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Urinary proteomics in biomarker discovery of kidney-related disorders: diabetic nephropathy and drug-induced nephrotoxicity in chronic headache

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ABSTRACT

Objective

Urinary proteomics is primarily applied to the study of renal and urogenital tract disorders. Here are reported two distinct successful examples of this approach for the discovery of early urinary biomarkers of kidney-related dysfunctions: diabetic nephropathy (DN), a well-known complication of diabetes frequently leading to dialysis, and drug-induced nephrotoxicity, a possible condition caused by medication-overuse headache (MOH). Early detection of kidney disorders based on selective biomarkers could permit to diagnose patients at the initial stage of the disease, where the therapy may be suspended or prevent disease advancement.

Methods

Urine samples were first concentrated and desalted. Subsequently, they were subjected to two-dimensional gel electrophoresis (2-DE) coupled to mass spectrometry (MS) for protein identification. Furthermore,

some proteins were verified by Western blot and ELISA test.

Results

In diabetes-related study, 11 differentially expressed proteins were detected (8 up-regulated and 3 down-regulated) in type 2 diabetic (T2D) and T2DN patients compared to the healthy control subjects. In the MOH study, a total of 21 over-excreted proteins were revealed in urine of non-steroidal anti-inflammatory drugs (NSAIDs) and mixtures abusers vs controls. Particularly, 4 proteins were positively validated by immunoblotting and ELISA.

Conclusion

Urinary proteomics allows non-invasive assessment of renal diseases at an early stage by the identification of characteristic protein pattern.

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INTRODUCTION

Proteomics is the study of protein expression in a definite tissue, cell type or biological body fluid. The comparison of protein patterns between healthy subjects and patients with a given pathological condition can be useful to identify specific diagnostics or prognostics biomarkers of diseases. Particularly, urinary proteomics has rapidly developed and has been extensively applied in the field of early diagnostics and differentiation of renal damage (1).

Urine is a valuable source of proteins and peptides; it has the advantage of being obtained non-invasively, easily and frequently, and in a large quantity. It has been defined as a fluid biopsy of the kidney and urogenital tract, thus providing considerable information about these organs. Consequently, many changes in kidney and urogenital tract function may be detected in the urinary proteome (2).

Urine proteomics studies were conducted in the search for early biomarkers of renal changes in:

1. type 2 diabetic nephropathy (T2DN), a complication linked to diabetes, which leads to end-stage renal disease (3);
2. drug-induced nephrotoxicity in medication-overuse headache (MOH), a chronic disorder associated with overuse of analgesic drugs or compounds for acute headache (4-6).

MATERIALS AND METHODS

Subjects

1) Diabetic nephropathy

Diabetic patients were enrolled from the "Division of Nephrology, Dialysis and Renal Transplantation" of the University-Hospital of Modena and Reggio Emilia, Italy: 10 normoalbuminuric patients with type 2 diabetes (T2D), 12 T2DN patients with microalbuminuria (range 130-280 mg/mL) and/or proteinuria (>10 mg/dL), and a control group of 12 healthy volunteer subjects with a history of regular renal function.

The duration of diabetes was similar in the two patients groups. Moreover, all groups were matched for age and gender (3).

2) Drug-induced nephrotoxicity

A total of 87 MOH patients were recruited from the "Headache and Drug Abuse Center", University-Hospital of Modena and Reggio Emilia, Italy. They were divided into three groups, according to the type of the primary abused drug, as follows: 31 patients who consumed exclusively triptans, 27 non-steroidal anti-inflammatory drugs (NSAIDs) and 29 taking mixtures. Healthy volunteers (n=30) were enrolled as controls. Each group was matched for age and gender; moreover, patients showed similar MOH duration, days with headache/month and about daily drug intake. Kidney diseases and urogenital tract dysfunctions,

together with other important illness, were considered as exclusion criteria (5).

Both studies were in compliance with the ethical principles for medical research involving human subjects, in accordance with the Declaration of Helsinki. Written informed consent were received from both, patients and healthy subjects.

Urine proteomics analysis

Morning midstream urine samples were collected and immediately centrifuged at 800 g for 10 min at +4C°, to remove cell debris and contaminants. Commonly, human urine has a very diluted protein concentration and, at the same time, a high-salt content, which hampers the proteomic analysis. Sample preparation is therefore a pivotal step in urinary proteomics, especially during two-dimensional polyacrylamide gel electrophoresis (2-DE) (2). In order to concentrate proteins, eliminating the interfering salts, urine samples were treated with filter devices, 3 kDa MW-cut off (Merck Millipore). Subsequently, total protein content was estimated by the

Table 1 **Differentially expressed proteins detected in T2D and T2DN patients by MS**

