Detection of Subclinical Tubular Injury After Renal Transplantation: Comparison of Urine Protein Analysis With Allograft Histopathology

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Background. Tubulointerstitial injury due to rejection leads to tubular atrophy (TA)/interstitial fibrosis (IF) followed by deterioration of allograft function. This study investigated whether urinary tubular injury biomarkers can detect subclinical tubulitis found in protocol biopsies allowing for a noninvasive screening procedure.

Methods. Four rigidly defined groups (stable transplants with normal tubular histology [n = 24], stable transplants with subclinical tubulitis [n = 38], patients with clinical tubulitis Ia/Ib [n = 18], and patients with other clinical tubular pathologies [n = 20]) were compared for differences in urinary intact/cleaved β2-microglobulin (i/cβ2m), retinol-binding protein (RBP), neutrophil-gelatinase-associated lipocalin (NGAL), and α1-microglobulin (α1m).

Results. Tubular proteinuria was present in 38% (RBP) to 79% (α1m) of patients in the stable transplant with normal tubular histology group. The stable transplant with subclinical tubulitis group had slightly higher levels of i/cβ2m (P = 0.11), RBP (P = 0.17), α1m (P = 0.09), and NGAL (P = 0.06) than the stable transplant with normal tubular histology group with a substantial overlap. The clinical tubulitis Ia/Ib and the other clinical tubular pathology groups had significantly higher levels of RBP, NGAL, and α1m than stable transplants with normal tubular histology or stable transplants with subclinical tubulitis (P < 0.002).

Conclusions. None of the investigated biomarkers allow for clear differentiation between stable transplants with normal tubular histology and stable transplants with subclinical tubulitis. Therefore, the protocol allograft biopsy currently remains the preferred tool to screen for subclinical tubulitis. Further longitudinal studies should determine whether tubular proteinuria in stable transplants with normal tubular histology indicates a clear risk for early development of TA/IF.

Keywords: Renal allograft rejection, Tubular injury, Urine proteins, Protocol biopsy, Noninvasive monitoring.

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The tubulointerstitial compartment is a major target for the alloimmune response. In addition, ischemia-reperfusion, calcineurin inhibitors, and infections (e.g., polyomavirus associated nephropathy [PVAN]) can damage tubular epithelial cells. As a consequence of persisting injury tubular atrophy (TA) and interstitial fibrosis (IF) can develop (1–4). Coexisting TA/IF and inflammation (i.e., tubulitis) are associated with progression of tubulointerstitial pathology, subsequent deterioration of allograft function, and finally allograft failure (5–8). It is tempting to speculate that undetected continuous or intermittent injuries in the tubulointerstitial compartment could partly explain why long-term allograft survival has only marginally improved in recent years despite a significant reduction of acute clinical rejection episodes (9). Therefore, early detection of tubulointerstitial injury followed by adequate therapy might be important to further improve long-term renal allograft survival.

Several protocol biopsy studies have demonstrated that serum creatinine underestimates the extent of both tubulointerstitial inflammation (i.e., subclinical tubulitis) and TA/IF supporting a protocol biopsy-based strategy for patient management (1, 5, 6, 10–12). However, protocol biopsies cannot be performed on a regular basis throughout the lifetime of an allograft due to the associated inconvenience, morbidity, and costs. Therefore, screening for tubulointerstitial injury by noninvasive biomarkers would be useful to select patients that could benefit from an allograft biopsy to determine the cause of injury. Furthermore, such biomarkers could be used to noninvasively monitor the response to therapeutic interventions.

Previously, using an unbiased proteomic-based strategy, we identified in a rigidly defined patient cohort cleaved urinary β2-microglobulin (cβ2m) as a potential biomarker for acute clinical tubulointerstitial injury related to rejection (13, 14). The aim of this study was to investigate whether cβ2m and other urine biomarkers for tubular injury (i.e., retinol-binding protein [RBP], neutrophil-gelatinase-associated lipocalin [NGAL], and α1-microglobulin [α1m]) allow detection of subclinical tubulitis found in protocol biopsies allowing for a noninvasive screening procedure.
MATERIALS AND METHODS

Patient Population

All urine samples analyzed in this study were obtained with informed consent and ethics approval by the local institutional review boards. From 2003 to 2005, midstream urine samples from 214 patients were collected at the University Hospital Basel immediately before a protocol (n=249) or clinically indicated (n=207) renal allograft biopsy was performed. These 214 patients represent 90% of all patients who had an allograft biopsy within this time frame. Protocol biopsies were scheduled at months 3 and 6 posttransplant. A clinically indicated biopsy was performed when serum creatinine was elevated from baseline by more than 20%. All biopsy specimens (two cores obtained with a 16-gauge needle) were evaluated by light microscopy, immunofluorescence (C4d), and immunohistochemistry (SV40 antigen). Findings were graded.

