Proteomic identification of vanin-1 as a marker of kidney damage in a rat model of type 1 diabetic nephropathy

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At present, the urinary albumin excretion rate is the best noninvasive predictor for diabetic nephropathy (DN) but major limitations are associated with this marker. Here, we used in vivo perfusion technology to establish disease progression markers in an animal model of DN. Rats were perfused with a reactive ester derivative of biotin at various times after streptozotocin treatment. Following homogenization of kidney tissue and affinity purification of biotinylated proteins, a label-free mass spectrometry-based proteomic analysis of tryptic digests identified and relatively quantified 396 proteins. Of these proteins, 24 and 11 were found to be more than 10-fold up- or downregulated, respectively, compared with the same procedure in vehicle-treated rats. Changes in the expression of selected differentially regulated proteins were validated by immunofluorescence detection in kidney tissue from control and diabetic rats. Immunoblot analysis of pooled human urine found that concentrations of vanin-1, an ectoenzyme pantetheinase, distinguished diabetic patients with macroalbuminuria from those with normal albuminuria. Uromodulin was elevated in the urine pools of the diabetic patients, regardless of the degree of albuminuria, compared with healthy controls. Thus, in vivo biotinylation facilitates the detection of disease-specific changes in the abundance of potential biomarker proteins for disease monitoring and/or pharmacodelivery applications.


KEYWORDS: diabetic nephropathy; diagnosis; podocyte; vascular access

Diabetes mellitus is one of the most common chronic diseases affecting 285 million patients worldwide. Classically, diabetes has been divided in two main subtypes type 1 diabetes (T1D) and type 2 diabetes (T2D), as well as secondary diabetes and some monogenic forms of diabetes.

Although T1D only accounts for 5–10% of patients, its incidence continues to increase worldwide.1 The susceptibility for the disease is largely inherited, residing predominantly in two genotypes of the human leukocyte antigen, but it is largely unknown which factors trigger the onset of clinical symptoms.2 After disease onset, an autoimmune reaction results in the destruction of pancreatic β-cells and in an absolute insulin deficiency.

In the etiopathology of T1D as well as T2D, several complications are likely to occur. The chronic elevation of blood glucose levels together with the lack of insulin and other metabolic consequences leads to damage of blood vessels, hence called diabetic micro- and macroangiopathy. Diabetic angiopathy of the capillaries in the kidney glomeruli is most likely involved in the general diabetic nephropathy (DN), a progressive kidney disease that is characterized by a set of structural and functional kidney abnormalities.3 Structural abnormalities include kidney hypertrophy in the early stage of the disease, continuous overproduction of extracellular matrix leading to increased glomerular basement membrane thickness, nodular and diffuse glomerulosclerosis, tubular atrophy, and interstitial fibrosis.4 In an early stage, the functional kidney abnormalities include hyperfusion and intraglomerular hypertension.5 Subsequently, increasing proteinuria, abnormalities of intraglomerular pressure regulation, systemic hypertension, and continuous decrease of glomerular filtration rate characterize DN.6 Currently, the earliest clinical evidence of DN is microalbuminuria, which is defined as urinary albumin excretion between 30 and 300 mg/day. Without specific interventions, ~40% of subjects with T1D progress to the stage of overt nephropathy (or clinical albuminuria; defined as urinary albumin excretion >300 mg/day) over a period of 10–15

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The availability of rodent models of T1D and its complications, such as rats with streptozotocin (STZ)-induced DN and OVE26 transgenic mice, facilitate these investigations. Sharma et al. have analyzed the urinary proteome of patients with DN and healthy controls, resulting in the identification of alpha-1 antitrypsin being 19-fold upregulated in the diabetic group. In another study, the C-reactive protein has been identified as possible serum biomarker when comparing STZ-induced diabetic rats with healthy controls. The comparison of whole-kidney lysates derived from OVE26 transgenic mice with the ones of background FVB non-diabetic mice allowed the identification of tubulointerstitial elastin depositions in DN and indicated the potential value of proteomics analyses in defining features of the pathophysiological processes in diabetes. Furthermore, techniques for the proteomics-based identification of differentially expressed proteins in glomeruli from mouse kidneys and human biopsies were developed, either embolizing magnetic beads into the glomerular capillaries, followed by magnetic separation of bead-filled glomeruli or by laser capture microdissection, respectively. Finally, the analysis of cultured podocytes grown under different glucose concentrations contributed to the identification of novel targets involved in DN.

