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Urinary exosomes and diabetic nephropathy: a proteomic approach†

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Urinary exosomes (UE) are nanovesicles released by every epithelial cell facing the urinary space and they are considered a promising source of molecular markers for renal dysfunction and structural injury. Exosomal proteomics has emerged as a powerful tool for understanding the molecular composition of exosomes and has potential to accelerate biomarker discovery. We employed this strategy in the study of diabetic nephropathy (DN) and the consequent end stage renal disease, which represent the dramatic evolution of diabetes, often leading the patients to dialysis or kidney transplantation. The identification of DN biomarkers is likely to help monitoring the disease onset and progression. A label free LC-MS/MS approach was applied to investigate the alteration of the proteome of urinary exosomes isolated from the Zucker diabetic fatty rats (ZDF), as a model of type 2 DN. We collected 24 hour urine samples from 7 ZDF and from 7 control rats at different ages (6, 12 and 20 weeks old) to monitor the development of DN. Exosomes were isolated by ultracentrifugation and their purity assessed by immunoblotting for known exosomal markers. Exosomal proteins from urine samples of 20 week old rats were pooled and analyzed by nLC-ESI-UHR-QToF-MS/MS after pre-filtration and tryptic digestion, leading to the identification and label free quantification of 286 proteins. Subcellular localization and molecular functions were assigned to each protein by UniprotKB, showing that the majority of identified proteins were membrane-associated or cytoplasmic and involved in transport, signalling and cellular adhesion, typical functions of exosomal proteins. We further validated label free mass spectrometry results by immunoblotting, as exemplified by: Xaa-Pro dipeptidase, Major Urinary Protein 1 and Neprilysin, which resulted increased, decreased and not different, respectively, in exosomes isolated from diabetic urine samples compared to controls, by both techniques. In conclusion we show the potential of exosome proteomics for DN biomarker discovery.

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Introduction

Exosomes are 30–100 nm vesicles, derived from the endosomal compartment and released *via* fusion of multivesicular bodies with the plasma membrane.[1] They comprise a lipid bilayer membrane, an array of membrane and cytosolic proteins, and selected RNA species.[2] This molecular complexity suggests that exosomes may mediate a variety of physiological and pathological functions. An increasing body of evidence indicates that they play a pivotal role in cell-to-cell communication, and immuno-modulatory activity,[3] but may also help in regulating the molecular composition of original cells.[4] Exosomes are released by most cell types in the extracellular space; moreover, the presence of exosomes *in vivo* in many body fluids, including blood and urine, makes them readily accessible. Actually, human urine contains large numbers of exosomes, released from every renal epithelial cell type facing the urinary space: thus, their study opens the possibility of obtaining information on the cell of origin and of discovering molecular markers of renal dysfunction and structural injury.[5,6] In fact different cell types release distinct populations of vesicles, harboring a common set of proteins, but also proteins linked to cell-type

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associated functions. Digging out the molecular composition of urinary exosomes under physiological and pathological conditions may be useful in order to reveal different cell behavior, such as in the field of cell communication, and to identify the candidate biomarker of disease.[7]

To date, proteomic strategies for biomarker discovery in urine have primarily included top-down approaches, for example two-dimensional gel electrophoresis or liquid chromatography coupled with mass spectrometry.[8,9] Recent advances in both chromatography and mass spectrometry have enabled bottom-up approaches to identify and to quantify proteins at the peptide level.[10,11] Among approaches to bottom-up proteomics, the label free approach takes advantage of the highly reproducible chromatography and high mass accuracy available in current LC/MS systems. Relative peptide quantification is obtained from their intensities and alignment of each peptide across individual samples based on their accurate mass measurement and LC retention time.[12,13] Moreover, the label free approach overcomes the need of chemical or metabolic labeling steps, simplifying the overall procedures.

Diabetic nephropathy (DN) is a major microvascular complication of type 2 diabetes. It is strictly associated with obesity and insulin-resistance and is the main cause of end stage renal disease, requiring dialytic treatment.[14] However, less than half of all type 2 diabetic subjects develops this complication. The cellular and molecular mechanisms that lead to diabetic nephropathy are incompletely identified. It is known that the renal functional changes are associated with cellular and extracellular derangements in both the glomerular and tubulo-interstitial compartments,[15] which could be reflected by urinary exosomes. Traditionally, incipient nephropathy is defined by the appearance of microalbuminuria,[16] but it does not correlate well with underlying glomerular damage, since diabetic subjects with microalbuminuria display tremendous heterogeneity when concomitant biopsies are examined.[15,17] Therefore, new specific indicators of diabetic nephropathy might be useful to monitor the disease progression or regression and are required to accurately target these patients for therapeutic intervention earlier in the course of the disease.