Eighty-seven of 214 patients (41%) were included because serum creatinine was elevated from baseline by more than 20%. All biopsy specimens (two cores obtained with a 16-gauge needle) were evaluated by light microscopy, immunofluorescence (C4d), and immunohistochemistry (SV40 antigen). Findings were graded according to the updated Banff ’97 classification (15). Eighty-seven of 214 patients (41%) were included because they met defined criteria regarding key elements of tubulointerstitial pathology as described in detail below. Thirteen patients with subclinical tubulitis Ia/Ib, who had not been previously analyzed, were added from the Winnipeg transplant cohort to increase this diagnostic category. In total, 100 patients were analyzed in this study. Every patient contributed only one urine sample to the study. The investigated groups were rigidly defined based on the allograft biopsy results, the allograft function, and the clinical course as follows:

1. Normal tubular histology group: Consists of 24 urine samples from 24 patients, who had stable transplant function with a Modification of Diet in Renal Disease (MDRD) glomerular filtration rate (GFR) ≥40 mL/min, no history of clinical or biopsy-proven rejection, and a protocol biopsy with no tubular, glomerular, or vascular infiltrates (i.e., Banff i0−t0g0v0) as well as negative C4d-staining in peritubular capillaries (PTC).

2. Subclinical tubulitis group: Consists of 38 urine samples from 38 patients, who had stable transplant function with a MDRD-GFR ≥40 mL/min and a protocol biopsy demonstrating one of the two following grades of tubulitis. Subgroup 1: borderline tubulitis (n=15); subgroup 2: tubulitis Ia/Ib (n=23; 13 patients were from the Winnipeg transplant cohort).

3. Clinical tubulitis Ia/Ib group: Consists of 18 urine samples from 18 patients, who had a clinically indicated biopsy (serum creatinine >20% above baseline) demonstrating tubulitis Ia/Ib.

4. Other clinical tubular pathology group: Consists of 20 urine samples from 20 patients, who had a clinically indicated biopsy (serum creatinine >20% above baseline) demonstrating one of the two following tubular pathologies. Subgroup 1: PVAN defined by positive staining for SV40 antigen and polyoma BK-virus plasma polymerase chain reaction (PCR) >10,000 copies/mL (n=7); subgroup 2: moderate to severe TA/IF in the absence of tubular, glomerular, or vascular infiltrates (i.e., Banff i0−t0g0v0, ci2−3ct2−3; n=13).

Urine Collection

Midstream urine samples collected in Basel (n=87) were centrifuged at 1500g for 5 min and supernatants were used for further analysis. Measurements of creatinine, total protein, albumin, RBP, and α1m were performed immediately as part of clinical routine. Measurement of cβ2m and intact β2m (iβ2m) with surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) and NGAL by enzyme-linked immunosorbent assay (ELISA) were done on aliquots stored at −80°C. Midstream urine samples collected in Winnipeg (n=13; subclinical tubulitis Ia/Ib group) were stored noncentrifuged at −70°C prior to all analysis.

Measurement of Total Urine Protein, Albumin, RBP, α1m, and NGAL

Measurement of total protein (benzethonium chloride method) and creatinine (enzymatic method) were performed on a Modula clinical chemistry analyzer (Roche Diagnostics, Roche, Switzerland). Albumin, RBP, and α1m were measured on a Beckman-Coulter array nephelometry system (Beckman-Coulter, Brea, CA). Antibodies used in these assays were purchased from Beckman-Coulter (albumin, α1m) and Dako A/S Glostrup, Denmark (RBP). The analytical sensitivity limits of the assays were 40 mg/L for total protein, 2 mg/L for albumin, 4 mg/L for α1m, and 0.3 mg/L for RBP. NGAL was measured with an ELISA kit (Antibodyshop, Gentofte, Denmark) according to the instructions of the manufacturer. In order to correct for different urine dilution, excretion of urine proteins are given in relation to urine creatinine (i.e., milligrams or micrograms of protein per mmol of creatinine). Normal values used in this study for total protein (<11.3 mg/mmol), albumin (<2.26 mg/mmol), α1m (<1.58 mg/mmol), and RBP (<0.08 mg/mmol) were determined in >400 healthy individuals (unpublished data) and used in our clinical practice for years (16, 17). Normal range for NGAL (<1.63 μg/mmol corresponding to <10 μg/L) is based on values observed in 92 healthy individuals who all had NGAL concentrations <10 μg/L (own data [n=8] and published data [18, 19]).

