Serum and urine markers of collagen degradation reflect renal fibrosis in experimental kidney diseases

Marios Papasotiriou1,2,3, Federica Genovese4, Barbara M. Klinkhammer1, Uta Kunter2, Signe H. Nielsen4, Morten A. Karsdal4, Jürgen Floege2 and Peter Boor1,2

1Institute of Pathology, RWTH University of Aachen, Aachen, NRW, Germany, 2Department of Nephrology, RWTH University of Aachen, Aachen, NRW, Germany, 3Department of Nephrology, University Hospital of Patras, Patras, Greece and 4Nordic Bioscience, 2730 Herlev, Denmark

Correspondence and offprint requests to: Peter Boor; E-mail: pboor@ukaachen.de

ABSTRACT

Background. The extent of renal fibrosis in chronic kidney disease (CKD) is the best predictor for progression of most renal diseases. To date, no established biomarkers of renal fibrosis exist.

Methods. We measured circulating and urinary-specific matrix metalloproteinase (MMP)-generated collagen type I and III degradation fragments (C1M and C3M) and an N-terminal propeptide of collagen III (Pro-C3), as markers of collagen type III production, in three rat models of CKD and fibrosis: renal mass reduction (5/6 nephrectomy), progressive glomerulonephritis (chronic anti-Thy1.1 nephritis) and adenine crystal-induced nephropathy. Healthy rats served as controls.

Results. In all three models, the animals developed significant CKD and renal fibrosis. Compared with healthy rats, serum C1M and C3M significantly increased in rats with 5/6 nephrectomy and adenine nephropathy (2- to 3-fold), but not with chronic anti-Thy1.1 nephritis. Urinary C1M and C3M levels increased 9- to 100-fold in all three models compared with controls. Urinary degradation markers correlated closely with renal deposition of collagen type I and type III. Pro-C3 was significantly increased only in the urine of 5/6 nephrectomy rats.

Conclusions. In particular, urinary markers of MMP-driven collagen degradation, rather than collagen production markers, may represent a novel, specific and non-invasive diagnostic approach to assess kidney fibrosis.

Keywords: biomarkers, interstitial fibrosis, rat models

INTRODUCTION

The extent of chronic injury to both native and transplanted kidneys is best reflected by renal interstitial fibrosis, a common pathological process in chronic kidney disease (CKD). The hallmark of renal fibrosis is exaggerated deposition of extracellular matrix (ECM) by expanded fibroblasts and myofibroblasts. The degree of renal interstitial fibrosis is a reliable and almost universal predictor of progressive renal functional decline [1, 2]. Thus, an assessment of renal fibrosis would be of utmost importance for therapeutic and prognostic decisions in clinical practice but also for advancing translational research [3, 4]. At present, the gold standard, and the only way to specifically assess renal fibrosis, is kidney biopsy. The invasive nature of this procedure and its associated possible complications, sampling errors due to the focal nature of renal fibrosis and the limited possibility for sequential assessments are important limitations hampering its use in monitoring fibrosis. In other organs, in particular in the liver, several non-invasive biomarkers for monitoring the degree of fibrosis were developed; however, no such fibrosis markers exist for kidneys [5].

During fibrosis, ECM undergoes remodelling and turnover, e.g. via matrix metalloproteinase (MMP)-mediated degradation of collagen [6, 7]. MMPs were shown to be dysregulated in various experimental models and human renal diseases, and their increased expression accompanies renal fibrosis [8, 9]. MMP-mediated degradation of collagens results in fragments with neo-epitopes, which can be detected in the urine or circulation using specific antibodies. We have developed several enzyme-linked immunosorbent assays (ELISAs) for the
assessment of such neo-epitopes in serum and urine [6, 10]. We previously showed that in liver disease, two specific MMP-2- and MMP-9-generated fragments of type I and III collagen (C1M and C3M) were sensitive and specific markers associated with the extent of fibrosis in liver [6, 11, 12].

Here we tested the diagnostic value of these collagen type I and III degradation markers, compared with a collagen type III production marker, as potential biomarkers for renal fibrosis in experimental renal disease associated with renal fibrosis.