Here we use a comparative label free LC-MS/MS approach to investigate the alteration of the proteome of urinary exosomes isolated from the Zucker diabetic fatty rats (ZDF), a model of type 2 diabetes mellitus. These rats carry a mutation in the gene coding the leptin receptor (fa/fa) that results in a phenotype very similar to humans with type 2 diabetes mellitus, including the existence of diabetes nephropathy.[18,19] We describe further validation of our results confirming the observed changes for some proteins by immunoblotting.

Materials and methods

Chemicals

Milli-Q water and HPLC-grade water (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) (for MS analysis) are used for all home-made solutions. BCA protein assay, trifluoroacetic acid, ammonium bicarbonate, porcine trypsin, DTT, IAA, acetonitrile, acetone, methanol and Trizma-base were from Sigma-Aldrich. The Hybond-ECL nitrocellulose membrane was from GE (Little Chalfont, Buckinghamshire, United Kingdom). NuPAGE® SDS-PAGE Gel Electrophoresis System Components (mini gels, running and loading buffer, molecular weight markers and coomassie blue staining) were supplied by Life Technologies (Paisley, Renfrewshire, UK). Anti-protease inhibitor cocktail (Complete) was purchased from Roche (Monza, Italy). Monoclonal anti-Alg-2 interacting protein X (Alix) antibody was from Cell Signaling Technology (Danvers, MA, USA); monoclonal anti-Aquaporin1 (AQP1) and polyclonal anti-MUP antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); monoclonal anti-tumor susceptibility gene 101 (TSG10) and anti-neprilysin (CD10) antibodies from Abcam (Cambridge, UK); monoclonal antibody against Xaa-Pro peptidase (PEPD) was from Abgent (San Diego, CA, USA). Species-specific secondary peroxidase conjugated antibodies and ECL reagents were from Pierce (Rockford, IL, USA).

Animal model and experimental protocol

Animal husbandry was in conformity with the Institutional Guidelines in compliance with National laws and policies (D.L.n. 116, Gazzetta Ufficiale della Repubblica Italiana, suppl. 40, Feb.18, 1992). Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Experiments were carried out on male Zucker Diabetic Fatty rats (ZDF, fa/fa; n = 7) and lean male control (fa/+, n = 7) (Charles Rivers, Calco, Italy), maintained on a Purina 2008 diet (Charles Rivers), with free access to tap water. During the experimental period rats were individually housed with a 12 : 12 h light–dark cycle under temperature-controlled ambient conditions (22 °C).

At 5, 11 and 19 weeks of age rats were individually housed in metabolic cages and 24 h urine samples were collected for 5 consecutive days. Body weight (BW, g) and blood glucose (mg dl^{-1}) were measured once a week. Blood glucose was measured using OneTouch Ultra system, LifeScan Inc, Milpitas CA, USA.

Rats were sacrificed at two time points, 12 weeks (ZDF, n = 3; Lean, n = 4) or 20 weeks (ZDF, n = 4; Lean, n = 3), by an overdose of anesthesia (sodium pentobarbital). The kidneys were immediately excised, weighed, and stored for histopathological (formalin 10%) analysis.

Histopathological examination

For each experimental group, renal transmural sections (4 μm) were deparaffinized, rehydrated and stained with hematoxylin–eosin (H&E), following standard techniques (Leica Microscope, Wetzlar, Germany).

Urine samples treatment and exosome isolation

Urine samples were collected in the presence of protease inhibitors (Complete, Roche), subjected to a low spin (1000 × g 10 min) to remove cellular debris and then stored at −80 °C. Exosomes were prepared by ultracentrifugations:[20] urine samples were centrifuged for 15 min at 17 000 × g, 4 °C, to eliminate large

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membrane fragments and other debris. Supernatants were subjected to ultracentrifugation for 1 h at 200 000 × *g*, 4 °C: crude exosome pellets were washed in PBS and then resuspended in bidistilled water, in the presence of protease inhibitors. The samples were stored at −80 °C until use.

Moreover, we prepared urine protein samples (U), after sediment removal, and supernatant of 200 000 × *g* ultracentrifugation (Sn). In order to concentrate proteins, urine and supernatant samples were subjected to ultrafiltration: briefly, 500 μL of urine were loaded onto concentrator devices, VivaSpin 500 (3000 MW cut-off PES membrane, Sartorius), pre-treated with 5% Triton-X100 for improved recovery of low-concentrated samples, according to the manufacturer's instructions. After a 45 min centrifugation at 15 000 × *g* (4 °C), the concentrate was collected and lyophilized. Protein concentration was measured by BCA protein assay.