Measurement of Cleaved and Intact β2m With SELDI-TOF-MS

Urine samples were thawed on ice and vortexed. Analysis was performed as described previously (13). Briefly, 5 μL of urine was applied to normal phase chips (ProteinChip NP20; Ciphergen, Freemont, CA) and incubated for 30 min in a humidity chamber. Spots were then washed three times with 5 μL high-performance liquid chromatography grade water and air-dried for 10 min. Then 1 μL of 35% α-cyano-4-hydroxycinnamic acid (CHCA; Ciphergen) was applied to each spot and air-dried. Chips were read with a SELDI-TOF-MS instrument (ProteinChip Reader II; Ciphergen) in the positive ion mode with the following settings: laser intensity, 215; detector sensitivity, 6; detector voltage, 1700 V; 240 shots were collected per sample. Calibration was done externally with a mixture of four proteins with masses ranging from 2 to 16 kDa. For comparison, spectra were normalized by total ion current and transformed into gel-view. Estimation of the amount of urinary cβ2m and iβ2m is semiquan-
titative based on peak intensities. The detection limit for iβ2m and cβ2m by SELDI-TOF-MS is 0.1 mg/L. The upper limit of normal urinary β2m measured by immunoassays has been reported to be 0.2 mg/L. Therefore, lack of detection of iβ2m and cβ2m by SELDI-TOF-MS is consistent with concentrations in the normal range.

Statistical Analysis

We used JMP software version 6.0 (SAS Institute Inc., Cary, NC) for statistical analysis. Data are given as median (range). For categorical data, Fisher’s exact test or Pearson’s chi-square test was used. As all continuous data were not normally distributed Wilcoxon or Kruskal-Wallis rank sum tests were used for analysis. Significant results in the Kruskal-Wallis rank sum test were further analyzed with pairwise non-parametric tests. A P-value <0.05 (two-sided test) was considered to indicate statistical significance.

RESULTS

Patient Characteristics

The characteristics of the patient groups are detailed in Table 1. Patients in the normal tubular histology group (n=24) were different from those in the subclinical tubulitis group (n=38) regarding the extent of acute tubulointerstitial inflammation, but equal with respect to GFR (P=0.90), immunosuppressive regimen (P>0.24), time posttransplant (P=0.25), and TA-/IF-scores (P>0.10). Patients with clinical tubulitis Ia/Ib (n=18) had the same acute and chronic histological Banff-scores as patients with subclinical tubulitis Ia/Ib.

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<th>Table 1. Baseline characteristics</th>
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<sup>a</sup> Most patients were either on mycophenolate–mofetil or azathioprine.

<sup>b</sup> GFR calculated with the MDRD study equation: 186 × serum creatinine<sup>−1.154</sup> × age<sup>−0.203</sup> × (if subject is female).

<sup>c</sup> P=0.025 vs. subclinical tubulitis, P=0.15 vs. clinical tubulitis Ia/Ib, and P=0.0001 vs. other clinical tubular pathology.

<sup>d</sup> P=0.81 vs. subclinical tubulitis, P<0.0001 vs. clinical tubulitis Ia/Ib, and P<0.0001 vs. other clinical tubular pathology.