**METHODS**

**Animal experiments**

Animals were housed in rooms with standard lighting conditions (12 h/12 h light–dark cycles) and in an environment with constant temperature and humidity as well as free access to drinking water (ozone-treated and acidified). Standard ad libitum rat chow was used in the control rats and rats with 5/6 nephrectomy and chronic anti-Thy 1.1 nephritis. All animal experiments and protocols were approved by the local review boards and authorities.

We used three different models of renal fibrosis in rats, 5/6 nephrectomy, chronic anti-Thy-1.1 nephritis and adenine nephropathy. The 5/6 nephrectomy model is a well-established model of progressive glomerulosclerosis and renal fibrosis due to critical renal mass reduction. Seven F344 rats, weighing 150–180 g (Charles River, Erkrath, Germany), were used. The 5/6 nephrectomy was performed by right-sided uninephrectomy followed by ligation of two of three renal arteries of the left kidney or sham operation as described previously [13, 14], and the animals were sacrificed 20 weeks after disease induction.

The chronic, progressive anti-Thy 1.1 nephritis model is a model of mesangio proliferative glomerulonephritis that is aggravated by urine proteinuria. Six male Wistar rats weighing 200 g (Charles River, Sulzfeld, Germany) were used in this study. We induced the model by unilateral nephrectomy followed by injection of 1 mg/kg monoclonal anti-Thy 1.1 antibody (clone OX-7; European Collection of Animal Cell Cultures, Salisbury, England) as described previously [15, 16], and all of the animals were sacrificed on Day 56 after disease induction.

The rat adenine nephropathy leads to progressive renal fibrosis due to formation of renal crystals (nephroliths) and resulting tubular injury. We induced this model in five male F344 rats weighing 200–220 g (Charles River, Erkrath, Germany) by administration of an adenine-rich diet (supplemented with 0.75% adenine) (Altromin, Lage, Germany) [13, 17], and all rats were sacrificed at the age of 26 weeks after 14 weeks of adenine diet (the following protocol was used: 3 weeks adenine, 5 weeks normal rat chow, 3 weeks adenine and 1 week normal rat chow).

Renal function was tested (s-creatinine and s-urea) using an autoanalyser and 24-h urine for proteinuria was collected from animals housed in metabolic cages for 16 h prior to sacrifice. Renal tissues for histological evaluation were collected and venous blood samples were drawn from the inferior vena cava at sacrifice.

**Histology and immunohistochemistry**

One-micrometre sections of methacarn-fixed renal tissues were processed and immunohistochemistry was performed as previously described [18]. Primary antibodies included a murine monoclonal antibody (clone 1A4) to α-smooth muscle actin (α-SMA), affinity-purified polyclonal goat antibodies against human type I and type III collagens (Southern Biotechnology, Birmingham, AL) plus appropriate negative controls. The stains for cortical type I and type III collagens and α-SMA were evaluated by computer-based morphometry using the ImageJ software as previously described [13]. In short, the positively stained interstitial area in each tissue was calculated (in %) in almost the whole cortical area. All analyses were performed in a blinded fashion.