To establish new vascular markers of pathology, our group has previously used chemical proteomic techniques, based either on the in vivo perfusion of tumor-bearing animals or on the ex vivo perfusion of surgically resected human organs with cancer, followed by the subsequent capture of biotinylated proteins on streptavidin resin. These studies revealed that the in vivo perfusion procedure facilitates the study of the vascular proteome in cancer, allowing the identification of markers of angiogenesis, which can be drugged with monoclonal antibodies.

In this study, we used the in vivo perfusion technology to establish disease progression markers of DN in an animal model of T1D. For this, we performed a comparative proteomic analysis of the accessible proteins of kidneys in the STZ model of non-insulin-treated rats by in vivo protein biotinylation and mass spectrometry-based label-free protein identification and quantification. The accessible kidney proteomes of citrate-treated rats as well as rats terminally perfused either 4, 8, or 12 weeks after STZ injection were compared using the in-house-developed software suite DeepQuanTR. The proteomic analysis resulted in the identification and relative quantification of 396 proteins, whereof 24 and 11 have been found more than 10-fold up- or downregulated, respectively. Selected differentially regulated proteins were validated by immunofluorescence detection in rat kidney tissue. Interestingly, our results show typical leakage pattern of the respective proteins into the urine of patients with T1D and different levels of DN.

RESULTS

STZ-induced rat model of DN
Following STZ injection, animals developed severe diabetes with very high postprandial blood glucose values compared with citrate-treated control animals. At 12 weeks after citrate injection, blood glucose was normal as assessed by the oral glucose tolerance test. In contrast, STZ-treated rats showed a slightly elevated glucose level after fasting and developed very high serum glucose levels following oral glucose tolerance test. The hyperglycemia was lowered only very slowly, and was still high at 2 h after glucose gavage (Supplementary Table S1 online).

Furthermore, STZ-induced diabetes resulted in severe renal damage. Urinary albumin excretion rarely exceeded 250 mg/day in citrate-treated control rats, whereas animals treated with STZ developed progressive albuminuria, reaching a UAER of 1500 mg/day (Supplementary Table S1 online). Albuminuria was accompanied by reduced renal excretory function at 12 weeks after STZ treatment. Blood urea and creatinine values were about four times higher in STZ rats compared with normal values measured in citrate-injected controls.

In vivo perfusion of rats and chemical proteomic analysis
Twenty-one-week-old rats at 4, 8, and 12 weeks after STZ treatment as well as age-matched citrate-injected control rats were subjected to a terminal perfusion procedure with a 0.5 mg/ml solution of the reactive biotin ester Sulfo-NHS-LC-biotin (Pierce, Rockford, IL), followed by a quenching step with 50 mmol/l Tris-HCl solution. Immediately after perfusion, the renal capsules were removed, the kidneys cut in half, and the two halves separately frozen in liquid nitrogen. After the first thawing, a cross-section was cut out of the middle of each kidney half and embedded in OCT.
medium for histochemical analyses, whereas the rest of the kidney was homogenized. The resulting protein extract was loaded in the presence of 2% SDS onto a streptavidin-affinity resin to purify biotinylated proteins. The following on-resin tryptic protein digestion yielded proteolytic peptides suitable for the mass spectrometry-based comparative proteomic analysis (Figure 1a). Successful biotinylation of accessible structures in the kidney was assessed by histochemical staining of biotin using the streptavidin–biotinylated alkaline phosphatase complex and fast red as phosphatase substrate (Figure 1b).

Tryptic peptides derived from the on-resin digestion of purified biotinylated proteins of individual kidneys were separated by reversed-phase nanocapillary high-performance liquid chromatography. The resulting fractions were mixed with constant amounts of four internal standard peptides and matrix, robotically spotted on MALDI-target plates (Foster City, CA) and analyzed by MALDI-TOF (Foster City, CA) and MALDI-TOF/TOF MS (Foster City, CA) for quantification and identification purposes, respectively. The DeepQuanTR analysis allowed the relative quantification of a total of 396 proteins identified with at least two proteotypic peptides (Figure 2 and Supplementary Table S2 online). The number of identified proteins is similar to experiments performed for the analysis of tumors and normal organs in mice.20,21

