Early Urinary and Plasma Biomarkers for Experimental Diabetic Nephropathy

MARKUS L. ALTER 1,2,4, AXEL KRETSCHMER 3, KAROLINE VON WEBSKY 1,2, OLEG TSUPRYKOV 1,2, CHRISTOPH REICHTZEDER 1,2, ALEXANDRA SIMON 1,2, JOHANNES-PETER STASCH 5, BERTHOLD HOCHER 2

1 Center for Cardiovascular Research/Institute of Pharmacology, Charité, Campus Mitte, Berlin, Germany
2 Institute for Nutritional Science, University of Potsdam, Germany
3 Global Biomarker, Bayer HealthCare, Wuppertal, Germany
4 Department of Nephrology, Charité, Campus Benjamin Franklin, Berlin, Germany
5 Cardiology Research, Bayer HealthCare, Wuppertal, Germany

SUMMARY

Background: As the prevalence of diabetes rises, its complications such as diabetic nephropathy affect an increasing number of patients. Consequently, the need for biomarkers in rodent models which reflect the stage and course of diabetic nephropathy is high. This article focuses on Heart-type fatty acid binding protein (H-FABP), osteopontin (OPN), nephrin, and Neutrophil gelatinase-associated lipocalin (NGAL) in urine, and kidney injury molecule (KIM)-1, clusterin, and tissue inhibitor of metalloproteinases (TIMP) 1 in plasma in uni-nephrectomized rats with streptococcyin-induced type 1 diabetes mellitus, a common animal model to explore renal impairment in the setting of diabetes mellitus.

Methods: 23 male Wistar rats were uni-nephrectomized and subsequently divided into two study groups. The diabetic group received streptozotocin (STZ) via tail-vein injection, the non-diabetic group received citrate buffer without STZ. Subsequently, blood glucose, body weight, and blood pressure were checked regularly. After 18 weeks, animals were placed in metabolic cages, blood and urine obtained and subsequently organs were harvested after sacrifice.

Results: Blood glucose levels were highly increased in diabetic animals throughout the experiment, whereas systolic blood pressure did not differ between the study groups. At study end, classical biomarkers such as urinary albumin and protein and plasma cystatin c were only slightly but not significantly different between groups indicating a very early disease state. In contrast, urinary excretion of H-FABP, OPN, nephrin, and NGAL were highly increased in diabetic animals with a highly significant p-value (p<0.01 each) compared to non-diabetic animals. In plasma, differences were found for calbindin, KIM-1, clusterin, TIMP-1, and OPN. These findings were confirmed by means of the area under the receiver operating characteristic curve (ROC-AUC) analysis.

Conclusions: In summary, our study revealed elevated levels of new plasma and urinary biomarkers (urinary osteopontin, urinary nephrin, urinary NGAL, urinary H-FABP, plasma KIM-1, plasma TIMP-1) in uni-nephrectomized diabetic rats, an established rat model of diabetic nephropathy. These biomarkers appeared even before the classical biomarkers of diabetic nephropathy such as albuminuria and urinary protein excretion. The new biomarkers might offer an advantage to urinary albumin and plasma cystatin c with respect to early detection.


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diabetic nephropathy, urinary biomarker, blood biomarker, heart-type fatty acid binding protein, osteopontin, nephrin, neutrophil gelatinase-associated lipocalin, kidney injury molecule 1, clusterin, tissue inhibitor of metalloproteinases 1

