38.0)	10.3 $\pm$ 7.0, 12.7 (6.7, 15.1)	<0.001

Continued

Table 2. Continued

	Total (n = 165)	Non-CKD (n = 33, 20.0%)	CKD 1 (n = 21, 12.7%)	CKD 2 (n = 27, 16.4%)	CKD 3 (n = 43, 26.1%)	CKD 4 (n = 38, 23.0%)	CKD 5 (n = 3, 1.8%)	P-value
Cystatin C (nmol/L)	147.1 ± 93.8, 113.8 (72.7, 206.3)	66.7 ± 10.1, 68.2 (62.9, 73.4)	62.1 ± 10.9, 66.7 (55.4, 70.4)	91.5 ± 9.5, 89.1 (85.0, 98.9)	151.9 ± 27.4, 147.6 (127.0, 176.4)	273.8 ± 39.3, 271.1 (247.9, 304.3)	453.1 ± 70.5, 445.7 (408.2, 494.3)	<0.001
Creatinine (µmol/L)	176.9 ± 126.1, 127.0 (93.0, 223.3)	96.7 ± 22.1, 94.0 (80.0, 117.0)	82.4 ± 16.8, 76.9 (72.0, 93.0)	107.0 ± 21.8, 105.0 (91.5, 119.2)	169.3 ± 52.7, 160.9 (125.0, 206.0)	333.5 ± 131.5, 316.5 (246.1, 383.1)	669.5 ± 153.0, 670.0 (575.9, 763.3)	<0.001
BUN (mmol/L)	11.9 ± 8.0, 8.4 (5.9, 16.6)	5.9 ± 1.6, 5.7 (4.9, 6.8)	5.1 ± 1.2, 4.9 (4.6, 5.9)	7.3 ± 3.4, 6.2 (6.0, 7.4)	11.9 ± 4.4, 11.8 (7.9, 15.5)	21.8 ± 6.5, 20.2 (16.7, 26.7)	28.3 ± 4.3, 29.4 (26.0, 31.2)	<0.001
eGFR <sub>H<sub>2</sub>Ook</sub> (mL/min)	64.2 ± 37.9, 57.7 (29.7, 92.9)	104.6 ± 20.7, 99.3 (91.9, 108.0)	114.0 ± 26.8, 101.0 (96.0, 123.2)	73.6 ± 7.8, 74.9 (67.1, 78.8)	43.7 ± 8.7, 43.6 (35.7, 51.1)	22.0 ± 3.8, 21.8 (19.1, 24.3)	11.8 ± 2.5, 12.0 (10.3, 13.4)	<0.001
eGFR <sub>CKD-EPI</sub> (mL/min)	49.4 ± 31.1, 44.0 (20.5, 71.5)	78.8 ± 17.5, 76.5 (65.1, 86.2)	92.8 ± 18.3, 88.9 (85.1, 97.0)	60.1 ± 11.5, 61.6 (51.5, 69.5)	32.5 ± 8.7, 31.4 (25.7, 39.5)	14.5 ± 4.0, 14.2 (11.6, 16.9)	6.7 ± 1.2, 7.0 (6.0, 7.5)	<0.001

NIDDM, non-insulin-dependent diabetes mellitus type II; IDDM, insulin-dependent diabetes mellitus type I; GN, glomerulonephritis; biopsy proven; pHPT, primary hyperparathyroidism; obstrN, obstructive nephropathy; toxN, CKD due to former nephrotoxic chemotherapy; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

steepest decline in sUmod, occurred within the 'creatinine blind range' (Figure 2).

All traditional kidney markers significantly differentiated between all CKD stages from 1 to 5, but none showed a conclusive statistical difference between non-CKD and CKD 1. Creatinine and cystatin C were even higher in non-CKD compared with CKD stage 1 [creatinine: median 94.0 µmol/L (IQR: 80.0, 117.0) versus 76.9 µmol/L (72.0, 93.0),  $P = 0.011$ ; cystatin C: median 68.2 nmol/L (IQR: 62.9, 73.4) versus 66.7 nmol/L (IQR: 55.4, 70.4),  $P = 0.153$ ].

ROC analysis of sUmod to differentiate between non-CKD and CKD 1 resulted in an AUC of 0.90 ( $P < 0.001$ , 95% CI 0.808 vs 0.990) at an optimal cut-off of 163 ng/mL (sensitivity 57.1%, specificity 100%). None of the other parameters including eGFR discriminated between stages non-CKD and CKD 1 (cystatin C: AUC 0.39; creatinine: AUC 0.29; BUN: AUC 0.37; eGFR<sub>CysC</sub>: AUC 0.39; eGFR (CKD-EPI): AUC 0.26) (Figure 3).