Exosome proteome analysis by nLC-ESI-MS/MS

For MS analysis, 24 hours urine samples were collected from 7 different 20-week-old rats (3 controls and 4 diabetics) and the related isolated exosomes for each group were then pooled. Each of two resulting pools, from diabetic and control rats, were lysated and the extracted proteins digested with trypsin enzyme adapting the Filter Aided Sample Preparation (FASP) technique.[21] Briefly, exosome lysis was performed through a 30 min incubation in lysis buffer (50 mM Tris–HCl pH7.4, NP40 1%, DOC 0.25%, NaCl 150 mM, EDTA 1 mM). About 150 μg of protein pellet for each pool were then re-suspended in NH_4HCO_3 50 mM and submitted to disulfide bonds reduction with DTT 400 mM (95 °C for 5 min). The supernatants were then transferred to spin ultrafiltration units of nominal molecular weight cutoff of 10 000 (Amicon Ultra 0.5 mL 10 K, Millipore). After sample cooling, lysis buffer and DTT were discharged, by centrifugation for 15 min at 14 000 × rpm. Thus, filters were washed with 300 μL of 50 mM NH_4HCO_3 five-times (10 min at 14 000 × rpm). Then 80 μL of 100 mM iodoacetamide was added to the filters and the samples were incubated in darkness for 30 min. Filters were washed with 200 μL of 50 mM NH_4HCO_3. Protein digestion was conducted overnight at 37 °C adding 6 μg of porcine trypsin for each sample. After digestion, the filtrated tryptic peptides were collected by centrifugation with two washes of water (40 μL and subsequently 100 μL). Digested samples were injected into the EasynLC™ system (Proxeon Biosystems, Odense, Denmark) coupled with a MaXis hybrid UHR-QToF system (Bruker Daltonics, Germany). For the label free quantification each sample was analyzed at least three times. Peptide mixtures were diluted about 1 : 3, before LC-MS analysis. This dilution avoids the saturation of the detector, which causes a truncate peak shape and an incorrect peak area/intensity, without greatly affecting the number of identified peptides. LC-MS analysis was carried out as already described by Mainini *et al.*[22] with small adjustments. MS level measurements were all performed on a predefined 50–2200 *m*/*z* acquisition window at 5000 TOF summations (approximately 1 Hz of spectral rate). Isolation width and collision energy were applied on the basis of the isolation mass value and the charge state against a table of isolation and fragmentation lists fitted for tryptic peptides. Accumulation times for MS/MS were also intensity binned at a maximum of 10 000 summations (approximately 0.5 Hz, if the precursor was at 3000 ion counts) to a minimum of 5000 summations (approximately 1 Hz, if the precursor was at 10^5 ion counts) with a total cycle time range of 6–11 seconds.

Peptide identification by nLC-ESI-MS/MS

Raw MS/MS data obtained by nLC-ESI-MS/MS analysis were lock-mass corrected (at *m*/*z* 1221.9906), then deconvoluted and converted to XML peaklists *via* Compass DataAnalysisTM v.4.0 Sp4 (Bruker Daltonics, Germany). Peakfinder (sumpeaks) was set to exclude any ion with <1 S/N and <20 counts intensity. XML data resulting from DataAnalysis elaboration for each analysis were interrogated using in house Mascot search engine (version: 2.3.02). Database searching was restricted to human Swissprot (accessed Feb 2012; 20 317 sequences) database. Trypsin as enzyme and carbamidomethyl as fixed modifications were set in search parameters. Mass tolerances for all identifications were generally set at 10 ppm for the precursor ions and 0.5 Da product ions. Mascot threshold scores for identity and decoy database were used as peptide level filters of peptide significance.

Label free expression evaluation

Protein abundance in the urinary exosome was determined by IDEAL-Q software (vers 1.6.0.2) (Academia Sinica, Taipei, TAIWAN). The raw data of the three analyses for each exosome preparation pool (diabetic and control rats) were converted to mzXML using CompassXport vers 3.0.4 (Bruker Daltonics, Germany). Peptides–proteins identity was obtained using Mascot (vers 2.3; Matrixscience, England) and the output of the DataAnalysis™ elaboration as Mascot generic format (mgf) file. In order to allow spectral data alignment (mzXML) with the search results, the compound number (Cmpd) in the mgf file was replaced with the scan number. Mascot search results obtained using the above described parameters were exported as an XML file format. The mzXML and its corresponding XML files were used to determine the diabetic/controls protein ratios in the urinary exosome. The IDEAL-Q data input was set to centroid MS1 data mode. Default parameters for Q-TOF instruments were used for data peak alignments. Normalization was performed using the median of peptide abundance and quantile of the peptide ratio level.[13] Protein abundances were then determined using all unique peptides belonging to each proteins. Moreover results obtained with IDEAL-Q were verified with those obtained by Progenesis LC-MS software vers 4 (Non-linear Dynamics, Newcastle, England) using default settings.