INTRODUCTION

The prevalence of diabetes mellitus is rising worldwide and its severe complications, such as diabetic nephropathy, affect an increasing number of patients. Hence, the need for reliable diagnostic tools and treatment strategies grows. However, sensitive markers to detect very early changes in kidney function and kidney injury are sparse. Even urinary albumin excretion, which is being early changes in kidney function and kidney injury are growing. However, sensitive markers to detect very early changes in kidney function and kidney injury are sparse. Even urinary albumin excretion, which is being widely accepted as one of the earliest and most sensitive markers detecting renal damage and predicting further cardiovascular morbidity [1-3], does not necessarily uncover renally impaired patients [4]. Consequently, the need for further biomarkers which reflect the stage and course of diabetic nephropathy is still high. Our study focuses on a number of urinary and plasma biomarkers in a renally impaired rat model with beginning diabetic nephropathy when urinary albumin is still unremarkable. The focus lies on Heart-type fatty acid binding protein (H-FABP), osteopontin (OPN), nephrin, and Neutrophil gelatinase-associated lipocalin (NGAL) in urine, and kidney injury molecule (KIM)-1, clusterin, and tissue inhibitor of metalloproteinases (TIMP) 1 in plasma. These proteins have been recently reported to be promising new biomarkers with the ability to reflect renal damage and impairment. Furthermore, we show the results of the other plasma biomarkers from Rules Based Medicine’s kidney panel (calbindin, cystatin c, glutathione S-transferase alpha, glutathione S-transferase Mu, vascular endothelial growth factor a). The fatty acid-binding proteins (FABPs) are small cytoplasmic proteins (14 kDa) which are expressed in huge amounts in tissues with an active fatty acid-binding metabolism. Recent studies suggest that both types, the liver (L-FABP) and heart type (H-FABP), may detect ongoing damages in heart and kidney and be useful as diagnostic markers [5-7]. In contrast to L-FABP, which reflects proximal tubule damage, H-FABP is mainly expressed in distal tubule cells and indicates injury thereat [8]. Osteopontin (OPN) is a calcium binding phosphoglycoprotein which is expressed in bone and endothelial tissues and in all glomerular cells [9,10]. It is implicated in bone remodeling and inflammation and was shown to be involved in atherosclerosis. Therefore, it is regarded as a biomarker of vascular calcification [11] and is associated with fibrosis and tissue remodeling [12]. In patients with type 2 diabetes mellitus (T2DM), plasma osteopontin levels significantly correlated with the severity of diabetic nephropathy (sCr, eGFR) and coronary artery disease (CAD) [13]. In urine, increased OPN levels were detected in animal models of acute kidney injury (AKI) even before urinary albumin levels rose [14]. Concerning diabetic nephropathy, the diagnostic value of urinary OPN remains unclear. Nephrin is a transmembrane protein and obviously plays an important functional role as a molecular scaffold at the slit diaphragm between neighboring podocytes [15-17]. It was found to be involved in several proteimuric diseases such as the congenital nephrotic syndrome of the Finnish type, hypertension, and diabetes mellitus [16,18-20]. Human neutrophil gelatinase-associated lipocalin (NGAL) is a 25 kDa protease resistant polypeptide that was initially identified bound to gelatinase in specific granules of the neutrophil [21]. It is involved in ischemic renal injury and repair processes [22]. Serum and urine NGAL levels gained diagnostic value with respect to early prediction and severity of multiple kidney disorders, such as AKI [23,24] and chronic dysfunction due to cardiovascular morbidity [25] and diabetes [7,26]. It is regarded as a marker of proximal tubular damage. Kidney injury molecule (KIM)-1 is a type I cell membrane glycoprotein, and its mRNA levels were reported to be upregulated after proximal tubular injury with a consecutive increase of urinary KIM-1 excretion [27]. Furthermore, elevated urinary KIM-1 was measured in patients with T1DM with or without albuminuria [26]. Concerning the diagnostic value in plasma, however, the published literature is sparse. Clusterin is a lipoprotein with anti-complement effects. Urinary excretion was to be an early reflection of proximal tubular damage in the setting of nephropathy and AKI in rats [28-30]. In humans, reduced urinary and plasma levels were associated with active nephrotic syndrome [31]. Moreover, increased plasma clusterin was associated with vascular damage, such as CAD and myocardial infarction. In kidneys of diabetic rats, clusterin expression is enhanced and associated with glomerular and proximal tubular damage [32]. Furthermore, clusterin is linked to high-density lipoprotein (HDL) and, therefore, underlies metabolic changes associated with hyperglycemia [33]. Tissue inhibitor of metalloproteinases (TIMP)-1 is a biomarker of extracellular matrix remodeling and tubulointerstitial fibrosis [34]. Urinary excretion was elevated in rat models of nephrotoxicity, whereat the focus was on proximal tubules [28]. Furthermore, plasma and urinary levels showed a significant association with the progress of glomerular diffuse lesions in diabetic patients [35]. The aim of the present study was to measure the urinary excretion of H-FABP, OPN, nephrin, and NGAL in uninephrectomized rats with streptococcal-induced type 1 diabetes mellitus, a common animal model to explore renal impairment in the setting of diabetes mellitus [36]. Furthermore, we detected the plasma concentration of several kidney biomarkers (calbindin, clusterin, cystatin...
Materials and methods