### sUmod in autoimmune diseases

Three patient cohorts with autoimmune diseases, including pulmorenal syndromes ( $n = 10$ ), SLE ( $n = 53$ ) and PLA2-R Ab-positive glomerulonephritis (mGN,  $n = 20$ ) were assessed. Patients with acute anti-GBM nephritis of CKD 3–5 had much lower sUmod compared with blood donors (median 37 ng/mL,  $P < 0.001$ , Supplementary data, Figure S1). Patients with SLE-nephritis (CKD 2–4) had median sUmod of 101 ng/mL,  $P < 0.001$ , while those without kidney involvement had similar sUmod levels compared with blood donors (median: 259 ng/mL,  $P = 0.485$ ). Patients with mGN had median sUmod of 81 ng/mL,  $P < 0.001$ .

### sUmod after KTX

As shown in Figure 4 prior to surgery sUmod was not detectable in all patients due to end-stage renal disease (ESRD). Immediately after transplantation sUmod rapidly increased in recipients with IGF and DGF until post-KTX day 5, reaching 43 ng/mL (median) in IGF and 33 ng/mL (median) in DGF, respectively. Thereafter, levels diverged from day 7 (IGF: 59.5 ± 41.2 ng/mL, DGF: 33.1 ± 20 ng/mL;  $P = 0.033$ ) to day 25. The cohort with DGF failed to reach sUmod levels above 40 ng/mL (cut off 39.26 ng/mL). From day 5 on, the RR and OR to develop DGF rose dramatically on the basis of sUmod (day 5: RR 1.3, OR 1.5; day 9: RR 2.9, OR 6.4, etc.; day 25: RR 8.2, OR 26; Supplementary data, Table S2). Sixteen of 17 patients with DGF needed 3–24 postoperative haemodialysis treatments. Patients with IGF were discharged from hospital after a median of 25 days, with a median creatinine of 118 µmol/L and a stable 'high' sUmod (76.6 ± 46.6 ng/mL). Patients with DGF and additional complications were discharged from hospital much later, at a median of 51 days, with a median creatinine of 180 µmol/L and lower sUmod concentration (33.9 ± 19.7 ng/mL,  $P < 0.001$ ).

### Umod distribution in normal human kidney

Labelling with anti-Umod antibodies was confined to the distal tubule (Figure 5). Umod was seen throughout the cytoplasm and in apical and basolateral membranes of the distal tubule cells. The images illustrate the transition from the unlabelled thin

**Table 3. Kidney allograft recipient's demographics**

Parameter	Total (n = 44)	Immediate graft function (n = 27)	Delayed graft function (n = 17)	P-value
Age (years)	42.0 ± 10.8, 41.0 (33.8, 51.3)	43.1 ± 10.6, 44.0, (34.5, 52.0)	43.0 ± 11.0, 39.0, (32.0, 48.0)	0.421
Gender				
Male	27 (61.4)	16 (59.3)	11 (64.7)	0.761
Female	17 (38.6)	11 (40.7)	6 (35.3)	
Kind of donation				
Deceased donor	44 (100)	27 (100)	17 (100)	1.000
Cold ischaemia time (min)	952.8 ± 455.3, 859.5 (609.8, 1227.0)	803.1 ± 347.9, 720.0 (570.5, 1005.0)	1190.6 ± 502.1, 1200.0 (794.0, 1500.0)	0.007
Maximum panel cytotoxicity (>5%)	11 (25.0)	7 (25.9)	4 (23.0)	1.000
Immunosuppression—Induction therapy				
ATG Fresenius, 9 mg/kg body weight intraoperatively (i.op.)	30 (68.2)	19 (70.4)	11 (64.5)	0.124
Lymphoglobulin Merieux, 30 mg/kg body weight i.op.	11 (25.0)	8 (29.6)	3 (17.6)	
ATG Biotest, 1.5 mg/kg body weight i.op.	1 (2.3)	0 (0)	1 (5.9)	
None	2 (4.5)	0 (0)	2 (11.8)	
Replacement (or additional therapies) therapy after Tx				
None	27 (61.4)	27 (100)	0 (0)	<0.001
Haemodialysis	16 (36.4)	0 (0)	16 (94.1)	
Peritoneal dialysis	1 (2.3)	0 (0)	1 (5.9)	
Number of dialysis sessions	9.1 ± 5.5	0	9.1 ± 5.5	
Patient with rejection during the first year	16 (36.4)	8 (29.6)	8 (47.1)	0.337
Patient with Cytomegalovirus infections during the first year	8 (18.2)	4 (14.8)	4 (23.5)	0.690
Biomarker levels at day of discharge				
Day of discharge after Tx	40.7 ± 21.3, 37.5 (23.8, 50.3)	30.4 ± 13.2, 25.0 (20.0, 42.5)	56.9 ± 21.5, 51.0 (38.0, 63.0)	0.004
Uromodulin (ng/mL)	60.1 ± 43.1, 47.6 (29.3, 75.8)	76.6 ± 45.7, 63.0 (45.0, 95.1)	33.9 ± 19.1, 29.4 (21.2, 39.0)	<0.001
Creatinine (µmol/L)	146.0 ± 53.5, 140.0, (107.8, 170.3)	124.7 ± 30.6, 118.0 (105.0, 145.5)	179.8 ± 63.7, 180.0, (161.0, 191.0)	<0.001
eGFR (MDRD) (mL/min)	49.2 ± 17.0, 47.1 (36.7, 58.9)	55.4 ± 15.5, 53.6, (44.6, 66.5)	39.4 ± 14.5, 37.4 (32.3, 47.0)	0.004

