Pursuing type 1 diabetes mellitus and related complications through urinary proteomics

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Diabetes mellitus is a chronic metabolic disease with multiple complications, and its successful management requires early diagnosis, to allow timely interventions. Here, we have comprehensively analyzed the proteome changes in urine of type 1 diabetic subjects with and without complications such as retinopathy and nephropathy. Gel electrophoresis combined to liquid chromatography-tandem mass spectrometry (GeLC-MS/MS) analysis of midstream urine highlighted the mechanisms involved in disease pathogenesis as, for instance wound healing and blood coagulation in all diabetics or altered ganglioside metabolism in retinopathy, and also some urinary proteins with potential diagnosis value. From these, gelsolin and antithrombin-III appear as promising diagnosis markers for type 1 diabetes mellitus (T1DM), whereas ephrin type-B receptor 4 and vitamin K-dependent protein Z seem to be promising markers for advanced T1DM disease state presenting retinopathy and nephropathy (T1DM-R + N). Data also suggest urinary ganglioside GM2 activator and beta-hexosaminidase subunit beta as potential urinary markers of retinopathy in diabetics. Taken together, the present exploratory urinary proteomic analysis might be seen as an important starting point for studies targeting specific urinary proteins aimed at the implementation of new biomarkers for the early detection of T1DM-related microvascular complications. (Translational Research 2014;163:188–199)

Abbreviations: SDS = Sodium dodecylsulphate; GeLC-MS/MS = Gel electrophoresis combined to liquid chromatography-tandem mass spectrometry; SDS-PAGE = Sodium dodecylsulphate-polycrylamide gel electrophoresis; LC-MS/MS = Liquid chromatography-tandem mass spectrometry; ID = Identification; CHCA = alpha-Cyano-4-hydroxycinnamic acid; ACN = Acetonitrile; TFA = Trifluoroacetic acid; TOF = Time-of-flight; LC-MALDI = Liquid chromatography-matrix assisted laser desorption ionization; S/N = Signal/noise

Diabetes mellitus is a chronic metabolic disease leading to life-threatening complications with the potential for morbidity. Indeed, the hyperglycemia and metabolic dysregulation found in diabetes often gives rise to microvascular complications, such as diabetic nephropathy, diabetic retinopathy, and...
AT A GLANCE COMMENTARY

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Background

Proteomics based on mass spectrometry hold special promise for the identification of novel biomarkers in biofluids that might form the foundation for new clinical tests. By studying the proteins that are excreted into the urine, one can learn more about the mechanisms of diabetes and related complications, and develop the tests using this knowledge for translational medicine.

Translational Significance

This study highlights the contribution of ganglioside metabolism in retinopathy as well as gelsolin and antithrombin-III that seem to be promising markers for the diagnosis of type 1 diabetes mellitus (T1DM) and ephrin type-B receptor 4 and vitamin K-dependent protein Z seem to be promising markers for advanced disease states.

Proteomics based on mass spectrometry hold special promise for the identification of novel biomarkers in biofluids that might form the foundation for new clinical tests. In particular, urinary proteomics has gained attention in biomedical research with the ultimate goal of biomarker discovery for diagnosis and prognosis of kidney- and non-kidney diseases. Urine is an attractive sample for biomarker identification as it can be obtained in large quantities using noninvasive procedures, and contains stable proteins and polypeptides of lower molecular mass in a highly soluble form, facilitating its analysis. Nevertheless, until recently urinary proteomics has been considered an arduous task with limited outcomes, particularly for type 1 diabetes mellitus (T1DM). The goal of our study was to identify urinary proteins modulated by T1DM and related microvascular complications, specifically nephropathy and retinopathy, using a gel electrophoresis combined to liquid chromatography-tandem mass spectrometry (GeLC-MS/MS) protein profiling approach of clinically well-defined groups of diabetic subjects. More than identifying urinary proteins modulated by T1DM, with our without microvascular complications, bioinformatic analysis of unique proteins per patient groups or proteins present in significant altered levels among groups also highlighted the biological processes underlying disease pathogenesis.