Study design

Rats were kept under standard conditions with respect to temperature and humidity, and were housed on a 12 hour light/12 hour dark cycle in cages with 2 to 3 animals with food (commercial standard diet) and water ad libitum. Animal housing, care, and applications of experimental procedures complied with the Guide for the Care and Use of Laboratory Animals of the State Government of Berlin, Germany. All procedures were performed under inhalation anesthesia with isoflurane. 23 male Wistar Hannover rats with a body weight of 250 - 300 grams were obtained from Charles River Laboratories (Sulzfeld, Germany). In order to perform uninephrectomy, the animals were anesthetized with isoflurane and placed on a heated table to maintain normal body temperature. The right kidney was exposed via flank incision and removed (day 0). After a one week period to enable animals to recover from surgery (day 8), rats were randomly divided in two groups: 14 animals received a single tail-vein injection of streptozotocin (STZ, 35 mg/kg body weight) to induce diabetes, nine animals received only citrate buffer (0.1 mM; pH = 4.5) without STZ in equal volumes (non-diabetic controls). 10 days later (day 17), non-fasting blood glucose levels were measured by tail vein puncture. Hyperglycemia was considered at values above 250 mg/dL, whereas values below 200 mg/dL were considered normoglycemic. Further non-fasting blood glucose testing occurred on day 22 and in weeks 6, 10, 14, and 18. Glucose testing was performed with a Contour glucometer (Bayer, Leverkusen, Germany) with a results range up to 450 mg/dL. The duration of the study was 18 weeks; the hyperglycemic period lasted at least 15 weeks. During the study period the animals were weighed twice weekly, blood pressure was assessed via the tail-cuff method during weeks 4, 8, 12, and 16. The animals were placed in metabolic cages to obtain 24 hour urine samples before sacrifice at week 18; at the same time blood was taken from a tail vein. Afterwards, animals were killed, kidneys were harvested for histological studies, and organ weights were measured.

Histological studies

Tissue samples were all embedded in paraffin, cut into 3 µm and 1 µm sections, submitted to hematoxylin-eosin (HE), Sirius red, periodic acid-Schiff (PAS) and Elastica van Gieson staining. Renal morphology (interstitial fibrosis, perivascular fibrosis, glomerulosclerosis, and media/lumen ratio of blood vessels) was measured as recently described [37]. In brief, glomerular matrix expansion was evaluated on PAS-stained slides by rating the percentage of the PAS-positive areas within the glomerulus using a subjective, semi-quantitative score system (grade I - IV) by two investigators who were blinded to the study groups of the animals. The severity of interstitial fibrosis was evaluated after Sirius Red staining using computer-aided histomorphometry devices. In brief, at least 30 microscopic pictures per kidney section were transferred to a PowerMAC via CFW-1310C (Scion Corporation) camera. After manually setting a threshold using a randomly chosen subset of the pictures, we measured the relationship of SR-stained area (connective tissue) to total area of the picture using ImageJ, an image processing software (shareware from the NIH). Accordingly, microscopic pictures of kidney sections after Elastica-van Gieson staining showing perivascular fibrosis was judged after Sirius-Red staining using a semiquantitative score by one independent investigator blinded to the groups to which the animals belonged.

Plasma and urinary analyses

All other plasma measurements were performed using the Rules Based Medicine (Austin, TX, USA) platform. Urinary concentrations of H-FABP (heart muscle-type fatty acid binding protein), nephrin, NGAL (neutrophil gelatinase-associated lipocalin), and osteopontin were determined in urine samples collected in a six hour time period in metabolic cages. Urine samples were centrifuged to separate cells and particulate debris prior to analyses by commercially available ELISA kits. H-FABP (Hycult Biotech, kit no. HK403, Uden, The Netherlands), nephrin (USCN Life Sci. Inc. kit no. E90937Ra, Burlington, NC, USA), osteopontin (R&D Systems, kit no. MOST00, Wiesbaden, Germany), and NGAL (BioPorto Diagnostics, kit no. KIT041, Gentofte, Denmark) ELISA were conducted according to the protocols provided by the suppliers of the assays. Optical density of the samples was measured at 450 nm using an automatic ELISA reader (Tecan Infinite M200, Tecan Group Ltd., Männedorf, Switzerland). Concentrations were calculated using a four-parameter curve fitting. Urinary creatinine, albumin, and protein were quantitatively determined in the samples using a Cobas Integra 400 Plus analyzer (Roche Diagnostics Deutschland GmbH, Germany) according to the manual of the manufacturer.
Statistical analyses
All values are given as means ± standard deviation (SD). Statistical analyses were performed with SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA). For comparisons between the two groups, Student’s t-test was used if variables were parametric and approximately normally distributed, which was tested by the Kolmogorov-Smirnov test. Otherwise, the Wilcoxon-Mann-Whitney U-test was used instead. A biomarker’s predictive value with regard to hyperglycemia was calculated by means of the area under the receiver operating characteristic curve (ROC-AUC). A value of 0.60 - 0.69 (0.31 - 0.4) was defined as poor, 0.70 - 0.79 (0.21 - 0.30) as fair, 0.80 - 0.89 (0.11 - 0.20) as good, and 0.90 - 1.00 (0.00 - 0.10) as excellent (ranges in brackets for inverse correlations). A value of 0.50 corresponded to the null-hypothesis, i.e. without any predictive information, chance only, and calculated ROC-AUCs were statistically compared with this value. Mortality calculations were performed using the Mantel-Cox log rank test. Differences were considered significant if p<0.05 and highly significant if p<0.01.