Electrophoresis and immunoblotting

Protein separation was performed using the NuPAGE® electrophoresis system (Life Technologies), using 4–12% pre-cast gels, and staining by Coomassie Blue (SimplyStain, Life Technologies). For western blotting analysis equal amounts of proteins were applied to 4–12% NuPAGE and transferred to nitrocellulose membranes, using a mini transfer tank (Hoefer). After blocking with 5% free-fat milk/0.2% Tween 20 in PBS solution, the blots were incubated with the

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respective primary antibodies followed by peroxidase-conjugated secondary antibodies (Pierce) and signals were detected by a CCD camera (Kodak ds Image Station 2000 R) after enhanced chemiluminescence assay (SuperSignal West ECL, Pierce). Densitometric analysis was performed by molecular Imaging Software (Kodak) after normalization by Ponceau Red staining of the blot.[23,24]

Statistics

Statistical analysis was performed by the unpaired t-test and using GraphPad Prism 5 software (GraphPad Software, Inc.).

Results and discussion

Metabolic characteristics of the experimental animals and renal histopathological examination

Data obtained from ZDF and control lean rats are shown in Table 1. As expected, ZDF rats at 6 weeks of age, before the onset of diabetes, had blood glucose levels similar to control lean rats, whereas at 12 and 20 weeks they showed hyperglycemia. At 12 and 20 weeks of age, proteinuria and diuresis significantly increase in ZDF rats; also their body weight was higher than that in control rats at 6 and 12 weeks of age, while at 20 weeks it did not increase any more, indicating the progression of the disease. The kidney weight/body weight ratio was increased both in 12 and 20 week-old ZDF rats (Table 1). Results show that the use of an animal model, together with a correct setting of urine collection times, allows a suitable representation of the natural DN progression, in the absence of any therapy, as needed for humans.

As compared to control lean rats (Fig. 1A and C), in both diabetic groups (Fig. 1B and D), the renal histological sections show interstitial mononuclear inflammatory cells, dilated tubules and tubules with degenerative epithelium. ZDF diabetic rats at 20 weeks of age (Fig. 1D) show focal fibrosis associated with atrophic tubules, thickening of the glomerular basement membrane and an increase of the mesangial matrix (H&E, 10X).

Table 1 Metabolic features of the experimental animals

Age	6 weeks		12 weeks		20 weeks	
Rat type	Lean n = 7	ZDF n = 7	Lean n = 7	ZDF n = 7	Lean n = 3	ZDF n = 4
Blood glucose (mg dl^{-1})	127 ± 4.2	135 ± 6.5	131 ± 7.0	544 ± 49.3***	129 ± 4.1	530 ± 45.5***
Weight (g)	217 ± 4.4	266 ± 9.1***	322 ± 8.2	365 ± 7.4**	416 ± 18.2	379 ± 21.1
Proteinuria (mg per 24 h)	42.7 ± 5.2	86.8 ± 18.4*	74.7 ± 6.3	424 ± 74.1***	39.9 ± 2.1	411 ± 46.3***
Diuresis (ml per 24 h)	6.4 ± 0.17	11.3 ± 1.2***	9.8 ± 0.33	86.9 ± 12.8***	7.1 ± 0.94	119 ± 12.4***
			n = 4	n = 3	n = 3	n = 4
Kidney/body weight (mg g^{-1})	—	—	3.79 ± 0.05	4.14 ± 0.45	3.18 ± 0.20	4.67 ± 0.35*

t-test Lean *vs.* ZDF. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.