METHODS

Patients. Subjects enrolled in the present study included 15 patients with T1DM: 5 with retinopathy and nephropathy (T1DM-R + N), 5 with retinopathy (T1DM-R), and 5 without chronic complications (T1DM) followed-up by the internal medicine service of Hospital da Figueira da Foz, Portugal. Five healthy volunteers (control, CTRL) were also included in the present study. All groups were matched by sex and age. Diabetic patients presented disease duration of a minimum of 15 years, with HbA1c levels higher than 7.7%, significantly different from healthy individuals (T1DM-R + N vs CTRL groups, P < 0.01; T1DM-R and T1DM vs CTRL groups, P < 0.05). All T1DM patients included in this study were antibody positive for at least 1 of the following antibodies: anti-islet cell, anti-insulin, or anti-glutamic acid decarboxylase. The patients were all examined by the same internal medicine physician. The patients with nephropathy presented a urinary albumin excretion of more than 300 mg in a 24-hour collection period, and retinopathy was screened by an ophthalmologist. In T1DM-R + N, the average duration of retinopathy and nephropathy was 13 and 4 years, respectively. The duration of retinopathy in group T1DM-R was of 8 years in

macrovascular complications, such as coronary heart disease and strokes. Among microvascular complications, diabetic nephropathy is a progressive kidney disease of long-standing diabetes and is the most frequent motive for dialysis in many Western countries. In clinical practice, diabetic nephropathy is diagnosed by the presence of proteinuria and/or changes in the glomerular filtration rate. The development of this complication is characterized by a progressive increase of urinary albumin excretion rate from normo- to micro- to macroalbuminuria. Nevertheless, as a consequence of high interindividual variability, standard tests present major limitations for early diagnosis of disease. Diabetic retinopathy occurs in approximately 25% of patients with type 1 or type 2 diabetes and is the major cause of acquired blindness in working-age adults. This microvascular complication occurs as a result of weakened retinal capillaries promoted by hyperglycemia, causing leakage of blood into surrounding areas, leading to poor vision and even blindness. About one-third of the diabetic population present signs of retinopathy and approximately one-tenth have vision-threatening stages of retinopathy such as diabetic macular edema and proliferative diabetic retinopathy. It is, therefore, of great interest to recognize these diabetes-related complications in their early phases enabling the physicians to take the necessary medication and/or surgical intervention before the renal failure or vision loss occurs.
average. The demographic and clinical characteristics of enrolled subjects are summarized in Table I.

The present study was approved by the Hospital Ethics Committee and followed the Declaration of Helsinki. All subjects included in the study gave their written informed consent after being informed of the research project’s nature. Urine samples were collected from each individual.

**Sample collection.** A midstream urine collection was performed and the abnormal presence of leukocytes was screened with urinalysis test strips (Uritest Makromed C), and the supernatant was passed through a 10-kDa filter to exclude urinary tract infection. Urine samples were centrifuged at 1000 g for 10 minutes (4°C), and the resulting retentate was resuspended in 100 μL of 0.05 M Tris and 2% sodium dodecyl sulphate (SDS). Total protein content was estimated in the fraction corresponding to the retentate using the DC protein assay kit (Bio-Rad, Hercules, Calif.).

### Table I. Clinical characteristics of control (CTRL) and type 1 diabetes subjects with retinopathy and nephropathy (T1DM-R + N), with retinopathy (T1DM-R), and with no complications (T1DM)