RESULTS

Survival rate
One animal from the diabetic group died before sacrifice, presumably from hyperglycemia, whereas none of the non-diabetic animals died prior to regular sacrifice. There is no significant difference in mortality between the study groups (p = 0.25, log-rank test).

Hemodynamics and blood glucose
Systolic blood pressure was measured four times during the course of the experiment, and at no point in time was there a significant difference between the two study groups (Figure 1). The first non-fasting blood glucose testing was performed one week after administration of STZ. 7 out of 14 animals (50%) from the STZ-treated group (diabetic group) showed hyperglycemia (>250 mg/dL). Five days later, hyperglycemia was confirmed in all 14 animals from the diabetic group. These animals stayed hyperglycemic in all further testing throughout the experiment, whereas the 9 animals from the non-diabetic group all showed normoglycemic values. The differences between the two study groups were highly significant (Figure 2).

Body and organ weights
Non-diabetic animals were gaining body weight during the entire experiment, whereas the STZ-treated animals remained at a stable level after STZ application (Figure 3). Differences between the study groups began to be significant four weeks after surgery and two weeks after STZ/citrate buffer application. Highly significant differences in body weight after sacrifice (p<0.000001) are most likely to have caused significantly different relative liver weights, whereas absolute liver weights were statistically similar (Table 1). Diabetes led to significantly higher kidney weights (relative to body and absolute weight). In contrast, absolute heart weight was significantly lower in diabetic animals, whereas relative weight was significantly higher (Table 1).

Urinary parameters and kidney function
Urinary and blood samples were obtained at the end of the study (Table 2 and Table 3). Urinary albumin and protein excretion and albumin/creatinine ratio showed no significant differences between the study groups (Figure 4 and Table 3). ROC-AUC analyses showed unremarkable values close to 0.50, signaling no predictive information (Table 3). Furthermore, plasma cystatin c levels tended to be slightly lower in diabetic animals (p = 0.056; Table 2) indicating that the kidney still hyperfiltrated due to hyperglycemia. However, these parameters denoted neither an impairment of kidney function nor kidney injury in diabetic animals. In contrast, urinary levels of Heart-type fatty acid binding protein (H-FABP), osteopontin (OPN), nephlin, and Neutrophil gelatinase-associated lipocalin (NGAL) showed clear differences between diabetic and non-diabetic animals (Figure 5). Urinary excretion amounts per 24 hours of each of these parameters were highly increased in diabetic animals with a highly significant p-value (p<0.01 each) compared to non-diabetic animals (Table 3). Calculation of the ratios to urinary protein and creatinine concentrations confirmed significant differences for H-FABP, OPN, and nephlin, whereas NGAL/protein and NGAL/creatinine ratios no longer differed significantly (p>0.1; see Figure 5 and Table 3). Concerning the area under the receiver operating characteristic curve (ROC-AUC), H-FABP, osteopontin, and NGAL achieved excellent values with regard to predictive information, whereas the value of nephlin can be regarded as good (Table 3). In contrast, creatinine, albumin, and protein excretion did not achieve any predictive value which statistically differed from the null hypothesis (ROC-AUC = 0.5, i.e. no predictive value).