**Enzyme-linked immunosorbent assay**

The competitive ELISAs detecting the specific MMP-generated neo-epitope of collagen type I and III in serum and urine, namely C1M and C3M, were based on monoclonal antibodies detecting two specific neo-epitopes generated by MMP cleavage of the α1 chain of collagen type I and the α1 chain of collagen type III. Specifically, the detected sequences were GSPGKDGVGR_{764} in collagen type I and KNGETFPQGP_{310} in collagen type III, with the neo-epitope positioned in the C- and N-terminal, respectively. These sequences are conserved in the rat and human, and can therefore be used for translational research. The protocols for the serum assays have been previously described [11, 12]. The urinary C1M and C3M assay used mAb clones specifically selected for their reactivity in urine samples, NB105-4D2 and NB51-134, respectively. The urine C1M ELISA was developed as follows: a 96-well plate pre-coated with streptavidin was further coated with 100 μL of 20 ng/mL synthetic peptide biotin-GSPGKDGVGR dissolved in 10 mM PBS–BTB, pH 7.4, buffer at 20°C for 30 min by constant shaking at 300 rpm. The plate was then washed five times in washing buffer. Thereafter, 20 μL of sample was added, followed by 100 μL of 50 ng/mL peroxidase-conjugated mAb-NB105-4D2 in 50 mM Tris–BTB 50 mM, 2 g/l NaCl, pH 8.0. The free peptide GSPGKDGVGR was used as standard peptide at 250, 125, 62.5, 31.25, 15.62, 7.81, 3.91, 1.95, 0.97 and 0 ng/mL. The plate was incubated for 3 h at 4°C by constant shaking at 300 rpm. The plate was again washed five times. Finally, 100 μL tetramethylbenzidine (TMB) (Kem-En-Tec) was dispensed and the plate incubated for 15 min in darkness and shaken at 300 rpm. Then, 100 μL of stopping solution (0.1% H_{2}SO_{4}, Merck) was finally added and the plate analysed in the ELISA reader at 450 nm with 650 nm as the reference. The urine C3M ELISA was developed as follows: a 96-well plate pre-coated with streptavidin was further coated with 100 μL of 20 ng/mL synthetic peptide biotin-KNGETFPQGP dissolved in 10 mM PBS–BTB, pH 7.4, buffer at 20°C for 30 min by constant shaking at 300 rpm. The plate was then washed five times in washing buffer. Thereafter, 20 μL of sample was added, followed by 100 μL of 50 ng/mL peroxidase-conjugated mAb-NB105-4D2 in 50 mM Tris–BTB 50 mM, 2 g/l NaCl, pH 8.0. The free peptide KNGETFPQGP was used as standard peptide at 250, 125, 62.5, 31.25, 15.62, 7.81, 3.91, 1.95, 0.97 and 0 ng/mL.
The plate was incubated for 20 h at 4°C by constant shaking at 300 rpm. The plate was again washed five times. Finally, 100 μL TMB (Kem-En-Tec) was dispensed and the plate incubated for 15 min in darkness and shaken at 300 rpm. One hundred microlitres of stopping solution (0.1% H₂SO₄, Merck) were finally added and the plate analysed in the ELISA reader at 450 nm with 650 nm as the reference. The urine C1M and C3M concentration was normalized for urine creatinine measured by the QuantiChrom Creatinine Assay Kit (Bioassay System).

The competitive ELISA-detecting the neo-epitope of collagen type III pro-peptide was used for measurement in both serum and urine and was performed as described previously [19].

The competitive ELISA-detecting soluble α-SMA in serum targets the acetylated N-terminal of the molecule, specifically the sequence Ac-EEEDSTALV. The assay was developed as follows: a 96-well plate pre-coated with streptavidin was coated with 100 μL of 0.078 ng/mL synthetic peptide Ac-EEEDSTALV-K-biotin dissolved in 50 mM TBS–BTB with 2 g/l NaCl, pH 8.0, buffer at 20°C for 30 min by constant shaking at 300 rpm. The plate was then washed five times in washing buffer. Thereafter, 20 μL of sample was added, followed by 100 μL of 12.5 ng/mL mAb-NB552-2A11 in 50 mM TBS–BTB with 2 g/l NaCl, pH 8.0. The free peptide Ac-EEEDSTALV was used as standard peptide at 62.5, 31.25, 15.6, 7.8, 3.9, 1.95, 0.98 and 0 ng/mL. The plate was incubated for 1 h at 20°C by constant shaking at 300 rpm. The plate was washed again five times. Then, 100 μL rabbit anti-mouse (Jackson Immunoresearch Inc.) diluted 1 : 5000 in 50 mM TBS–BTB with 2 g/l NaCl, pH 8.0, was added to the plate and incubated for 1 h at 20°C by constant shaking at 300 rpm. Finally, 100 μL TMB (Kem-En-Tec) was dispensed and the plate incubated for 15 min in darkness and shaken at 300 rpm. Finally, 100 μL of stopping solution (0.1% H₂SO₄, Merck) was added and the plate analysed in the ELISA reader at 450 nm with 650 nm as the reference.

**Statistical analysis**

Data are given as means ± SD. Kolmogorov–Smirnov test was used to test normal distribution of variables. Student’s t-test and Mann–Whitney U-test were used as appropriate. Spearman’s correlation was used to test the association between the variables in a pooled analysis of all animals. All tests were two-tailed, and statistical significance was defined as P < 0.05.