<table>
<thead>
<tr>
<th></th>
<th>T1DM-R + N</th>
<th>T1DM-R</th>
<th>T1DM</th>
<th>CTRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>41.31 ± 11.16</td>
<td>46.07 ± 12.28</td>
<td>38.94 ± 13.86</td>
<td>44.75 ± 4.66</td>
</tr>
<tr>
<td>Sex (M/F sex ratio)</td>
<td>4/1</td>
<td>3/2</td>
<td>3/2</td>
<td>3/2</td>
</tr>
<tr>
<td>Duration of diabetes (y)</td>
<td>29.80 ± 8.80</td>
<td>31.40 ± 11.64</td>
<td>17.40 ± 5.38</td>
<td>–</td>
</tr>
<tr>
<td>Duration of retinopathy (y)</td>
<td>12.6 ± 10.78</td>
<td>7.80 ± 6.14</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Duration of nephropathy (y)</td>
<td>4.05 ± 3.35</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.92 ± 1.61*</td>
<td>8.18 ± 0.60†</td>
<td>7.71 ± 0.09‡</td>
<td>5.17 ± 0.42</td>
</tr>
<tr>
<td>Albumin excretion rate</td>
<td>&gt;300 mg/24 h</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Abbreviations: CTRL, control; HbA1c, hemoglobin A1c; T1DM, type 1 diabetes mellitus; T1DM-R, type 1 diabetes mellitus with retinopathy; T1DM-R + N, type 1 diabetes mellitus with retinopathy and nephropathy.

The mean value of each characteristic is presented together with its corresponding standard deviation.

*P < 0.01 (T1DM-R + N vs CTRL).
†P < 0.05 (T1DM-R vs CTRL).
‡P < 0.05 (T1DM vs CTRL).

sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and in-gel digestion. The proteome sample (50 μg) was separated using SDS-PAGE in a 12.5% gel prepared as previously described. The gel was stained with Colloidal Coomassie Blue G-250. The samples were prepared in duplicate. Following SDS-PAGE of the protein samples, complete lanes were cut out of the gel and sliced into 16 sections. Each section was in-gel digested with trypsin. The resulting peptide mixture was then extracted from the gel fractions and dried using vacuum centrifugation.

liquid chromatography-tandem MS (LC-MS/MS) analysis and protein identification (ID). The dried extracted peptides were dissolved in 10 μL of mobile phase A (0.1% trifluoroacetic acid, 5% acetonitrile, 95% water). All peptide mixtures were analyzed twice. The tryptic digests were then separated using an Ultimate 3000 (Dionex, Sunnyvale, Calif) onto a 150 mm × 75 μm Pepmap100 capillary analytical C18 column with 3 μm particle size (Dionex/LC Packings) at a flow rate of 300 nL/min. The gradient started at 10 minutes and ramped to 50% buffer B (85% acetonitrile, 0.04% trifluoroacetic acid) over a period of 45 minutes. The chromatographic separation was monitored at 214 nm using a ultraviolet detector (Dionex/LC Packings) equipped with a 3 nL flow cell. The peptides eluting from the column were mixed with a continuous flow of matrix solution (270 nL/min, 2 mg/mL alpha-Cyano-4-hydroxycinnamic acid in 70% acetonitrile/0.3 % trifluoroacetic acid and internal standard Glu-Fib at 15 fmol) in a fraction microcollector (Probot; Dionex/LC Packings) and directly deposited onto the Liquid chromatography-matrix assisted laser desorption ionization plates at 12-second intervals for each spot (150 nL/fraction). For every chromatographic run, a total of 156 fractions were collected.

Samples were analyzed using a 4800 MALDI-time-of-flight (TOF)/TOF Analyzer (Ab SCIEX, Canada). A signal/noise threshold of 50 was used to select peaks for MS/MS analyses. The spectra were processed using the TS2Mascot (v1.0; Matrix Science Ltd, London, UK) and submitted to Mascot software (v.2.1.0.4; Matrix Science Ltd) for peptide/protein identification. Searches were performed against the SwissProt protein database (March 2013) for Homo sapiens. Two approaches were performed: 1 search for each individual band and a merged search including data from all slices for global protein identification and exponentially modified protein abundance index (emPAI) calculation. A MS tolerance of 30 ppm was found for precursor ions and 0.3 Da for fragment ions, as well as 2 missed cleavages and methionine oxidation as variable modification. The confidence levels accepted for positive protein identification were above 95%. A minimal Mascot
A peptide score of 30 was determined by a reverse database search, which revealed a false positive rate below 5% for identified proteins. Furthermore, proteins identified with 1 peptide were manually validated when MS/MS spectra presented at least 4 successive amino acids covered by b or y fragmentations.