Plasma parameters
Plasma samples were obtained at the end of the experiment. Neither clear differences nor predictive value with respect to ROC-AUC could be observed for plasma glutathione S-transferase Mu (GST-Mu) and vascular endothelial growth factor (VEGF)-a (overview of all mentioned parameters in Table 2). Unlike in urine, plasma concentration of NGAL tended to be lower in diabetic animals (p = 0.078), whereas plasma OPN levels non-significantly appeared to be higher (p = 0.170). Both biomarkers showed good predictive values with regard to ROC-AUC (0.139 and 0.870 for NGAL and OPN, respectively). Interestingly, plasma clusterin and plasma TIMP-1 levels were significantly higher in the diabetic group with good and fair predictive values, respectively (p = 0.018 and ROC-AUC = 0.829 for clus-
Table 1. Organ weights right after euthanasia. Values of the left kidney are not available due to its removal at the beginning of the experiment.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>non-diabetic</th>
<th>diabetic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>mean ± SD</td>
<td>n</td>
</tr>
<tr>
<td>dead body weight [g]</td>
<td>9</td>
<td>576.2 ± 51.8</td>
<td>13</td>
</tr>
<tr>
<td>liver weight [g]</td>
<td>9</td>
<td>16.4 ± 2.26</td>
<td>13</td>
</tr>
<tr>
<td>relative liver weight [mg/g]</td>
<td>9</td>
<td>28.4 ± 3.16</td>
<td>13</td>
</tr>
<tr>
<td>kidney weight [g]</td>
<td>9</td>
<td>2.42 ± 0.186</td>
<td>11</td>
</tr>
<tr>
<td>relative kidney weight [mg/g]</td>
<td>9</td>
<td>4.23 ± 0.493</td>
<td>11</td>
</tr>
<tr>
<td>heart weight [g]</td>
<td>9</td>
<td>1.39 ± 0.154</td>
<td>13</td>
</tr>
<tr>
<td>relative heart weight [mg/g]</td>
<td>9</td>
<td>2.43 ± 0.343</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 2. Plasma parameters at the end of the experiment (after 18 weeks). P values refer to parametric comparison between the two study groups (using Student’s t-test). ROC-AUC reflects the area under the curve of the receiver operating characteristic; * p<0.05; ** p<0.01 denying the null hypothesis (ROC-AUC = 0.5).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>non-diabetic</th>
<th>diabetic</th>
<th>p</th>
<th>ROC-AUC</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>mean ± SD</td>
<td>n</td>
<td>mean ± SD</td>
</tr>
<tr>
<td>Plasma calbindin [ng/mL]</td>
<td>9</td>
<td>0.290 ± 0.483</td>
<td>13</td>
<td>5.07 ± 7.65</td>
</tr>
<tr>
<td>Plasma clusterin [µg/mL]</td>
<td>9</td>
<td>158.1 ± 19.5</td>
<td>13</td>
<td>196.08 ± 41.1</td>
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<tr>
<td>Plasma cystatin c [ng/mL]</td>
<td>9</td>
<td>845.9 ± 69.3</td>
<td>13</td>
<td>696.31 ± 211.7</td>
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<tr>
<td>Plasma glutathione S-transferase alpha (GST-alpha) [ng/mL]</td>
<td>9</td>
<td>92.5 ± 113.3</td>
<td>13</td>
<td>22.7 ± 25.2</td>
</tr>
<tr>
<td>Plasma glutathione S-transferase Mu (GST-Mu) [ng/mL]</td>
<td>9</td>
<td>189.0 ± 363.3</td>
<td>13</td>
<td>88.3 ± 98.8</td>
</tr>
<tr>
<td>Plasma kidney injury molecule-1 (KIM-1) [ng/mL]</td>
<td>9</td>
<td>0.191 ± 0.0521</td>
<td>13</td>
<td>1.37 ± 2.57</td>
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<tr>
<td>Plasma neutrophil gelatinase-associated lipocalin (NGAL) [ng/mL]</td>
<td>9</td>
<td>277.3 ± 72.9</td>
<td>13</td>
<td>204.5 ± 100.2</td>
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<td>Plasma osteopontin [ng/mL]</td>
<td>9</td>
<td>21.4 ± 2.35</td>
<td>13</td>
<td>39.1 ± 37.1</td>
</tr>
<tr>
<td>Plasma tissue inhibitor of metalloproteinases 1 (TIMP-1) [ng/mL]</td>
<td>9</td>
<td>8.05 ± 1.23</td>
<td>12</td>
<td>9.88 ± 2.23</td>
</tr>
<tr>
<td>Plasma vascular endothelial growth factor a (VEGF-A) [pg/mL]</td>
<td>9</td>
<td>149.8 ± 25.2</td>
<td>13</td>
<td>182.1 ± 135.1</td>
</tr>
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</table>

terin and p = 0.034 and ROC-AUC = 0.787 for TIMP-1). With respect to the aforementioned levels of OPN in urine and plasma, the plasma level of the calcium-binding protein calbindin tended to be higher in diabetic animals (p = 0.078) with a good predictive value (0.875). Plasma KIM-1 levels were non-significantly higher in diabetic animals (p = 0.187), but this parameter was excellent at predicting diabetes (ROC-AUC = 0.907). In contrast to plasma GST-Mu, plasma GST-alpha levels were significantly lower in diabetic rats (p = 0.043) without any predictive value (ROC-AUC = 0.431).