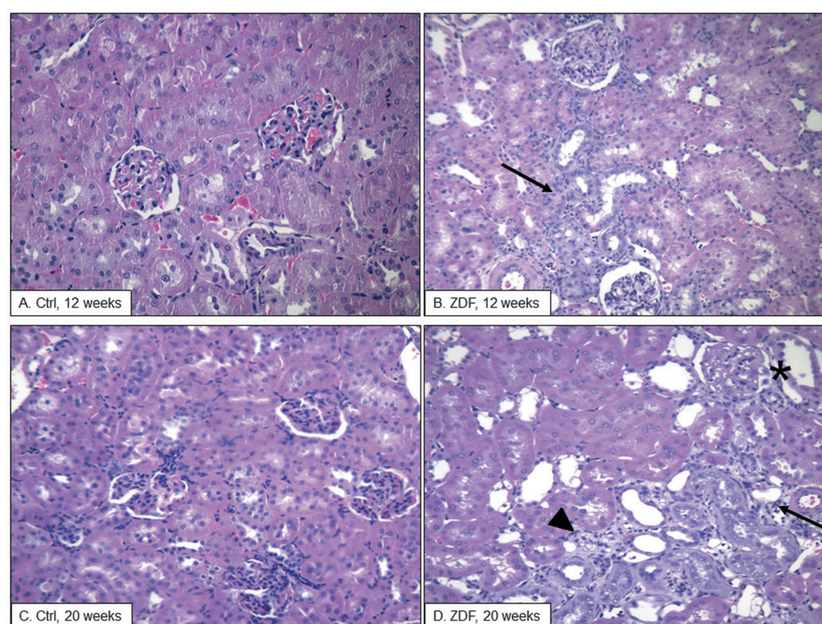

Fig. 1 Representative microphotographs of kidneys in ZDF and control lean rats. (↑) interstitial mononuclear inflammatory cells, dilated tubules and tubules with degenerative epithelium; (▶) focal fibrosis associated with atrophic tubules; (*) thickening of the glomerular basement membrane and increase of the mesangial matrix.

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Urinary exosome characterization

After urinary vesicles isolation, we measured their protein content (Fig. 2A). It appears evident that, under any conditions, the amount of vesicle-associated proteins is negligible (0.1–0.4%), compared to the corresponding total urinary protein amount (Fig. 2B): this means that the vesicle proteome is quite compartmentalized and that also low abundant proteins can be focused. However, a slight interference is likely to be provided by the massive plasmatic protein loss occurring during nephropathy progression, as suggested by the increase in vesicle protein content in diabetic urine samples at 12 and 20 weeks (Fig. 2A).

Moreover, vesicle protein profiles were characterized by comparison with starting urine samples and supernatant ones, after 1D SDS-PAGE separation and CBB staining (Fig. 3). This result shows that the protein pattern of vesicles is completely different from that of starting urine samples and supernatant, which in turn are very similar to each other. Furthermore, albumin represents the main protein species encountered in the total urinary profile of diabetic rats, while it is barely visible in the vesicle protein profile. This observation confirms that vesicles from diabetic urine samples retain a specific and distinct proteome, in spite of advanced proteinuria, and suggests that it may represent glomerular and/or tubular cell changes more faithfully than the whole urinary proteome.

Finally, immunoblotting with antibodies directed against known exosomal markers (Alix, TSG101 and AQP1)[3] demonstrates that they are highly enriched in the vesicle fraction,

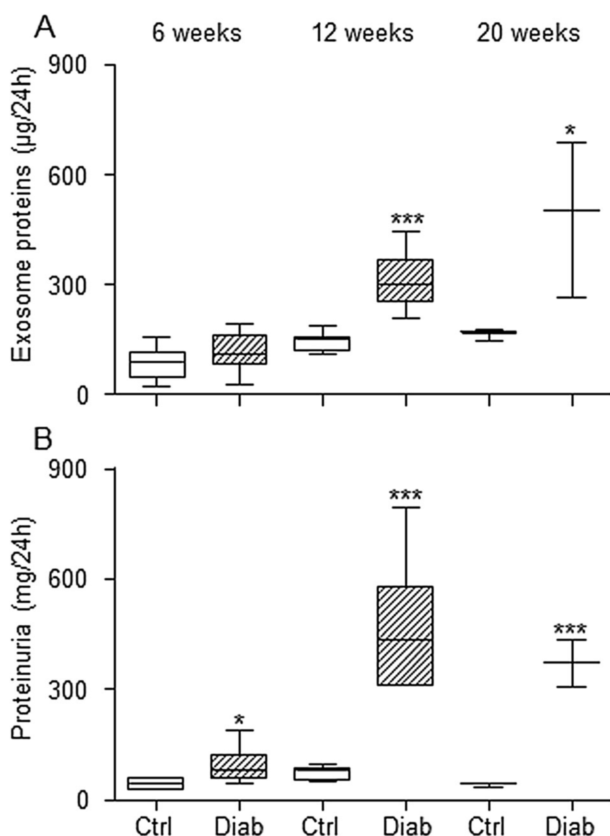

Fig. 2 Exosome-associated proteins (A) and Total urinary proteins (B). Data are represented as box-and-whisker diagram (box-plot).