Protein name	Acc. number[(a)]	MW (kDa)	Function
Up-regulated proteins			
Transthyretin precursor	P02766	16.0	Hormone-binding
Ig Kappa chain C-region	P01834	11.8	Immune response
Ig Kappa chain V-II region Cum	P01614	12.8	Antigen-binding
Ig Kappa chain V-II region SIE	P01620	11.9	Immune response
Carbonic anhydrase 1	P00915	28.8	Miscellaneous
Plasma retinol-binding protein	P02753	23.3	Transport
Beta-2-microglobulin precursor	P61769	13.8	Immune response
Beta-2-glycoprotein 1	P02749	39.6	Binding protein
Down-regulated proteins			
Prostatic acid phosphatase precursor	P15309	44.9	Dephosphorylation
Ribonuclease 2	P10153	18.9	Miscellaneous
Kallikrein-3	P07288	29.3	Hydrolysis

(a) Primary accession number from the SwissProt database.

spectrophotometric Bradford method, and 100 mg of protein was premixed with a specific lysis buffer. The first-dimension separation (isoelectrofocalization) was performed using IPG strips 17 cm long, wide pH range 3-10 (Bio-Rad), while in the second-dimension separation, 8-16% polyacrylamide gradient gels were used, that finally were staining with a silver-nitrate staining protocol. Afterwards, gels images were acquired with a calibrated densitometer (GS800 model, Bio-Rad) and analyzed by a specific image analysis software (PDQuest, Bio-Rad), to reveal differentially expressed protein spots among the patients and control groups (3, 5). The spots of interest were cut from the gels and subjected to trypsin digestion. Peptides were finally extracted and analyzed by the mass spectrometry using a quadrupole-time of flight-liquid chromatography mass spectrometer (Q-ToF-LC/MS, Agilent-Technologies).

In the second study (drug-induced nephrotoxicity), the results obtained by proteomic analysis were further confirmed and validated by Western blot and ELISA test (6).

RESULTS AND DISCUSSION

1) Diabetic nephropathy

Comparing the urinary proteomic profiles obtained by 2-DE analysis, 11 differential proteins were identified that progressively changed between controls and T2D and T2DN patients. Precisely, 8 proteins were significantly up-regulated: transthyretin precursor, Ig k chain C region, Ig k chain V-II region Cum, Ig k-chain V-III region SIE, carbonic anhydrase 1, retinol binding protein, beta-2-microglobulin precursor and beta-2-glycoprotein 1.

Except for the last one, all the other proteins were in the low MW range (<30 kDa). Three proteins were found down-regulated: prostatic acid phosphatase precursor, ribonuclease 2 and kallikrein-3 (Table 1).

Proteomic analysis allowed to detect alterations of urinary proteins in both T2DN and T2D

Table 2 Differentially expressed proteins identified by Q-ToF-LC/MS

Protein name	Acc. number[(a)]	MW (kDa)	Over-expression *vs* controls[(b)]		
			Mixtures	*NSAIDs*	*Triptans*
Low - MW proteins					
Prostaglandin-H2-D-isomerase	P41222	18.7	x	x	x
Ig kappa chain C region	P01834	11.8	x	x	NS
Perlecan (fragment)	P98160	479.2	x	x	x
Transthyretin	P02766	15.9	NS	x	NS
Proactivator polypeptide	P07602	9.11	x	x	x
Nuclear transport factor 2	P61970	14.6	x	x	x

Fatty acid-binding protein	Q01469	15.5	NS	x	x
Beta-2-microglobulin	P61769	11.7	NS	x	x
Protein S100-A11	P31949	11.8	NS	x	x
Non-secretory ribonuclease	P10153	18.9	x	x	NS
Cystatin-C	P01034	13.3	x	x	NS
Protein S100-A8	P05109	10.8	x	x	x
Medium - MW proteins					
Alpha-1-antitrypsin	P01009	46.9	x	x	NS
Actin, cytoplasmic1	P60709	42.1	x	x	x
Alpha-1-microglobulin	P02760	39.9	x	x	NS
Apolipoprotein H	P02749	38.3	NS	x	NS
Serpin B3	P29508	44.6	x	x	x
Annexin A1	P04083	38.6	x	x	x
High - MW proteins					
Serum albumin	P02768	66.5	x	x	NS
Uromodulin	P07911	69.7	x	x	x
Inter-α-trypsin inhibitor heavy chain H4	Q14624	70.6	x	x	NS

(a) Primary accession number from the SwissProt database
(b) Expression difference calculated by the PDQuest software: x: significant, NS: not-significant

normoalbuminuric patients. Thus, this protein pattern might be of potential interest to identify diabetic patients prone to develop nephropathy, contributing to a better understanding of diabetic-related renal damage. The strength of proteomics in this research area has been confirmed also by recently published review articles (7, 8).