Histopathology
Histopathological evaluation of the kidneys with respect to glomerulosclerosis, interstitial and perivascular fibrosis revealed no differences between diabetic and non-diabetic animals. Solely the media-to-lumen ratio of diabetic animals was higher than in non-diabetic ones (p = 0.010). The overview of these parameters is depicted in Table 4.
Table 3. Urinary parameters at the end of the experiment (after 18 weeks). Visualization of these parameters can be found in Figure 4 and Figure 5. P values refer to parametric comparison between the two study groups (using Student’s t-test). ROC-AUC reflects the area under the curve of the receiver operating characteristic; * p<0.05; ** p<0.01 denying the null hypothesis (ROC-AUC = 0.5).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>non-diabetic</th>
<th>diabetic</th>
<th>p</th>
<th>ROC-AUC</th>
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<tbody>
<tr>
<td>n mean SD</td>
<td>n mean SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary H-FABP excretion per 24 hrs [ng]</td>
<td>9 25.9 ± 20.1</td>
<td>12 454.7 ± 328.3</td>
<td>&lt;0.001</td>
<td>1.000**</td>
</tr>
<tr>
<td>Urinary H-FABP/creatinine ratio [ng/mL:mmol/L]</td>
<td>9 0.251 ± 0.141</td>
<td>12 3.70 ± 2.53</td>
<td>&lt;0.001</td>
<td>1.000**</td>
</tr>
<tr>
<td>Urinary osteopontin excretion per 24 hrs [ng]</td>
<td>9 41.0 ± 66.3</td>
<td>13 1396.0 ± 1064.2</td>
<td>0.001</td>
<td>0.988**</td>
</tr>
<tr>
<td>Urinary osteopontin/creatinine ratio [ng/mL:mmol/L]</td>
<td>9 0.409 ± 0.542</td>
<td>13 14.2 ± 12.5</td>
<td>0.004</td>
<td>0.988**</td>
</tr>
<tr>
<td>Fractionary osteopontin clearance [mL/h]</td>
<td>9 0.080 ± 0.13</td>
<td>13 1.85 ± 1.50</td>
<td>0.002</td>
<td>0.552</td>
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<tr>
<td>Urinary nephrin excretion per 24 hrs [ng]</td>
<td>9 209467 ± 144535</td>
<td>13 1047563 ± 788998</td>
<td>0.005</td>
<td>0.788*</td>
</tr>
<tr>
<td>Urinary nephrin/creatinine ratio [ng/mL:mmol/L]</td>
<td>9 2140.0 ± 1953.7</td>
<td>13 9248.4 ± 7274.1</td>
<td>0.010</td>
<td>0.800*</td>
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<tr>
<td>Urinary NGAL excretion per 24 hrs [U]</td>
<td>9 12649 ± 7493.9</td>
<td>13 38782 ± 21614</td>
<td>0.002</td>
<td>0.988**</td>
</tr>
<tr>
<td>Urinary NGAL/creatinine ratio [U/mL:mmol/L]</td>
<td>9 118.9 ± 38.5</td>
<td>13 548.3 ± 951.6</td>
<td>0.194</td>
<td>0.963**</td>
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<tr>
<td>Fractionary NGAL clearance [mL/h]</td>
<td>9 2.00 ± 1.28</td>
<td>13 8.26 ± 3.45</td>
<td>&lt;0.001</td>
<td>0.643</td>
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<td>Urinary creatinine excretion per 24 hrs [µmol]</td>
<td>9 101.2 ± 46.9</td>
<td>13 117.8 ± 32.7</td>
<td>0.338</td>
<td>0.613</td>
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<td>Urinary creatinine/protein ratio [mmol/g]</td>
<td>9 4.15 ± 3.40</td>
<td>13 3.29 ± 2.74</td>
<td>0.518</td>
<td>0.500</td>
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<td>Urinary protein excretion per 24 hrs [mg]</td>
<td>9 46.7 ± 44.8</td>
<td>13 86.2 ± 121.4</td>
<td>0.365</td>
<td>0.513</td>
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<td>Urinary protein excretion per 24 hrs &amp; body weight [µg/g]</td>
<td>9 2.42 ± 1.53</td>
<td>13 1.01 ± 1.53</td>
<td>0.046</td>
<td>0.112**</td>
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<tr>
<td>Urinary albumin excretion per 24 hrs [mg]</td>
<td>9 21.6 ± 16.8</td>
<td>13 71.8 ± 113.8</td>
<td>0.207</td>
<td>0.563</td>
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<tr>
<td>Urinary albumin excretion per 24 hrs &amp; body weight [µg/g]</td>
<td>9 36.7 ± 28.2</td>
<td>13 232.5 ± 439.0</td>
<td>0.200</td>
<td>0.700</td>
</tr>
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<td>Urinary albumin/creatinine ratio [1/100]</td>
<td>9 19.9 ± 20.7</td>
<td>13 55.4 ± 95.2</td>
<td>0.286</td>
<td>0.587</td>
</tr>
</tbody>
</table>