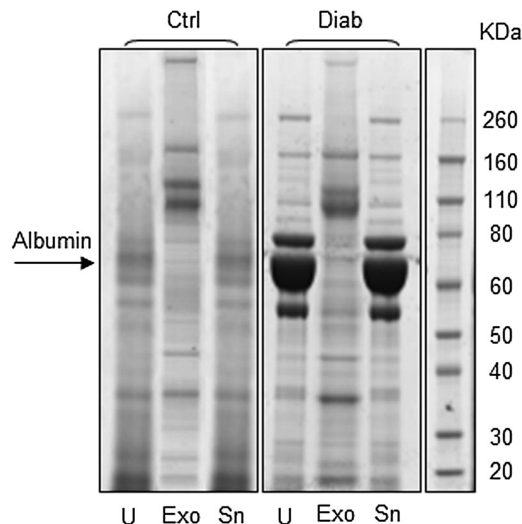

Fig. 3 Protein profiles by NuPAGE 4–12% of vesicle fractions (Exo), compared with total urine samples (U), after sediment removal, and with supernatants (Sn), obtained after 200 000 × g ultracentrifugation, from representative 12 week old rats.

indicating that the protocol of ultracentrifugation provides good quality exosomes, not different when applied to control or DN urine samples, and suitably reproducible (Fig. 4).

LC-MS protein identification and label free quantification

We identified more than 3400 and 4000 peptides in exosomes isolated from urine samples of diabetic and control rats, respectively, with a score above peptide identity threshold, and with a false discovery rate below 2.6% and 1.8%, respectively.[25] These peptides belong to more than 280 protein species that were identified and characterized for their relative content (Tables S1–S3, ESI†). Identification was accepted with at least one unique peptide exceeding the Mascot score of identity cut-off. We included in the list also the proteins identified with one peptide to ensure a better comprehension of exosome proteome. Proteins were considered up or down-expressed when the ratio was higher than 1.50 and lower than 0.50.[22,26] According to these criteria, the content of 76 protein species out of 286 resulted increased in diabetic exosomes, and 68 reduced, compared to urinary exosomes from control lean rats, while the majority of proteins (n = 143) were unchanged (Fig. S1 in ESI†).

Bioinformatic analysis

The identified proteins were investigated to assess their sub-cellular localization and molecular function by UniprotKB. Results show that the majority of identified proteins (66% and 52% in CTRL and DIAB, respectively) were membrane-associated or cytoplasmic (Fig. 5A), correctly reflecting the exosome usual composition.[7] The higher representation of secreted proteins in the diabetic exosomal protein profile may be due to entrapping/adherence of some plasmatic proteins filtered by the damaged glomeruli. Regarding molecular functions, many proteins are involved in transport, signalling and cellular adhesion, typical functions of exosomal proteins (Fig. 5B).

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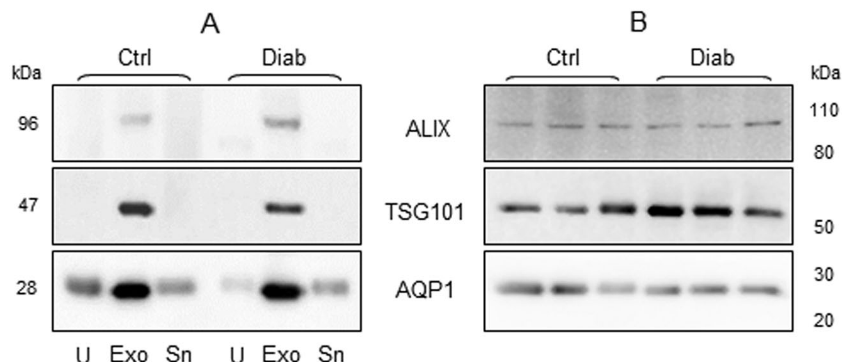

Fig. 4 Urinary exosome protein markers. (A) Immunoblotting for known exosomal markers (Alix, TSG101 and AQP1) in vesicle fractions (Exo), in comparison with total urine samples (U), after sediment removal, and with supernatants (Sn), obtained after 200 000 × *g* ultracentrifugation from representative (12 week old) rats. (B) Immunoblotting for the same markers in exosomal samples from 3 different (12 week old) control and diabetic rats. Equal amounts of proteins were loaded on the lanes of each gel.