Data are presented as mean ± SD and median with IQR in brackets or as absolute numbers (n) with percentage (%) in brackets. Tx, transplantation; MDRD, Modification of Diet in Renal Disease.

ascending limb to the intensely labelled TAL. In the macula densa, the cells were largely unlabelled or weakly labelled, whereas the other distal tubule cells were intensely labelled.

### Umod location in kidney diseases

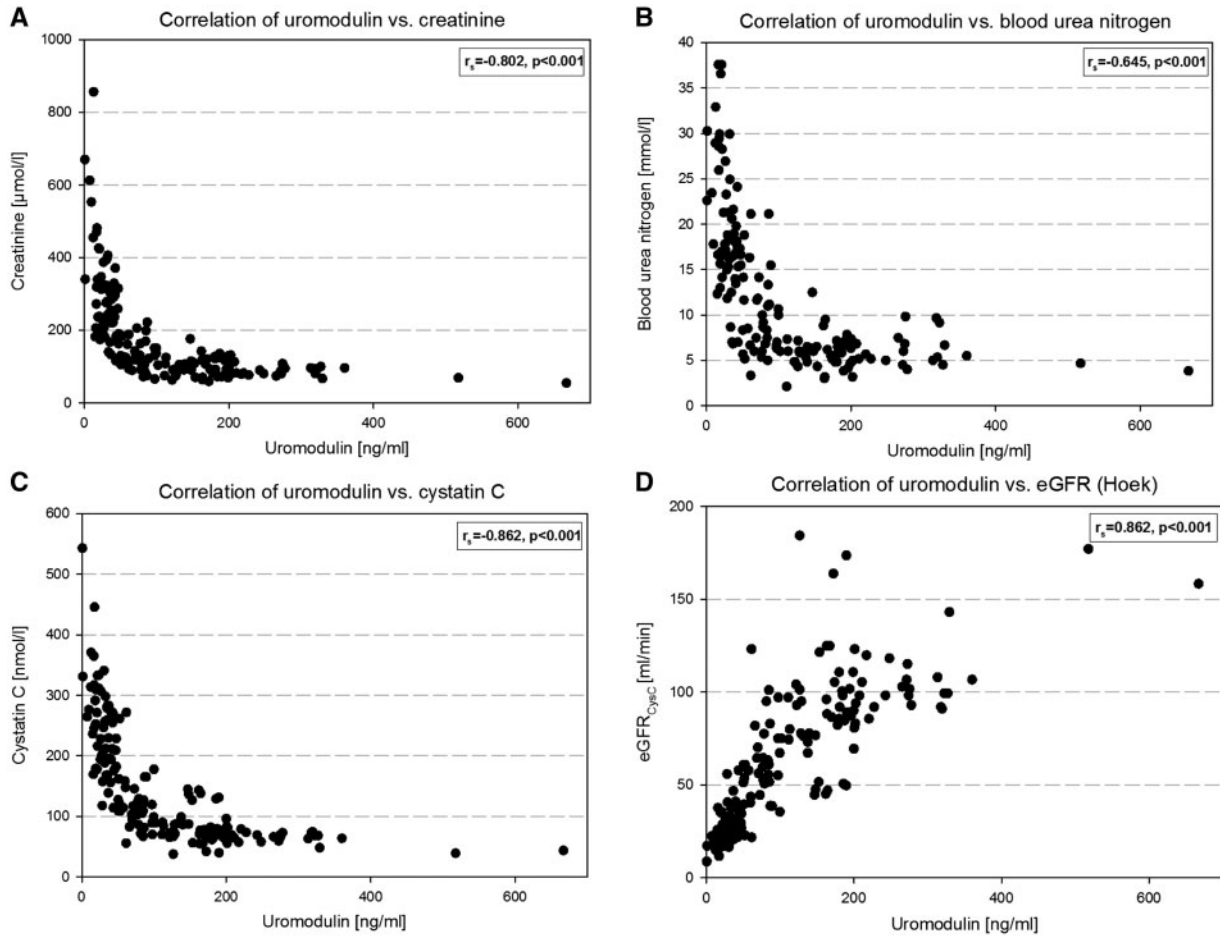
Umod staining of biopsy material from patients with proven kidney diseases and of grafts that had to be explanted due to ESRD showed an irregular pattern and increased intracellular expression of the antigen compared with healthy controls (Supplementary data, Figure S2). In kidney biopsies, TAL segments were under-represented compared with other tubular segments and were not accessible to further morphometric analyses. In most Umod-positive cells, the antigen appeared to accumulate and condense around the nuclei, the endoplasmic reticulum and the Golgi apparatus. The cytoplasm of TAL cells from explanted kidney grafts stained for Umod with a dramatically increased intensity; here potential changes in Umod expression of apical and basolateral membranes could not be assessed (Supplementary data, Figure S2).