Table 4. Histopathological evaluation of the kidneys included assessment of glomerulosclerosis, interstitial fibrosis and perivascular fibrosis as well as media to lumen ratio of intra-renal arteries.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>non-diabetic</th>
<th>diabetic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n mean SD</td>
<td>n mean SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glomerulosklerosis kidney (score)</td>
<td>8 3.62 ± 0.139</td>
<td>12 3.64 ± 0.256</td>
<td>0.270</td>
</tr>
<tr>
<td>Media-to-lumen ratio kidney (ratio)</td>
<td>8 1.38 ± 0.349</td>
<td>12 1.93 ± 0.457</td>
<td>0.010</td>
</tr>
<tr>
<td>Interstitial fibrosis kidney [%]</td>
<td>8 0.107 ± 0.0611</td>
<td>12 0.0914 ± 0.0470</td>
<td>0.517</td>
</tr>
<tr>
<td>Perivascular fibrosis kidney (ratio)</td>
<td>8 2.81 ± 0.481</td>
<td>12 3.11 ± 0.660</td>
<td>0.473</td>
</tr>
</tbody>
</table>
Figure 1. Systolic blood pressure measured by tail-cuff method during the experiment. The values did not differ significantly between the study groups. Values are given as means ±SD in mmHg.

Figure 2. Non-fasting blood glucose values during the experiment. The results range of the glucose tester reached only up to 600 mg/dL and results above this threshold value were calculated as if they were 601 mg/dL. Highly significant differences were detected between the study groups. * p<0.001. Values are given as means ±SD in mg/dL.
Figure 3. Body weights during the experiment. Values differed significantly from week 4 on until sacrifice. * p<0.05. Values are given as means ±SD in grams.

Figure 4. Overview of basic urinary parameters at the end of the experiment (after 18 weeks). None of the mentioned parameters showed significant difference between the study groups (p values in brackets). Values are given in means ±SD For detailed values, see Table 3. A, urinary albumin excretion per 24 hours (p = 0.141). B, urinary albumin/creatinine ratio (p = 0.286). C, urinary protein excretion per 24 hours (p = 0.365).
Figure 5. Overview of the urinary biomarkers Heart-type fatty acid binding protein (H-FABP), osteopontin, nephrin, and Neutrophil gelatinase-associated lipocalin (NGAL) (from top to bottom) as excretion per 24 hours, and creatinine ratio (from left to right). Urine was obtained at the end of the experiment after 18 weeks. ** p<0.01; *** p<0.001. Values are given as means ±SD For detailed values, see Table 3. A, H-FABP excretion per 24 hours. B, H-FABP/creatinine ratio. C, osteopontin excretion per 24 hrs. D, osteopontin/creatinine ratio. E, nephrin excretion per 24 hours. F, nephrin/creatinine ratio. G, NGAL excretion per 24 hours. H, NGAL/creatinine ratio.
DISCUSSION

In this study we show for the first time in uninephrectomized rats with STZ-induced type 1 diabetes mellitus that a number of urinary and plasma biomarkers (urinary osteopontin, urinary nephrin, urinary NGAL, urinary H-FABP, plasma KIM-1, plasma TIMP-1) precede plasma cystatin c, albuminuria, and proteinuria, which are well-established, sensitive, and early. Thus, those biomarkers in urine and plasma might offer new diagnostic perspectives in renally impaired rodent models of early stage diabetes mellitus.