2) Drug-induced nephrotoxicity

In this study, both qualitative and quantitative differences in urine of MOH patients were studied and revealed. Interestingly, by 2-DE combined with MS analysis, 21 over-excreted proteins and a significantly higher number of total protein spots were identified in the urine of NSAIDs, mixtures

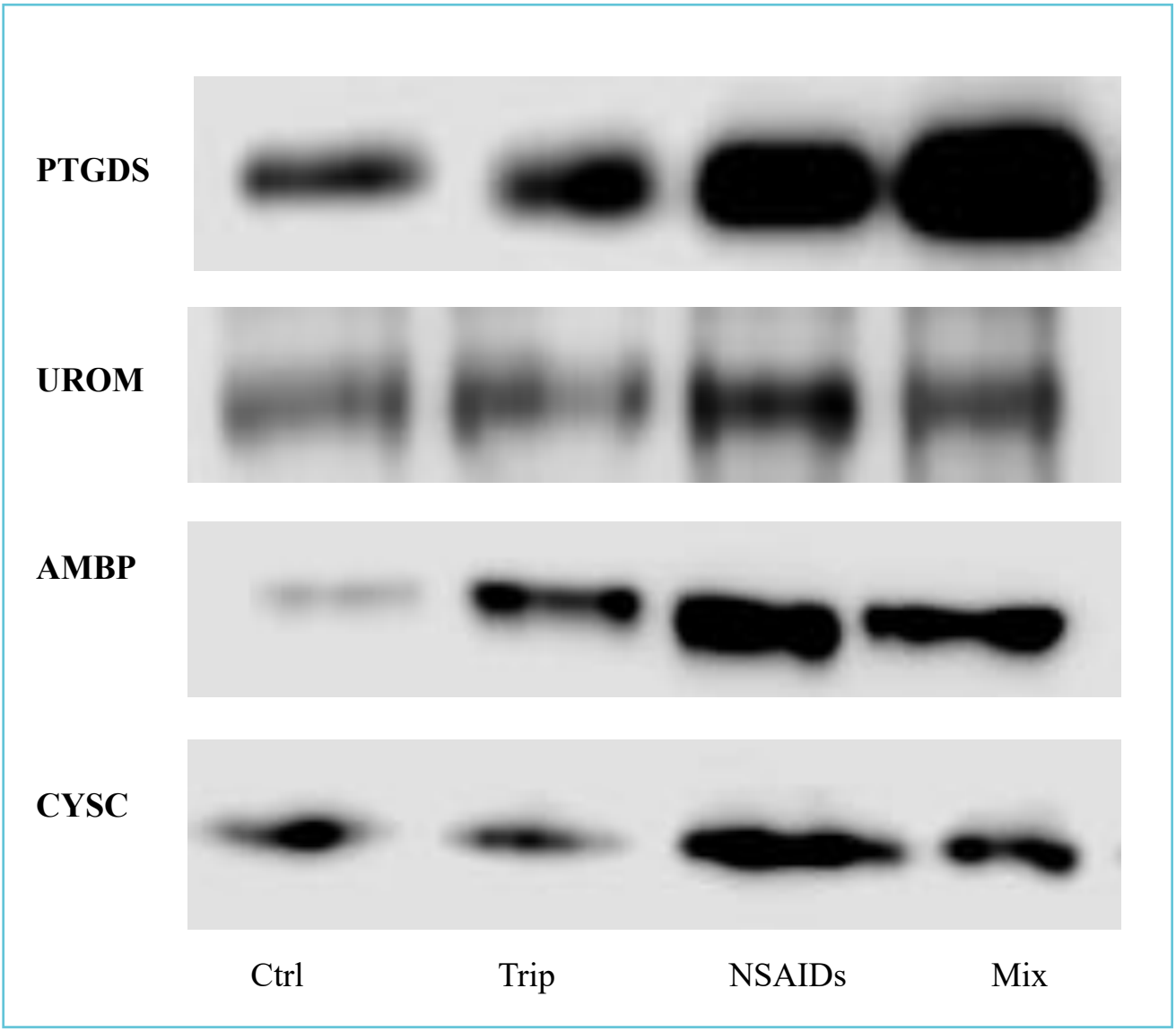
and triptans abusers compared to the controls (Table 2).

Some differentially expressed proteins detected by proteomic analysis were found to be strongly related to renal injury (9), as assessed by an extensive literature review. Particularly, 4 proteins were validated by Western blot: prostaglandin-H2 D-synthase (PTGDS), uromodulin (UROM), alpha-1-microglobulin (AMBP) and cystatin-C (CYSC), as shown in Figure 1.

Immunoblotting allowed to confirm previous data of over-expression of these proteins in urine of MOH patients (especially NSAIDs and mixtures abusers) vs normal controls (6).