**The accessible kidney proteome of STZ-treated and citrate-injected control rats**

The chemical proteomic analysis revealed a number of proteins with known expression on vascular endothelial cells, in the apical and the lateral membrane area of podocytes, in the glomerular basement membrane, and in the proximal tubule (Table 1), defining therefore the kidney substructures and analyzed by MALDI-TOF (Foster City, CA) and MALDI-TOF/TOF MS (Foster City, CA) for quantification and identification purposes, respectively. The DeepQuanTR analysis allowed the relative quantification of a total of 396 proteins identified with at least two proteotypic peptides (Figure 2 and Supplementary Table S2 online). The number of identified proteins is similar to experiments performed for the analysis of tumors and normal organs in mice.20,21

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**Figure 1** Schematic depiction of the chemical proteomic workflow and validation of the in vivo biotinylation efficacy. (a) The abdomen of anesthetized rats was cut open and two ligations (L1 on the hepatic artery, L2 on the abdominal artery, and vein below the branching points of the kidney arteries) were placed. Using a buttoned cannula, the kidneys were perfused through the aorta abdominalis using a solution containing a reactive ester derivative of biotin. Following removal of the kidneys, tissue homogenization and protein extraction, biotin-tagged proteins were enriched on a streptavidin-affinity resin. A tryptic on-resin digestion resulted in proteolytic peptides submitted to the comparative proteomic analysis. B, biotin; SA, streptavidin. (b) The in vivo biotinylation efficacy was validated by a histochemical staining of biotin using the streptavidin–biotinylated alkaline phosphatase complex and fast red. Scale bars: 200 μm. Cit, citrate-treated control; Neg, phosphate-buffered saline-perfused control rat; 4W, 8W, and 12W, 4, 8, and 12 weeks after streptozotocin treatment.
readily accessible for the charged biotinylation reagent used in the perfusion experiments. During perfusion with the reactive ester derivative of biotin and subsequently the quenching solution soluble proteins are removed from the kidneys. Out of the 396 identified and quantified proteins, 24 and 11 proteins were found more than tenfold upregulated and downregulated in the diseased kidneys compared with the citrate-treated kidneys, respectively (Supplementary Figure S1 online). Some of

Table 1 | Localization of selected proteins identified in the comparative proteomic analysis of citrate-treated and STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Gene name</th>
<th>SwissProt acc. nr.</th>
<th>Location</th>
<th>Reference</th>
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<tr>
<td>Platelet endothelial cell adhesion molecule</td>
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<td>Q3SWT0</td>
<td>EM</td>
<td>Woodfin and co-workers(^5^6)</td>
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<td>Nrp1</td>
<td>Q9QWJ9</td>
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<td>Pellet-May et al.(^5^1)</td>
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<td>Endothelial cell-selective adhesion molecule</td>
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<td>P31977</td>
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<td>Low-density lipoprotein receptor-related protein 2 (megalin)</td>
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</tbody>
</table>

Abbreviations: AMP, apical membrane area of the podocyte; EM, endothelial membrane; GBM, glomerular basement membrane; LMP, lateral membrane area of the podocyte; PT, proximal tubule; STZ, streptozotocin.
these have previously been described to be involved and/or regulated in DN (Supplementary Table S3 online).

**Validation of accessible targets in DN**

The selection of proteins used for the validation of the DeepQuanTR results was based both on the protein regulation determined by this proteomic investigation and on the availability of antibodies recognizing the rat protein. Four differentially regulated proteins (Figure 2, bold) were selected for validation by immunofluorescence: (1) vanin-1 (*Vnn1*, upregulated in diseased), (2) Ennp6 protein (*Enpp6*, upregulated in diseased), (3) alpha-1-acid glycoprotein (*Orm1*, slightly upregulated in diseased), and (4) uromodulin (*Umod*, downregulated in diseased). The immunofluorescence analysis of rat kidney tissue derived from citrate-injected control rats and the STZ-injected diabetic rats (4, 8, and 12 weeks after STZ injection) confirmed the mass spectrometry-based findings (Figure 3). Unfortunately, vanin-1-specific antibodies that cross-react with the rat protein are not commercially available. Antibodies against *Enpp6* and *Umod* stained tubular structures, whereas *Orm1* was found in glomeruli. The differential expression of the three analyzed proteins shown by immunostaining confirmed the results obtained by the proteomic analysis. Scale bars: 200 μm. Neg, negative.