**RESULTS**

We first confirmed the induction of chronic renal failure and fibrosis in all three models. Compared with control sham rats, serum creatinine concentrations were significantly increased in all three models (7-fold in 5/6 nephrectomy, 4-fold in chronic anti-Thy 1.1 nephritis and 6-fold in adenine nephropathy; Figures 1G, 2Ga and 3G). Proteinuria was also significantly increased in 5/6 nephrectomy (8-fold, Figure 1H) and in chronic anti-Thy 1.1 nephritis (7-fold, Figure 2H). Rats with adenine nephropathy showed no pathological urinary protein excretion compared with controls despite a pronounced renal failure (Figure 3H), in line with a different mechanism of renal injury not involving the glomeruli.

![Figure 1](https://example.com/figure1.png)

**FIGURE 1:** 5/6 nephrectomy. Compared with sham animals (A–C), rats with 5/6 nephrectomy (D–F) had significantly increased renal deposition of collagen type I (I) and III (J) and significant expansion of α-SMA-positive cells (K). Renal function was significantly reduced in diseased animals as shown by increased serum creatinine (G) and proteinuria (H). Scale bars represent 100 μm. **P < 0.01 versus sham; ***P < 0.001 versus sham. α-SMA, α-smooth muscle actin; sG, serum creatinine.
Next we assessed the major hallmarks of renal fibrosis, i.e. renal cortical expression of collagen type I, type III and α-SMA. Expression of all these markers was significantly up-regulated in all rat models of CKD, albeit to a different extent. The collagen type I-positively stained renal area was increased 6-, 4- and 7-fold, whereas collagen type III was increased 7-, 3- and 7-fold in 5/6 nephrectomy, chronic anti-Thy1.1 nephritis and adenine nephropathy, respectively (Figures 1–3). These
findings reflected closely the degree of renal failure in each animal model and correlated closely with the increase in serum creatinine, but not proteinuria (Table 1). α-SMA, a marker of myofibroblasts, the principal ECM-producing cell in renal fibrosis, showed a 10-, 16- and 9-fold increase in 5/6 nephrectomy, chronic anti-Thy1.1 nephritis and adenine nephropathy, respectively (Figures 1–3).

We then measured two specific MMP-generated fragments, C1M and C3M, of the major scar collagens type I and III in serum and urine. Compared with healthy rats, serum concentrations were significantly increased in 5/6 nephrectomy and adenine nephropathy: serum C1M increased up to 2- and 3-fold and serum C3M up to 2.5- and 2-fold, respectively (Figure 4A, B and E, F). Despite increased deposition of both collagens in the kidney, we observed no such increase in serum C1M or C3M in chronic anti-Thy 1.1 nephritis (Figure 4C and D). Compared with healthy rats, both urinary markers C1M and C3M were highly increased in all three models: 13- and 40-fold in 5/6 nephrectomy, 20- and 32-fold in chronic anti-Thy1.1 nephritis and 9- and 100-fold in adenine nephropathy, respectively (Figure 5).

Table 1. Correlations (Spearman’s r coefficient) of C1M, C3M and Pro-C3 in serum and urine with renal immunohistochemical expression of markers of renal fibrosis (collagen type I, type III and α-SMA) quantified by computer-based morphometry

<table>
<thead>
<tr>
<th></th>
<th>Col. I</th>
<th>Col. III</th>
<th>α-SMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary C1M/Cr</td>
<td>0.581</td>
<td>0.569</td>
<td>0.885</td>
</tr>
<tr>
<td>Urinary C3M/Cr</td>
<td>&lt;0.001</td>
<td>0.632</td>
<td>0.737</td>
</tr>
<tr>
<td>Urinary Pro-C3</td>
<td>−0.087</td>
<td>0.208</td>
<td>0.397</td>
</tr>
<tr>
<td>Serum C1M</td>
<td>0.511</td>
<td>0.551</td>
<td>0.571</td>
</tr>
<tr>
<td>Serum C3M</td>
<td>0.328</td>
<td>0.593</td>
<td>0.271</td>
</tr>
<tr>
<td>Serum Pro-C3</td>
<td>−0.034</td>
<td>−0.261</td>
<td>−0.377</td>
</tr>
<tr>
<td>sCr</td>
<td>0.694</td>
<td>0.722</td>
<td>0.688</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>−0.140</td>
<td>−0.086</td>
<td>0.343</td>
</tr>
</tbody>
</table>

All animals (sham control, 5/6 nephrectomy, chronic anti-Thy1.1 nephritis and adenine nephropathy) were pooled for this analyses. Significant correlations are highlighted in italic. α-SMA, α-smooth muscle actin; Col., collagen; n.s., not significant; sCr, serum creatinine.