Protein quantification and abundance measurement. The abundance of identified proteins was estimated by calculating the emPAI. The emPAI is an exponential form of PAI \(^{-1}\) (the number of detected peptides divided by the number of observable peptides per protein, normalized by the theoretical number of peptides expected via in silico digestion) defined as \(\text{emPAI} = 10^{\text{PAI}^{-1}}\) and the corresponding protein content in mole percent is calculated as \(\text{mol \%} = (\text{emPAI} / \sum \text{emPAI}) \times 100\). Microsoft Office Excel was used to calculate the mole percent. The theoretically observable peptides were determined by the in silico digestion of mature proteins using from the output of the program Protein Digestion Simulator (http://panomics.pnnl.gov/software/). The observed peptides were unique parent ions including those with 2 missed cleavage. Mean protein emPAI values were log2 transformed for protein ratio calculation.

Immunoblotting analysis. Immunoblotting analysis was performed according to Caseiro et al\(^{20}\) with slight modifications. In brief, concentrated urine samples were diluted in Tris buffered saline to a final protein concentration of 0.01 \(\mu g/\mu L\) and a volume of 100 \(\mu L\) was slot-blotted into a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were blocked with 5% (w/v) dry non-fat milk in Tris buffered saline-Tween and then incubated overnight at 4°C with the primary antibodies anti-osteopontin (anti-Osteopontin antibody [ab91655], anti-S100A9 antibody [ab92507], Abcam) diluted 1:500 in blocking solution. The membranes were washed 3 times, 10 minutes each, with Tris buffered saline-Tween and incubated 2 hours with secondary antibody (horseradish-conjugated anti-mouse, GE Healthcare, Buckinghamshire, UK) in a dilution of 1:10000. Detection was carried out with enhanced chemiluminescence according to manufacturer’s instructions (GE Healthcare). Film images were acquired using GelDoc XR system (Bio-Rad) and quantitative analysis of optical density (OD) was performed with Quantity One 1-D Analysis Software (Bio-Rad).

Statistical data analysis. Analysis of the statistical significance of differences between groups in relation to OD measures were performed with the GraphPad Prism v. 5.0 for Windows (GraphPad Software, Inc; Translational Research Volume 163, Number 3 Caseiro et al 191
San Diego, CA). Mean and standard deviation were calculated and a Kruskal-Wallis test followed by Dunn’s multiple comparison post-hoc test was performed. Differences were considered statistically significant at $P$ values lower than 0.05. In case of protein data analysis, a comma separated values dataset containing relevant information pertaining to all identified proteins was analyzed to extract meaningful information. An in-house developed C# program using Language Integrated Query (Microsoft Visual Studio 2012; Microsoft, USA) was used for data-mining the dataset. The output of the program has given several statistics which were then used by R Language scripts to produce heat maps and emPAI distribution facilitating the dataset analysis. The recovered statistics were (1) the protein abundance as a function of individual group (Fig 1); (2) dendrograms for normalized emPAI values to analyze their similarity/dissimilarity between groups, (3) protein abundance ratio distribution as a function of each group (Fig 2).

**RESULTS**

To identify type 1 DM-related proteins in urine, we performed a large scale GeLC-MS/MS approach for
quantitative comparison among subjects. Table I shows the clinical characteristics of subjects enrolled in this study: 5 controls (CTRL) and 15 diabetic patients those with nondiagnosed complications (T1DM, n = 5), with retinopathy (T1DM-R, n = 5), and those with retinopathy and nephropathy (T1DM-R + N, n = 5). Subjects included in T1DM-R + N group showed macroalbuminuria, and the remaining evidenced normoalbuminuria. Desalted urine samples from all subjects were analyzed by SDS-PAGE, and the results showed inter-individual biological variations (Supplementary Fig S1). However, band OD analysis highlighted 2 sections with significant variations among groups (Fig 3). Gel section 4 (estimated MW of 97 kDa) presented higher OD in T1DM and T1DM-R whereas for section 6 (estimated MW 66 kDa) significantly higher values were observed for T1DM-R + N (Fig 3). LC-MS/MS analysis of these sections retrieved 82 and 104 distinct proteins for sections 4 and 6, respectively. From the 82 urinary proteins detected in section 4, uromodulin, galectin-3-binding protein and plasma protease C1 inhibitor were the most prevalent. In section 6, albumin, kininogen-1, and osteopontin were among the most widespread (Supplementary Table SI).