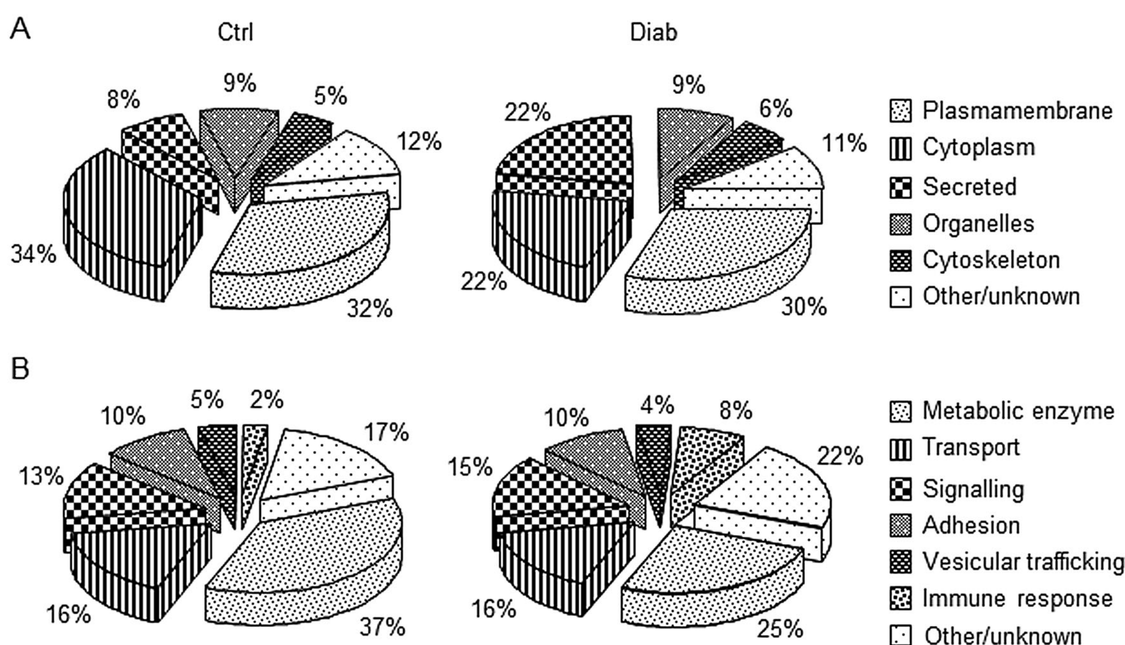

Fig. 5 Bioinformatic analysis of exosome proteins. (A) Subcellular localization; (B) molecular functions.

The greatest difference between control and diabetic protein classification concerns the category of ''immune response'', and possibly derives from the increase in immunoglobulin species and complement components, categorized as secreted proteins, in agreement with the above observation.

Validation of differential exosomal protein content by immunoblotting analysis

Relative quantitation results obtained by the label-free approach were verified by immunoblotting on selected renal proteins (Xaa-Pro dipeptidase; MUP-1; and CD10) not only at 20 weeks but also at 6 and 12 weeks. Band intensities were quantified by densitometry, after protein loading normalization, and results are shown as a box plot (Fig. 6A–C).

Xaa-Pro dipeptidase (or Prolidase, PEPD) is a member of the matrix metalloproteinase family, is expressed at the level of kidney tubules and has an important role in the recycling of proline for collagen synthesis.[25] This enzyme levels are found significantly increased in diabetic rat exosomes both after label free quantification and immunoblotting (Fig. 6A, and Table S1 in ESI†). Moreover, the increased PEPD content of diabetic urinary exosomes seems to correlate with DN severity, being much higher in older Zucker rats (Fig. 6A). Serum PEPD activity was recently found elevated in diabetic patients with neuropathy, in comparison with both diabetics without neuropathy and the control group.[27] This is considered of potential interest, since PEPD activity, reflecting collagen breakdown, can be viewed as a promising candidate marker for diabetic microangiopathy and other tissue changes involving the extracellular matrix.[27]

The Major Urinary Protein 1 (MUP-1) is a low molecular weight protein belonging to the lipocalin family, which carries