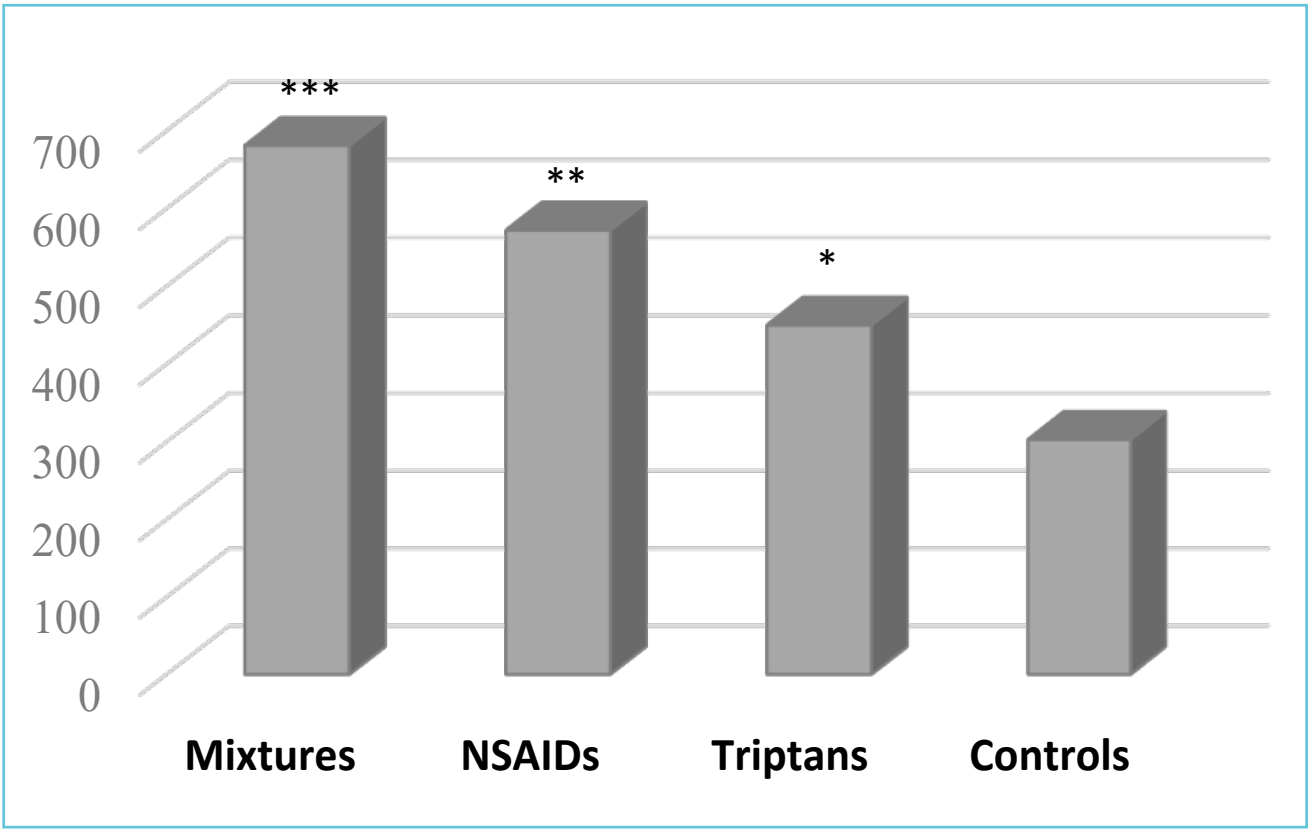
Finally, PTGDS was further quantified by the ELISA test (Figure 2), which proved its significant increase in all MOH groups: mixtures (681 ± 218 ng/mL), NSAIDs (572 ± 135 ng/mL,) and triptans (450 ± 116 ng/mL), compared to the controls (303 ± 130 ng/mL). These data points,

Figure 1 Western blot analysis



The protein expression was evaluated on urine samples in controls (Ctrl), triptans (Trip), NSAIDs and mixtures group (Mix). For each protein tested the signal is higher in NSAIDs and mixtures abusers compared to controls.

Figure 2 ELISA test of PTGDS protein



*Significant difference was estimated by the Student's t-test (*p<0.01, **p<0.0001, ***p<1.00^{-06} vs controls).*

expressed as mean ± standard deviation, were in strict accordance with MS and Western blot results (6).

The results of this study allowed to define the urinary protein profile of MOH, in relation to the type of drug abused. The use of powerful proteomic methodologies could permit to identify promising candidate biomarkers of kidney dysfunctions, and consequently those chronic headache patients at risk to develop drug-induced nephrotoxicity.

CONCLUSIONS

In conclusion, urinary proteomics proved to be a suitable tool in nephro-toxicological research. Actually, its application may be useful in the search of early biomarkers, providing important diagnostics and prognostics indications. Additionally, the study of the urinary proteome can offer significant data for a better understanding of renal pathophysiology.

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