The large-scale LC-MS/MS analysis of all gel bands retrieved 219 unique proteins (Supplementary Table SII). Most of these proteins possess antigen-, carbohydrate-, collagen-, calcium-, and cell adhesion molecule-binding. The majority of these proteins have extracellular origin and are mainly involved in biological processes such as protein activation cascades, complement activation, platelet degranulation, and immune response (Supplementary Table SIII). Protein-protein interaction analysis performed with Cytoscape v3.0.1 evidenced 2489 proteins from 34 clusters, suggesting the contribution of players from several cellular pathways to whole urine proteome (Supplementary Fig S2).

Figure 4 illustrates the distribution of identified proteins per group. From all urinary proteins detected, only 75 were common to all subjects studied, including albumin, pancreatic amylase, AMBP, uromodulin, apolipoprotein D, and osteopontin. These common proteins are mainly involved in platelet and complement
activation. Thirty-one proteins were only identified in the urine of healthy individuals, most of which related to metabolism. Only 8 proteins were common to all diabetics, namely immunoglobulin kappa variable 2–30, gelsolin, proline rich 4, antithrombin, carboxyl-terminal PDZ ligand, contactin-1, dyslexia-associated protein KIAA03119, and hornerin (Supplementary Table SIV). Protein-protein interaction analysis (performed with STRING v9.05) of these T1DM-related proteins evidenced wound healing and regulation of coagulation as the main clusters involved. Curiously, the analysis of proteins per group evidenced an enrichment of unique proteins in the urine of T1DM-R subjects (59 in T1DM-R vs 9 in T1DM-R + N vs 11 in T1DM). T1DM-R specific urinary proteins are mainly involved in ganglioside metabolism and glycosphingolipid catabolic process, whereas T1DM-R + N-related proteins participate in platelet degranulation and wound healing. The unique proteins identified in the urine of T1DM patients without diagnosed complications are enrolled in proteolysis, namely through proteasomal ubiquitin-independent pathway. Protein-protein interaction analysis of unique proteins from CTRL and T1DM (with and without complications) groups evidenced a T1DM-related enrichment of proteins belonging to ceramide catabolic processes, pyrimidine-containing compound biosynthetic processes, and regulation of neurotransmitter transport. Contrarily, in healthy subjects there is a clear prevalence of proteins associated with fatty acid biosynthetic processes (Fig 5).

The comparative abundance of the 75 common proteins based on emPAI values evidenced a normal distribution of log2 values for CTRL and T1DM-R groups. A slight overexpression of emPAI values were observed for T1DM and an opposite behavior was noticed for T1DM-R + N (Fig 1). Protein distribution among groups was pictured in a heat map based on their normalized abundances (Fig 2) and 2 main clusters (CTRL and T1DM-R vs T1DM-R + N, T1DM) were highlighted. Moreover, 4 distinct protein nodes might be observed which include proteins involved in platelet degranulation and regulation of cell adhesion (node 1), lipid metabolism (node 2), blood coagulation vs fibrinolysis (node 3), and polysaccharide catabolism and complement activation (node 4). Cell growth-inhibiting gene 2 protein (CD9), cadherin-2 (CADH2), extracellular matrix receptor III (CD44), and endosialin (CD248) were included in node 4. Node 1 includes IGKC, albumin apolipoprotein D, and uromodulin, which are linked to MyD88-independent toll-like receptor signaling pathways and regulation of interferon-beta biosynthetic processes, according to STRING analysis.