<sup>e</sup> P=0.90 vs. subclinical tubulitis, P<0.0001 vs. clinical tubulitis Ia/Ib, and P<0.0001 vs. other clinical tubular pathology. GFR between the two subgroups within the subclinical tubulitis group (i.e., borderline tubulitis, tubulitis Ia/Ib) were not different (P=0.32).
Correlation of Tubular Pathology With Urinary \( \beta 2m \) Detected by SELDI-TOF-MS

Detectable \( \beta 2m \) (\( \beta 2m \) and/or \( \beta 2m \)) by SELDI-TOF-MS indicating an amount \( >0.1 \) mg/L was observed in 11 of 24 patients (46%) in the normal tubular histology group and in 26 of 38 patients (68%) in the subclinical tubulitis group (\( P=0.11 \)). In the clinical tubulitis \( Ia/Ib \) group, 17 of 18 patients (94%) had detectable \( \beta 2m \) (\( P=0.001 \) vs. normal tubular histology; \( P=0.04 \) vs. subclinical tubulitis); in the other clinical tubular pathology group, 14 of 20 patients (70%) had detectable \( \beta 2m \) (\( P=0.14 \) vs. normal tubular histology; \( P=1.0 \) vs. subclinical tubulitis; \( P=0.09 \) vs. clinical tubulitis \( Ia/Ib \); Fig. 1).

Influence of Urine pH on Generation of \( \beta 2m \)

Urine pH values of the four different groups were equal with an overall median of 5.6 (range: 4.9–7.9; \( P=0.43 \)). SELDI-TOF-MS analysis illustrated that in some patients only \( \beta 2m \) was detectable, whereas others showed predominantly \( \beta 2m \). The relative distribution of \( \beta 2m \) and \( \beta 2m \) was related to a urine pH above or below 6 (Figs. 1 and 2).

Correlation of Tubular Pathology With Total Urine Protein, Albuminuria, RBP, \( \alpha 1m \), and NGAL

Total protein/creatinine ratios in the normal tubular histology group (11.4 mg/mmol, range: 5.3–49.8) were lower than in the subclinical tubulitis group (17.6 mg/mmol, range: 4.9–74.5) with a substantial overlap (\( P=0.05 \)). The clinical tubulitis \( Ia/Ib \) group had higher total protein/creatinine ratios (28.3 mg/mmol, range: 7.4–401.8) than the normal tubular histology group (\( P<0.0001 \)) and the subclinical tu-

\( n=23; \ P>0.11 \), but their GFR was significantly lower (32 mL/min (8–51) vs. 53 mL/min (40–93); \( P<0.001 \)).
bulitis group ($P=0.002$), but they were not different from those in the other clinical tubular pathology group (39.1 mg/mmol, range: 7.3–694.5; $P=0.84$; Fig. 3A).

Albumin/creatinine ratios in the normal tubular histology group (2.6 mg/mmol, range: 0.4–30.7) were statistically not different from the subclinical tubulitis group (3.8 mg/mmol, range: 0.5–52.9; $P=0.28$). The clinical tubulitis Ia/Ib group had higher albumin/creatinine ratios (15.3 mg/mmol, range: 0.5–327) than the normal tubular histology group ($P=0.0009$) and the subclinical tubulitis group ($P=0.0004$), but they were not different from those in the other clinical tubular pathology group (12.1 mg/mmol, range: 2.4–596; $P=0.96$).

RBP/creatinine ratios in the normal tubular histology group (0.04 mg/mmol, range: 0.01–1.95) were statistically not different from the subclinical tubulitis group (0.09 mg/mmol, range: 0.01–3.49; $P=0.17$). The clinical tubulitis Ia/Ib group had higher RBP/creatinine ratios (1.05 mg/mmol, range: 0.02–8.82) than the normal tubular histology group ($P<0.0001$) and the subclinical tubulitis group ($P<0.0001$), but they were not different from those in the other clinical tubular pathology group (1.46 mg/mmol, range: 0.01–10.96; $P=0.43$; Fig. 3B).

$\alpha$1m/creatinine ratios in the normal tubular histology group (3.5 mg/mmol, range: 0.4–18.4) were statistically not different from the subclinical tubulitis group (4.9 mg/mmol, range: 0.3–24.7; $P=0.09$). The clinical tubulitis Ia/Ib group had higher $\alpha$1m/creatinine ratios (14.9 mg/mmol, range: 3.1–65.9) than the normal tubular histology group ($P<0.0001$) and the subclinical tubulitis group ($P=0.0002$), but they were not different from those in the other clinical tubular pathology group (14.6 mg/mmol, range: 2.3–86.2; $P=0.67$; Fig. 3C).