### Immunoelectron microscopy

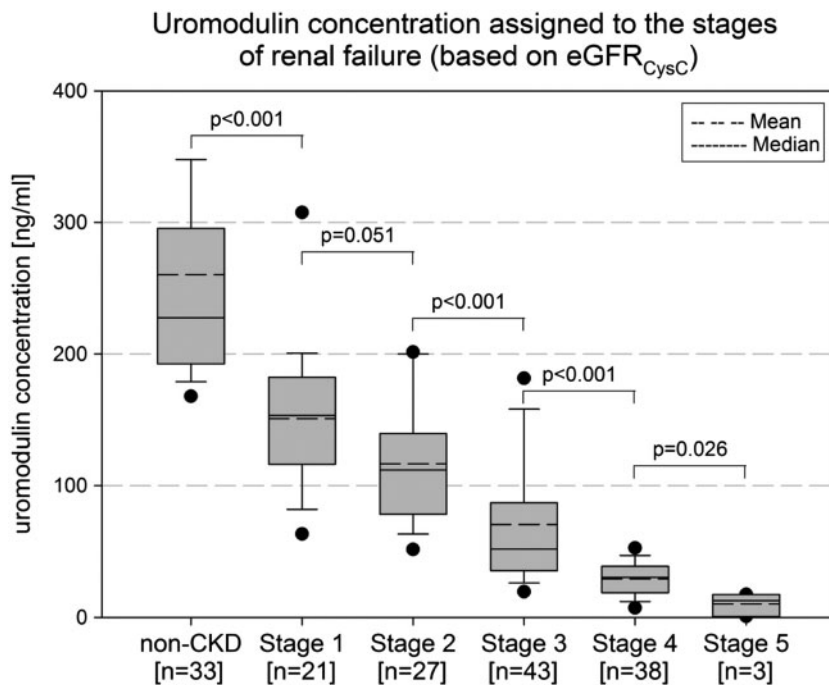
Immunogold labelling of Umod was seen at the apical and basolateral membrane, in the cytoplasm, the granular endoplasmic reticulum, the Golgi apparatus and in vesicles located apically but also in vesicles close to the basement membrane (Figure 6). Labelling was largely confined to the external layer of the apical and external parts of the basolateral plasma membrane including their infoldings. No labelling was seen in the collecting duct either in principal or in intercalated cells.

### DISCUSSION

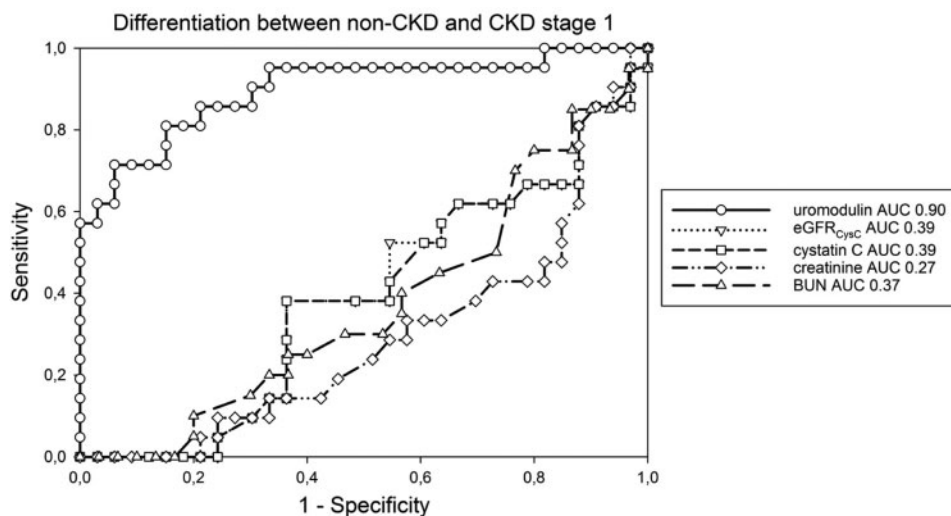
The majority of studies on Umod to date focus on the urinary form of Umod. sUmod in contrast has rarely been investigated [25, 26] and its clinical significance, molecular structure and function in blood are poorly understood. Brezis *et al.* first emphasized that the renal medulla is a neglected tissue compartment, not adequately reached by renal biopsy. Umod is a major secreted protein produced by the renal medulla and