![Figure 3 Validation of DeepQuanTR results](image-url)

**Figure 3** Validation of DeepQuanTR results. Immunostainings of kidney sections derived from citrate-injected control rats and streptozotocin-injected diabetic rats killed at 4, 8, and 12 weeks after the end of the pharmacological treatment using antibodies against *Enpp6*, *Orm1*, and *Umod* are presented. Additionally, negative controls omitting the primary antibody are shown (using only the secondary anti-mouse antibody as negative control for *Enpp6* and *Umod*; and only the secondary anti-rabbit antibody for *Orm1*). Antibodies against *Enpp6* and *Umod* stained tubular structures, whereas *Orm1* was found in glomeruli. The differential expression of the three analyzed proteins shown by immunostaining confirmed the results obtained by the proteomic analysis. Scale bars: 200 μm. Neg, negative.
antibody stained the glomeruli. The albumin band is indicated with an arrowhead. Urines is shown. The albumin band is indicated with an arrowhead. The urine of 6 healthy individuals and 10 diabetic patients were performed using antibodies against albumin excretion (normo) and with macroalbuminuria (macro) were collected for an analysis of interindividual variability of urinary albumin and vanin-1 in samples of diabetic patients with elevated UAER did not show a significant increase of vanin-1 concentration in the urine.

**Comparison with published microarray data**
To get further insight into the mechanism of protein regulation, we compared our proteomics findings with publicly available microarray data (see Supplementary Materials online). Owing to the different nature of the samples (renal tubular cells for microarray experiments versus the cell surface proteome of endothelial cells, podocytes, and tubular cells, as well as extracellular matrix in the proximity of these cells for our proteomic study), there was no global correlation between the proteomics and the microarray data (Supplementary Figure S2 online). Noteworthy, neither VNN1 nor UMOD was found to be significantly regulated in these studies. Furthermore, microarrays used in both studies lacked in probes against ENPP6. Although Rudnicki et al. analyzed patient samples from proteinuric nephropathies, but not from DN, we estimate that the lack of correlation emphasizes the unique nature of the proteomics analysis presented here.

**DISCUSSION**

The proteomic analysis described in this article was based on the terminal perfusion of citrate-treated rats and rats treated with STZ using a reactive ester derivative of biotin (Figure 1a). The terminal perfusion of rodents has previously shown to be a useful technique to identify markers of tumor neo-vasculature, which can be drugged with monoclonal antibodies. The results of this study show that in vivo biotinylation facilitates the detection of disease-specific changes in the abundance of accessible proteins in rat kidneys after STZ-induced diabetes. STZ is an analog of N-acetylglucosamine, readily transported into pancreatic β-cells by the glucose transporter GLUT-2 causing β-cell toxicity, leading to insulin deficiency and elevated blood glucose levels. A known limitation of this model is the toxicity of STZ to renal tubular cells. For this study, the standard STZ-induced diabetes model was modified to avoid excessive initial toxic effects and thus to obtain a more manageable diabetes. Further, the earliest time point for the perfusion of rat kidneys was set to 4 weeks in which tubular epithelial cells could regenerate.

Compared with citrate-treated control rats, which showed normal UAERs of about 200 μg per 24 h, the UAER of STZ-treated rats was in the range of 900–1500 μg per 24 h (Supplementary Table S1 online), which is in line with values previously published and which indicates severe kidney damage. The successful in vivo protein biotinylation, which was assessed by histochemistry, showed a uniform labeling of glomeruli, blood vessels, and certain tubular structures (Figure 1b). The subsequent mass spectrometry-based

**Figure 4** Validation of selected proteins in human urine. (a) Western blot analyses using urine derived from healthy individuals and from diabetic patients with normal urinary albumin excretion (normo) and with macroalbuminuria (macro) were performed using antibodies against VNN1, UMOD, and ALB. (b) A Ponceau S-stained western blot of creatinine-normalized urines is shown. The albumin band is indicated with an arrowhead.

*Umod* stained tubular structures, whereas the anti-Orml antibody stained the glomeruli.