The animal model used in this experiment reflects the impact of type 1 diabetes mellitus in the setting of renal impairment, which was induced by removal of one kidney. This approach is well-established and was previously used [36,38,39]. This rat model is characterized by the development of typical laboratory as well as morphological alterations seen in diabetic nephropathy such as progressive proteinuria, decline in kidney function, and glomerular sclerosis and hypertrophy.

As expected, STZ treatment led to a robust hyperglycemia (Figure 2), and those rats showed significantly lower body weights as compared to control rats (Figure 3). In contrast, systolic blood pressure did not differ significantly between the study groups. Hence, our findings resulted from diabetes not from hypertension. Plasma cystatin c tended to be lower (p = 0.056) in hyperglycemic animals suggesting that kidneys still persisted in a hyperfiltrating state which is typical in early diabetes [40]. Urinary protein and albumin excretion ratio were numerically higher in diabetic animals, although these differences were not significant indicating again an early disease state.

In other words, plasma cystatin c, albuminuria, and proteinuria as standard biomarkers appear not to be sensitive enough at this very early stage of disease. Our basic histological evaluations are in line with these laboratory findings (Figure 4).

Plasma glutathione S-transferase alpha (GST-alpha), plasma glutathione S-transferase Mu (GST-Mu), and plasma vascular endothelial growth factor a (VEGF-A) have not been shown to have predictive information with regard to ROC-AUC. In contrast, urine osteopontin (OPN) values were clearly higher in diabetic animals (p<0.01) with an excellent ROC-AUC value of 0.988 (p<0.01). Plasma OPN showed a good predictive ROC-AUC of 0.870 (p<0.01). Recently, OPN was identified as a key contributor to glomerular damage even before albuminuria and without albuminuria but not in healthy controls [47]. Unfortunately, urinary nephrin excretion was not measured in either study [20,46]. Assuming that the urinary nephrin detected is of glomerular origin, these findings would suggest that urinary nephrin levels decline with the duration of hyperglycemia and increasing proteinuria. Hence, elevated urinary nephrin appears to be characteristic for early stage diabetic nephropathy rather than for advanced stages. Moreover, our results suggest that urinary nephrin excretion increases directly reflecting glomerular damage even before albuminuria emerges, which is a result of the damage. In humans, these findings were confirmed by Pátrai et al. who found significant nephrinuria in T1DM patients with and without albuminuria but not in healthy controls [47].

Similar to nephrin, our diabetic rats showed significantly higher (p = 0.002) urinary NGAL levels (ROC-AUC = 0.988). In patients with T1DM and T2DM, urinary and plasma NGAL were significantly associated with albuminuria and eGFR [7,26] and significantly differed from those in healthy control subjects, indepen-
Plasma TIMP-1 levels were significantly higher in diabetic animals with a fair predictive value of 0.787. As plasma TIMP-1 appears to be a biomarker of cardiac, renal, and vascular fibrosis [28,34,35,50,51], our results probably reflected ongoing reno-cardiovascular fibrosis and remodeling processes under hyperglycemic conditions.

In summary, our study revealed elevated levels of numerous plasma and urinary biomarkers in uni-nephrectomized STZ-treated diabetic rats compared to non-diabetic controls. Except for H-FABP, which reflects distal tubule damage, all biomarkers reflect glomerular and proximal tubule damage (OPN, nephrin, NGAL, KIM-1) and general cardiovascular, not specifically renal, processes as a result of hyperglycemia (calbindin, clusterin, TIMP-1). At this early stage, classical biomarkers such as albuminuria and cystatin c and also histopathology showed no or just a trend difference between control rats and rats that will later develop full blown diabetic nephropathy. The new biomarkers may thus offer earlier detection of overt diabetic nephropathy. Our study should stimulate clinical studies aiming to confirm that these new biomarkers appear earlier than the traditional ones in human diabetic nephropathy as well. Biomarker research is important for the preclinical as well as clinical development of new drugs for the treatment of diabetic nephropathy [52,53]. Some of the above described biomarkers are for example used for the evaluation of the safety and cardiovascular as well as renal efficacy of new antidiabetic drugs such as DPP4 inhibitors [54,55,56].

**Declaration of Interest:**
There is no conflict of interest for any of the authors

**References:**


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Correspondence: Prof. Berthold Hocher, MD, PhD
Institute of Nutritional Science
University of Potsdam
D-14558 Nuthetal Potsdam, Germany
Email: hocher@uni-potsdam.de
Homepage: http://www.uni-potsdam.de/eem