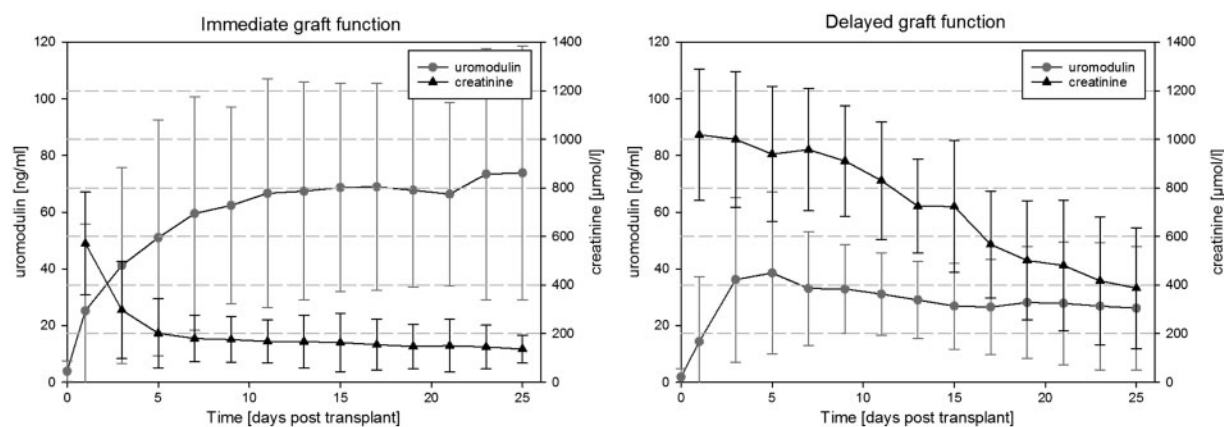
**FIGURE 1:** Relationship between serum uromodulin and creatinine (A), blood urea nitrogen (B), cystatin C (C) and eGFR<sub>CysC</sub> (D).  $r_s$ : Spearman's rank correlation coefficient.



**FIGURE 2:** Serum uromodulin concentration of patients without kidney diseases (non-CKD) and patients with CKD (stage 1 to stage 5) based on eGFR<sub>CysC</sub> assignment.



**FIGURE 3:** ROC analysing the ability of different parameters to differentiate patients without kidney disease (non-CKD) and CKD stage 1; BUN = blood urea nitrogen, eGFR = estimated glomerular filtration rate, AUC = area under the curve.



**FIGURE 4:** Post-kidney transplant comparison of serum uromodulin and creatinine in patients with immediate and delayed graft function.

experimental data support the pivotal role of medullary hypoxic injury in kidney diseases [12, 18–23, 27].

We assessed sUmod in patients with different CKD stages by ELISA. sUmod levels inversely correlated with creatinine and creatinine/cystatin C-based eGFR. The lowest sUmod was measured in patients with CKD stages 4 and 5. We speculate that low sUmod results from a disturbed biosynthesis in cells of the TAL. Patients with renal injury also show a decrease in urinary Umod levels [28, 32–34]. sUmod is not measurable in anephric patients [24]. In elderly patients, sUmod displayed inverse relationships with creatinine, cystatin C and urea, and correspondingly, a positive relation with eGFR [26]. Contradicting all recent data, Prajczek *et al.* report that sUmod is very low in healthy controls and increased in patients with CKD 1–5 [25]. We can only speculate that these conflicting results depend on the design of their ELISA and potential additional preanalytic factors.

Addressing the limitations of our study we did not measure GFR by inulin or iothexol clearance. Instead we calculated eGFR

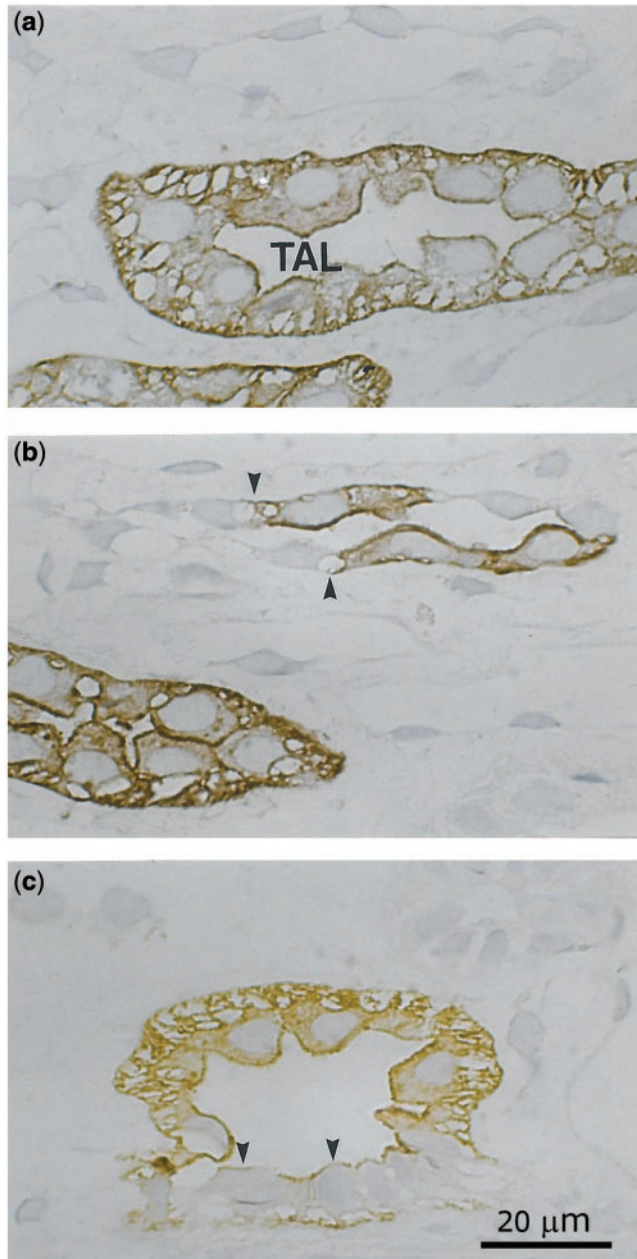
on a creatinine and cystatin C basis, which is presently still the most accepted method under clinical routine conditions.