**Analysis of protein expression in human urine**
To investigate whether the differential protein expression of the four selected regulated proteins could be translated to the human pathology, urine sample pools derived from healthy individuals and from T1D diabetes patients without (<30 mg/day) and with highly (>300 mg/day) elevated UAER were analyzed. To standardize the spot urine samples, all urine pools were normalized to the creatinine concentration before analysis, analogous to standard clinical procedures. Western blots of the three urine pools confirmed the upregulation of Vnn1 and Umod in the macroalbuminuria samples and in the diabetic samples, respectively (Figure 4). Compared with albumin, which can be identified both in urine of healthy individuals and diabetic patients (Figure 4), uromodulin was found to be strongly upregulated in diabetic samples and vanin-1 in samples of diabetic patients with macroalbuminuria.

**Analysis of protein expression in individual human urine**
The urine of 6 healthy individuals and 10 diabetic patients was collected for an analysis of interindividual variability of VNN1 and UMOD excretion (Figure 5a). UMOD levels in all diabetic patients were found to be elevated, similar to what was observed in pooled urine samples. The increase was most pronounced in diabetic patients with elevated UAER (Figure 5b). In contrast, the vanin-1 concentration in the urine samples of individual patients was found to be highly variable (Figure 5c). Patients with elevated UAER did not show a significant increase of vanin-1 concentration in the urine.
proteomic analysis resulted in the identification and label-
free relative quantification of 396 proteins, whereof 24 and
11 proteins were found more than 10-fold upregulated and
downregulated in the diseased kidneys compared with the
healthy citrate-treated kidneys, respectively (Figure 2, Supple-
mentary Figure S1 online and Supplementary Table S2 online).
Several proteins could be mapped to distinct locations in
the kidney, such as the glomerular basement membrane, the
apical as well as the lateral membrane of the podocyte, the
endothelial membrane, and the proximal tubule (Table 1),
thereby defining the kidney substructures in which proteins
were chemically modified by the reactive ester derivative
of biotin. Importantly, most serum components, such as
albumin (a known marker for accelerated plasma protein
leakage across the glomerular basement membrane in DN),
transferrin, and fibrinogen, were not found to be regulated
in this proteomic analysis. In contrast, serum components,
which were previously identified to be either upregulated in
the serum of patients with diabetes (kallikrein, haptoglo-
bin, and coagulation factor XII) or to be associated
with DN (kininogen 1), were found among the most
differentially expressed proteins.

The upregulated proteins vanin-1 (Vnn1), Enpp6 protein
(Enpp6), and alpha-1-acid glycoprotein (Orm1) were further
validated. Although no correlation between the upregulation
of Enpp6 and diabetes or DN was found in literature, the
upregulation of Vnn1 in T1D and of Orm1 in DN has
previously been described. An immunofluorescence analysis
of Enpp6 and Orm1 on rat kidney section revealed their
upregulation in tubular structures and glomeruli in STZ-
treated diabetic rats, respectively (Figure 3).

Orm1 could be detected by immunofluorescence staining,
which was also confirmed by western blot of rat kidney tissue
homogenates (data not shown) but could not be detected in
human urine samples.

Enpp6 was found to be expressed on tubular epithelium in
kidney sections from STZ-treated rats, but both commercial
antibodies tested were reactive against human immunono-
globulin G heavy chain, as demonstrated by western blot,
detecting purified human immunoglobulin G (data not

![Figure 5](image-url)  
**Figure 5** Quantitative assessment of VNN1 and UMOD in individual urine samples. (a) Western blot analyses using urine from healthy individuals (healthy), patients with T1D but normal albumin excretion rates (T1D), and patients with elevated (DN1) and pronounced (DN2) urinary albumin excretion rate (UAER) were performed with antibodies against UMOD, VNN1, and ALB. VNN1 (b) and UMOD (c) bands were quantified using Image J software (US National Institutes of Health, Bethesda, MD). The area under the curve of bands from individual urine samples is displayed as circles, whereas the average in a pathological group is displayed with a horizontal line. For this analysis, all diabetic patients with elevated UAER were included in one group (T1DDN) to have a higher number of measurements per group.
shown). Owing to the similar size of the heavy chain (~55 kDa) and ENPP6 (~52 kDa), and the upregulation of human immunoglobulin G fragments in DN, it was impossible to discriminate between specific and nonspecific signals. Interestingly, ENPP6 was identified in a study of urinary exosomes, suggesting that this protein is also expressed in healthy kidneys, making it a top candidate for follow-up studies.