NGAL/creatinine ratios in the normal tubular histology group (1.5 $\mu$g/mmol, range: 0.2–17.4) were statistically

![FIGURE 3](image_url). Urine proteins measurements. Urine protein concentrations are normalized to urine creatinine and given as mg or $\mu$g/mmol creatinine. Data are presented as box and whiskers plots. The box represents the interquartile range with the median. Individual points represent outliers. Gray areas indicate normal ranges for healthy individuals. (A) Total protein concentration. (B) RBP concentration. (C) $\alpha$1m concentration. (D) NGAL concentration. Differences across all groups were statistically significant (total protein: $P<0.0001$; RBP: $P<0.0001$; $\alpha$1m: $P<0.0001$; NGAL: $P<0.0001$; Kruskal-Wallis rank sum tests). Individual comparisons noted in the figures were done by Wilcoxon rank sum tests.
not different from the subclinical tubulitis group (2.4 μg/mmol, range: 0.3–29.9; \( P = 0.06 \)). The clinical tubulitis la/lb group had higher NGAL/creatinine ratios (9.2 μg/mmol, range: 1.3–261.0) than the normal tubular histology group (\( P = 0.0002 \)) and the subclinical tubulitis group (\( P = 0.002 \)), but they were not different from those in the other clinical tubular pathologies group (9.8 μg/mmol, range: 0.7–101.3; \( P = 0.48 \); Fig. 3D).

**Subgroup Analyses**

There were no differences within the two subgroups of the subclinical tubulitis group (i.e., borderline tubulitis vs. tubulitis la/lb) regarding total urine protein, albuminuria, RBP, α1m, and NGAL (\( P > 0.10 \)). Furthermore, total urine protein, albuminuria, RBP, α1m, and NGAL were not different between patient from Basel (\( n = 10 \)) and Winnipeg (\( n = 13 \)) in the subclinical tubulitis la/lb group (\( P > 0.08 \)). However, patients with clinical tubulitis la/lb had significantly higher total urine protein, RBP, α1m, and NGAL values than patients with subclinical tubulitis la/lb (total urine protein: 28.3 mg/mmol [range: 7.4–401.8] vs. 17.6 mg/mmol [range: 8.3–73.9], \( P = 0.01 \); RBP: 1.05 mg/mmol [range: 0.02–8.82] vs. 0.23 mg/mmol [range: 0.01–3.49], \( P = 0.002 \); α1m: 14.9 mg/mmol [range: 3.1–65.9] vs. 5.8 mg/mmol [range: 0.4–24.6], \( P = 0.002 \); NGAL: 9.2 μg/mmol [range: 1.3–261.0] vs. 2.5 μg/mmol [range: 0.7–29.9], \( P = 0.02 \)).

**Correlation of Tubular Injury Biomarkers (RBP, α1m, and NGAL) With Total Urine Protein and Albuminuria**

RBP and α1m (\( r^2 = 0.85, P < 0.0001 \); Fig. 4A), RBP and NGAL (\( r^2 = 0.47, P < 0.0001 \)), as well as α1m and NGAL (\( r^2 = 0.53, P < 0.0001 \)) were closely associated. Total urine protein excretion correlated very strongly with albuminuria (\( r^2 = 0.98, P < 0.0001 \); Fig. 4B), but weakly with α1m (\( r^2 = 0.12, P = 0.0005 \); Fig. 4C), RBP (\( r^2 = 0.13, P = 0.0002 \)) and NGAL (\( r^2 = 0.12, P = 0.0004 \)). We found no correlation between albuminuria and α1m (\( r^2 = 0.02, P = 0.24 \); Fig. 4D), RBP (\( r^2 = 0.02, P = 0.19 \)), and NGAL (\( r^2 = 0.03, P = 0.11 \)).
DISCUSSION

The results of this study indicate that tubular epithelial dysfunction defined by elevation of tubular injury biomarkers (i/cβ2m, RBP, α1m, and NGAL) is very common after renal transplantation. Indeed, more than 40% of stable transplants with normal tubular histology had tubular injury biomarkers above the normal range of healthy individuals (RBP: 38%, NGAL: 42%, i/cβ2m: 46%, α1m: 79%). Furthermore, all tubular injury biomarkers were significantly higher in patients with clinical tubular pathologies (i.e., tubulitis Ia/Ib, PVAN, and moderate to severe TA/IF) than in stable transplants with normal tubular pathology or stable transplants with subclinical tubulitis. However, none of the investigated biomarkers allowed for clear differentiation of stable transplants with normal tubular histology from those with subclinical tubulitis.