**Figure 4:** Serum C1M and C3M in sham animals and in the different models of renal fibrosis. Both markers were significantly increased in the serum of 5/6 nephrectomy rats (5/6 Nx; A and B) and adenine nephropathy rats (Adenine; E and F), but not in rats with chronic anti-Thy 1.1 nephritis (a.Thy 1.1, C and D). *P < 0.05 versus sham; ***P < 0.001 versus sham.
Serum C1M correlated with renal collagen type I, type III and α-SMA, whereas serum C3M only correlated with renal collagen III expression (Table 1). Urinary C1M and C3M both correlated significantly with renal expression of collagen I, III and α-SMA (Table 1). Both markers in serum and urine correlated positively with renal function (serum creatinine), whereas apart from urinary C1M, none of the markers correlated with proteinuria (Table 2).

Next we evaluated the levels of N-terminal pro-peptide of collagen III (Pro-C3), a marker of collagen type III de novo formation, in both serum and urine. Serum Pro-C3 concentrations were not different (or even slightly lower) in all the different disease models compared with control animals (Figure 6A–C). Urinary Pro-C3 concentrations were increased, however, significantly only in rats with 5/6 nephrectomy (3-fold), while a

| Table 2. Correlation (Spearman’s ρ coefficient) of C1M, C3M and Pro-C3 in serum and urine with proteinuria and serum creatinine |
|---------------------------------|-------------|--------|---------------|-----|
|                                | Proteinuria | P      | sCr           | P   |
| Urinary C1M/Cr                  | 0.585       | 0.007  | 0.771         | <0.001 |
| Urinary C3M/Cr                  | 0.035       | n.s.   | 0.899         | <0.001 |
| Urinary Pro-C3                  | 0.870       | <0.001 | 0.425         | n.s. |
| Serum C1M                       | −0.235      | n.s.   | 0.675         | <0.001 |
| Serum C3M                       | 0.159       | n.s.   | 0.612         | 0.001 |
| Serum Pro-C3                    | −0.640      | 0.014  | −0.266        | n.s. |

All animals (sham control, 5/6 nephrectomy, chronic anti-Thy1.1 nephritis and adenine nephropathy) were pooled for this analysis. Significant correlations are highlighted in italic. n.s., not significant; sCr, serum creatinine.

non-significant increase was observed in the chronic anti-Thy1.1 animals and no change occurred in adenine nephropathy (Figure 6D–F). Pro-C3 both in serum and urine neither

FIGURE 5: Urinary C1M and C3M in sham animals and in the different models of renal fibrosis. Urinary excretion of both markers was highly increased in all models of renal fibrosis as shown for 5/6 nephrectomy (5/6 Nx; A and B), chronic anti-Thy1.1 nephritis (a.Thy1.1, C and D) and adenine nephropathy (Adenine; E and F). All values were normalized to urinary creatinine. *P < 0.05 versus sham, **P < 0.01 versus sham; ***P < 0.001 versus sham.
correlated with collagen type I and type III renal expression (Table 1) nor with serum creatinine (Table 2). Pro-C3 concentration in urine correlated positively with proteinuria, while serum Pro-C3 correlated negatively with proteinuria (Table 2).

Since fibrosis is characterized by prominent expansion of α-SMA-positive myofibroblasts, as was the case in all three models in this study, we analysed serum levels of α-SMA as a potential marker of myofibroblast turnover. Compared with sham animals, we found no significant differences in serum α-SMA in any of the groups (Figure 7).