With the help of our sensitive assay, to the best of our knowledge, we first demonstrated Umod as a physiological and very stable antigen circulating in the blood of healthy adults and children without any known kidney problems.

Importantly, we defined conspicuous sUmod at values below the fifth percentile range of healthy individuals and found different thresholds for children and males (<80 ng/mL) and for females (<100 ng/mL). Higher sUmod levels in healthy female compared with men were also observed in a previous study [26] and parallel similar sex differences in the urinary excretion of Umod and other tubular markers [43–46]. Although gender-dependent endocrine, haemodynamic, metabolic and genetic factors, expression of receptors, as well as sex differences in the susceptibility of cell integrity may contribute to this observation, most questions are still unanswered [47, 48].

The clinical data presented here are limited to patients with CKD (apart from the cohorts after grafting) and need to be





**FIGURE 5:** Immunohistochemical distribution of Umod in the normal human kidney (a–c); semithin cryosections. (a) Apical, basolateral and cytoplasmic staining. (b) transition of the thin ascending limb (negative) to the TAL (positive, arrows), (c) macula densa reveals a faint staining pattern only (arrowheads).

extended to cases suffering from AKI, which are now under study. In contrast to Risch *et al.* [26] we could not find a correlation between sUmod and age (age range from 18 to 60 years;  $r = -0.60$ ,  $P < 0.001$ ). A possible explanation is that Risch *et al.* focused on elderly participants (mean age  $71 \pm 7$  years) and the physiological decrease of sUmod probably starts at the age of 60 years. Age-related changes in the healthy kidney assessed by multidetector CT scans showed diverged alterations (disequilibrium) of renal compartments, where cortical volume (and GFR in parallel) declines, whereas medullary parenchymal volume

increases [49]. This suggests that synthesis and release of Umod by TAL into the blood might be maintained over a longer period.