Intact β2m, RBP, and α1m are low molecular weight proteins (11.7, 21, and 27kDa, respectively) that are freely filtrated through the glomeruli, almost completely reabsorbed by tubular epithelial cells and degraded in lysosomes (22). As long as tubular epithelial cell function is not impaired, only trace amounts of i/cβ2m, RBP, and α1m are excreted in urine. NGAL, which was recently introduced as a very early and sensitive biomarker for tubular injury, is actively secreted from damaged tubular epithelial cells and therefore assesses a different aspect of tubular epithelial cell function (18, 23, 24). However, all tubular injury biomarkers correlated strongly with each other and provided similar information. Because the patients investigated in this study were highly selected, we have avoided calculating parameters that are used to characterize and compare individual biomarkers (e.g., sensitivity, specificity, positive and negative predictive value, receiver operating characteristic curve) as this might be misleading (25).

RBP (26), α1m (22) and NGAL (19) are very resistant to proteolytic degradation allowing for reliable measurement by ELISA. In contrast, β2m can remain intact or can be partially/completely transformed into cβ2m which might not be detectable by ELISA (22, 26–28). Due to this limitation measurements of “β2m” by ELISA have to be interpreted with caution. The cleavage of β2m is dependant on the amount and activity of aspartic proteases in the urine, which is almost completely inhibited at pH ≥6 and influenced by the temperature (high at body temperature, low at room temperature, no activity at 4°C) (14, 29). Therefore, urine pH and the time period the urine has resided in the bladder prior to voiding are important confounding factors, which can not be easily controlled in a clinical setting. Adding protease inhibitors postvoid can only prevent further cleavage happening ex vivo. Although proteins prone to proteolytic cleavage may be difficult to use as reliable biomarkers depending on their relative stability in the urine, the cleaved protein may define a specific pathologic pathway and the actual cleavage process itself may reflect disease related changes (30).

Ideally, a noninvasive screening biomarker for tubular injury should be able to detect subclinical but relevant tubular pathology prior to the graft sustaining sufficient injury to impact serum creatinine. Notably, patients in the normal tubular histology and the subclinical tubulitis groups were only different regarding the extent of tubulointerstitial inflammation, but equal with respect to GFR, immunosuppressive regimen, time posttransplant, and TA/IF scores. Therefore, these well defined groups allow assessing whether the investigated tubular injury biomarkers are able to predict subclinical tubulointerstitial inflammation. Although elevated levels of these biomarkers were very common in the subclinical tubulitis group, they were only slightly higher than in the normal tubular histology group. Although the differences approached (RBP: P=0.17; α1m: P=0.09; NGAL: P=0.06) statistical significance, the overlap was large limiting their clinical utility to separate these two groups. There are two central questions that arise from the observation that tubular injury biomarkers were similar in stable transplants with normal tubular histology and subclinical tubulitis: 1) what is the nature of tubular proteinuria detected in patients with normal tubular histology? and 2) does tubular proteinuria in the presence of normal tubular histology represent in fact evidence of tubular injury capable of progression to acute or chronic tubular pathology?

To address the first issue, one possible explanation is the persistence of tubular proteinuria due to ischemia-reperfusion injury. While tubular proteinuria is extremely high immediately after transplantation, it decreases steadily and would reach normal values around 2 months posttransplant if no additional injury occurs (31, 32). In our study population, only 8 of 100 samples (8%; subclinical tubulitis Ia/Ib, n=5; clinical tubulitis Ia/Ib, n=3) were obtained within the first 2 months, which excludes ischemia-reperfusion injury as a relevant confounding factor. Another possible explanation is the persistence of tubular proteinuria originating from the native kidneys. Although this can not be excluded, a recent study demonstrated that pretransplant proteinuria rapidly declined within 3 weeks after transplantation (33). Therefore, tubular proteinuria in stable transplants with normal histology most likely reflects actual tubular injury in the allograft due to the alloimmune response or other causes (e.g., drug toxicity). If this is true, then why is the process not histologically evident? It is likely that subclinical tubular pathologies early on are patchy and as such are missed even with an adequate biopsy sample defined by the Banff criteria (i.e., sampling error) (34, 35). Alternatively, the injury may precede visible histological changes.