**DISCUSSION**

The major and novel finding of our study is that serum, and in particular urinary, markers of MMP-driven collagen type I and III degradation (C1M and C3M) closely reflect experimental renal fibrosis. In contrast, serum α-SMA and a urinary and serum collagen formation marker (Pro-C3) were not diagnostic. These data suggest that C1M and C3M could be novel specific biomarkers of renal fibrosis. The serum concentrations of both markers were significantly increased in serum; however, the urinary markers showed much more prominent increases during fibrosis (up to 100-fold) and therefore seem to have superior diagnostic value. Importantly, C3M in urine significantly correlated with renal function but not with proteinuria. This suggests that the increased urinary excretion of C3M is independent of glomerular and tubular mechanisms of proteinuria in diseased animals and does not represent ‘spillage’ from serum or result from impaired tubular absorption of proteins. This notion is further supported by our data obtained in adenine nephropathy where highly increased urinary C1M and C3M occurred despite the absence of proteinuria.

In this study, we have focused on turnover of collagen type I and III since both are the prototypical scar collagens across species, deposited already in early stages of fibrosis and progressively increase during renal fibrosis [6]. C1M and C3M increased very similarly in all models and in both urine and serum. Some minor differences, however, were observed, in particular with regard to numerical changes and a closer correlation of serum C1M with fibrosis parameters compared with serum C3M. This might have been due to the relatively low number of animals used in this study, which was sufficient for detection of the highly significant differences in C1M and C3M but might have been low for the correlation analyses. In our experience, and as documented by immunohistochemistry in this study, both collagen type I and III are upregulated and deposited in a comparable fashion during renal fibrosis, further suggesting a mathematical rather than biological cause of the different correlation. In all the models, urinary C3M showed more profound changes compared with C1M. At the moment, we have no mechanistic explanation for this finding,
but from a diagnostic perspective, urinary C3M might be superior to C1M.

One of the strengths of our study is that we used three different models with distinct pathophysiological mechanisms leading to renal fibrosis. Chronic anti-Thy 1.1 represents a model of progressive glomerulonephritis [20], the 5/6 nephrectomy model is the most frequently used animal model for focal segmental glomerulosclerosis associated with progressive hypertensive renal injury [21] and adenine nephropathy is a model with crystal-induced tubular injury leading to interstitial fibrosis without involving the glomerular compartment and without development of proteinuria [22].

Few studies to date have analysed the feasibility of using ECM components as biomarkers of renal fibrosis, mostly focusing on collagen type IV and collagen type III amino-terminal pro-peptide (PIIINP) [6]. In patients with CKD of different aetiologies, urinary PIIINP, which is released following maturation of newly formed collagen type III, was increased and correlated with interstitial fibrosis [23]. In human renal transplant recipients urinary PIIINP levels correlated with the degree of fibrosis [24]. Another study in CKD patients suggested that serum PIIINP, but not C-terminal telopeptide of collagen type I, was associated with arterial stiffness [25]. In our study, serum Pro-C3 concentrations were not significantly different from controls in all three rat models we used. Our assay specifically detects a neo-epitope on collagen type III N-terminal pro-peptide after cleavage by N-terminal peptidase, and is therefore found in biological fluids mainly during active synthesis of collagen type III. In contrast, the commercial PIIINP assay detects an internal epitope of the pro-peptide and is therefore not able to distinguish between synthesis versus release of collagen type III. It is therefore not unexpected to detect differences in the performance of these assays. In addition, differences between animal models and patients with fibrosis are another likely explanation. Several studies suggested that urinary collagen type IV, a typical and major component of renal basement membranes, might be a promising marker of renal injury, in particular in diabetic nephropathy [6, 26, 27]. Tissue and ECM remodelling is very low in physiological conditions, but increases during injury and involves increased ECM production and degradation, the latter, e.g. via increased expression of proteases such as MMPs [28]. Compared with the previous studies, our approach was based on analyses of such specific, MMP-driven degradation fragments that are released into urine and circulation. Our and previous data suggest that this approach might be more specific for pathological ECM turnover during fibrosis since it is dependent on the local protease activity [7]. Taken together, compared with available collagen biomarkers, our assays are highly specific and only detect the MMP-cleaved degradation products of collagen type I or III. To our knowledge, no comparable assay is currently available or published. Commercial assays for measurements of collagens in serum or urine most often use polyclonal antibodies, i.e. they are not directed to specific epitopes. Our C1M and C3M assays allow us, for the first time, to assess with very high specificity collagen degradation by MMPs, a process associated with pathological remodelling during fibrosis [29].