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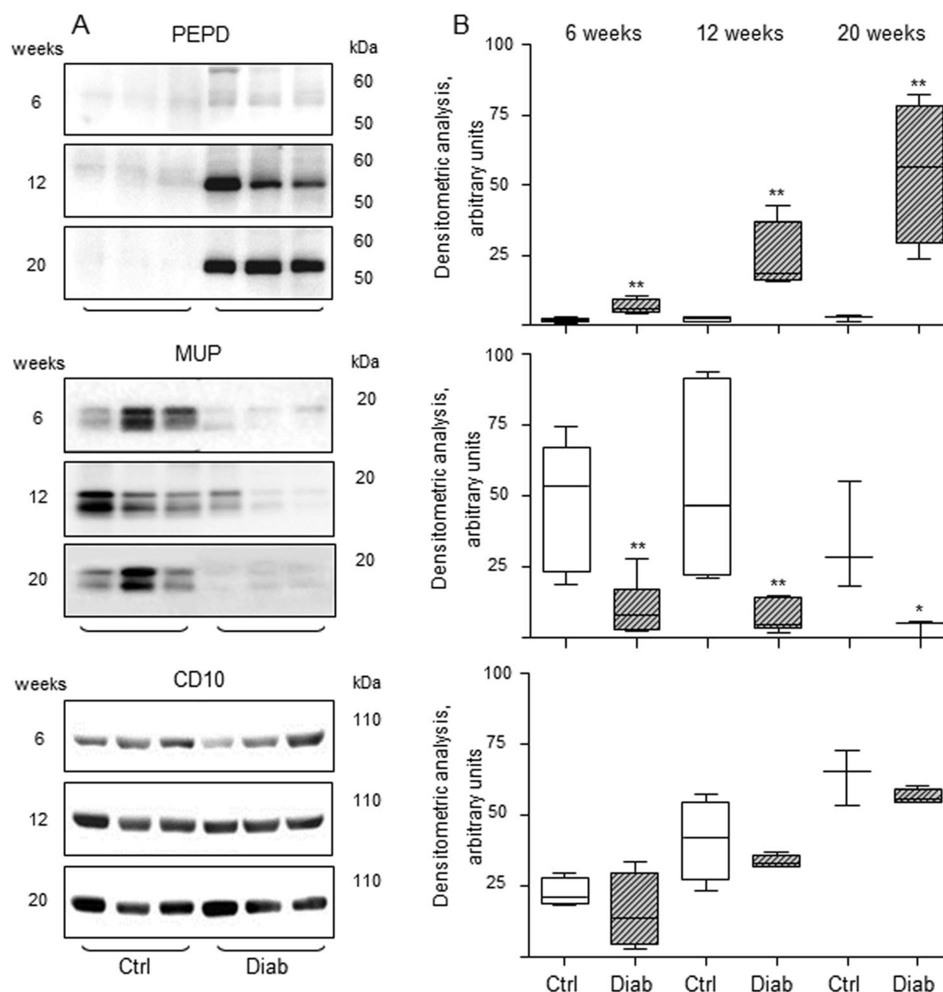

Fig. 6 Validation of exosome protein content by western blotting. Panel (A) PEPD; panel (B) MUP1; panel (C) CD10. For each protein, membranes representing the three time points were ECL incubated and CCD exposed together. Data are represented as box-plot. *t*-test Ctrl *vs.* Diab: $*p < 0.05$; $**p < 0.01$.

small hydrophobic ligands such as pheromones. However, the physiological functions of MUP-1 remain poorly understood.[28] MUP-1 was demonstrated to have a reduced content in DN exosomes, compared to control ones, by immunoblotting, confirming the data found by the label free approach (Fig. 6B and Table S2 in ESI†) at the time of later sacrifice. MUP-1 has already been reported as an important player in regulating energy expenditure and metabolism in mice and it has been suggested that MUP-1 deficiency might contribute to the metabolic dysregulation in obese/diabetic mice.[11] Interestingly, the human genome contains a gene (accession number: XM_001723632), which is predicted to encode a MUP-1like molecule (designed as hMUP) and ~50% identical to mouse MUP1 in amino acid sequence. It was predicted that hMUP also regulates glucose metabolism by a similar mechanism to that of MUP1.[28]

Finally, CD10 is a 100 kDa urinary exosomal transmembrane glycoprotein[29,30] also known as neprilysin, involved in the cleavage and inactivation of multiple physiologically active peptides.[31] Label free quantification showed that CD10 is unchanged in the 20 week old rat samples, and also this result was confirmed by immunoblotting performed at the three time points (Fig. 6C and Table S3 in ESI†). Accordingly, the exosome content of this protein may be used as a standard for normalization, a sort of housekeeping protein, between two different experimental sets (diabetic and control rats). Moreover, it guarantees once again that the burden of plasmatic proteins present in DN urine samples (proteinuria) does not importantly affect exosome proteome.

Concluding remarks

We show here that the application of a label free LC-MS/MS approach can reliably assess differential proteome of urinary exosomes isolated from an animal model of type 2 DN. The use of a urine subfraction, like exosomes, ensures proteome simplification and relative high consistency, despite the fluctuations in the environmental conditions. In fact, our proteomic results show that exosomes isolated from urine samples of diabetic subjects are only minimally affected by massive proteinuria. Moreover, the label free approach allows obtaining protein identification and quantification at the same

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time, without the use of chemical labelling or modification and it is suitable for application to small amounts of sample. We are confident that digging out the molecular composition of urinary exosomes may be useful in order to reveal pathophysiological alterations occurring in DN progression, and in order to discover biomarker candidates for this condition.

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