Although the immunofluorescence staining of Vnn1 could not be performed because of the unavailability of antibodies recognizing the rat protein, it showed to be upregulated in pooled urine of T1D patients with macroalbuminuria (Figure 4). In contrast, the analysis of spot urine from individual patients presented a variable VNN1 concentration, without a significant upregulation in DN patients. These findings could be explained in terms of patient improvement as a result of pharmacological intervention (formerly overt albuminuria indeed reverted to UAER below 300 mg/l). Alternatively, if this protein is indeed of tubular origin, timed urine collections should be considered, as recently proposed, to more reliably assess the rate of protein release. Both issues can only be resolved by studying a larger patient population, with additional time points for urine collection.

Uromodulin (UMOD; also known as Tamm–Horsfall urinary glycoprotein) was identified in the proteomic analysis to be downregulated in the diabetic rat samples when compared with the citrate-treated control rats. This is in line with the observation of Chakraborty and co-workers who showed that a decreased immunogold labeling of UMOD in human kidney specimens is associated with renal damage and with our immunofluorescence analysis on rat kidneys (Figure 3). The upregulation of UMOD in individual and pooled patient urine (Figure 4) seems to be contradictory at first but can be explained by its shedding into the urine, which was proposed to have a role in water/salt balance. The observation that this balance is altered in DN suggests that uromodulin shedding is increased under pathological conditions.

In this proteomics-based study of a T1D rat model and its complications, we have identified 396 proteins and followed their expression over a time course of 12 weeks. The protein most significantly upregulated in the diabetic rats was vanin-1, a glycosylphosphatidylinositol-anchored epithelial pantetheinase, which has proinflammatory and cytoprotective effects in some tissues. Modulation of the vanin-1 activity has been suggested as alternative strategy to maintain islet cell homeostasis. Although vanin-1 is indeed upregulated in a pool of urine from patients with macroalbuminuria, an analysis of individual urine samples showed large interindividual variability, indicating the need for dedicated studies with large numbers of individual urine samples to properly assess the value of this candidate marker for the monitoring of DN progression in patients.

Recently, serum cystatin C has been proposed for the early detection of DN, and is currently evaluated for the detection of mildly to moderately impaired kidney function in diabetic patients. In full analogy to the development of cystatin C, vanin-1 could be validated as marker for the early detection of impaired kidney function in diabetes patients. For this purpose, large-scale studies using clinically validated urine samples of healthy individuals and diabetic patients with different stages of impaired renal function have to be carried out. To perform such studies, specific high-affinity monoclonal antibodies as well as validated antibody-based tests (for example, enzyme-linked immunosorbent assay) are essential and have to be developed, challenges which are clearly beyond the scope of this study.

**MATERIALS AND METHODS**

**Animals**

Nine-week-old male Sprague-Dawley rats (Charles River, Wilmington, MA) with known sensitivity to STZ-induced diabetes weighing 268 ± 11 g were used. All animals were housed under standard conditions (light on 08:00-20:00 h; 40–70% relative humidity, 22 ± 1 °C), and had free access to water and chow (Altromin standard diet, Lange, Germany). All procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Semmelweis University.

**DN model**

STZ diabetes was induced, as described previously. Briefly, rats obtained a single intraperitoneal injection of 55 mg STZ (N-(methylnitrosocarbamoyl)-α-D-glucosamine; Sigma-Aldrich, St Louis, MO) per kg body weight dissolved in citrate buffer (pH 4.5). Rapid administration of STZ and solution pH is important to achieve diabetes. A total of 17 animals were stratified into four groups (n = 4/group) with equal average initial body weight: four groups of animals were injected with STZ, and 5 age-matched control rats were injected with the citrate buffer only.

**In vivo biotinylation**

In vivo biotinylation experiments were performed similarly, as previously described. Briefly, rats were anesthetized with a combination of ketamin (Vétouquinol, Ittingen, Switzerland), xylazin (Streuli, Uznach, Switzerland), and acepromazine (Arovet, Dietikon, Switzerland). The abdomen was opened and two ligations (the first on the abdominal artery and vein below the branching points of the kidney arteries and the second on the hepatic artery) were placed. Following a median sternotomy, a buttoned cannula was inserted into the aorta abdominalis and fixed with medical wire. To allow the biotinylation reagent to flow out, the vena cava was cut open. The perfusion was carried out at 150 mm Hg column using 20 ml prewarmed (40 °C) biotinylation solution (0.5 mg/ml Sulfo-NHS-LC-Biotin (Pierce) and 10% Dextran-40 (GE Healthcare, Chalfont St Giles, UK) in phosphate-buffered saline, followed by 20 ml of prewarmed (40 °C) quenching solution (50 mmol/l Tris–HCl and 10% Dextran-40 in phosphate-buffered saline). After perfusion, kidneys were excised, cut in half, and separately freshly snap frozen in liquid nitrogen-cooled isopentane for preparation of organ homogenates. A total of nine STZ rats and five citrate-treated control rats were terminally perfused. An unperfused citrate-treated rat was used as negative control for the proteomic analysis.