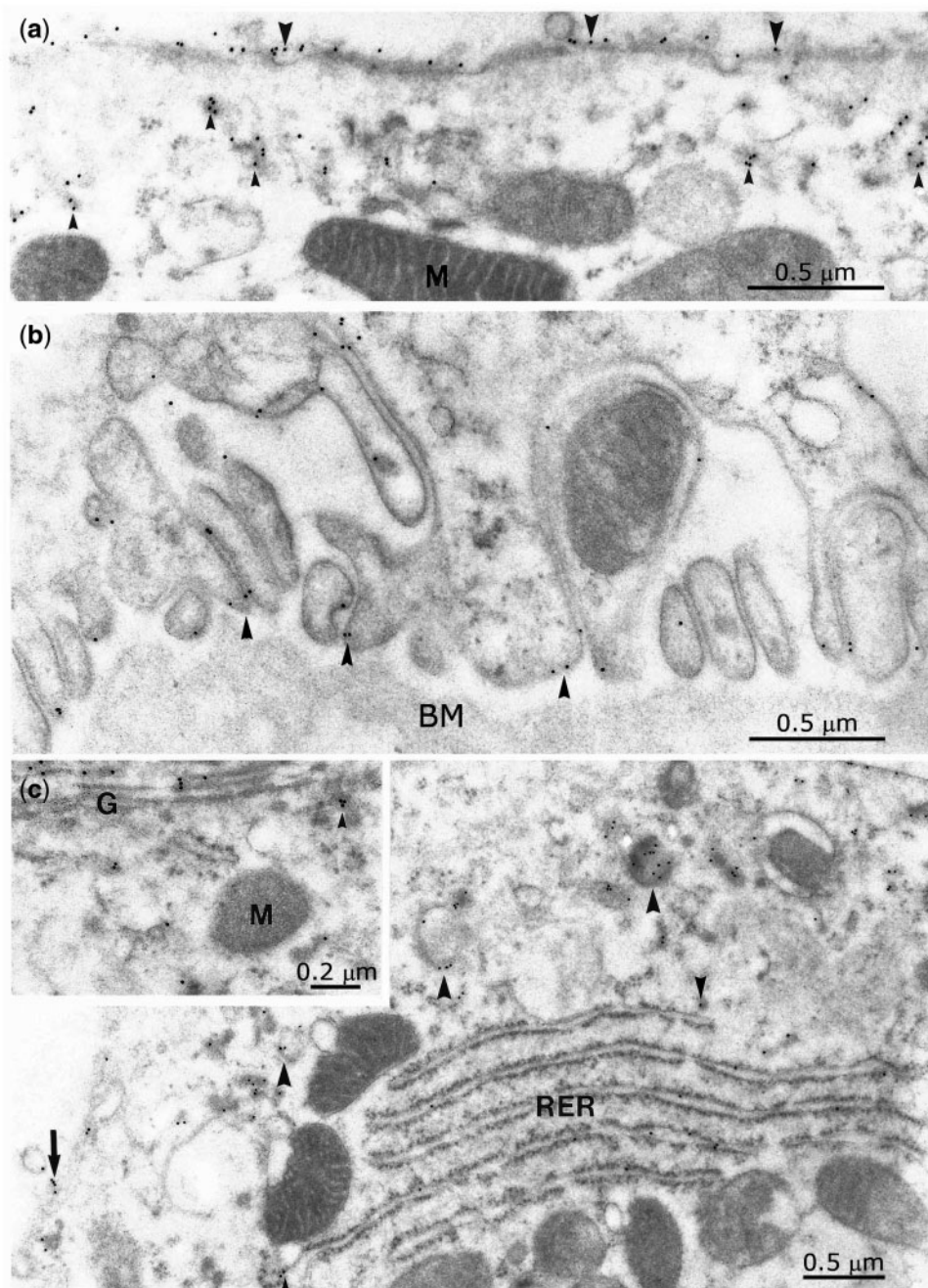
According to as yet unpublished studies, we solely found monomeric sUmod and so far did not observe any aggregation processes in serum. As a key novel finding we noticed extraordinarily stable signals over weeks even at increased temperatures. In contrast, Umod from urine (uUmod) lacks stability depending on the storage conditions, centrifugation, vortexing, pH, electrolytes and osmolality [31, 43]. The changing conformation of uUmod and the varying degree of polymerization, possibly modulating antigenic binding sites, may impede the reproducibility of the results.

A major finding is the comparative analysis of sUmod with creatinine, cystatin C and eGFR indicating that lower sUmod reflects a decline in kidney function and may precede the onset of CKD, as was shown by us in another patient cohort [50].

Tubulointerstitial injury is closely linked to progression of renal disease [51, 52]. We assume that low sUmod parallels a structural derangement of the TAL. sUmod levels may drop due to altered intracellular processing and reduced basolateral exocytosis and mirror the reduced quantity of intact TAL cells. Acute and chronic renal injury characterized by epithelial atrophy and tubular loss of both cortical and medullary segments may also parallel changes of the vascular microarchitecture [53]. Interstitial vascular remodelling may influence the structural integrity of tubular cells, e.g. vulnerable TAL segments, and change glomerular morphology and haemodynamics, which would link sUmod levels with eGFR (CKD stages). In the present study, we could not assess the relation between histological alterations of Umod-expressing cells and sUmod. The amount of TAL segments varied greatly among the biopsies, and Umod-positive cells appear considerably under-represented in renal clinical pathology.

One known secretory pathway of Umod occurs at the apical membrane of the TAL cells. Umod concentrations in urine are approximately 1000-fold higher than in serum. Umod's apical secretion is based on exocytosis and the formation of a GPI anchor, followed by its release from the membrane via proteolytic cleavage [3, 27]. However, besides the luminal secretion process of Umod an additional basolateral pathway was suggested [54, 55]. Our data confirm the view that Umod is synthesized in the endoplasmic reticulum, transferred to the Golgi apparatus and transported to the apical membrane by vesicles. Finally it sheds into the luminal space. In addition, we clearly demonstrated a second, basolateral pathway of Umod secretion (Figure 6), which apparently contributes to sUmod levels (Figure 7).

We interpret sUmod as an early indicator defining the integrity and the functional viability of the TAL. In contrast, the traditional endogenous filtration markers are not related to defined renal structures and need mathematical transformation. Established renal markers have limitations in detecting early or slight changes in kidney function, particularly in a range when approximately half of the filtration capacity is lost [56]. Following the analyses and conclusions from experimental animal data on TAL segments [18–23] we assume that sUmod