In support of the argument that tubular proteinuria in the presence of normal tubular histology may represent tubular injury that can evolve into TA/IF there are three indirect lines of evidence to date. First, two studies have demonstrated the prognostic significance of tubular proteinuria in patients with good and stable allograft function (36, 37). Elevated levels of RBP (>0.4 mg/L corresponding to >0.06 mg/mmol creatinine) or α1m (>5 mg/mmol creatinine) at 6 months posttransplant were associated with subsequent deterioration of allograft function, development of TA/IF, and in some cases allograft failure. Unfortunately, in these studies either protocol biopsies were not performed (36) or a multivariate analysis was absent (37) to determine whether RBP or α1m were predictive for poor outcome in the presence of normal allograft histology. Thus, at best one can conclude that in stable grafts defined by functional parameters (i.e., creatinine) the presence of tubular proteinuria is predictive of a poor outcome.

The second line of support comes from two independent studies presented at the World Transplant Congress in
Boston in 2006 (38, 39). The common finding presented in these studies is that despite a low prevalence of tubular pathology detected by protocol biopsies within the first 3 months posttransplant, 21–37% of subsequent protocol biopsies demonstrated significant TA/IF. The question remains whether the cohort that progressed to TA/IF had had tubular proteinuria in the face of normal histology prior to the evolution of TA/IF. In the microarray study by Vitalic et al., 3-month protocol biopsies manifesting TA/IF expressed genes associated with tubular injury and fibrosis and 90% of these genes were already upregulated in the 1-month protocol biopsy prior to the development of TA/IF (39).

The third line of support comes from a study evaluating risk factors for early epithelial to mesenchymal transition (EMT), which is involved in the pathogenesis of TA/IF (40). Hertig et al. found that 23 of 56 patients (41%) showed expression of markers for EMT in protocol biopsies obtained at 3 months posttransplant. Interestingly, only 10 of those 23 patients (43%) had acute subclinical tubular pathologies supporting the concept that tubular injury on a molecular level may exist with normal histology by standard light microscopy. Clearly, these lines of reasoning are only suggestive and longitudinal studies are required to assess whether tubular proteinuria in the presence of normal tubular histology indicates a significant risk for development of TA/IF. In this context, the responsible processes (e.g., EMT) and the contribution of immune and nonimmune factors have to be further studied.

To date, no specific urine biomarker assessing tubulo-interstitial rejection/injury has found an application in daily patient management despite encouraging results in initial, discovery phase studies (41). This clearly highlights that biomarker development is a difficult venture and requires an iterative approach (42). As demonstrated by this study, validation in a clearly independent sample set with refined control groups (i.e., subclinical tubulitis) and side-by-side evaluation of comparable biomarkers is essential to confirm or discard conclusions drawn from the discovery phase study (13, 14). In fact, we could confirm the prevalence of $\beta$2m in patients with clinical tubulitis Ia/Ib and stable transplants with normal tubular histology (stable transplant with normal tubular histology: Winnipeg 4/22 [18%], Basel 8/24 [33%], $P=0.32$; clinical tubulitis Ia/Ib: Winnipeg 17/18 [94%], Basel 15/18 [83%], $P=0.60$ [13]). However, the validation study revealed that $\beta$2m is: 1) expected not specific for rejection as all other tubular injury biomarkers, 2) unable to distinguish normal tubular histology from subclinical tubulitis, 3) similar to the other investigated biomarkers (RBP, $\alpha$1m, NGAL), and 4) confounded by urine pH restricting its clinical usefulness. Ultimately, the success of biomarker development will depend on the quality of the patient cohorts used for the initial biomarker discovery phase and the subsequent independent validation study, as well as the commitment to design prospective trials implementing promising biomarkers into patient management to determine their clinical utility (25).

In conclusion, proteinuria associated with tubular injury is common after renal transplantation and can be assessed noninvasively with specific tubular injury biomarkers. However, none of the investigated biomarkers can clearly differentiate between stable transplants with normal tubular histology and stable transplants with subclinical tubulitis allowing for a noninvasive screening procedure. Therefore, the next important step will be to determine whether tubular proteinuria in stable patients with normal tubular histology indicates a significant risk for early development of TA/IF. Concurrently, studies are required to evaluate whether a combination of biomarkers can predict relevant subclinical tubulitis. In the interim, the protocol allograft biopsy remains the preferred tool to screen for subclinical tubulitis.

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