**Purification of biotinylated proteins and proteomic analysis**

Purification of biotinylated proteins on streptavidin resin, the tryptic on-resin digestion, and the subsequent proteomic analysis of resulting tryptic peptides were performed according to standard
procedures. A detailed description can be found in the Supplementary Materials online.

**Relative protein quantification by DeepQuanTR software**

The DeepQuanTR software has been described in detail elsewhere. Briefly, after mass spectrometry acquisition, data related to the individual peaks (fractions, intensities, and mass-to-charge ratios) were loaded into the DeepQuanTR software, which performed a normalization of individual signal intensities to the internal standard peptides and an annotation (peptide identification and association with a parent protein). Normalized intensities for the individual peptides from all samples of each group (that is, citrate control, 4, 8, and 12 weeks after STZ injection) were used for the computation of DeepQuanTR peptide and protein scores, indicating the relative abundance of individual peptides and proteins in the various groups of samples.

**Human urine samples**

Human urine samples from healthy individuals were obtained from male volunteers of the lab (midstream morning urine, supplemented with 1 × complete proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), centrifuged at 3300 × g for 10 min at 4 °C, and the supernatant was aliquoted and stored at −80 °C. Human urine from patients with T1D were obtained from the Finnish Diabetic Nephropathy (FinnDiane) Study. Six patients with normal albumin excretion rate (<30 mg/24 h) and six with macroalbuminuria (≥300 mg/24 h) were used for urine from two pools of urine, respectively. All patients exhibited similar creatinine concentrations. The pools were aliquoted and stored at −20 °C.

For the analysis of interindividual variability of VNN1 and UMOD levels, urine of 10 additional patients was collected, as described above. Five patients were diabetic with UAER <30 mg/l and two patients exhibited elevated albumin levels (≥30; <200 mg/l). Three patients suffered from overt albuminuria but because of successful medication, their UAER fell below the threshold of macroalbuminuria at the time of sample collection. All relevant data for the 16 individual urine donors can be found in Supplementary Table S5 online.

A detailed description of the functional measurements, the histochemistry, the preparation of protein extracts, the purification of biotinylated proteins, the proteomic analysis, the immuno-fluorescence analysis (Supplementary Table S4 online), and the western blot analysis can be found in the Supplementary Materials online.

**DISCLOSURE**

All the authors declared no competing interests.

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**AUTHOR CONTRIBUTIONS**

TF performed experiments, analyzed data, and wrote the manuscript. BB performed perfusion experiments. CsR set up the rat diabetes model. MG performed the rat in vitro functional analysis. CF provided validated urine samples of diabetic patients. PH planned and supervised the setup of the rat diabetes model and wrote the manuscript. DN designed the project, analyzed data, wrote, and reviewed the manuscript. CHr designed the project, performed experiments, analyzed data, and wrote the article.

**SUPPLEMENTARY MATERIAL**

Table S1. Specification of functional data obtained at 12 weeks after streptozotocin (STZ) or citrate treatment in the Sprague–Dawley rats.

Table S2. Total list of proteins identified with at least two proteotypic peptides in the comparative proteomic analysis.

Table S3. Comparison of protein regulation in diabetic nephropathy between a transcriptomic study (data based on human mRNA expression profiles of diabetic glomeruli and glomeruli from healthy individuals) and this proteomic study (data based on protein expression in STZ rats and healthy citrate-treated rats).

Table S4. Summary of the antibodies used for immunofluorescence analysis and/or western blotting.

Table S5. Clinical data of healthy individuals and patients with type 1 diabetes.

**Figure S1.** Cluster analysis.

**Figure S2.** Correlation between microarray data (Rudnicki et al.) and the proteomics data presented in this work.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

**REFERENCES**