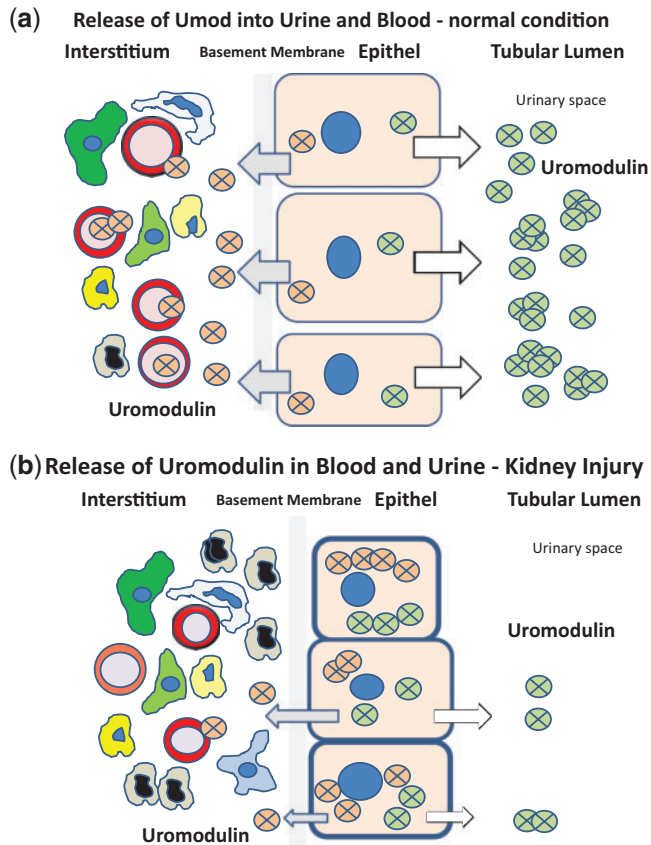


**FIGURE 6:** Immunogold labelling of Umod revealing the transport of the antigen within cytoplasmatic vesicles (small arrowheads) to the apical (a) as well as to the basolateral cell pole (b). Shuttle of uromodulin (c) from the endoplasmatic reticulum (RER) to the Golgi apparatus (G) (inset to C), through cytoplasmic vesicles (arrowheads). Basolateral membrane (BM) labelling (arrow). M, mitochondria.

levels may somehow reflect the amount of intact nephrons being linked to the 'functioning kidney mass'.

Another important new finding is that, in accordance with eGFR, sUmod recovers to higher levels as shown in transplant patients, and may distinguish IGF from DGF. Worsening of graft function was always accompanied by long-lasting reduced sUmod levels and predictable after the seventh postoperative day. It is a subject of future research to clarify whether low sUmod levels in patients with DGF are predictive for later chronic graft dysfunction and serious patient outcome [57].

In conclusion, we have demonstrated that sUmod concentrations decrease in line with eGFR and with biopsy-classified stages of renal failure. Our data strengthen the link between sUmod and kidney disease and suggest that sUmod might be superior compared with the traditional filtration markers to detect early stages of CKD, independent of the underlying disease. sUmod should not only be interpreted as a complementary parameter describing global 'kidney function', but may shift our focus to potential structural and metabolic lesions of TAL cells. These encourage more extensive elucidation of the role of



**FIGURE 7:** Cartoon demonstrating the proposed bidirectional physiological transfer of Umod to the urinary space and to the tubulo-interstitial space. As shown in Figure 6b, Umod is physiologically released from basolateral plasma membranes and membrane infoldings into the interstitium and enters the blood compartment (a). Umod serum levels are correlated with eGFR. sUmod concentrations may depend on the cellular integrity of the TAL segment. Therefore, sUmod might be released into serum at a decreased rate in renal injury (b).

sUmod as a diagnostic tool in comparison with, and likely in concert with, other novel renal biomarkers [58–61].

## SUPPLEMENTARY DATA

Supplementary data are available online at <http://ndt.oxfordjournals.org>.

## ACKNOWLEDGEMENTS

We thank Prof. Walter Nathrath, Institute of Pathology, Prof. Oliver Reich, Department of Urology, Klinikum Harlaching, LMU München, and Dr Florian Laenger, Institute of Pathology, Goethe-University Frankfurt am Main and Hannover Medical School, for supplying paraffin-embedded material and analysing tissue staining patterns. Parts of the work were presented in abstract form at the congress of the German Society of Nephrology, Berlin, September 2015, and the congress of Deutsche Transplantationsgesellschaft, Dresden, October 2015, and the 10th International Congress

on Autoimmunity, Leipzig, April 2016. We thank Prof. Seymour Rosen, Beth Israel Deaconess MC, Harvard Medical School, Boston, Prof. Anna Koettgen, Medizinische Klinik IV, Department of Nephrology, University of Freiburg, and Dr Ulrike Krummrei, Euroimmun, for critical proofreading of the manuscript, and Prof. Olivier Devuyst, University Zürich, Switzerland, and Prof. Hans-Joachim Anders, LMU Munich for collaboration and valuable discussions.

## CONFLICT OF INTEREST STATEMENT

J.E.S. received travel support from Hoffmann-La Roche and Euroimmun, R.G. received travel support from Roche and ThermoScientific, V.H., M.B. and W.S. are full time employees of Euroimmun and J.K. is member of the supervisory board of Euroimmun. W.A.N., E.I.C. and H.S. declare no potential conflicts of interest.

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Received: 14.8.2016; Editorial decision: 7.11.2016