Normalized abundance analysis evidenced albumin, serotransferrin, and orosomucoid-1 as the top 3 most abundant proteins in T1DM-R + N and leukocyte-associated immunoglobulin-like receptor 1 (CD305), deoxyribonuclease-1 and Pro-epidermal growth factor as the lowest abundant ones (Fig 6, A). CD305 and serotransferrin were also in the top 3 prevalent proteins in T1DM-R. Glutaminyl-peptide cyclotransferase and deoxyribonuclease-1 were among the lesser abundant ones (Fig 6, B). Orosomucoid-I was found in low levels in the urine of T1DM patients (Fig 6, C). Regarding osteopontin, no abundance differences were observed between T1DM and CTRL, whereas lower levels were observed for T1DM-R + N. To validate emPAI data of urine proteome, immunoblotting analysis of target proteins was performed in individual samples. In this sense, osteopontin and S100-A9 expressions were evaluated in triplicate in all urine samples (Fig 7), and data obtained highlight a similar trend to that observed for the emPAI distribution. Protein-protein interaction analysis performed with ClueGo and CluePedia of the most abundant proteins in the urine of diabetics (normalized values higher than 1.5) evidenced the prevalence of phospholipid and sterol homeostasis, regulation of cholesterol transport, and other processes related to lipid metabolism. Among the biological processes involving the less abundant proteins (normalized values lower than −1.5), negative regulation of epidermal growth factor and negative regulation of protein catabolic processes were highlighted (Fig 8).

Considering the biological origin of urine, it is somehow expected to find a high number of protein fragments compared with other fluids. Some of these are expected to be abundant plasma-derived peptides and proteins such as albumin and various globulins that can pass the glomerular filter in substantial amounts.
Gel band-by-band analysis evidenced some proteins more susceptible to proteolysis, including uromodulin, albumin, and osteopontin. In all subjects, we identified mannan-binding lectin serine protease 2 and Basement membrane-specific heparan sulfate proteoglycan core protein fragments corresponding to the N-terminal and C-terminal, respectively (Supplementary Fig S3). According to Pfam, mannan-binding lectin serine protease 2 is a serum protease that regulates complement system and the maintenance of glomerular permeability, and its CUB domain at the N-terminal has a major role in protein function. PGBM (basement membrane specific heparin sulfate proteoglycan core protein) is a component of the glomerular basement membrane, and laminin G_2 domain at C-terminal is important for maintenance and survival of tissues. No T1DM-related specific protein fragments were identified.

**DISCUSSION**

Our study highlights the effect of T1DM and related microvascular complications (specifically retinopathy and nephropathy) on urinary proteome, allowing the envisioning of potential disease biomarkers with diagnosis and prognosis significance, and the improved elucidation of disease pathogenesis. From the 219 proteins identified in the urine of all subjects studied, only 8 proteins were exclusively assigned to diabetic patients,
Fig 6. Normalized abundance of urinary proteins in T1DM-R + N (A), T1DM-R (B), and T1DM (C) groups. Protein accession number has correspondence to protein name at Supplementary Table SII. CTRL, control; T1DM, type 1 diabetes mellitus; T1DM-R, type 1 diabetes mellitus with retinopathy; T1DM-R + N, type 1 diabetes mellitus with retinopathy and nephropathy.
which integrated analysis with STRING bioinformatic tool strongly suggest their involvement in wound healing and blood coagulation processes. Indeed, clinical studies have confirmed the hypercoagulability and platelet hyperactivity seen in diabetics, namely in type diabetes mellitus with increased urinary thromboxane excretion particularly related to nephropathy and retinopathy. From proteins involved in the regulation of the blood coagulation cascade, serine protease inhibitor SERPIN C1 (also known as antithrombin III) was exclusively identified in the urine of diabetics. Other diabetes-related identified protein was gelsolin, a critical regulator for actin filament assembly and disassembly, previously identified as an invasion modulator in many cancers, and detected in acute kidney injury-related high urinary levels. Gelsolin was also reported to inhibit retinal cell growth in diabetic retinopathy.