Expansion of α-SMA-positive myofibroblasts, the main cells responsible for collagen deposition, is a hallmark of renal fibrosis. In this study, we analysed serum α-SMA concentrations, and apart from a slight and non-significant increase in the most advanced model (5/6 nephrectomy), we found no differences compared with healthy animals. Since α-SMA is an intracellular protein, these data suggest that during renal fibrosis, in the models and time-points we used, there is no significant cell death of myofibroblasts. Since the ELISA for α-SMA was only developed for serum, we were not able to measure urinary α-SMA. Although discouraging at the moment, this biomarker could be of interest in monitoring therapeutic approaches, specifically inducing apoptosis or other types of cell death in myofibroblasts in renal fibrosis.

The ELISA assays for C1M and C3M detect and quantify exclusively fragmented and non-intact collagen type I and III [11, 12]. These assays were developed and validated previously in animal models of liver fibrosis and patients with liver and lung fibrosis [12, 30–33]. Serum C1M was increased in patients with pancreatic cancer [34] and in patients with idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD) compared with age- and sex-matched normal controls [35]. Elevated serum C3M was also found in IPF and COPD patients [35]. In line with previous experimental studies on liver and lung fibrosis, we found a very close correlation of renal expression of fibrosis markers with C1M and C3M concentrations, in particular in the urine [31, 36]. Previous reports suggested that fragments of collagen type I were significantly lower in urine of patients with diabetic nephropathy [37, 38]. Contrary to this observation, in all our animal models, we have found significantly increased concentrations of both C1M and C3M. We speculate that our findings highlighted a previously unrecognized difference between fibrosis found in patients versus that found in animal models, whereby the fibrosis in our rat models is a much more active process compared with patients, in which fibrosis is established over longer periods of time and likely undergoes modifications like cross-linking, rendering them resistant to proteolytic cleavage. The reasons for such differences are most likely due to the high severity of the initiating injury, rapid onset and a fast progression of fibrosis in the animal models compared with the rather slow progression in patients (most of whom also receive some sort of treatment). Furthermore, our own data in IgA nephropathy patients support this hypothesis and show a significant and progressive reduction of urinary C1M and C3M with increasing CKD stage (unpublished results and manuscript in revision).

To further support the renal source of C1M and C3M, we have attempted to establish immunohistochemical and immunofluorescence staining methods using our specific antibodies for C1M and C3M. However, even after extensive testing with three different tissue fixatives and unfixed tissues, as well as a number of various protocols, we were not able to obtain a specific result. This is likely due to the fact that both C1M and C3M antibodies were raised to detect fragments in the fluid phase and thus are best suitable for ELISA of body fluids but not for tissue analyses.

In conclusion, we show that specific degradation, rather than formation fragments of collagen type I and III, measured using a specific ELISA in serum and in particular in urine, is
highly increased in experimental renal fibrosis. C1M and C3M appear to be exclusively related to the dysregulated ECM turnover during renal fibrosis. In particular, urinary C3M seems to be a unique candidate for a novel, specific and non-invasive biomarker of kidney fibrosis.

**FUNDING**

This work was supported by financial research grants TP25, TP19 and Q1 of the SFB/Transregio 57 ‘Mechanisms of organ fibrosis’ (to PB, JF and UK), BO 3755/1-1 and BO 3755/2-1 (both to PB) all from the German Research Foundation (‘Deutsche Forschungsgemeinschaft – DFG’), the Else-Kröner Fresenius Stiftung (EKFS 2012_A216 to PB), the Danish Research Fund (‘Den Danske Forskningsfond’ to FG and SHN) and the Hellenic Society of Nephrology to MP.

**CONFLICT OF INTEREST STATEMENT**

FG, SHN and MAK are full-time employees at Nordic Bioscience and developed the ELISAS and measured the serum and urine samples in this study. MAK holds stock in Nordic Bioscience. MP, BM K, UK, JF and PB have had no other involvements that might raise the question of bias in the work reported or in the conclusions, implications, or opinions stated.

**REFERENCES**


Received for publication: 8.1.2015; Accepted in revised form: 19.2.2015