Our data showed the presence of this protein in all diabetics, with no association to a particular T1DM-related complication.

Bioinformatic analysis of the 8 unique urinary proteins identified in diabetics with retinopathy and nephropathy also retrieved wound healing and platelet degranulation as the core biological processes. From these, ephrin type-B receptor 4 plays a role in postnatal blood vessel remodeling and makes part of the Eph-ephrin system used by pancreatic β cells for the regulation of insulin secretion. Vitamin K-dependent protein Z is mainly synthesized and γ-glutamylcarboxylated in the liver and inhibits the activity of the coagulation protease factor Xa in the presence of SERPINA10, calcium, and phospholipids. High levels of serum alpha-2-macroglobulin were traditionally linked to the incidence of peripheral vascular complications in diabetic patients because of its intimately association to vascular endothelium, and more recently, urinary levels of this protein were suggested as a hematuria location marker. The somewhat low number of exclusive T1DM-R related proteins seem to be justified by the high levels of urinary albumin, considering the diagnosed macroalbuminuria and also the intense gel band displayed in SDS-PAGE (Table I and Fig 3). Far more exclusive proteins were identified in the urine of T1DM-R, most of which assigned to ganglioside metabolism, as ganglioside GM2 activator and beta-hexosaminidase subunit beta. In fact, early stages of retinopathy seem to be characterized by death of pericytes, the retinal microvascular cells. Masson et al reported the involvement of gangliosides in mediating the adverse glucosamine effects on pericyte proliferation. Pericyte loss and subsequent modification of endothelial cell proliferation and function, combined with basement membrane thickening, trigger structural and functional alterations of capillaries that ultimately can lead to blindness. Additionally, one of the urinary proteins only identified in diabetics with retinopathy was prolactin-inducible protein, which is expressed in pathologic conditions of exocrine tissues as lacrimal glands. The members of prolactin family are potential regulators of angiogenesis, acting as circulating hormones in several stages of formation and remodeling of new blood vessels. Prolactin-like proteins were previously detected in serum and related to retinopathy.

G-protein coupled receptor family C group 5 member
C, a retinoic acid-inducible G-protein coupled receptor, was also detected in the urine of diabetic subjects with this microvascular complication. To the best of our knowledge, no previous association of this protein to diabetes-related retinopathy was reported. Besides emphasizing the molecular mechanisms underlying disease, the results highlight the usefulness of urine for the characterization of T1DM-related retinopathy, not traditionally assessed through the analysis of this biofluid.

The analysis of urinary proteins shared by all subjects based on protein relative abundance (emPAI) highlighted T1DM-R + N-related low levels of osteopontin, which acts as an immune modulator. The inflammatory marker orosomucoid-1 is a glycoprotein synthesized by hepatic cells, the serum concentration of which increases during inflammation. Inflammatory factors like this acute-phase protein modulate endothelial cell function and prothrombotic properties, underlying microvascular complications. However, elevated urinary orosomucoid excretion by subjects exhibited normal glomerular and tubular function were reported, suggesting the possibility of local renal production of orosomucoid because of chronic low-grade inflammation rather than hyperfiltration. Curiously, when no complications were diagnosed, lower levels of orosomucoid-1 were detected in the urine of diabetics.

In conclusion, GeLC-MS/MS analysis of urine from T1DM subjects with and without microvascular complications highlight the mechanisms involved in disease pathogenesis as, for instance wound healing and blood coagulation in all diabetics or altered ganglioside metabolism in retinopathy. Indeed, the analysis of urine proteome presents the added advantage of giving an integrated perspective of the molecular events underlying diabetes mellitus and related microvascular complications. However, some urinary proteins might present potential diagnosis value. From the identified urinary proteins, gelsolin and antithrombin-III seem to be promising markers for the diagnosis of T1DM and ephrin type-B receptor 4 and vitamin K-dependent protein Z for advanced disease states. Data here presented might be an important starting point for studies targeting specific urinary proteins aiming the prevention of T1DM-related microvascular complications through their early detection.

**Acknowledgments**

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.trsl.2013.09.005.

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